Genetics of Cutaneous Malignant Melanoma

Julie M S Lang BSc (Hons) MSc (Dist)

For the Degree of

Doctor of Philosophy (PhD)

Thesis submitted to

University of Glasgow

Faculty of Medicine

Division of Developmental Medicine

November 2004

UNIVERSITY
of
GLASGOW

Acknowledgments

It is a pleasure to thank the many people who have made this thesis possible.

I would especially like to thank my PhD supervisor Professor Rona MacKie for all her inspiration and enthusiasm. Throughout my research she has provided both encouragement and support, for which I am extremely grateful.

I would like to thank Dr Maureen Boxer for stimulating and technical discussions and for her helpful guidance when it was most required. My advisor Professor Fiona Lyall deserves special acknowledgement for her support and sound advice. I am thankful to Professor Michael Connor for giving me the opportunity to carry out this research within his department. I am especially grateful to Dr Vincent Macaulay for helping me to understand correlations and statistical significance. I am also grateful to Professor William Hunter for help with molecular modelling and relevant discussions.

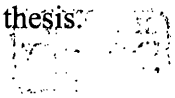
Many thanks to Professor David Hogg from Canada, Dr Nick Hayward from Australia and Dr Hensin Tsao from the U.S.A, for kindly sending me DNA samples from their M53I mutation carrying families.

All the staff in the DNA lab are thanked for help with experimental setup, technical problems and general advice. In particular, I would like to acknowledge the help of Ann Weber with DNA extractions. I would like to thank Dr Gordon Graham for his kind assistance with computing. My appreciation goes to the university staff members in the Department of Medical Genetics for their encouragement and support.

I would like to thank the Shaw Melanoma Trust, which has supported me during my three years of research and for the award of travel grants.

Lastly, I wish to thank my family, whose constant encouragement, love and emotional support, I have relied on throughout my time at university.

It is to my gran that I dedicate this thesis.



List of Contents

List of Figures **Page Number**

List of Tables **Page Number**

List of Publications

Excerpts and preliminary results of this thesis have been published as detailed below:

Lang, J., Boxer, M., & MacKie, R. 2003, "Absence of exon 15 BRAF germline mutations in familial melanoma", *Hum.Mutat.*, vol. 21, no. 3, pp. 327-30.

Lang, J., Boxer, M., Hunter, W., & MacKie, R. 2004, "Analysis of CDKN2A mutations in 42 Scottish families with cutaneous melanoma: evidence that the M53I mutation originated in Scotland", *Clinical Molecular Genetics Society*, pp. 27.

Lang, J., Boxer, M., & MacKie, R. 2003, "Studies on B-RAF in familial and sporadic melanoma", *International Melanoma Consortium Annual Scientific Meeting.*

Lang, J., Boxer, M., & MacKie, R. 2003, "Familial Melanoma: Absence of exon 15 BRAF germline mutations", *Eur.J.Hum.Genet.*, vol. 11, suppl. 1, pp. 72.

Lang, J., Boxer, M., & MacKie, R. 2002, "Variations in the CDKN2A Gene in Familial Melanoma in Scotland", *J.Med.Genet.*, vol. 39, suppl. 1, pp. S69.

List of Abbreviations

+ve	positive
-ve	negative
α	alpha
β	beta
Acral	Acral Lentiginous Melanoma
ASO	allele-specific oligonucleotides
BRAF	v-raf murine sarcoma viral oncogene homolog B1
CDK4	cyclin-dependent kinase 4
CDKN2A	cyclin-dependent kinase inhibitor 2°
CI	confidence intervals
°C	degrees centigrade
dHPLC	denaturing high performance liquid chromatography
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetra-acetic acid
EtBr	ethidium bromide
F	female
FAM	6-Carboxy-fluorescein
FISH	fluorescence in situ hybridisation
g	grams
g	gravitational force
LMM	Lentigo Maligna Melanoma
log	logarithm
M	male
MC1R	melanocortin 1 receptor
MPM	multiple primary melamomas
mins	minutes
Mucosal	Mucosal Melanoma
Nodular	Nodular Melanoma
NRAS	neuroblastoma ras viral oncogene homolog
NS	not significant
nt	nucleotides

OD	optical density
O/N	overnight
OR	odds ratio
p	significance value
PCR	polymerase chain reaction
rpm	revolutions per minute
RT	room temperature
UV	ultraviolet
SE	standard error
secs	seconds
SNP	single nucleotide polymorphism
SSM	Superficial Spreading Melanoma
TAE	tris acetate EDTA
TEMED	N,N,N',N'-Tetramethylethylenediamine
WT	wild type
l	litre
ml	millilitre
µl	microlitre
b	base
bp	base pairs
kb	kilobase pairs
kDa	kilodalton
g	gram
mg	milligram
µg	microgram
ng	nanogram
M	molar
mM	millimolar
µM	micromolar
mm	millimetre

Declaration

Excerpts from the results of this thesis have been published as detailed on page 22. I certify that this thesis does not contain any other material published or written by another person except where due reference is made in the text. The results presented in this thesis have not been submitted for any other degree or diploma.

Julie M S Lang

Summary

Cutaneous malignant melanoma has doubled in incidence in many countries over the past 20 years. The majority of melanomas are sporadic, but up to 5% of melanoma patients have one or more first-degree relatives also affected, and genetic analysis of these families shows germline mutations of *CDKN2A* in 20-30% of patients.

This thesis has investigated 48 Scottish families with cutaneous malignant melanoma and also 28 patients with sporadic melanoma for mutations in the *CDKN2A, MC1R* and *BRAF* genes using molecular genetic techniques.

The work presented in this thesis adds 32 new melanoma families to the 16 already reported from Scotland (MacKie et al. 1998). In total, 13/48 (27%) Scottish families have detectable *CDKN2A* mutations. One of the mutations detected (H83N) has not previously been reported in melanoma, and molecular modelling suggests the likely functional result of this mutation.

In the course of studying the *CDKN2A* gene the M53I mutation was identified in six Scottish families making it the most common *CDKN2A* mutation identified in this thesis from the Scottish melanoma families studied. Haplotype analysis surrounding *CDKN2A* was therefore performed on the six Scottish families and also examined in 12 M53I carrying families from around the world.

This study provides further evidence that the M53I mutation appears to have originated from a common founder and provides further evidence demonstrating common founders for most of the recurrent mutations in the *CDKN2A* gene.

The aim of the analysis of the *MC1R* gene was to investigate any underlying genetic relationship between *MC1R* variants and *CDKN2A* mutations. In total, 13 *MC1R* variants leading to an amino acid substitution were detected, three of which have not been described elsewhere (L44I, M128K, A171G) and appear to be very rare. Collectively, both familial and sporadic melanoma patients are more likely to carry a *MC1R* variant than normal control subjects (P = 0.006; OR = 4.485; CI 1.492-12.883) and melanoma patients carry significantly more *MC1R* variants than control subjects (P = 0.003; OR = 2.696; CI 1.384-5.253).

Familial melanoma patients are more likely to carry the R151C variant than normal control subjects (P = 0.043; OR = 2.406; CI 1.062-5.452). *CDKN2A*

positive familial melanoma patients are more likely to carry the V60L and R151C variants than *CDKN2A* negative familial melanoma patients (P = 0.017; OR = 3.818; CI 1.315-11.084 and P = 0.029; OR = 3.515; CI 1.160-10.650, respectively). Conversely, *CDKN2A* negative familial melanoma patients are more likely to carry the V92M variant than *CDKN2A* positive familial melanoma patients (P = 0.021; OR = 9.308; CI 1.112-77.888).

The variant R160W is significantly correlated with skin type 1 (P < 0.001) and variants R142H (P = 0.019) and R160W (P = 0.018) are significantly correlated with red hair, although this significance is lost after the Bonferroni correction.

During the time of this study Davies et al. (2002) reported that the most frequently targeted gene in melanoma is *BRAF*. All mutations were within the kinase domain, with a single substitution in exon 15 (V599E) accounting for 80% of mutations. *BRAF* mutations in germline DNA from familial melanoma patients had not been investigated, and the number of melanoma tissue samples investigated for *BRAF* mutations was low. One of the aims of this thesis therefore was to screen exon 15 of *BRAF* to determine if the V599E mutation would contribute to melanoma predisposition in familial melanoma as a germline mutation. The study of this thesis also investigated primary and secondary melanomas for exon 15 *BRAF* mutations.

DNA from the peripheral blood of 42 familial melanoma cases contained no exon 15 *BRAF* mutations. DNA from two samples of secondary melanoma from two individuals with a family history of melanoma also failed to show exon 15 *BRAF* mutations. These results therefore suggest that exon 15 *BRAF* mutations are not causative germline mutations in melanoma.

The V599E substitution was however detected in formalin fixed paraffin embedded primary tumour DNA from 13/52 sporadic cases (25%). The V599E substitution was also detected in secondary tumour DNA from 6/22 sporadic cases (27%) of frozen secondary melanoma.

CHAPTER 1

INTRODUCTION

1.1 Introduction

In 1820 William Norris first reported an inherited predisposition to develop melanoma. In describing a case of malignant melanoma, Norris wrote: 'It is remarkable that this gentleman's father, about thirty years ago, died of a similar disease. A surgeon of this town attended him, and he informed me that a number of small tumours appeared between the shoulders...This tumour, I have remarked, originated in a mole, and it is worth mentioning, that not only my patient and his children had many moles on various parts of their bodies, but also his own father and brothers had many of them. The youngest son had one of these marks exactly in the same place where the disease in his father first manifested itself. These facts, together with a case that has come under my notice, rather similar, would incline me to believe that this disease is hereditary.' Quoted in Hecht (1989).

Cutaneous Malignant Melanoma is the most lethal form of skin cancer, MIM# 155601 (MIM - Mendelian Inheritance in Man is a catalog of genetic disorders named after Gregor Mendel, who determined the basic principles of inheritance in the latter half of the 19th century. Each MIM entry is given a unique six-digit number whose first digit indicates the mode of inheritance of the gene involved). The risk of developing melanoma increases with age, but nonetheless the disease may affect young, otherwise healthy people.

Melanoma is a malignant tumour of melanocytes or nevus cells, arising either de novo or from a pre-existing benign nevus. Melanocytes produce a brown-black pigment called melanin, which helps to protect against the damaging rays of the sun and to determine skin colouring. Melanocytes are found in the basal layer of the epidermis, comprising 5-10% of cells in the basal layer with approximately 1000-2000 melanocytes per square millimetre of skin. Melanin production is stimulated by ultraviolet (UV) radiation. Melanocytic nevi are most prevalent in the second and third decades of life. Malignant transformation of the melanocyte to melanoma is a rare event. Most melanomas originate in epidermal melanocytes, but they can arise from ocular melanocytes and rarely in the membranes of the nasal passages, oral, pharyngeal mucosa, anal and vaginal mucosa.

1.2 Anatomy of the Skin (Figure 1.1)

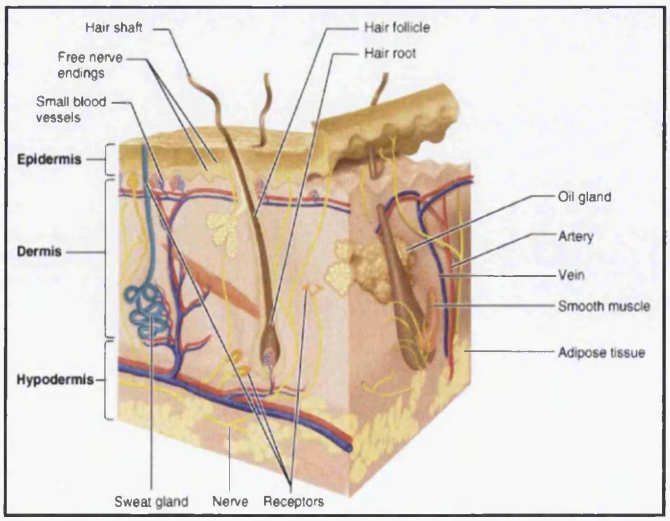
The skin is the largest organ of the body, forming a protective barrier against infection and helping to maintain a constant body temperature. Skin is only about 2mm thick and is made up of two layers that cover a third fatty layer. The outermost layer is called the epidermis, which is up to 20 cells deep or 0.1 to 1.5 millimetres thick. It is made up of five layers: the basal cell layer, the squamous cell layer, the stratum granulosum, the stratum lucidum, and the stratum corneum. These layers maintain the skin's strength, protect against UV radiation and are constantly self-renewing. The basal cell layer, which is constantly producing new basal cells, also contains the melanocytes. When skin is exposed to the sun, the melanocytes increase melanin production to protect the skin from damaging UV radiation.

Below the epidermis lies the dermis, ranging in thickness from one to four millimetres. The dermis contains nerve endings, eccrine sweat glands, sebaceous glands, hair follicles and blood and lymph vessels, which increase in number deeper in the skin. The main components of the dermis however are collagen and elastin, which support the epidermis and keep the skin flexible.

Under the epidermis and dermis layers is a fatty subcutaneous tissue layer acting as an insulator and protecting the internal organs from damage. The blood and lymph vessels also cross this layer.

FIGURE 1.1

Cross Section of the Skin



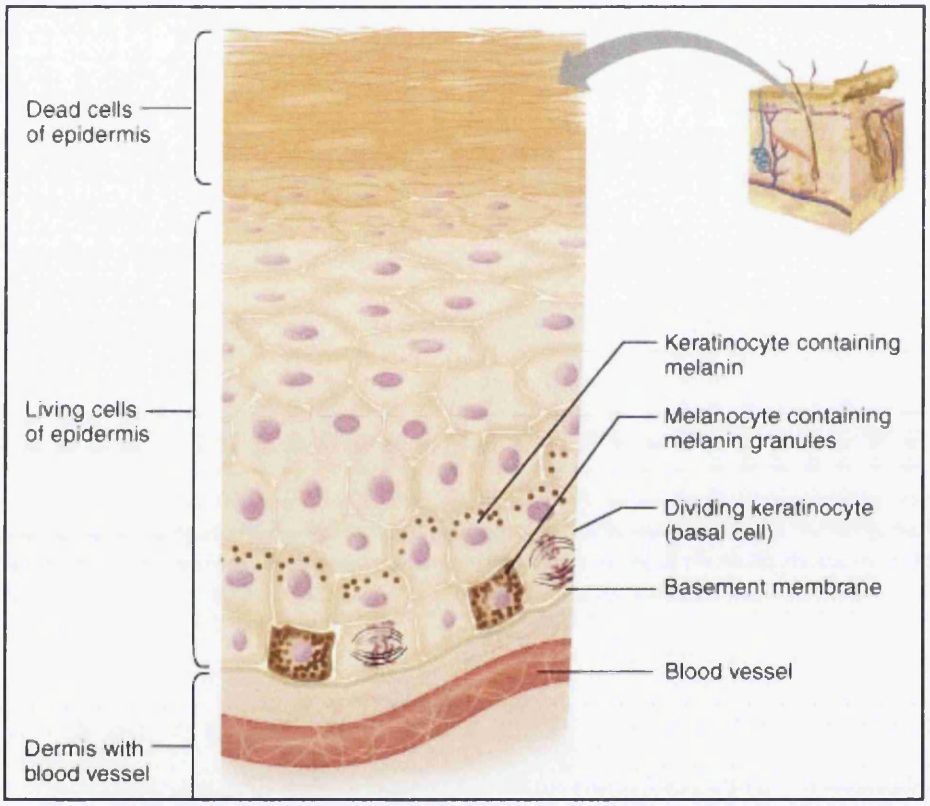
This image was taken from the Department of Biochemistry and Molecular Biology, The Pennsylvania State University website.

1.3 Melanin Pigment

Melanin is a pigment that gives colour to hair, skin and the iris of the eye. It is a water-insoluble polymer derived from the amino acid tyrosine. It is found in melanosomes produced by melanocytes, which are found in the basal layer of the epidermis (Figure 1.2).

FIGURE 1.2

Melanocytes in the Skin



This image was taken from the Department of Biochemistry and Molecular Biology, The Pennsylvania State University website.

Skin colour is determined by the various degrees of pigmentation found in the skin, which is directly related to the number, size and distribution of melanosomes within the melanocytes. There are two types of melanin, the red/yellow phaeomelanin and the brown/black eumelanin. Variation in both skin and hair pigmentation is due to varied amounts of eumelanin and phaeomelanin. Eumelanin is thought to give protection against UV radiation. Individuals with red hair have a predominance of phaeomelanin in their hair and skin and have an impaired ability to produce eumelanin in response to UV light.

The relative proportions of eumelanin and phaeomelanin are controlled by the melanocyte-stimulating hormone (MSH) by binding to its receptor, melanocortin 1 receptor (MC1R) on the surface of melanocytes (Thody et al. 1991, Burchill et al. 1993, Hunt et al. 1995). MC1R controls the level of tyrosinase production therefore stimulating melanocytes to synthesise eumelanin. Only when the MSH binds to the MC1R does the receptor stimulate the melanocyte to increase the level of tyrosinase, causing the cell to produce eumelanin. In the absence of signalling, melanocytes make phaeomelanin (Burchill et al. 1993).

Variants in *MC1R* affect the pigmentation phenotype between phaeomelanin and eumelanin. Valverde et al. (1995) reported 21/30 (70%) of the individuals with red or fair hair and a poor tanning response analysed from their British and Irish population, had at least one variant of the MSH-receptor. In this paper, *MC1R* variants were even more abundant in those individuals with skin types 1 and 2 (see section 2.4.1 for skin type groups according to Fitzpatrick 1988).

1.4 Melanoma Growth Pattern

Initially invasive melanoma cells expand laterally across the superficial or papillary dermis. This step is called the radial growth phase and at this early stage, of unknown duration, surgical excision of the melanoma gives an excellent chance of a complete cure. However, if not excised, the melanoma progresses to the vertical growth phase, invading downward into the deeper dermis as clusters of tumour cells which can invade the lymph and blood vessels, greatly increasing the likelihood of metastases. Primary melanomas that have become raised indicate that downward growth or the vertical growth phase has occurred. In some cases, this growth pattern is very rapid. Removal of the primary melanoma before it penetrates to the deeper layers of the skin is therefore crucial for achieving a cure.

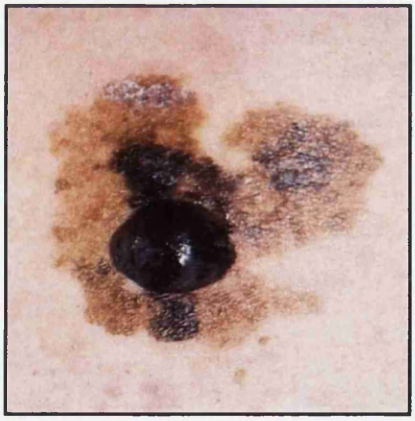
1.5 Types of Melanoma

Although there is some controversy, most pathologists classify melanoma into four major clinicopathologic types (lentigo maligna melanoma, superficial spreading melanoma, nodular melanoma, and acral lentiginous melanoma) (Clark et al. 1969).

1.5.1 Superficial Spreading Melanoma (SSM) (Figure 1.3) is the most common type of melanoma. It is usually flat, asymmetrical, unevenly coloured with varying shades of black and brown and grows laterally across the surface of the skin. SSM can have a relatively long radial growth phase occurring over months to years. It may occur at any age or site and is most common in Caucasians. The greatest increase for histogenetic type in Scotland over the past 20 years is in superficial spreading melanoma, which is the most common type of melanoma (MacKie et al. 2002).

FIGURE 1.3

Superficial Spreading Melanoma

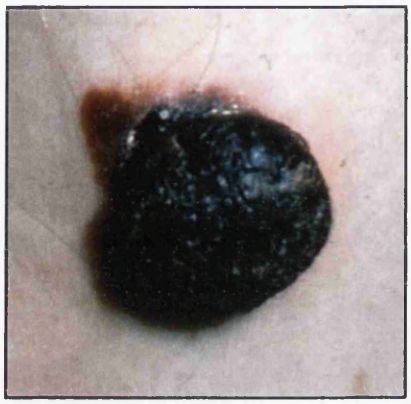


Large Superficial Spreading Melanoma with central raised nodule. Note the irregular edge and colour.

1.5.2 Nodular Melanoma (NM) (Figure 1.4) appears as a fast-growing brown or black raised nodule, although some lack melanin pigment. It is the second most common subtype of melanoma. NM can occur at any body site although the trunk appears to be more frequently involved than with other types of melanoma.

FIGURE 1.4

Nodular Melanoma

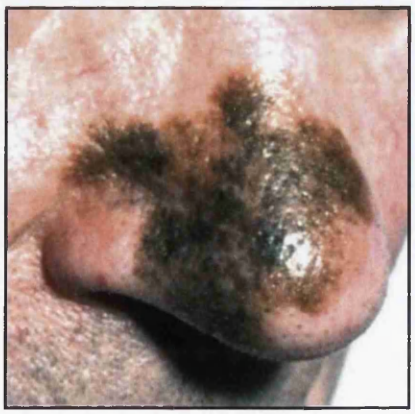


Note the black raised nodule.

1.5.3 Lentigo Maligna Melanoma (LMM) (Figure 1.5), sometimes called Hutchinson's freckle, usually occurs in the elderly. The lesions are large and flat with intermixed mottled areas of brown and black with irregular borders. These lesions most often appear on the face. The radial growth phase may last for years before invasion occurs.

FIGURE 1.5

Lentigo Maligna Melanoma

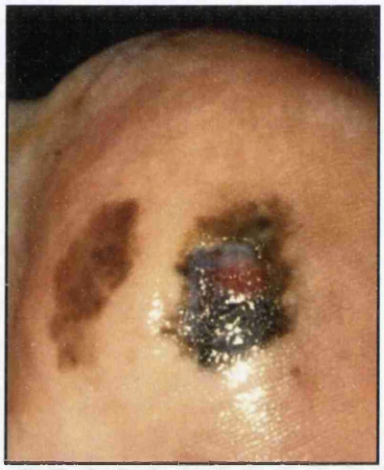


Extensive lentigo maligna on the nose.

1.5.4 Acral Lentiginous Melanoma (ALM) (Figure 1.6) is the least common form of melanoma in Caucasians, although it is the most common melanoma among African and Asian populations. It usually occurs as a dark patch on the palms, soles, or under the nails.

FIGURE 1.6

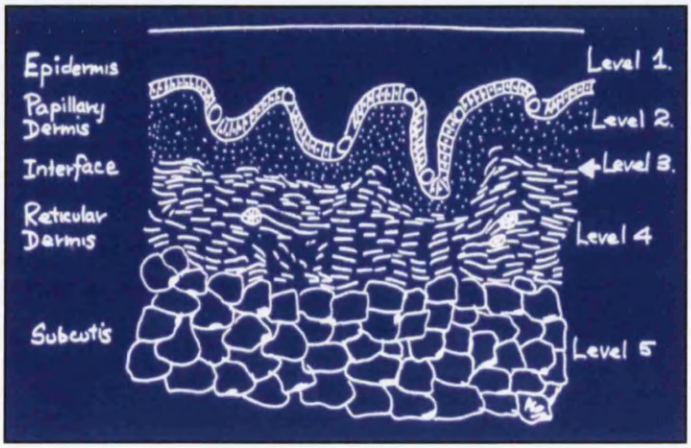
Acral Lentiginous Melanoma



The prognosis for a melanoma patient is mainly determined by the Breslow tumour thickness measured in millimetres (Breslow 1970) or by levels of invasion related to skin structures (Clark et al, 1969). All patients presented in this thesis have invasive melanoma of Clark level 2 or deeper. Clark level 2 is defined as melanoma cells that have extended into the papillary dermis (Figure 1.7).

FIGURE 1.7

Clark Levels



1.6 Body Site

Common sites for melanoma in men are the head, neck, and trunk, and in women, the arms or legs. The most common body site for melanoma in Scotland is the female leg. In Scottish male patients, the melanoma is most commonly located on the back (MacKie 2002). Any area of the skin may be affected however, in either gender.

1.7 Ultraviolet Radiation

Epidemiologic studies suggest that exposure to UV radiation is the major environmental risk factor for melanoma, but the association is complex. Melanoma occurs more frequently in areas of the body receiving intermittent sun exposure (Elwood and Jopson 1997).

The relationship between UV radiation and melanoma risk was investigated in Denmark using a population-based case-control study of 474 patients with melanoma and 926 population controls (Osterlind 1988). A significantly increased risk was associated with severe sunburn before the age of 15 years, indicating that exposure to intermittent intense sunlight is an important risk factor for melanoma.

The association of melanoma risk with various measures of intermittent and chronic exposures to UV radiation was evaluated by analysing 583 melanoma cases and 608 controls from Ontario, Canada (Walter 1999). Significant increased risk was identified from several measures of intermittent exposure, including beach holidays during adolescence and in the past 5 years and previous sunburn. Chronic exposure, measured by days of outdoor activity during adolescence and by outdoor activity during occupation in adult life, was associated with a significantly reduced risk. This work supports the hypothesis of increased risk associated with intermittent UV exposure. This suggests that UV radiation may mutate melanocytes at an early stage.

Gilchrest et al. 1999 provided a potential explanation for the epidemiology of melanoma. Melanocytes transfer melanin to surrounding keratinocytes through their dendrites. When UV radiation penetrates the basal layer of skin it interacts with the DNA. When the damage to the DNA is extensive, keratinocytes undergo apoptosis and are eliminated. When the damage to the DNA is minimal the keratinocytes either repair the damage or give

rise in the next round of DNA replication to a mutation induced by UV radiation. The skin responds to UV radiation with increased melanin production (tanning) and increases the capacity to repair DNA in the surviving cells. In the case of melanocytes, the amount of initial damage to DNA is also directly proportional to the dose of UV radiation, but the greater resistance to UV radiation-induced apoptosis leads to the survival of virtually all melanocytes. Melanocytes that are damaged extensively are therefore at high risk for incorporating mutations into their DNA, and clonal expansion of these melanocytes with UV induced mutations may follow during melanocyte division. In contrast to its effect on keratinocytes, intense high-dose UV radiation results in many more mutated melanocytes than does low-dose UV radiation. Furthermore Gilchrest suggests that while the skin is tanned it has an increased capacity to repair DNA. The increased capacity to repair DNA results in nearly complete repair, although cell division stimulated by UV radiation may result in clonal expansion of the cells that are already mutated.

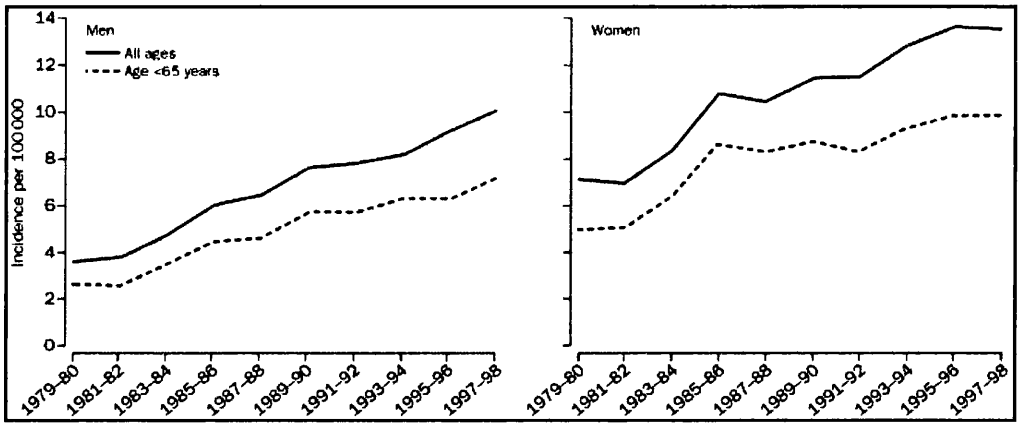
1.8 Changes in Incidence

Although a relatively uncommon cancer, melanoma has become an important public health issue because of the steady rise in incidence in Caucasian populations in all parts of the world including Australia, the U.S. and the U.K. over the past 70 years (Jemal et al. 2001; Marrett et al., 2001; Mackie et al., 2002). The increase in melanoma incidence in the U.S. is the greatest for any cancer. It is estimated that over the last 50 years, the incidence has risen by approximately 6% every year, leading to a 10-fold increase in incidence since the late 1950s (Weinstock 1998).

This increase in incidence is mainly attributed to greater intermittent periods of sun exposure over the last 30 years. The Scottish Melanoma Group recorded changes in melanoma incidence and survival in relation to prognostic factors. They investigated the incidence of melanoma in both sexes throughout Scotland during 1979-98 showing that there has been a steady and continuing rise in the incidence of melanoma in both sexes (Figure 1.8). They showed an incidence per 100,000 of population in 1998 of 10.6 in men and 13.1 in women (MacKie et al. 2002).

FIGURE 1.8

Incidence of Invasive Cutaneous Malignant Melanoma



Incidence of invasive cutaneous malignant melanoma in Scotland from 1979 to 1998. Every point is an average of 2 consecutive years.

Incidence rates and increases in incidence rates differ in different populations. There is an obvious difference in melanoma incidence between Australia (melanoma incidence in Queensland is 50/100,000) and Europe (melanoma incidence in UK is 10/100,000). Melanoma is the fourth most common cancer in Australia compared with the U.K. where it is the 11th most common cancer (World Health Organisation 2002, http://www-depdb.iarc.fr/globocan/GLOBOframe.htm). Even within Europe, there are differences between incidence rates. These differences in melanoma incidence rates may be relative to UV radiation exposure by geographic latitude.

1.9 Genetics of Familial Melanoma

A familial inheritance in malignant melanoma has been reported by many authors (Cawley 1952; Smith et al. 1966; Andrews 1968). Anderson et al. (1967) described 15 individuals with malignant melanoma in three generations of a pedigree. They showed an early age of onset and many had multiple primary melanomas. Patients have been reported from 36 pedigrees in which 106 members had cutaneous melanoma (Anderson 1971). Clark et al. (1978) first described kindreds with melanoma in one or more first-degree relatives who had a large number of melanocytic nevi some of which were atypical and variable in size. They termed these features the 'B-K mole syndrome' after the family names of two patients. At the same time, Lynch et al. (1978) reported similar families. Lynch et al. (1980) referred to the 'B-K mole syndrome' as FAMMM (familial atypical mole--malignant melanoma syndrome). They studied three pedigrees with FAMMM. Father-to-son transmission was observed and one patient had nine separate primary melanomas in 18 years.

In 1983 Trent et al. identified a deletion and translocation in the 6q15-q23 chromosome region in four of five cases of melanoma. Becher et al. (1983) also found chromosome aberrations of 6q (q11-q31 region) in a small number of families.

In 1989 attention moved to a locus on chromosome 1p36a, thought to be involved in melanoma. Bale and colleagues performed linkage analyses using 26 markers on the short arm of chromosome 1 on 99 relatives and 26 spouses from six families with a predisposition to melanoma. From the linkage analysis they found evidence that a familial melanoma gene maps to 1p36 (Bale et al. 1989). Dracopoli et al. (1989) found loss of heterozygosity at loci on 1p in 43% of melanomas and 52% of melanoma cell lines and they showed this loss of heterozygosity to be a late event in tumour progression. Kefford et al. (1991) assessed 119 family members, 30 of whom had cutaneous malignant melanoma, from eight Australian families and analysed the cosegregation of the cutaneous malignant melanoma with eight markers on the short arm of chromosome 1. No evidence for linkage was found. Goldstein et al. (1993) extended this linkage analysis work with the addition of seven new families. Their results showed significant evidence for a melanoma locus linked to marker D1S47.

Recently, Gillanders et al. (2003) have identified a novel familial melanoma susceptibility locus on chromosome 1p22. They performed a genome-wide scan for linkage in 49 Australian pedigrees containing three or more cases of melanoma, in which no cyclin-dependent kinase inhibitor-2A (*CDKN2A*) or cyclin-dependent kinase-4 (*CDK4*) mutations had been found. The highest LOD score was obtained at D1S2726, which maps to the short arm of chromosome 1 (1p22). Furthermore they analysed 33 additional melanoma families from around the world to provide further evidence for linkage to the 1p22 region. The highest LOD score of 6.43 was obtained at D1S2779. These data provide significant evidence of a novel susceptibility gene for melanoma located within chromosome band 1p22.

To map the locus more finely, Walker et al. (2004) looked for loss of heterozygosity (LOH) across the region in a panel of melanomas from 1p22-linked families, sporadic melanomas, and melanoma cell lines. Eighty percent of familial melanomas exhibited LOH making it likely that the susceptibility locus is a tumour suppressor. They screened the candidate genes in this region, but no coding mutations were detected. The Melanoma Genetics Consortium continues to search for the susceptibility gene for melanoma located within chromosome band 1p22.

1.10 The *CDKN2A* Gene

In 1986 Bergman et al. studied families from a village in the Leiden region, The Netherlands. In six pedigrees, 33 patients were reported with melanoma. In 1994 Bergman et al. extended their studies on their Dutch melanoma families and found by linkage analysis that a melanoma gene (*CDKN2A*) was linked to markers on chromosome 9p21. This familial melanoma gene is one of the three investigated in this thesis.

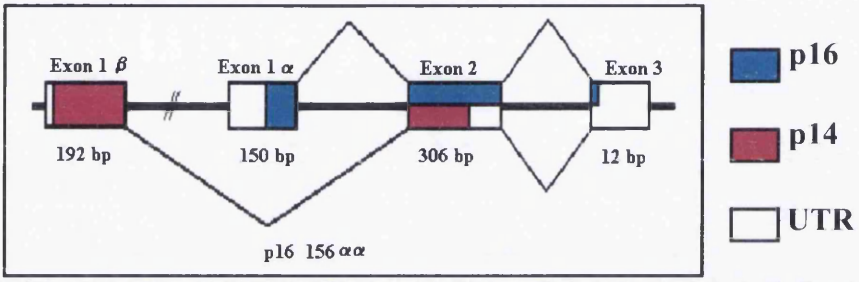
The *CDKN2A* locus was mapped to chromosome 9p21 (Hussussian et al. 1994; Kamb et al. 1994; Nobori et al. 1994) and its sequence as determined by Kamb et al. (1994) was identical to that of the sequence as cloned by Serrano et al. (1993).

The *CDKN2A* locus is unique in the genome, because it encodes two overlapping tumour suppressor genes, *CDKN2A* also known as *INK4A* and the alternative reading frame *ARF* (Stone et al. 1995) (Figure 1.9). These genes

encode two distinct cell cycle regulatory proteins, p16INK4A and p14ARF (Ruas and Peters 1998). The 16kDa protein p16INK4A is encoded by exons 1α, 2 and 3, comprising 156 amino acids. An alternative first exon (1β) is located approximately 22kbp upstream of the *CDKN2A* coding sequence. Transcripts initiating from exon 1β are spliced onto the common exon 2 and 3, giving rise to an alternative mRNA that encodes p14ARF in a different reading frame.

FIGURE 1.9

***CDKN2A* Locus**



The *CDKN2A* locus encodes two gene products involved in cell growth regulation. Transcription from exons 2 and 3 is shared by p16INK4A and p14ARF, in different reading frames. p16INK4A initiates within unique exon 1α and p14ARF within exon 1β, which is located approximately 20 kbp upstream of exon 1α.

Both of these proteins have a crucial role in cell death and apoptosis via the retinoblastoma pathway for p16INK4A and p53 pathway for p14ARF (Zhang et al. 1998). The p16INK4A protein acts as a tumour suppressor and a cell-cycle regulator that induces G1 cell-cycle arrest by binding to and inhibiting the kinase activities of the CDK4 (MIM# 123829)/cyclin D and CDK6 (MIM# 603368)/cyclin D complexes (Serrano et al. 1993). When CDK4 and cyclin D are in this complex they initiate the phosphorylation of the retinoblastoma protein, which permits cells to enter the DNA replication stage in the S phase of the cell cycle.

Mutations in the *CDK4* gene on chromosome 12, which encodes the cyclin-dependent kinase 4 protein that is normally inhibited by p16INK4A, have only been reported worldwide in three melanoma-prone kindreds (Zuo et al. 1996; Soufir et al. 1998). All the mutations occur in codon 24, with two families carrying an R24C mutation (Zuo et al. 1996) and another family with an R24H substitution (Soufir et al. 1998). This must mean that this residue interacts with

p16INK4A. p16INK4A binds to CDK4 at the arginine and so mutations at this residue will disrupt binding to CDK4 resulting in an activated CDK4. The activating mutations in *CDK4* are oncogenic, since this kinase negatively regulates the retinoblastoma protein by phosphorylation, causing it to release transcription factors.

The majority of melanomas are sporadic, but up to 5% of melanoma patients have one or more first-degree relatives also affected (Hayward, 2003). Genetic analysis of these melanoma families shows germline mutations of *CDKN2A* (MIM# 600160) in 20-30% of patients (Harland et al. 1997; Kefford et al. 1999; Hayward et al. 2000; Goldstein et al. 2001; Bishop et al. 2002). *CDKN2A* mutations are rare but highly penetrant and are transmitted in an autosomal dominant fashion. The estimated frequency of the mutated *CDKN2A* gene in the general population is 0.01% (Bishop et al. 2002). In a study of sporadic melanoma cases from Queensland, Australia, Aitken et al. (1999) estimated that 0.2% of all melanoma cases are caused by *CDKN2A* mutations.

The first germline mutation in *CDKN2A* was discovered in large melanoma pedigrees in 1994 by the Utah group. Hussussian et al. (1994) identified six probable disease-related mutations in the *CDKN2A* gene in families linked to 9p21. A variety of germline mutations within the coding sequences of exons 1α and 2 of *CDKN2A* have now been observed in affected members of more than 100 malignant melanoma families worldwide, including deletions, duplications and missense mutations (Harland et al.1997; Pollock et al. 1998; Ruas and Peters 1998), a selection of which will be discussed below. Mutations in exon 1α affect only the *CDKN2A* transcript, whereas those mutations in exon 2 can affect both *CDKN2A* and *ARF*.

The International Melanoma Genetics Consortium has established an online locus-specific variant database for familial melanoma called eMelanoBase which is accessible at www.wmi.usyd.edu.au:8080/melanoma.html (Fung et al. 2003). More recently, Murphy et al. (2004) introduced the *CDKN2A* Database, which is again an online database of germline and somatic variants of the *CDKN2A* tumour suppressor gene recorded in human disease, annotated with evolutionary, structural, and functional information. It is accessible at http://biodesktop.uvm.edu/perl/p16.

1.10.1 Commonly Detected CDKN2A Allelic Variants

R24P

In a patient with multiple primary melanomas, Monzon et al. (1998) identified an arginine 24 to proline mutation in the *CDKN2A* gene. This mutation had previously been reported in melanoma-prone families and was found to cosegregate with cases of melanoma (Holland et al. 1995). This mutation has also now been identified U.K. melanoma families (Harland et al. 1997; MacKie et al. 1998). Functional studies have shown the R24P mutant to behave normally in a CDK4 binding assay, despite cosegregation of this mutation with disease in nine melanoma families (Becker et al. 2001).

24bp Duplication

Pollock et al. (1998) identified two new melanoma kindreds that carried a duplication of a 24bp repeat present in the 5-prime region of the *CDKN2A* gene. This brought the total number of melanoma families described with this mutation to five, from three continents: Europe, North America, and Australasia. Previous families were reported by Goldstein et al. (1995), Walker et al. (1995), and Flores et al. (1997). This suggested to Pollock et al. (1998) that there had been at least three independent 24bp duplication events. The duplication was hypothesized to have arisen due to an unequal crossing-over between the two 24bp repeats naturally present in the wild-type sequence, possibly through polymerase slippage during replication. Further evidence that this repeat region is unstable and therefore prone to both meiotic and mitotic slippage was provided by the identification of a somatic 24bp deletion of one of these normally occurring repeats in a prostate tumour (Komiya et al., 1995).

Exon 1β Deletion

Randerson-Moor et al. (2001) described a family characterized by multiple melanoma and neural cell tumours segregating with a germline deletion of the *ARF*-specific exon 1β of *CDKN2A*. The deletion was approximately 14kb and did not affect the coding or minimal promoter sequences of either *CDKN2A* or *p14ARF*.

G67S

Holland et al. (1999) analyzed 131 Australian melanoma probands. They identified a glycine 67 to serine mutation. Glycine 67 is conserved, lying within the second ankyrin repeat and shares secondary structure alignment with glycine 101, a residue of proven significance to p16INK4A function. Glycine 67 is the first residue of loop 2 of p16INK4A (Byeon et al. 1998).

IVS2, A-G, -105

Harland et al. (2001) reported that affected individuals in six of 90 English melanoma pedigrees screened carried a transition (IVS2-105 A-G) deep in intron 2 of the *CDKN2A* gene. The mutation creates a false GT splice donor site 105 bases 5-prime of exon 3 and results in aberrant splicing of the mRNA. The authors proposed that this mutation and others similar to it might account for a significant proportion of 9p21-linked melanoma pedigrees with no detectable mutations in the coding region of *CDKN2A*.

R112G

Holland et al. (1999) analysed 131 Australian Melanoma Probands. They identified an arginine 112 to glycine mutation. Arginine 112, though not itself conserved in the INK4 family, is immediately flanked by several highly conserved residues. This mutation has also been observed as a somatic event in a sporadic metastatic melanoma (Platz et al. 1996a).

1.10.2 CDKN2A polymorphisms

In addition to mutations within the *CDKN2A* gene, the rare alleles in the 3' untranslated region of the gene, the C500G (Aitken et al. 1999) or C540T polymorphism (Kumar et al. 2001) have also been associated with risk of developing melanoma. Aitken et al. (1999) showed the frequency of the C500G polymorphism to increase with increasing familial risk and in those carrying mutations of *CDKN2A*. Kumar et al. (2001) analysed the C540T polymorphism and found the frequency to be significantly higher in the melanoma cases than in controls. Further studies are needed to confirm a possible association between the polymorphisms and melanoma risk.

1.10.3 CDKN2A mutations in Patients with Multiple Primary Melanomas

CDKN2A mutations have been detected in 9%-15% of patients with multiple primary melanomas and a family history of melanoma (Hayward, 2003). *CDKN2A* germline mutations have also been detected in 5/33 (15%) multiple primary melanoma cases irrespective of family history (Monzon et al., 1998) and in 2/17 (12%) multiple primary melanoma patients with a negative family history (MacKie et al., 1998).

1.10.4 CDKN2A AND UV Radiation

The exact sequence of the molecular changes in the melanocyte following UV induced damage is not yet understood. Pavey et al. (1999) reported increased levels of p16INK4A when human skin was exposed to UV radiation. The presence of a pathway involving p16INK4A, which can be stimulated by UV radiation, could provide a link between p16INK4A, UV radiation and melanoma. UV radiation causes both G1 and G2 phase checkpoint arrest in vitro cultured cells. Pavey et al. (2001) investigated the cell cycle responses to UV exposure on skin. Using skin cultures they demonstrated that melanocytes undergo a G2 phase cell cycle arrest for up to 48 hours following exposure and showed increased p16INK4A expression.

In addition Pavey et al. (2002) demonstrated that an increased expression of p16INK4A after exposure to UV radiation is influenced by α-MSH, a ligand for MC1R (page 35). This link between p16INK4A and MC1R may provide a molecular basis for the increased skin cancer risk associated with *MC1R* polymorphisms.

1.10.5 CDKN2A and Other Cancers

Somatic *CDKN2A* mutations have been reported in a number of different tumour types (Smith-Sorensen and Hovig 1996; Foulkes et al. 1997; Ruas and Peters 1998) and therefore it is possible that germline *CDKN2A* mutations could increase the risk of non-melanoma cancers in *CDKN2A* mutation-positive individuals. An increased risk of pancreatic cancer was first reported in melanoma families with *CDKN2A* mutations (Goldstein et al. 1995; Whelan et al. 1995).

Several other groups have since reported the occurrence of pancreatic cancer in mutation-positive melanoma families (Bergman et al. 1996; Ciotti et al. 1996; Soufir et al. 1998; Liu et al. 1999; Borg 2000; Lynch et al. 2002). The International Melanoma Genetics Consortium is currently collecting data on the risks of non-melanoma cancers in melanoma families carrying *CDKN2A* mutations.

1.11 Other Melanoma Susceptibility Genes

Familial melanoma is rare and the genes implicated in these melanoma families are likely to play a minor role in sporadic melanomas. Other high-penetrance melanoma predisposition genes are likely to exist and those that encode proteins involved in cell-cycle regulation will be strong candidates.

1.11.1 ARF

Melanoma segregates with chromosome 9p markers in a larger number of melanoma families than have been shown to carry mutations of *CDKN2A*. This high degree of linkage to 9p could be due to mutations affecting p14ARF, the product of the alternative transcript arising from the *CDKN2A* locus. Hewitt et al. (2002) described a melanoma family in which both mother and daughter carry a mutation in the last nucleotide of exon 1β. The substitution replaces a glycine with an arginine residue, which affects the splicing of the ARF mRNA, and leads to complete deletion of the mutant transcript.

An insertion of 16bp within exon 1β causing a frameshift in *ARF* but does not affect p16INK4A has been reported in a sporadic melanoma patient with multiple primary melanomas (Rizos et al. 2001).

1.11.2 MC1R

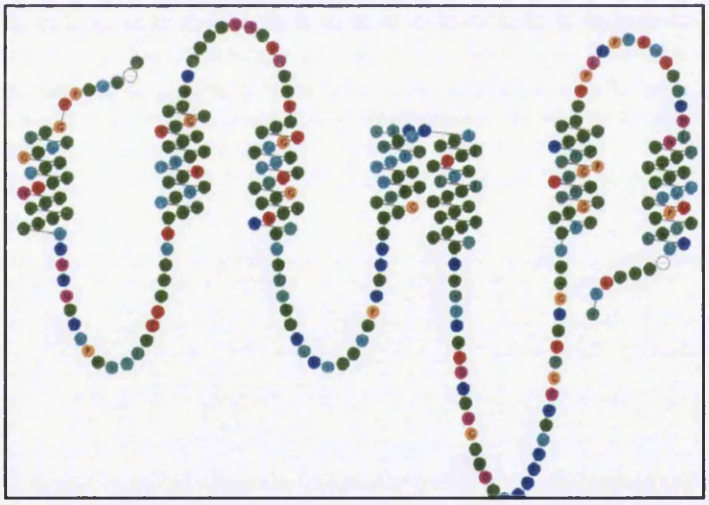
The suggestion of a low-penetrance susceptibility gene for sporadic melanoma was the association of variants of the *MC1R* gene with red hair and fair skin (Valverde et al. 1996).

The Melanocyte-stimulating hormone (MSH) regulates pigmentation by binding to its receptor Melanocortin 1 Receptor (MC1R) (MIM# 155555) as described previously. Chhajlani and Wikberg (1992) isolated the 317 amino acid

MC1R protein from human melanoma cells. The *MC1R* gene was mapped to chromosome 16q24.3 by fluorescence in situ hybridization (Gantz et al. 1994). This intronless gene encodes encodes a seven pass transmembrane G protein coupled receptor that controls melanogenesis (Figure 1.10). As described on pages 34-35, the relative proportions of pheomelanin and eumelanin are regulated by melanocyte stimulating hormone, which acts via its receptor (MC1R) on melanocytes, to increase the synthesis of eumelanin. Gene mutations that lead to a loss in function are associated with increased pheomelanin production, which leads to lighter skin and hair colour. Harding et al. (2000) have shown that there is extensive variation of the *MC1R* gene in European and Asian populations, but hardly any variation in African populations. This suggests the evolutionary conservation of the *MC1R* gene in Africa to retain eumelanin production.

FIGURE 1.10

The *MC1R* Gene



The *MC1R* gene has only one exon, which encodes a seven pass transmembrane G protein coupled receptor that controls melanogenesis.

Sequence variants in the *MC1R* gene were identified in over 80% of individuals with red hair and/or fair skin that tan poorly but in fewer than 20% of individuals with brown or black hair, and in less than 4% of those who showed a good tanning response (Valverde et al. 1995).

This indicated that MC1R is a control point in the regulation of pigmentation phenotype and that variations in this protein are associated with a

poor tanning response. From sequencing the entire *MC1R* gene from 30 unrelated British or Irish individuals with various degrees of red hair and 30 brown or black haired control subjects, nine sequence variants were identified. 53% of individuals with red hair were found to carry one variant allele and 29% had two variant *MC1R* alleles. No individual with any other hair colour was found to carry two variants. The majority of individuals (76.5%) with skin type 1 carried a *MC1R* variant, whereas no individuals with skin type 4 carried a variant. The frequencies were intermediate for individuals with skin types 2 (46.5%) and 3 (5%). 16 individuals carried the D294H variant, making it the most common variant identified. The variant was not present in the individuals with brown or black hair. D294 is in the seventh transmembrane domain and a mutation to D294H replaces an acidic residue with a basic one. Valverde also found that one of the most frequent mutations associated with red hair and a poor tanning capacity was V92M. Variant V92M and variants at codons 84 and 95, might be expected to alter the alpha-helix structure of the second transmembrane domain of the MSH receptor.

The link between *MC1R* and melanoma became apparent when Valverde et al. (1996) first reported certain variants of the M1CR gene to be more common in individuals with melanoma than in control subjects. They compared the *MC1R* genotype between 43 melanoma cases and 44 controls. They found that MC1R variants were significantly more common in cases than controls (P=0.0094). They found the D84E variant in 10/43 patients with melanoma with two individuals being homozygous for this substitution. The aspartate at codon 84 is highly conserved.

Following Valverde's two publications, there have been a number of publications looking both at *MC1R* variants in melanoma compared with healthy individuals, and also population studies looking at the association between *MC1R* variants and skin type and hair colour. This work has been carried out by Box et al, (1997); Ichii-Jones et al. (1998); Smith et al. (1998); Flanagan et al. (2000); Healy et al. (2000); Palmer et al. (2000); Kennedy et al. (2001).

Studies have also explored possible interactions between *CDKN2A* mutations and the *MC1R* variants by Box et al. (2001) in Australia and van der Velden et al. (2001) in The Netherlands.

There are a large number of *MC1R* variants and the relationship between *MC1R* variants and skin type and hair colour, as well as the relationship between *MC1R* variants and melanoma, are complex. Other genes responsible for pigmentation are likely to be involved as low-risk melanoma predisposition genes as well as modifier genes.

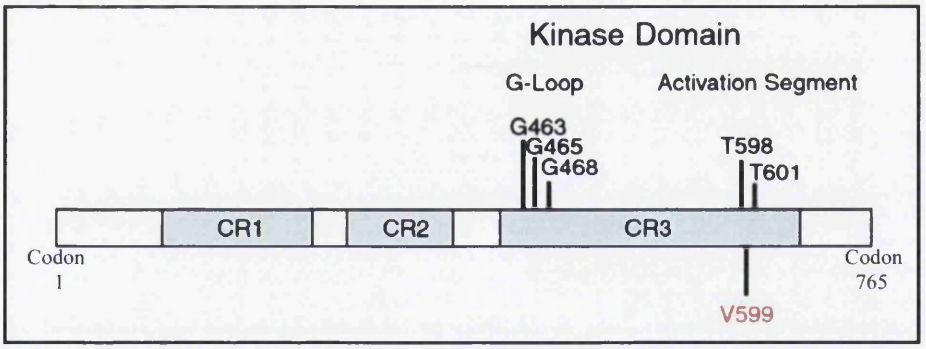
1.12 *BRAF*

In 1992 Eychene et al. identified two human *BRAF* loci by fluorescence in situ hybridization. The *BRAF* gene locus was mapped to chromosome 7q34 and encodes the functional gene product. *BRAF2*, located on Xq13, is an inactive pseudogene.

In 2002 Davies et al. reported for the first time *BRAF* (V-RAF Murine Sarcoma Viral Oncogene Homolog B1 (MIM# 164757) mutations in human melanoma. They reported a T to A transversion at nucleotide 1796 in exon 15 of the *BRAF* gene that leads to a valine-to-glutamic acid substitution at codon 599 (V599E) (Figure 1.11). This mutation accounted for 92% of the *BRAF* somatic mutations identified in the 20 of 34 (59%) human melanoma cell lines, short-term melanoma cultures, and 6/9 primary melanoma samples.

FIGURE 1.11

BRAF Structure



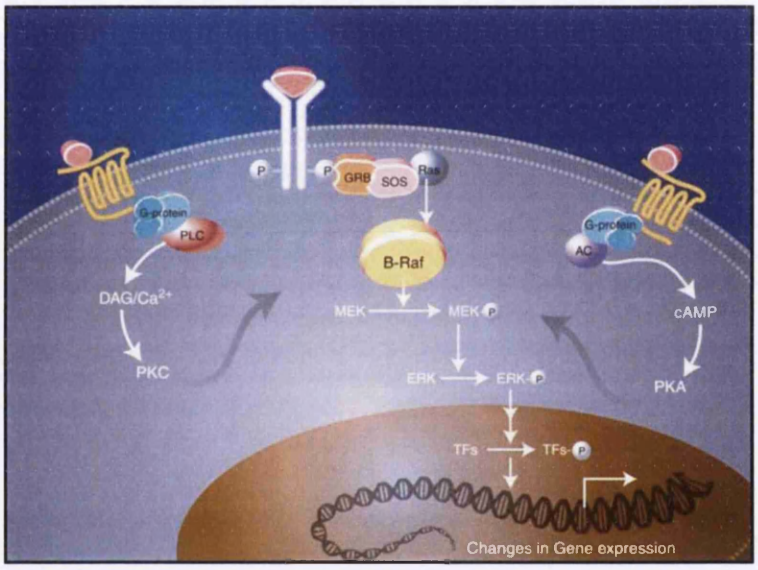
Schematic drawing of BRAF showing the location of the G-loop (exon 11) and activation segment (exon 15) of the kinase domain. Codon 599 is highlighted in red. The glycine residues that make up the backbone of the glycine loop and the phosphorylation sites in the activation segment are also shown.

No data was included either on cases of familial melanoma or on the prevalence of *BRAF* mutations in secondary melanoma tissue. We have therefore looked for exon 15 *BRAF* mutations in our material from these sources. We were particularly interested to see if exon 15 *BRAF* mutations were present in our families with no evidence of *CDKN2A* mutations.

The *BRAF* gene encodes a proto-oncogenic serine/threonine-protein kinase. Its normal function is to control proliferation and differentiation through the MAP kinase pathway (Figure 1.12).

FIGURE 1.12

Melanocyte Signal Transduction



BRAF functions downstream of Ras in the MAPK cascade. Signals from extracellular growth regulatory factors (pink) transduced through receptor tyrosine kinases (blue) or G protein-coupled receptors (yellow) result in activation of the MAPK pathway. This illustration was taken from Pollock and Meltzer (2002).

In the course of carrying out the studies reported in this thesis, further publications of *BRAF* in relation to melanoma have come from Meyer et al. (2003), Pollock et al. (2003), Edwards et al. (2004) and Wan et al. (2004). These are fully reviewed in Chapter 11, in relation to the findings in this thesis.

1.13 Aims of the Thesis

The aim of this study is to extend our knowledge of genetic factors in the development of melanoma. In this thesis I have investigated Scottish families with cutaneous malignant melanoma and also patients with sporadic melanoma for mutations in the *CDKN2A*, *MC1R* and *BRAF* genes using molecular genetic techniques. *CDKN2A* studies have been carried out in families with melanoma, and *MC1R* and *BRAF* variants investigated in familial melanoma, sporadic melanoma and phenotyped normal controls.

CHAPTER 2

DETAILS OF MELANOMA PATIENTS STUDIED AND CONTROL SUBJECTS INCLUDING PHENOTYPIC CHARACTERISTICS

2.1 Subjects

DNA for this study was extracted from peripheral blood and from tumour tissue of patients with melanoma, both with and without a family history of melanoma, from relatives in the case of familial melanoma, and from an appropriate control population. Ethical committee permission (reference number LREC 00/161(2)) was obtained to obtain peripheral blood and/or tumour tissue from all these groups and written informed consent given by all participants. To protect individual identity, no dates of birth are presented. All individuals have given written permission for the publication of their family trees.

2.1.1 Melanoma Families

Index cases of melanoma families were referred to the West of Scotland melanoma clinic, Department of Dermatology in the Western Infirmary, Glasgow, U.K., and family histories of other affected members confirmed by examination of the pathology slides from the primary tumour of the second and subsequent family members by Prof. MacKie. All melanoma patients in the study have primary cutaneous melanoma invasive to Clark level 2 or deeper (Clark et al. 1969). It was decided not to include patients who only had *in situ* melanoma because of the lack of concordance among pathologists in the discrimination between *in situ* melanoma and severely atypical nevi. While this may have excluded some families, it ensures that all families studied do indeed have potentially fatal melanoma.

Where possible, second and subsequent melanoma patients in affected families, and also unaffected family members have been personally examined by Prof. MacKie to establish information on the family pedigree, hair and eye colour and skin type with regard to sun exposure, sun bed use, and of other reported non melanoma cancers within the family. Since 1985 to date peripheral blood DNA has been collected from 48 melanoma families comprising 78 patients with melanoma and 28 currently unaffected family members. Forty-six families originated in the West of Scotland and two in the North East Grampian region who were referred by Dr M Nicholson of Aberdeen. Tumour DNA has also been obtained from secondary melanoma samples from two of these patients. In addition, DNA from five melanoma families from Toronto, four

from Brisbane and three from Boston (25 individuals) were made available for the M53I founder mutation study.

2.1.2 Sporadic Melanoma Cases

As with familial cases, all patients were referred to the West of Scotland melanoma clinic. A family history of melanoma was excluded in each case. Information on skin type, eye and hair colour with regard to sun exposure and sun bed use was collected as previously described. DNA was extracted from the peripheral blood of 28 patients with sporadic melanoma for the *MC1R* study. Peripheral blood DNA was also collected from eight patients with sporadic melanoma with three or more multiple primary melanomas for the *CDKN2A* study.

As with familial cases, all patients have invasive melanoma (Clark level 2 or deeper). Thick primary melanomas were used for the DNA extraction from paraffin-processed tissue. Fifty-two sections each 10μM thick were cut from the block containing the greatest density of melanoma tissue. DNA extracted from these sections was then screened for mutations in exon 15 of the *BRAF* gene.

DNA from patients with metastatic melanoma was obtained from frozen tumour tissue. In 22 cases this was from lymph nodes containing large melanoma metastases, and DNA was then extracted from the area in which nodal tissue was most obviously replaced by secondary melanoma. One of these patients gave two samples to the study because of two surgical procedures several months apart. A further patient had large soft tissue melanoma metastases and DNA was extracted both from two tumour deposits and also the peripheral blood.

All melanoma patients, both sporadic and familial, answered a detailed questionnaire at diagnosis. This included:

- Place of birth
- Whether or not childhood was spent in a high solar environment
- Occupational details, decade by decade, with regard to geographical location and whether their occupation was indoors or outdoors

- History of more than one sunny holiday per year
- History of any severe blistering or peeled sunburn
- Use of sunbeds

2.1.3 Control subjects

Individuals attending a local minor injury clinic were invited to contribute peripheral blood DNA to the study. They were from the same geographical area as the melanoma patients, and were matched for age and ethnic group. Past sun exposure and sun bed use, hair and eye colour was recorded, and skin type with regard to sun exposure was recorded. There is no need to believe that this population have any greater or lesser risk of melanoma than the general population.

2.2 Samples used in this study

2.2.1 Venous Blood Samples

Blood was collected by venous puncture into a 10ml EDTA coated vaccutainer or specimen tube (TEKLAB). Samples were routinely kept at room temperature for up to three hours then transferred to 4°C until extraction. Extraction was routinely carried out within 48 hours.

2.2.2 Paraffin Embedded Tissue Samples

Samples were obtained from the Department of Pathology at Glasgow Royal Infirmary. Routinely 10X 10μM sections of formalin fixed paraffin-embedded melanoma tissue were used for extraction. Samples were selected from thick melanomas to obtain adequate volumes of tumour tissue.

2.2.3 Fresh Tissue Samples

Frozen material was available from patients undergoing lymph node dissection for clinically enlarged lymph nodes or from patients having subcutaneous metastases excised at Gartnavel General Hospital. In every case the metastatic melanoma was confirmed by pathological examination. All samples were frozen and stored at -70°C.

2.3 Numbering of Families

The 48 families were numbered F1-F48. Where a sample from a family member was available, the family member was numbered. For example in family F11 there were three family member's samples available and therefore the family members were numbered F11.1, F11.2 and F11.3. A diagnosis of familial melanoma was made when two or more family members had a pathological confirmed diagnosis of invasive melanoma to Clark level 2 or deeper.

Of the 48 families in total, there are 106 family members with melanoma, 66 females and 40 males. As of June 2004, one family F19 has five affected members, one family F2 has four affected members, five families F1, F8, F20, F21 and F29 have three affected members, and the remaining 41 families have two affected members.

Thirteen DNA samples were available from familial melanoma patients with multiple primary melanomas, 9/66 (13.6%) females, and 4/40 (10%) males. In families F19, F30 and F31 there are a total of five cases of ovarian cancer, in families F11 and F21 there are a total of two cases of pancreatic cancer, in family F11 there is a case of breast cancer, in family F14 there is a case of lymphoma and in family F2 there is a case of oesophageal cancer. Details of currently affected family members, gender, age at diagnosis and other malignancies are presented in table 2.1.

Available samples were collected for this study from 106 members of these families of which 78 were affected members and 28 were currently unaffected members.

TABLE 2.1

Details of Scottish Families with Two or More Members with Invasive Melanoma

Scottish Family Number	Family Members with Melanoma	Age at Diagnosis (years), Sex M/F	Other Malignancies in the Pedigree
1	3	F48, F37*, F?	-
2	4	F39, M37, M31, M19	oesophagus
3	2	F55, F33*	-
4	2	M46*, F46*	-
5	2	M36*, F27	-
6	2	F34*, F21	-
7	2	F45, M43	-
8	3	F41, F23, M16	-
9	2	M56, F21	-
10	2	M53, F45	-
11	2	M63, F33*	breast, pancreatic
12	2	M69, F37	-
13	2	M34, M36	-
14	2	F68, F31	lymphoma
15	2	F61, M48	-
16	2	M34, F16	-
17	2	M34, M36	-
18	2	F42, F49	-
19	5	F66, F33*, F49*, M40, F40	ovarian
20	2	M38, M51	-
21	3	M34, F35, M46	pancreatic
22	3	F38, M33, M29	-
23	2	F48, F53	-
24	2	F39, F41	-
25	2	M45, F50	-
26	2	M70, M49	-
27	2	M43, F61	-
28	2	M34*, M37	-
29	3	M27, F50, M62	-
30	2	F20, F24	ovarian
31	2	F43, M61	ovarian
32	2	F38, F76	-
33	2	F25*, F28*	-
34	2	M23, M69	-
35	2	F44, F56	-
36	2	M43, F36	-
37	2	F24, F62	-
38	2	F43, F36	-
39	2	M27, F23	-
40	2	F27, F58	-
41	2	F18, F59	-
42	2	F39*, F62	-
43	2	F26*, M51*	-
44	2	F29, M66	-
45	2	F48, F21	-
46	2	F71, F79	-
47	2	F33, M?	-
48	2	F?, F?	-

* denotes a patient with multiple primary melanomas.

? denotes age unknown.

- denotes no other malignancy.

2.4 Phenotyping of Patient and Control Cohort

Sporadic cases are numbered S1-S28 and control subjects are numbered C1-C68.

2.4.1 Phenotype Grouping of Patients and Control Subjects for MC1R Study

Patients were asked to identify their skin type with regard to sun exposure as one of four groups according to Fitzpatrick (1988):

1 = never tans, always burns

2 = burns easily, tans rarely

3 = burns rarely, tans easily

4 = never burns, always tans

Eye colour was assessed into one of four categories at clinical examination by Prof. R. MacKie:

1 = blue

2 = green

3 = brown

4 = other

Patients were asked to classify their hair colour at 20 years of age into one of four groups:

1 = red

2 = blonde

3 = light brown

4 = dark brown/black

The phenotypic characteristics of all patients with melanoma, unaffected family members and of control subjects are summarised in tables 2.2, 2.3 and 2.4 respectively.

TABLE 2.2

Phenotypes of Familial Melanoma Patients & their Unaffected Relatives

Identifier	Skin Type	Eye Colour	Hair Colour
F1.1	1	1	2
F1.2	1	1	2
F1.3	2	1	3
F2.1	2	1	4
F2.2	2	1	3
F2.3	2	1	3
F2.4	2	1	2
F2.5	2	1	3
F2.6	2	4	2
F2.7	2	1	4
F2.8	2	1	3
F3.1	2	4	3
F3.2	2	1	3
F3.3	2	4	3
F4.1	2	4	3
F4.2	2	1	3
F4.3	2	1	3
F5.1	2	3	4
F5.2	2	4	4
F6.1	1	1	1
F6.2	1	1	1
F6.3	2	1	2
F6.4	2	1	2
F6.5	1	4	1
F7.1	1	4	1
F7.2	2	1	2
F8.1	2	3	4
F8.2	2	1	3
F9.1	1	1	1
F10.1	2	1	3
F10.2	2	1	2
F10.3	2	1	3
F11.1	1-2	1	1
F11.2	2	1	4
F11.3	2	4	2
F12.1	1	1	1
F13.1	3	3	4
F13.2	2	3	4
F13.3	2	4	2
F13.4	3	3	4
F14.1	2	1	3
F14.2	2	1	4

Identifier	Skin Type	Eye Colour	Hair Colour
F15.1	2	1	2
F15.2	2	1	2
F16.1	1	1	2
F17.1	2	1	2
F17.2	2	1	2
F17.3	1	4	1
F17.4	2	1	2
F17.5	2	1	2
F18.1	2	1	3
F18.2	2	1	3
F19.1	1	1	4
F20.1	3	4	4
F21.1	2	1	4
F21.2	2	3	4
F22.1	1	1	3
F22.2	1	2	1
F22.3	2	1	4
F22.4	2	2	3
F22.5	2	1	3
F22.6	2	2	3
F23.1	2	1	2
F23.2	2	4	2
F23.3	2	1	2
F23.4	2	1	3
F24.1	2	1	3
F24.2	2	1	3
F25.1	2	4	4
F25.2	1	2	2
F26.1	2	1	3
F26.2			
F27.1	1	1	1
F27.2	1	2	1
F28.1	2	1	3
F28.2	2	1	3
F29.1	2	1	3
F29.2	2	4	2
F30.1			
F30.2			
F31.1	1	1	1
F32.1	2	1	4
F32.2	1	1	3
F33.1	2	1	2
F33.2	2	1	2
F34.1	2	2	3
F35.1	2	2	3
F35.2	2	1	2

Identifier	Skin Type	Eye Colour	Hair Colour
F36.1	2	1	2
F37.1	2	1	3
F37.2	2	1	3
F38.1	2	1	3
F38.2	2	1	4
F39.1	1	1	2
F40.1	1	1	1
F41.1	2	3	4
F41.2			
F42.1	2	3	4
F42.2			
F43.1	3	1	4
F43.2	1	1	3
F44.1	2	1	3
F45.1	2	3	3
F46.1	2	1	2
F47.1	1	1	1
F48.1	2	1	3

TABLE 2.3

Phenotypes of Sporadic Melanoma Patients

Identifier	Skin Type	Eye Colour	Hair Colour
S1			
S2	1	1	1
S3	2	2	3
S4	1	1	1
S5	1	2	1
S6			
S7	2	1	3
S8	2	1	3
S9	2	1	2
S10	2	1	3
S11	2	2	1
S12	1	1	2
S13	3	1	1
S14	2	1	3
S15	2	1	2
S16	2	1	3
S17	3	1	2
S18	2	1	1
S19	3	1	2
S20	2	1	2
S21	2	1	2
S22	2	3	3
S23	2	1	2
S24	1	3	3
S25	2	1	3
S26	1	1	2
S27	2	1	3
S28	3	3	3

TABLE 2.4

Phenotypes of Control Subjects

Identifier	Skin Type	Eye Colour	Hair Colour
C1	2	4	4
C2	2	2	2
C3	3	3	4
C4	2	1	2
C5	2	1	4
C6	2	2	1
C7	2	1	4
C8	3	3	4
C9	3	3	4
C10	3	3	4
C11	2	1	2
C12	1	4	1
C13	2	4	4
C14	3	1	3
C15	2	1	3
C16	2	4	4
C17	2	1	3
C18	3	1	3
C19	2	1	2
C20	3	1	3
C21	3	1	2
C22	2	1	4
C23	1	1	1
C24	3	2	3
C25	3	1	2
C26	2	1	2
C27	1	1	2
C28	3	1	2
C29	3	3	4
C30	3	3	4
C31	2	1	2
C32	3	3	2
C33	3	3	4
C34	2	1	2
C35	2	1	3
C36	2	1	3
C37	2	1	2
C38	2	1	4
C39	1	2	3
C40	2	4	3
C41	3	1	4

Identifier	Skin Type	Eye Colour	Hair Colour
C42	1	3	1
C43	1	1	2
C44	2	3	2
C45	3	4	4
C46	2	1	2
C47	3	3	3
C48	1	1	1
C49	3	3	4
C50	1	1	1
C51	1	1	2
C52	3	3	3
C53	2	1	3
C54	3	3	4
C55	2	1	2
C56	3	1	2
C57	1	2	1
C58	3	3	4
C59	2	3	3
C60	2	1	3
C61	2	2	4
C62	3	1	2
C63	3	3	4
C64	1	1	2
C65	4	3	4
C66	2	1	2
C67	1	1	2
C68	4	1	2

2.5 Correlation between Phenotype and Melanoma

This section describes the phenotypic characteristics of melanoma patients and control subjects, aimed at confirming that the characteristics of both patients and control subjects were reasonably representative by comparing with large epidemiological studies (Palmer et al. 2000; Box et al. 2001; discussed in section 10.1).

2.5.1 Statistical Analysis

Statistical analysis was performed using the methods described in method 3.17. Due to the large quantity of results, all findings are reported in tabular form in Appendix 5. Significant results are only presented in this chapter.

2.5.2 Correlation between Skin Type and Melanoma

Among the four subsets of skin type, the most frequent phenotype observed in the melanoma patients is to burn easily and tan rarely 60/88 (68.2%) and also in 29/68 (42.7%) control subjects. Only 7/88 (7.9%) cases compared with 25/68 (36.8%) control subjects have skin type 3. A 2 × 4 table was constructed to include the four skin phenotypes described in section 2.4.1. A chi-squared test was performed to compare melanoma cases, both familial (60 cases) and sporadic melanoma patients (28 cases), with 68 control subjects. Familial and sporadic melanoma patients were pooled together as patient numbers are small. One of the potential problems of investigating both familial and sporadic patients together is that the genetic background of the different groups cannot be easily pooled together, because both specific mutation and genes involved may differ between the two groups. The correlations discussed in these sections do not investigate genetics status and therefore the error rate in pooling these two groups is thought to be very small.

Table 2.5 shows the very highly significant difference in skin types between melanoma patients, both familial and sporadic, and control subjects ($P < 0.001$). As expected, skin types 1 and 2 are significantly more common in patients than control subjects, compared with skin types 3 and 4, which are significantly more common in control subjects than in patients.

TABLE 2.5

Skin Types in Familial and Sporadic Melanoma Patients Compared with Control Subjects

	1 Never Tans Always Burns	2 Burns Easily Tans Rarely	3 Burns Rarely Tans Easily	4 Never Burns Always Tans
No. of Cases	21	60	7	0
No. of Control Subjects	12	29	25	2
P-value < 0.001 *** significant				

Skin type can be thought to have a natural scale going from type 1 to 2 to 3 to 4 and therefore to determine exactly which end of the skin type scale shows significance, similar skin types are grouped together, skin types 1 and 2 are grouped together and skin types 3 and 4 are grouped together, and compared against all melanoma patients, both familial and sporadic, and the control subjects by performing a two-tailed Fisher's exact test. The following table 2.6 shows the very highly significant difference between skin types 1 and 2 compared with skin types 3 and 4 in melanoma patients, both familial and sporadic, compared with control subjects. 81/88 (92%) cases have skin type 1 or 2 compared with 41/68 (60.3%) control subjects ($P < 0.001$; OR = 7.620; CI 3.060-18.974). These results confirm that skin types 1 and 2 are significantly seen more in patients than control subjects, compared with skin types 3 and 4, which are significantly seen more in control subjects than patients.

TABLE 2.6

Correlation between Skin Type 1 or 2 and Skin Type 3 or 4 and Melanoma Patients (Familial and Sporadic) and Control Subjects

	No. of Cases	No. of Control Subjects
Skin Type 1 or 2	81	41
Skin Type 3 or 4	7	27
P-value < 0.001 *** significant		
OR = 7.620, CI 3.060-18.974		

2.5.3 Correlation between Eye Colour and Melanoma

The most frequent eye colour observed in the melanoma patients is blue 63/88 (71.6%). Blue is also the most common eye colour in control subjects 38/68 (55.9%). 10/88 (11.4%) cases have brown eyes compared with 18/68 (26.5%) control subjects. A 2 × 4 table was constructed to include the four eye phenotypes described in section 2.4.1. A chi-squared test was performed to compare melanoma cases, both familial and sporadic melanoma patients together, with the control subjects. Table 2.7 shows the nearly significant observation (P = 0.095) in all melanoma patients, both familial and sporadic, compared to the control subjects.

TABLE 2.7

Correlation between Eye Colour and Melanoma Patients (Familial and Sporadic) and Control Subjects

	Blue	Green	Brown	Other
No. of Cases	63	7	10	8
No. of Control Subjects	38	6	18	6
P-value = 0.095 (not significant)				

To further consider if there is an association between an individual having a particular eye colour and having melanoma, a two-tailed Fisher's exact test was performed on cases and on control subjects for each eye colour against all other eye colours (Appendix 5, Table 9). The following table 2.8 shows the significant observations in all melanoma patients, both familial and sporadic, compared to the control subjects. Among the four different eye colours both blue and brown eyes returned a significant finding. 63/88 (71.6%) cases have blue eyes compared with 38/68 (55.9%) control subjects (P = 0.045; OR = 1.989; CI 1.022-3.873). Only 10/88 (11.4%) cases have brown eyes compared with 18/68 (26.5%) control subjects (P = 0.020; OR = 2.808; CI 1.199-6.574).

TABLE 2.8

Eye Colour in Melanoma Patients (Familial and Sporadic) Compared with Control Subjects

	No. of Cases	No. of Control Subjects
Blue Eyes	63	38
Other Eye Colour	25	30
P-value = 0.045 * significant OR = 1.989, CI 1.022-3.873		
Brown Eyes	10	18
Other Eye Colour	78	50
P-value = 0.020 * significant OR = 0.356, CI 0.152-0.832		

2.5.4 Correlation between Hair Colour and Melanoma

The most frequent hair colour observed in the melanoma patients is light brown 32/88 (36.4%) compared with 15/68 (22.1%) control subjects. The most frequent hair colour observed in control subjects is blonde 24/68 (35.3%) compared with 24/88 (27.3%) cases. Sixteen out of 88 (18.2%) melanoma patients have red hair compared with 7/68 (10.3%) control subjects. A 2 × 4 table was constructed to include the four hair phenotypes described in section 2.4.1. A chi-squared test of association was performed on melanoma cases, both familial and sporadic melanoma patients together and on control subjects. The following table 2.9 shows the significant observation (P = 0.042) in all melanoma patients, both familial and sporadic, and in the control subjects.

TABLE 2.9

Hair Colour in Melanoma Patients (Familial and Sporadic) Compared with Control Subjects

	Red	Blonde	Light Brown	Dark Brown/Black
No. of Cases	16	24	32	16
No. of Control Subjects	7	24	15	22

P-value = 0.042 * significant

To further investigate if there is a relationship between an individual having a particular hair colour and having melanoma, a two-tailed Fisher's exact test was performed on cases and on control subjects for each hair colour (Appendix 5, Table 8). The following table 2.10 shows the almost significant observations in all melanoma patients, both familial and sporadic, and in the control subjects. Among the four different hair colours only light brown hair and dark brown or black hair returned a near significant result. 32/88 (36.4%) cases have light brown hair compared with 15/68 (22.1%) control subjects (P = 0.056; OR = 2.019; CI 0.984-4.145). 16/88 (18.2%) cases have dark brown or black hair compared with 22/68 (32.4%) control subjects (P = 0.059; OR = 2.152; CI 1.024-4.523).

TABLE 2.10

Correlation between Hair Colour and Melanoma Patients (Familial and Sporadic) and Control Subjects

	No. of Cases	No. of Control Subjects
Light Brown Hair	32	15
Other Hair Colour	56	53
P-value = 0.056 (not significant) OR = 2.019, CI 0.984-4.145		
Dark Brown/Black Hair	16	22
Other Hair Colour	72	46
P-value = 0.059 (not significant) OR = 0.465, CI 0.221-0.976		

The phenotyping carried out for the work in this thesis can reassure the reader that the melanoma population presented in this thesis is not atypical with regard to skin type, eye or hair colour.

CHAPTER 3

LABORATORY METHODS

3.1 Extraction of Genomic DNA

3.1.1 DNA Extraction from Venous Blood

DNA was extracted using the Whatman BioScience Genomic DNA Purification System (England) according to the manufacturer's recommendations. The DNA was purified in approximately 30 minutes. Routinely 300μg of DNA per 10ml of blood was extracted with a concentration of 200ng/μl. The sample was stored at 4°C in a 2ml screw-cap tube (Sarstedt) for one month to allow complete dissolving of the sample. 10μl of each sample was loaded onto a 0.7% agarose gel containing ethidium bromide to estimate the amount of DNA of each sample. Samples were then transferred to -20°C for long-term storage.

3.1.2 DNA Extraction from Paraffin Embedded Tissue

Paraffin blocks of thick primary melanomas were selected from the files. These blocks were trimmed to remove as far as possible surrounding normal non melanoma tissue and 10 x 10μM sections were cut from the primary melanoma sample. Dissected tissue was deparaffinized in 1200μl of Histo-Clear (National Diagnostics) and vortexed vigorously using a rotamixer (Hook & Tucker Instruments) in a 1.5ml microcentrifuge tube. This was followed by centrifugation using a Micro Centaur (Scotlab) at 5,000 x g for 5 minutes at room temperature. The supernatant was removed by pipetting. 1200μl of 100% ethanol was added to the pellet and the sample vortexed at a low speed until the pellet was resuspended. This step was to ensure the removal of residual Histo-Clear. The sample was centrifuged at 5,000 x g for 5 minutes at room temperature to pellet the tissue and the ethanol was removed by pipetting. This step was repeated three times followed by incubation of the open microcentrifuge tube at 37°C for 15 minutes to evaporate residual ethanol. The tissue pellet was resuspended in 180μl of Buffer ATL from the QIAGEN QIAamp DNA Mini kit (England). 20μl of Proteinase K was added to the sample, mixed by vortexing and incubated at 56°C overnight until lysis had taken place. DNA was extracted using the QIAGEN QIAamp DNA Mini kit according to the manufacture's instructions.

3.1.3 DNA Extraction from Frozen Tissue

DNA was extracted from frozen tissue using the above method with the QIAGEN QIAamp DNA Mini kit.

3.1.4 DNA extraction from Bacterial Colonies

DNA was extracted from the cloning experiments using the QIAGEN QIAprep Spin Miniprep Kit according to the manufacture's instructions.

3.2 Storage of DNA

Two separate aliquots of extracted DNA were stored:

1) Stored at 4°C for at least 24 hours to allow complete dissolving of DNA. This was used as the working aliquot.

2) Stored separately at –20°C as a backup sample kept in case of contamination of one tube and also to avoid continual freeze thawing.

3.3 Determination of Concentration of DNA

The concentration of DNA was measured by absorbance at 260nm using the GeneQuant II spectrophotometer (Pharmacia Biotech) according to the manufacturer's instructions. 1ml of MqH_2O was aliquoted into a 10mm Silica cuvette (Amersham Pharmacia Biotech, Cambridge) to zero the spectrometer. Readings were taken at wavelength 260 nm (OD_{260}). The spectrometer was rezeroed between each wavelength reading. All DNA used has an absorbance reading at 260nm between the range of 1.5-2. An OD of 1 corresponded to approximately 50μg /ml for double stranded DNA.

3.4 The Polymerase Chain Reaction

3.4.1 Primer Design

Individual primers and primer pairs (Appendix 1) were designed with the aid of the Primer3 web site (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). Several variables were taken into account when designing primers. All oligonucleotides were designed to have a length between 18 and 24 bases in order for them to be sequence specific. When designing the primer pairs, both of the oligonucleotide primers were designed such that they had similar melting temperatures to allow for efficient amplification and rule out mis-priming at lower temperatures. The melting temperatures of the oligos were calculated using Primer3 and also by the Wallace formula:

$$T_m = 2(A+T) + 4(G+C)$$

The lowest melting temperature of each primer pair was then used for choosing the annealing temperature for PCR. Optimisation of PCR usually began with an annealing temperature 5°C lower than the melting temperature.

Primers were designed with no primer homology as partial homology in the middle regions of two primers could interfere with hybridization and no homology at the 3' end of either primer, as primer dimer formation would occur which could prevent the formation of the desired product via competition. The base composition of primers was designed to be between 45% and 55% GC with no polyG or polyC stretches as these could promote non-specific annealing. PolyA and PolyT stretches were also avoided as these would open up stretches of the primer-template complex, lowering the efficiency of amplification. Where possible a G or C residue at the 3' end of primers was included to help correct binding at the 3' end due to the stronger hydrogen bonding of G/C residues.

3.4.2 Primer Synthesis

All primer sequences were synthesised commercially (Sigma-Genosys). All primers were desalted, deprotected and at 0.03μM except the forward haplotyping primers which were at 0.05μM and 5′-end FAM fluorescently labelled.

3.4.3 Resuspension of Primers

Dried primers were resuspended in 1ml of sterile MqH_2O. Once suspended, primers stocks were stored at -20°C. A 5μM working stock of each primer was kept separately at -20°C. However, to minimize degradation due to freeze/thaw, aliquots were sometimes kept at 4°C for immediate, short term use.

3.4.4 PCR Mastermix

All PCR amplifications were carried out using the same mastermix made fresh each time. Each 25μl mastermix comprised the following reagents added to a 0.2ml sterile thin-walled tube with flat cap (ABgene). The master reaction mix was scaled up to provide sufficient quantities for the number of tubes required, plus a further tube was calculated for to allow for pippetting errors:

19μl MegaMix~W (Microzone Ltd, UK)
2.5μl Forward Primer (5μM)
2.5μl Reverse Primer (5μM)
24μl Total

3.4.5 Amplification

Routinely 1μl of genomic DNA or 2μl of tissue DNA (approximately 100ng/μl) was used as a template. 24μl of master mix was aliquoted into tubes on ice and DNA added to each tube. Mixing was by pipette. When multipore plates were used, the master reaction mix was aliquoted out first and then the appropriate DNA template added and mixed by swirling the pipette tip in the mixture, no pipette pumping action was used to reduce aerosol-contamination. The cycling parameters were determined for each primer pair (Appendix 2). Amplifications were performed using a Primus 96 Plus (MWG Biotech) and stored at 4°C.

5μl of PCR product was analysed by agarose gel electrophoresis for product amplification and size. When amplifying DNA from paraffin embedded tissue, the yield from a single PCR was insufficient to visualise in a gel, therefore a 2μl aliquot was used as a template for a second round of PCR amplification.

3.5 Gel Electrophoresis

Size separation of DNA fragments using a range of techniques were performed in the course of this study. Agarose gels were used for routine amplification checking and sizing of PCR products. Agarose or Spreadex gels were used in the sizing of digested PCR products. Polyacrylamide gel electrophoresis was used to visualise sequencing fragments.

3.5.1 Agarose

a) Agarose Gel Preparation

Routinely two types of agarose gels were used, UltraPure Agarose (Invitrogen Life Technologies, UK) or a mixture of UltraPure Agarose and NuSieve 3:1 agarose (BioWhittaker Molecular Applications, USA). 1X TAE buffer (Flowgen, UK) was used as a solute. The concentration of pure agarose gel varied depending upon the size of fragments to be separated. Both agarose and agarose/NuSieve were added to 1X TAE buffer in a large beaker. The mixture was heated on a high microwave setting for 1-2 min or until all solids or viscous agar had melted and the solution had started to boil. The mixture was than left to cool until hand hot or 65°C and then ethidium bromide (EtBr) (Sigma) was added to a final concentration of 0.3μg/ml and the solution poured into the appropriate gel mould to set (20 to 30 min).

b) Agarose Gel Electrophoresis

Agarose gel electrophoresis was carried out using either a RunOne System (Embi Tec, USA) or Sub Cell GT (Bio Rad). The polymerized gel was submerged to a depth of around 1mm in the appropriate horizontal electrophoresis tank, either RunOne System (Embi Tec, USA) or Sub Cell GT (Bio Rad), containing 1X TAE electrophoresis buffer. 2μl of loading mix was added to each sample and loaded into the sample wells. 10μl of an appropriate size marker, usually 1kb, 1kb plus or phi-X 174/Hinf I (Promega), was co-electrophoresed with the DNA samples to check for band presence, intensity and correct size. The DNA standards were loaded at one end of a row of samples. Electrophoresis was typically run at 100V for 20 minutes at room temperature

until the loading buffer dye front reached the base of the gel. During migration, the EtBr present in the gel intercalates with the amplicon nucleotides. The nucleotides are then visible when the gel is viewed under UV254 nm.

3.5.2 Spreadex

a) Spreadex Mini Gels

Ready prepared mini gels, Spreadex EL 600 and 1200, were purchased from Elchrom Scientific, Switzerland. These were used to separate digested PCR products, which were only a small number of bases difference. The gels were stored at 4°C until ready for use.

b) Spreadex Gel Electrophoresis

The gel was submerged to a depth of around 1cm in the submerged gel electrophoresis apparatus (Elchrom Scientific, Switzerland) containing 30mM TAE electrophoresis buffer. 3µl of loading mix was added to each 7µl sample and loaded into the sample wells. 10µl of an appropriate size marker, usually 1kb, 1kb plus or phi-X 174/Hinf I (Promega), was co-electrophoresed with the DNA samples. Electrophoresis usually was at 120V for 90 minutes at room temperature.

c) Ethidium Bromide Staining

After electrophoresis, the gel was placed on a shaker filled with 6µl of EtBr and 100ml 30mM TAE buffer and left to stain for 10 minutes.

3.5.3 DNA Visualisation

After electrophoresis, gels were placed on a Dual-Intensity Transiluminator (Alpha Innotech Corporation) and a picture of the fluorescent EtBr-stained DNA was captured with the AlphaArchiver 600 Documentation and Archiving System with the Mitsubishi P96 printer and video processor.

3.5.4 Polyacrylamide Gel Electrophoresis

Polyacrylamide gels for visualising sequencing products were prepared as follows:

50ml of Long Ranger PreMix Gel Solution (BioWhittaker Molecular Applications, USA), stored at -20°C in a falcon tube and thawed by placing in a beaker filled with warm water.
250µl of 10% ammonium persulfate, stored at -20°C.
30µl of TEMED (National Diagnostics, USA), stored at room temperature.

The above solutions were mixed together at room temperature in a 500ml glass beaker and used immediately. The mixture was poured using a syringe and capillary action to spread the acrylamide solution between two clamped 36cm, boiling water-cleaned glass plates for use in an ABI377 with 0.5mm spacers in the cassette. The plates were tipped at an angle to allow the acrylamide to move down between the plates. Any bubbles were carefully removed by slowly dragging them to the edge of the gel before inserting the straight blunt edge of a sharks tooth comb into the top of the gel. The gel was then left to polymerize for two hours. After 30 minutes paper towels soaked in electrophoresis buffer were placed over the ends of the plates to prevent moisture loss, which reduces the appearance of 'red rain' in the gel. The comb was removed from the gel and the wells flushed with copious amounts of dH_2O using a syringe. The polymerized gel was assembled into the ABI377 apparatus for electrophoresis. 2µl of sample was mixed with 3µl of loading dye and 5µl of formamide, denatured at 95°C for two minutes in a PCR block and then immediately placed on ice prior to loading. 0.5µl of each sample was loaded into the loading tray. A disposable comp was then dipped into the loading tray to soak up the sample. The comb was then inserted into the wells through the top buffer tank, which was filled with dH_2O and Ficol 1% Dextran Blue. A pre run was carried out for two minutes then the Ficol was removed and replaced with 10X TBE. Electrophoresis was carried out in 1X TBE for 9 hours.

3.6 Denaturing High Performance Liquid Chromatography (dHPLC) Analysis

dHPLC identified mutations by detecting sequence variation in reannealed DNA strands (heteroduplexes). dHPLC analysis was carried out using the Transgenomic WAVE 3500 machine. Optimum dHPLC temperatures were determined by a temperature scan, using the WAVEMaker 4.0 melting profile as a starting point. Control samples were originally run at four temperatures due to the large distribution of GC-rich regions. For the proband samples the two best temperatures were chosen for the dHPLC runs for exons 1α and 2. The amplicons were eluted with a linear acetonitrile (ACN) (Labscan, Ireland) gradient at a flow rate of 0.9 ml/min, 50% Buffer A:50% Buffer B. Using volumetric flasks the following buffer solutions were made up:

0.1M Triethylammonium acetate (TEAA) Buffer A
0.1M TEAA:25% ACN Buffer B
75% ACN cleaning solution Buffer C
8% ACN syringe wash solution

The gradient duration was adjusted according to each PCR product length. Each elution profile or chromatogram was compared with the profiles associated with homozygous wild type and mutated sequence controls.

3.7 Purification of PCR Products

Two methods of purification of PCR products were used during this study to remove nucleotides and primers from PCR products.

1) Amplified PCR products in microcentrifuge tubes were purified using the QuickStep PCR Purification Kit (Ver. 4) (EdgeBioSystems) according to the manufacturer's instructions.

2) 96 well MicroAmp plates containing amplified PCR products were purified using a shrimp alkaline phosphatase (SAP) method. SAP dephosphorylates nucleotides and ExoI degrades primers, which interfere with the primer extension reaction (Olsen et al. 1991).

3µl of 1µ/µl of shrimp alkaline phosphatase (Promega) was added to each sample along with 3µl of Exonuclease I which was diluted to 1:10 (Exonuclease I: 10X Exonuclease I Buffer (New England BioLabs)). The sample was then incubated at 37°C for 30 minutes, followed by 72°C for 15 minutes, followed by 8°C for 5 minutes.

3.8 Sequencing Reaction

Fluorescence-based cycle sequencing reactions on PCR fragments were performed using the ABI PRISM BigDye Terminator v3.1 Ready Reaction Cycle Sequencing Kit and BetterBuffer (Microzone Ltd, UK). This kit included the dye terminators, deoxynucleoside triphosphates, AmpliTaq DNA Polymerase, FS, magnesium chloride, and buffer premixed in a single tube of Ready Reaction Mix.

Each sample was mixed with 0.8µl of specific primer (5µM), 4µl of BigDye, 4µl of BetterBuffer, and sterile dH_2O to a final volume of 20µl. Samples were subjected to cycling parameters specified in Appendix 2 and then stored at 4°C until ready to purify.

3.9 Purification of Sequencing Products by Ethanol/EDTA Precipitation

All PCR products were purified to remove dNTPs, primers and unincorporated dye terminators before sequencing analysis.

5µl of 125 mM EDTA and 60µl of 100% ethanol was added to each well. The MicroAmp Optical 96-well reaction plate (ABI) was sealed with an adhesive plate seal (ThermoLife Sciences), mixed by inverting and then incubated at room temperature for 15 minutes. The plate was then spun at 3000 x g for 45 minutes. Following this, the plate was inverted and spun at 200 x g. 150µl of 70% ethanol was added to each well and the plate spun at 3000 x g for 15 minutes after which it was inverted and spun at 200 x g for about 10 seconds. The wells were covered with a paper towel and pellets left to dry for at least 30 minutes.

3.10 Sequence Analysis

12µl of Hi-Di Formamide was added to each cleaned pellet and incubated at room temperature for at least one hour before loading the plate into the ABI PRISM 3100 Genetic Analyzer. Sequence analysis was carried out using the ABI PRISM377 DNA Sequencer, and laterally the ABI PRISM 3100 Genetic Analyzer. Sequence analysis was performed with the ABI PRISM 3100 Genetic Analyzer because in contrast to the traditional slab-gel system which requires at least 9 hours of electrophoresis time for equivalent length of read in addition to significant time for pouring, setting up, loading and tracking lanes of sequence on the gel. With the 3100 Genetic Analyzer System 96 samples were electrophoresed in 4.5 hours, with minimal hands-on time.

For sequence analysis Dye Set Z with Mobility file DT3100POP4 (BDv3)v1, Run module UltraSeq36_POP4 and Analysis module BC3100UR were used. The ABI PRISM 3100 Genetic Analyzer contained 10X Buffer with EDTA, a 36cm capillary array length and POP-4 polymer. The total run time was 45 minutes per 16 samples.

3.11 Electropherogram Analysis

Forward and reverse sequence electropherograms were generated by ABI Sequence Analysis 3.4.1 and studied with ABI SeqEd 1.0.3 or latterly ABI SeqScape versions 1.1 and 2.0. The SeqScape software program contains basecalling, sequence assembly, alignment, and sequence comparison tools for fast sequence comparisons and accurate variant identifications based on a reference sequence with or without known nucleotide variant information. Unique to this tool are features such as per base and per sample quality values that allow a user to precisely detect polymorphisms including heterozygotes. Each sequence was read at least twice.

3.12 Haplotype Analysis

PCR reactions, using 5′-end fluorescently labelled primers (Appendix 1) were performed for eight microsatellite DNA markers, in the Genome Database (GDB) http://www.gdb.org/, D9S1604, D9S1748, D9S942, D9S974, RH103023, D9S2060, D9S2136 and D9S1749 from the region that contains the *CDKN2A* gene, which is localised on chromosome 9p21.

Microsatellite loci were PCR amplified (Appendix 2) and the PCR products were then analyzed by electrophoresis on an ABI PRISM 3100 Genetic Analyzer with GENESCAN Analysis 3.1 and GENOTYPER 1.1.1 software to size and quantitate DNA fragments. PCR-amplified microsatellite alleles were detected by fluorescent dye labeling.

The samples were placed in a thermal cycler for 3 minutes at 95°C to denature and then placed immediately on ice. 1µl of each PCR product and 0.5µl of the GeneScan ROX 500 Internal Lane Size Standard were diluted in 9µl of deionized formamide. Some samples were highly concentrated giving off the scale readings and therefore these were diluted to 1:20 (PCR product: sterile dH_2O).

For fragment analysis Dye Set 6-FAM (Blue) and ROX (Red, std) with Virtual Filter Set D, Run module GeneScan 36_POP4 and Analysis module GS500 were used.

3.13 Sizing of Fragments

The called size for a fragment can differ from its actual size due to different machinery setup, therefore fragment sizes were converted to alleles before comparing microsatellite data generated on different instruments used by other authors.

The ABI ROX 500 size standard was used to accurately calculate the sizes of the alleles. Alleles for each marker were numbered with allele 1 being the largest. The following table lists the lengths of the 16 fragments comprising the GeneScan ROX 500 size standard:

TABLE 3.1

ABI ROX 500 Size Standard Fragment Lengths

Fragment Lengths (nt)			
35	50	75	100
139	150	160	200
250	300	340	350
400	450	490	500

The accuracy of the sizing of alleles using the ABI ROX size standard was checked to ensure that allele sizes quoted in this thesis were comparable to other published work. The AMXY (amelogenin) marker, located on chromosomes Xp22.1-22.31 and Yp11.2, carries a small deletion of 6bp in the first intron on the X chromosome, facilitating the design of distinct X and Y specific PCR primers (Appendix 1) (Mann et al., 2001). The AMXY fragment identifies an X-specific 104bp fragment and a Y-specific 110bp (Appendix 2) (Nakahori et al. 1991). This AMXY marker of known size was checked against the ABI ROX size standard to confirm the ABI ROX size standard was sizing accurately.

Allele frequencies for the different markers were based on the typing of 86 unrelated, ethnically matched, noncarrier chromosomes of European ancestry and from previously published papers. The sizes of the different alleles were established by comparison with the ROX 500 standard sequence.

3.14 Digestion of Genomic DNA with Restriction Endonucleases

Restriction enzyme cutting sites were determined with the aid of the internet site http://darwin.bio.geneseo.edu/~yin/WebGene/temp/remain261.html. All enzymes were purchased from New England BioLabs (UK) and restriction enzyme digestion was performed according to the manufacturer's instructions by incubating the PCR product with the appropriate amount of restriction enzyme, its buffer and at the temperature specific for that enzyme (Appendix 3). Partially digested samples were digested to completion by adding 1 µl of restriction enzyme and incubating the reaction at the specified temperature for a minimum of 1h 30 min.

3.15 Cloning of PCR Products

50µl of PCR product and a 750bp control DNA template were cloned using the TOPO TA Cloning Kit for Sequencing Version H (Invitrogen Life Technologies) according to the manufacturer's instructions to produce colonies of single alleles of exon 2 of the *CDKN2A* gene. For each transformation, two LB plates containing 50µg/ml of kanamycin were used. Colonies were grown on imMedia Kan Agar and Liquid (Invitrogen Life Technologies). The control template was a plasmid that encodes ampicillin resistance and so was grown on kanamycin plates. The pUC19 plasmid was grown on LB plates containing100µg/ml of ampicillin to check the transformation efficiency of the One Shot competent cells.

LB-agar medium was cooled to 50°C before adding to falcon tubes containing ampicillin. The mixture was then poured into sterile plates. The kanamycin/ampicillin plates were stored at 4°C until needed when they were warmed to 37°C. The competent One Shot cells were removed from -70°C and thawed on ice for 5 minutes. 4µl of PCR product, 1µl of salt solution and 1µl of TOPO vector were gently mixed together and incubated for five minutes at room temperature before placing on ice. At the same time, 1µl of the control PCR product, 3µl of sterile water, 1µl of salt solution and 1µl of pCR4-TOPO were gently mixed together and incubated for five minutes at room temperature before placing on ice.

A vector only reaction was set up in the same way but omitting the control PCR product from the reaction. 2µl of these TOPO Cloning reactions were then mixed gently into a vial of One Shot TOP10 Chemically Competent *E.coli*. The vials were left on ice for 20 minutes. The vials were then heat-shocked by placing in a 42°C waterbath for 30 seconds. The vials were placed back immediately on ice for 2 minutes. The samples were taken off ice and 250µl of room temperature SOC medium was added to each tube. The tubes were gently inverted and placed in a 37°C waterbath for 1 hour. During this period the LB-agar/ampicillin/kanamycin petri dishes were removed from 4°C.

100µl of each transformation was pipetted to the middle of the sterile petri dishes and the solution spread over the plate using a disposable plastic sterile spreader. The petri dishes were then inverted and placed at 37°C over night. Ten colonies were picked, five from each plate and cultured overnight in 10ml of LB-kanamycin at 50µl/ml. The colonies were cultured in ten separate universals at 37°C in a shaker.

3.16 Molecular Modelling

Molecular Modelling was carried out in the laboratory of Professor William Hunter in the Division of Biological Chemistry and Molecular Microbiology in the School of Life Sciences at the University of Dundee.

The computer graphics program "O" (Jones et al. 1991) was used to visualize the structure of the protein p16INK4A and to investigate the potential effect of the mutations on the interactions formed between this tumour suppressor and the protein kinase CDK6. The crystalline structure for CDK4 is not yet available. Coordinates for the CDK6-p16INK4A complex were retrieved from the Protein Data Bank (http://www.rcsb.org/pdb/; code 1BI7) and supplied to Prof. Hunter. Each mutation was inserted into the model and the affect was observed. Molecular diagrams were produced with the program MOLSCRIPT.

3.17 Statistical analysis

Statistical analysis on the *MC1R* genotypes and phenotypes was carried out under the kind supervision of Dr. Vincent Macaulay, Department of Statistics, University of Glasgow.

3.17.1 Calculating P-values

The computer software, DNA Sequence Polymorphism (DnaSP), version 4 (University of Barcelona), www.ub.es/dnasp was used to perform Fisher's exact test, in order to test for associations between genetic and phenotypic variables. DnaSP allows testing of associations between 2 binary variables (in a 2×2 contingency table). The British geneticist and biostatistician R. A. Fisher (1890-1962) devised Fisher's exact test. Fisher's exact test tests the independence of rows and columns in a 2×2 contingency table (with two horizontal rows crossing two vertical columns creating four places for data) based on the exact sampling distribution of the observed frequencies. The test directly computes the probability, P, of getting a table less probable than the observed table, under the null hypothesis that the two variables are not associated. If this probability is sufficiently small, the null hypothesis can be rejected and a significant association declared. The well-known chi-squared test for independence in a 2×2 contingency table is an approximate form of this test. The probability of observing a given set of frequencies A, B, C, and D in a 2×2 contingency table, given fixed row and column marginal totals and sample size N, is:

$$P = \frac{(A + B)!(C + D)!(A + C)!(B + D)!}{A!B!C!D!N!}$$

If $P \leq 0.05$ we reject the null hypothesis of independence. Since the whole population cannot be tested, the P-value attempts to make a judgment about the effects from a sample of the population. If the P-value is > 0.05, there is no statistically significant association. The test assumes random sampling and that each observation is independent.

MINITAB Release 14 was used to perform a chi-squared test of independence in 2 × 4 contingency tables. P-values were only calculated when the expected frequency was ≥ 5 in every cell in the table. Due to this small data set, this was not always the case and therefore P-values were not always calculated.

3.17.2 Bonferroni Correction

Every statistical test has a probability that it will find a significant association even when one does not exist. The more tests one performs the more likely it is that some false positives will be declared. To try to compensate for this effect, the Bonferroni correction adjusts the statistical significance level (the value 0.05 above) when multiple tests are performed. This study has many cases of multiple tests e.g., analysing the associations between the various *MC1R* variants and the phenotypes and so the Bonferroni adjustment was required. If n tests are performed the value against which each P-value is compared in each test is reduced as follows:

$$\text{Bonferroni adjustment} = \frac{0.05}{n}$$

The disadvantage of the Bonferroni method however is that it can be over conservative (since it assumes that each test is independent) and so the analysis is in danger of finding no significant differences when in fact they could be there. If there are many tests, the Bonferroni criterion becomes so strict that it is very difficult to reject any null hypothesis and an important finding may be overlooked. Thus in an exploratory analysis, it is wise not to rule out possible associations which are significant without the correction, but which just fail to be significant after the correction. This is particularly true in this context where there is every reason to suppose that the individual tests will not be independent, owing to the correlations induced by linkage disequilibrium because of the shared ancestry of a short DNA segment.

3.17.3 Calculating Odds Ratios and Confidence Intervals

The odds ratio (OR) was also used to assess the risk of a particular outcome if a certain factor was present. Odds are the probability for an outcome occurring divided by the probability of them not occurring. The OR is the ratio of the odds for the particular trait in cases divided by the odds in controls. If the OR is > 1 then the event is more likely to happen in cases than controls. If the OR is < 1 then the event is less likely to happen in cases than controls. Since the OR is subject to sampling error, it is helpful to construct a 95% confidence interval for its true value. If this interval includes 1 then there is no significant difference between cases and controls (cf. the results of the Fisher's exact test). If the interval excluded 1 then there is a significant association and the magnitude of the OR gives a quantitative measure of the strength of the difference in risk. The following formulae were used to calculate an approximate 95% CI of the OR (using the same notation as above):

$$OR = (A/B) / (C/D)$$

$$\text{Upper limit of CI for } \ln(OR) = \ln(OR) + 1.96 \times SE(\ln OR)$$

$$\text{Lower limit of CI for } \ln(OR) = \ln(OR) - 1.96 \times SE(\ln OR)$$

$$\text{Upper limit of CI for OR} = \exp(\text{upper limit of CI for } \ln OR)$$

$$\text{Lower limit of CI for OR} = \exp(\text{lower limit of CI for } \ln OR)$$

$$\text{Standard error (SE) of } \ln(OR) = \sqrt{[(1/A) + (1/B) + (1/C) + (1/D)]}$$

For each Test of Independence in Chapter 6 a Two-tailed Fisher's Exact Test was performed, unless stated otherwise. Odds ratios (OR) are indicated with 95% confidence interval (CI) in all statistical analysis. Alleles were counted twice in homozygous individuals which increases the N value of subjects for certain variants.

CHAPTER 4

MUTATION SCREENING OF THE *CDKN2A* GENE

4.1 Families in Study

Members of 48 families were used in this section of the work. The families for identification purposes are numbered from F1-F48 throughout this thesis. *CDKN2A* results for patients F1-F16 have already been reported (MacKie et al. 1998). The *CDKN2A* studies carried out on these families in the course of work presented in this thesis is therefore confirmatory. All other results on families F1-F16 and all results on families F17-F48 are reported for the first time.

4.2 *CDKN2A* Mutation Detection

Familial cutaneous malignant melanoma can be caused by germline mutations in *CDKN2A*. The gene was therefore screened in Scottish familial melanoma patients. *CDKN2A* encodes a protein (p16INK4A) of 156 amino acids. The coding region for this protein product spans three exons: 150 bp in exon 1α; 306 bp in exon 2; and 12 bp in exon 3.

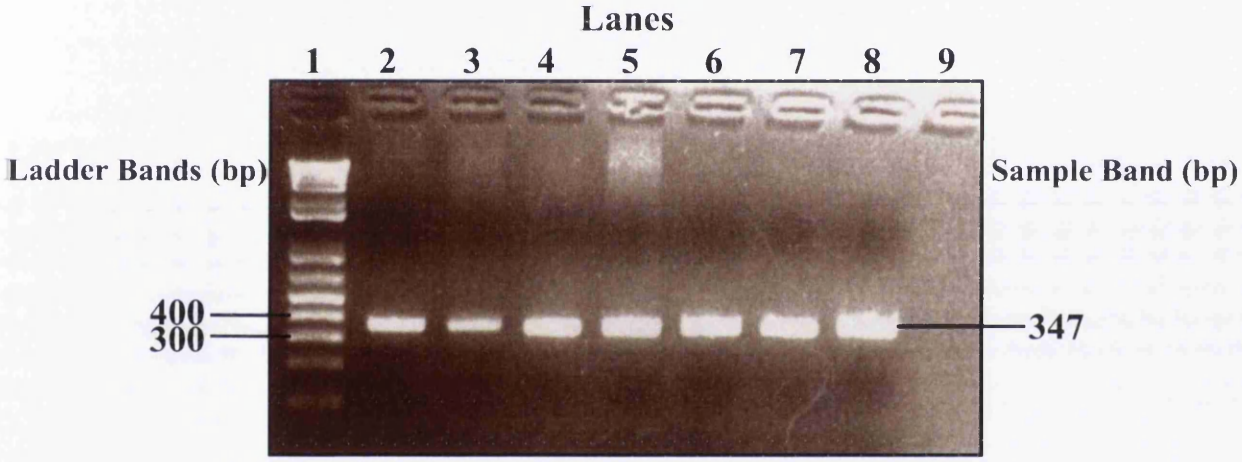
4.2.1 Mutation Detection Strategy Employed

Primers were designed (Methods 3.4.1-3.4.3) for independent amplification of the complete coding regions of exons 1α, 2 and 3 and their splice sites. PCR primers were situated up and downstream of each exon. The final amplification conditions are presented in Appendix 2. Routinely a 1μl aliquot of DNA (extracted as described in method 3.1a) from each patient was amplified (Method 3.4.5) with each of the three sets of primers. Fragment sizes of 347bp for exon 1α, 458bp for exon 2 and 254bp for exon 3 were generated. Negative controls (no DNA) were run along with each reaction. Once all the DNA samples had been transferred to PCR tubes, the same reagents were used for the negative control. The absence of amplicons in the negative control meant that there had been no cross-contamination between the samples or into the reagents. The distinct bands obtained from amplification were resolved on ethidium bromide stained agarose gels (Method 3.5) to verify the size of the amplified band and are presented in figures 4.1, 4.2 and 4.3.

FIGURE 4.1

Amplification of Exon 1α of *CDKN2A*

The expected 347bp amplicons of exon 1α of *CDKN2A* were confirmed by resolution on a 1.5% agarose gel with ethidium bromide. The amplicons were electrophoresed in 1X TAE buffer for 20 minutes. The size of the amplicons which are represented below shown in base pairs were deduced by comparison to the migration of the known size of the DNA ladder fragments in lane 1. 1µl of genomic DNA was routinely amplified by conditions specified in Appendix 2 and with primers specified in Appendix 1. The amplicons' size is consistent with the expected 347bp fragment and was sequenced to confirm this.



Electrophoresis Conditions: 1.5% agarose gel, 1X TAE, 10µl size marker, 5µl PCR product, 2µl loading mix, 100V, 20 minutes, room temperature

Lane 1 – 1kb Plus size marker

Lane 2 – PCR product from individual F2.1

Lane 3 – PCR product from individual F2.2

Lane 4 – PCR product from individual F2.6

Lane 5 – PCR product from individual F3.1

Lane 6 – PCR product from individual F3.2

Lane 7 – PCR product from individual F11.1

Lane 8 – PCR product from individual F11.2

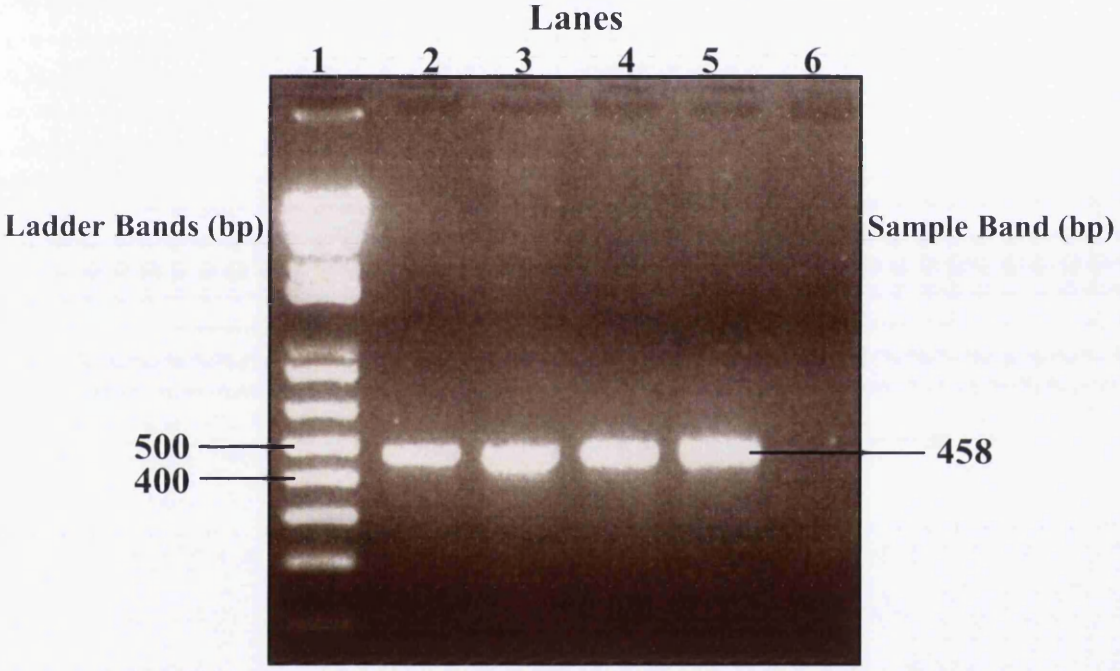
Lane 9 – negative control

See page 60 for patient details.

FIGURE 4.2

Amplification of Exon 2 of *CDKN2A*

The expected 458bp amplicons of exon 2 of *CDKN2A* were confirmed by resolution on a 1.2% agarose gel with ethidium bromide. The amplicons were electrophoresed in 1X TAE buffer for 20 minutes. The size of the amplicons which are represented below shown in base pairs were deduced by comparison to the migration of the known size of the DNA ladder fragments in lane 1. 1µl of genomic DNA was routinely amplified by conditions specified in Appendix 2 and with primers specified in Appendix 1. The amplicons' size is consistent with the expected 458bp fragment and was sequenced to confirm this.



Electrophoresis Conditions: 1.2% agarose gel, 1X TAE, 10µl size marker, 5µl PCR product, 2µl loading mix, 100V, 20 minutes, room temperature

Lane 1 – 1kb Plus size marker

Lane 2 – PCR product from individual F1.1

Lane 3 – PCR product from individual F17.1

Lane 4 – PCR product from individual F19.1

Lane 5 – PCR product from individual F33.1

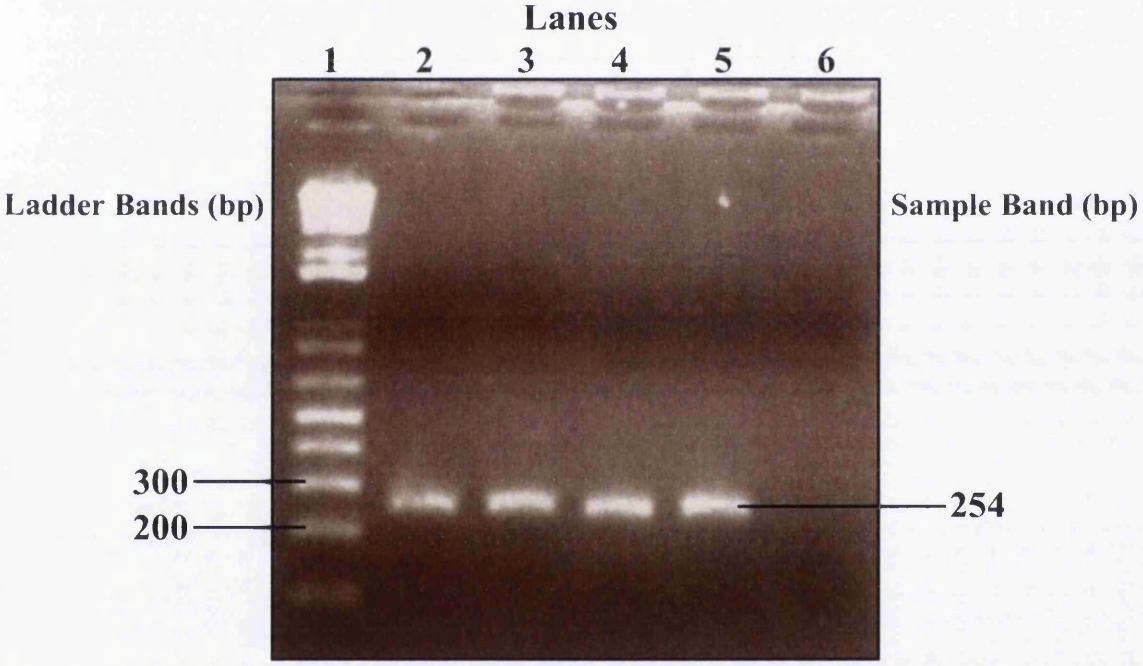
Lane 6 – negative control

See page 60 for patient details.

FIGURE 4.3

Amplification of Exon 3 of *CDKN2A*

The expected 254bp amplicons of exon 3 of *CDKN2A* were confirmed by resolution on a 1.5% agarose gel with ethidium bromide. The amplicons were electrophoresed in 1X TAE buffer for 20 minutes. The size of the amplicons which are represented below shown in base pairs were deduced by comparison to the migration of the known size of the DNA ladder fragments in lane 1. 1µl of genomic DNA was routinely amplified by conditions specified in Appendix 2 and with primers specified in Appendix 1. The amplicons' size is consistent with the expected 254bp fragment and was sequenced to confirm this.



Electrophoresis Conditions: 1.5% agarose gel, 1X TAE, 10µl size marker, 5µl PCR product, 2µl loading mix, 100V, 20 minutes, room temperature

Lane 1 – 1kb Plus size marker

Lane 2 – PCR product from individual F9.1

Lane 3 – PCR product from individual F12.1

Lane 4 – PCR product from individual F13.2

Lane 5 – PCR product from individual F14.1

Lane 6 – negative control

See page 60 for patient details.

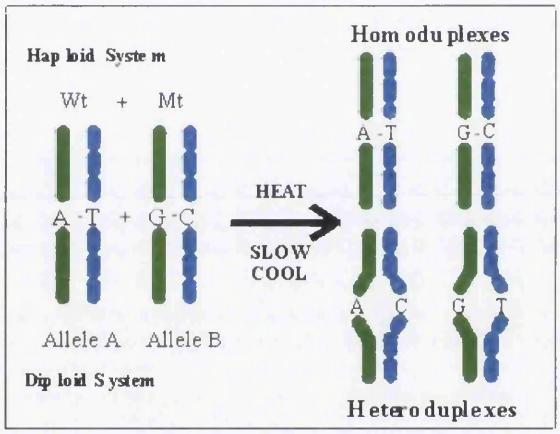
4.3 dHPLC Analysis Conditions

To identify the presence of mutant or polymorphic alleles a mutation screen of exons 1α and 2 was carried out using dHPLC analysis (Method 3.6). dHPLC analysis with the Transgenomic WAVE machine was a new technique in the laboratory and therefore both dHPLC and sequencing was performed to compare the sensitivity of dHPLC with sequencing. dHPLC analysis was not performed on exon 3 because exon 3 is only 12 bp long and direct sequencing of this exon together with SNPs 500 and 540 was thought to be more appropriate.

The detection of DNA changes by dHPLC is based on the formation of heteroduplexes of wild type and mutant amplicons (Figure 4.4).

FIGURE 4.4

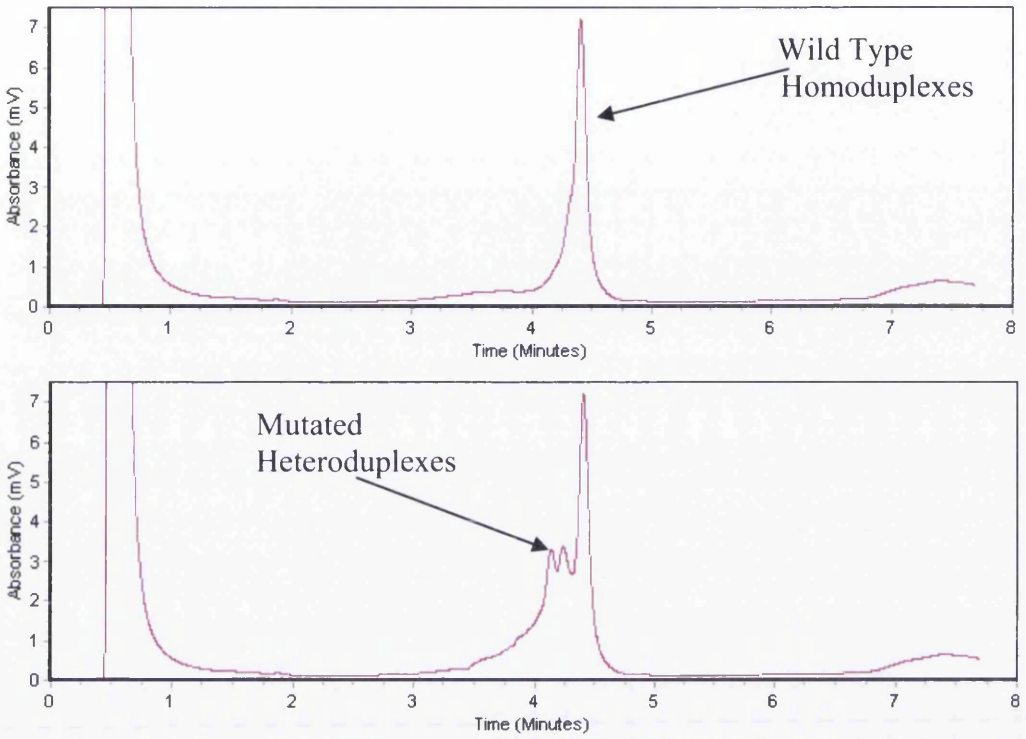
Heteroduplex Formation



Mutations are detected by comparison between the wild type and the sample chromatograms (Figure 4.5). Optimum dHPLC temperatures were determined by a temperature scan, using the WAVEMaker 4.0 melting profile as a starting point, which allowed the entire fragment to be screened at a minimum number of temperatures. Mutation positive control samples were originally run at four temperatures due to the large distribution of GC regions, 71.18% GC content for exon 1α and 66.15% GC content for exon 2. For the proband samples, the two temperatures that gave the clearest chromatogram results at which mutations were detected were chosen (Figures 4.6 and 4.7). For exon 1α, samples were run at 66°C and 68°C, although all mutations were detected at 68°C. For exon 2, samples were run at 63°C and 69°C. Mutations near the beginning of the exon 2 fragment were better detected at 63°C whereas mutations near the end of the exon 2 fragment were better detected at 69°C.

FIGURE 4.5

Heteroduplex and Homoduplex Elution Profiles

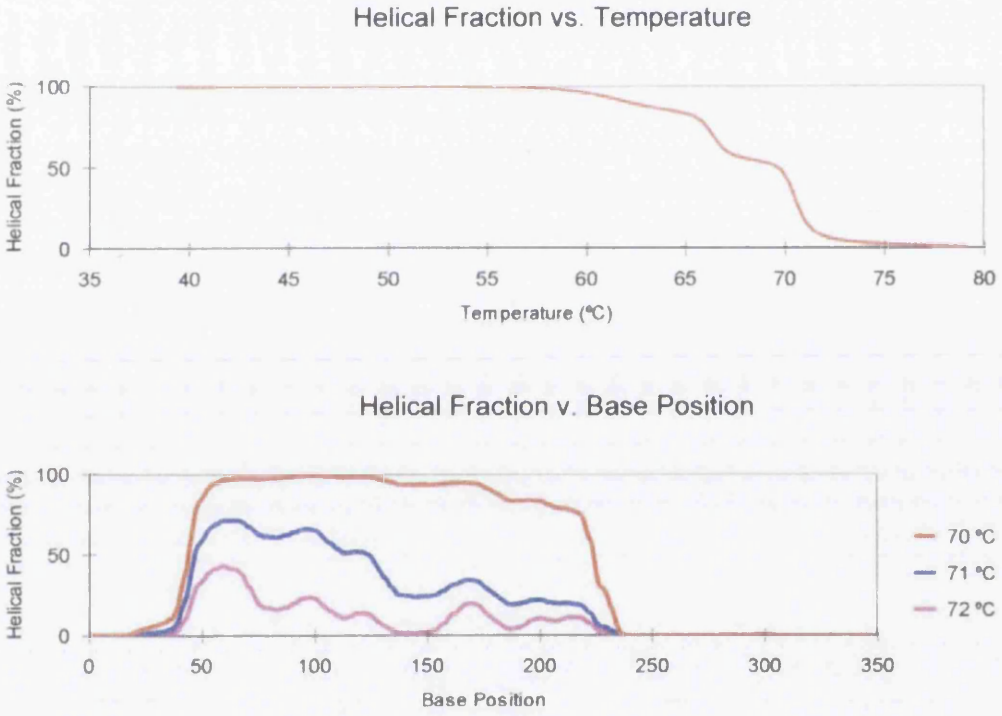


Elution profiles that differ from the wild type indicate the presence of a mutation. Heteroduplex peaks elute earlier than homoduplex, and can be observed as separate peaks or as shoulders. The numbers on the *x* axis correspond to the column retention time in minutes.

FIGURE 4.6

Exon 1α Optimum dHPLC Temperatures

The melting characteristics of the exon 1α fragment to determine the temperature at which mutations will resolve using dHPLC are illustrated. The software predicts the average Tm for the entire fragment was used as a midpoint for the temperature titration. Mutations are best detected if the helical fraction ranges between 70-85%.

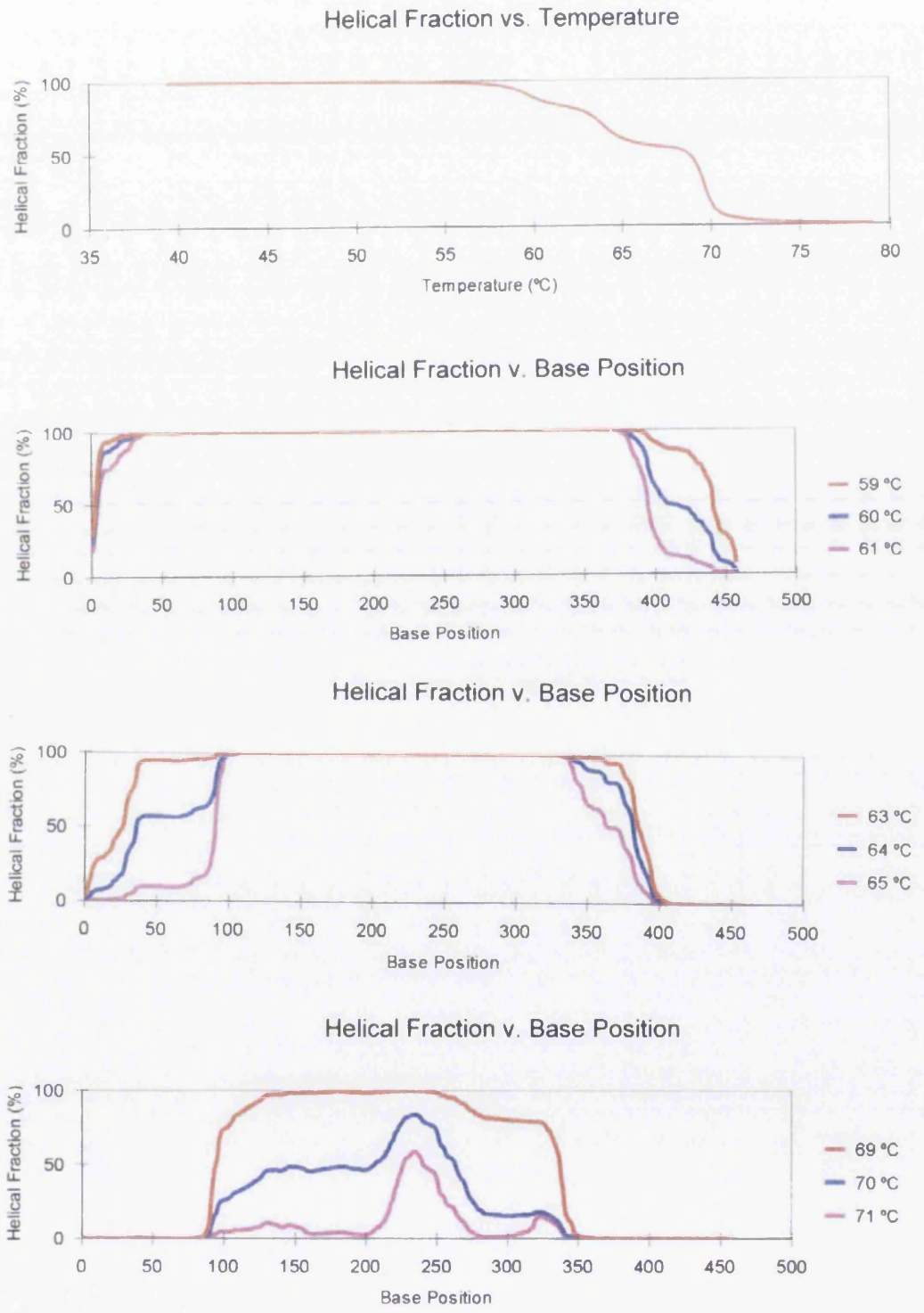


Predicted three temperature melting trial for exon 1α. For exon 1α, samples were run at 66°C and 68°C as the temperatures in the above graph were too high for best mutation detection.

FIGURE 4.7

Exon 2 Optimum dHPLC Temperatures

The melting characteristics of the exon 2 fragment to determine the temperature at which mutations will resolve using dHPLC is illustrated below.



Predicted nine temperature melting trial for exon 2.

For exon 2, samples were run at 63°C and 69°C. Mutations near the beginning of the exon 2 fragment were better detected at 63°C whereas mutations near the end of the exon 2 fragment were better detected at 69°C.

4.3.1 dHPLC Analysis of Proband Samples

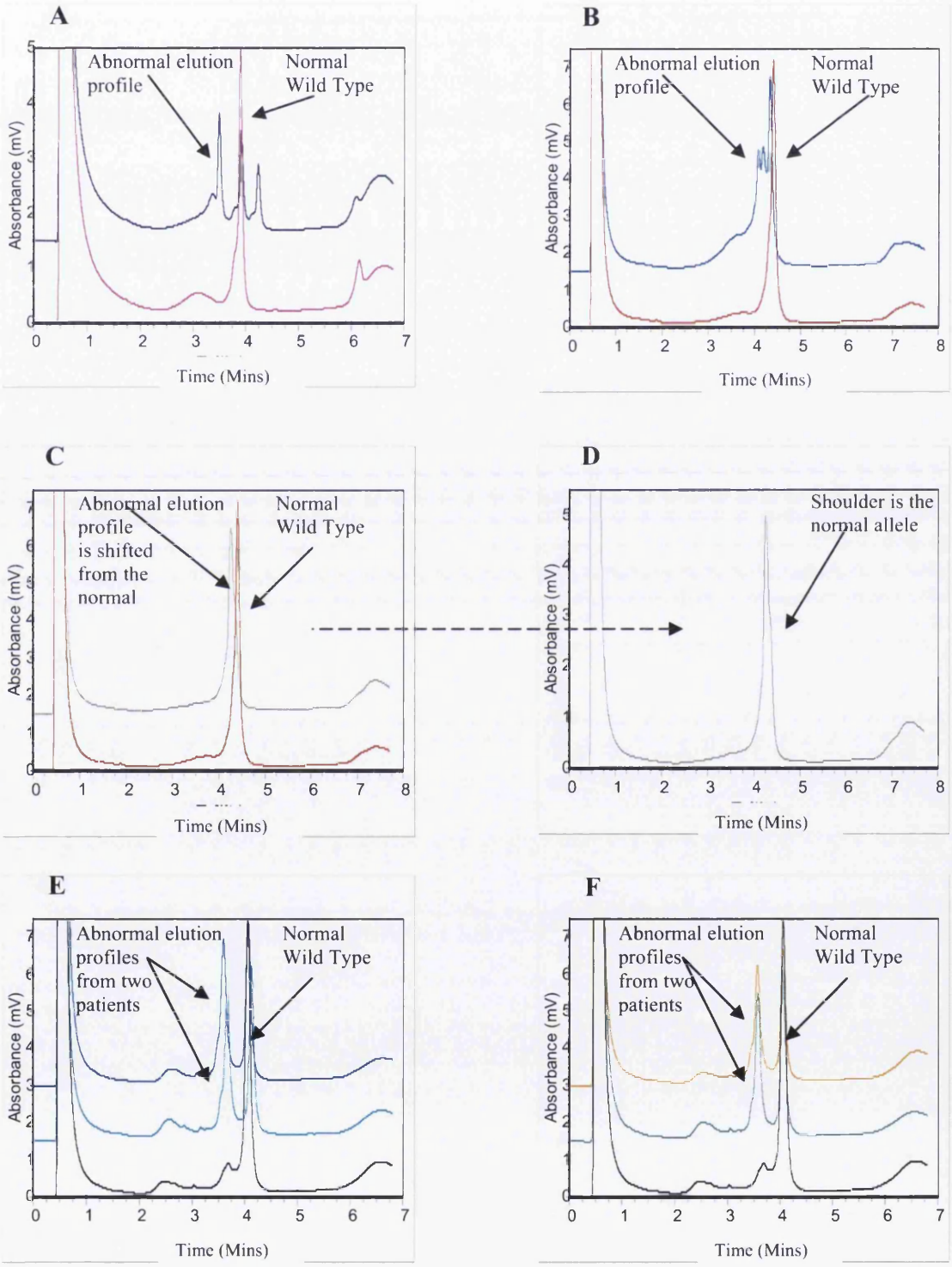
Amplified DNA samples from the 48 probands were subjected to dHPLC analysis (Method 3.6). Two optimum melting profiles were identified for use in the analysis of exons 1α (66°C and 68°C) and 2 (63°C and 69°C). Fifeteen abnormal chromatogram patterns were identified for exons 1α and 2 and their flanking sequences as shown in figure 4.8.

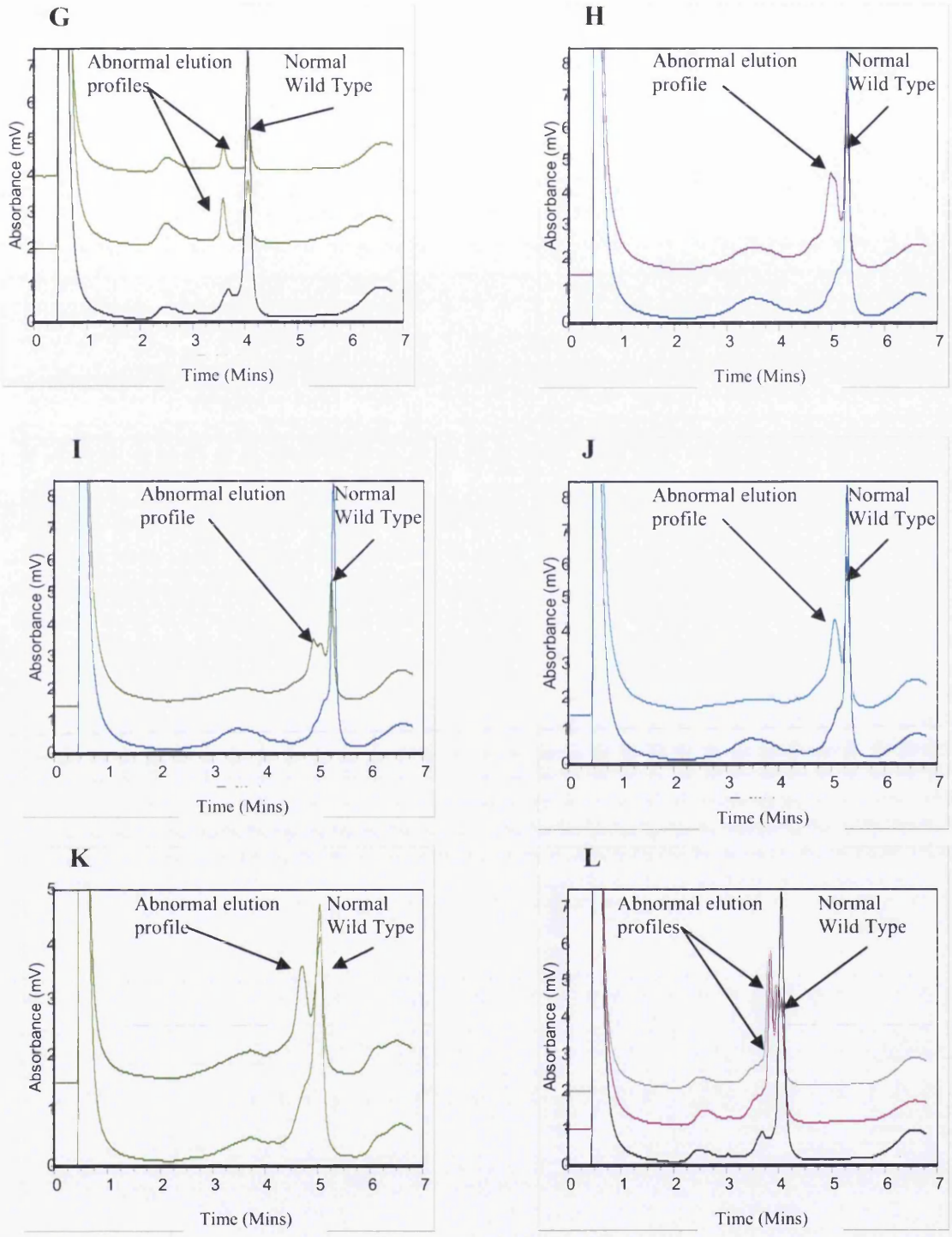
DNA from each abnormal sample was reamplified and subjected to direct sequence analysis to identify the cause of the chromatogram shift.

FIGURE 4.8

Detection of *CDKN2A* mutations by dHPLC

Elution profiles obtained in melanoma patients as compared with a wild-type elution profile are shown.





Exon 1α at 68°C is shown in panels A-D and exon 2 at 63°C and 69°C is shown in panels E-L. Representative normal profiles are depicted in each panel. Note the shoulder obtained for the sample in panel C which is shown more clearly in panel D. Additional peaks/shoulders were obtained in 15 proband samples. The numbers on the *x* axis correspond to the column retention time in minutes.

4.4 Direct Sequencing of Abnormal Amplicons

Each of the exons in which an aberrant chromatogram shift was identified was subjected to direct sequencing (Methods 3.7-3.11). The primers used for sequence analysis were the same as the amplification primers. DNA sequence of the complete exons and at least 20 base pairs of flanking DNA were routinely obtained and nucleic acid changes identified in all fragments sequenced. In total, 15 probands were found to have a nucleic acid change on sequencing. In exon 1α three distinct mutations were identified and in exon 2 five were found. All changes were confirmed by reamplification and sequencing of the mutated exons. The nature of these changes were analysed with respect to the amino acid change and whether they had been previously reported in melanoma patients (Table 4.2). Exon 3 was sequenced directly. No exon 3 mutations have been identified in any of these 48 families, however nine probands showed a change at SNP 500 and five probands showed a change at SNP 540.

4.5 Determining the Nature of the Nucleic Acid Change

The eight different nucleic acid changes were identified in samples from 15 probands. In three of these proband samples, individuals F2.1, F3.1 and F28.1, an exon 1α mutation was identified and in 13 of these proband samples, individuals F1.1, F4.1, F5.1, F6.1, F7.1, F13.1, F17.1, F19.1, F22.1, F29.1, F30.1 and F33.1, an exon 2 mutation was identified. No exon 3 mutations were identified in this study, however in individuals F7.1, F12.1, F13.1, F19.1, F25.1, F28.1, F31.1, F33.2 and F34.1, a polymorphism at the 3' end of exon 3, SNP 500, was found, and in individuals F9.1, F14.1, F27.2, F32.1, F36.1, a further downstream polymorphism, SNP 540, was identified. In two proband samples, individuals F7.1 and F13.1, the mutation identified, A148T, has been previously reported in melanoma families as a polymorphism (Bertram et al. 2002). The mutations 32-33ins9-32 24bp duplication, R24P, Y44stop, M53I, G67R and R112G, and SNPs 500C>G and 540C>T have been previously reported (Holland et al. 1995; Walker et al. 1995; Platz et al. 1997; MacKie et al. 1998; Newton Bishop et al. 1999; Kumar et al. 2001; Bertram et al. 2002), however mutation H83N has not to date been reported in melanoma families. The exon 1α 32-33ins 9-32 24 base pair duplication has previously been incorrectly reported as a 1-8 24 base pair duplication (Hussussian et al. 1994; Harland et al. 1997; Monzon et al. 1998).

4.6 Polymorphisms

The polymorphisms identified during this study have previously been reported (Ranade et al. 1995; Reymond and Brent 1995; Aitken et al. 1999). The site of three polymorphisms are one G>A A148T in exon 2, one 500C>G at the 3' end of exon 3 and a third 540C>T 40 base pairs downstream from this.

4.7 Confirmation of Mutation M53I by Restriction Digestion

A restriction assay was developed to test for the M53I mutation (Method 3.14), which is the most frequently occurring of the mutations in Scottish familial melanoma. Details of this assay are presented in Appendix 3.

The exonic sequence of both wild type and the heterozygous mutant was analysed to identify restriction enzyme sites (Method 3.14). For mutation M53I the sequence was altered such that a new restriction site was created. This assay allowed confirmation of the presence of the mutation and also permitted family studies to be carried out where the mutation was identified in the proband.

4.8 Summary of Findings

A summary of all mutations identified is listed below in table 4.1.

TABLE 4.1

***CDKN2A* Mutations Identified in Scottish Melanoma Families**

CDKN2A Mutation		Number of Scottish Melanoma Families with Mutation
Exon 1α		
32-33ins9-32	24bp dup	1
71G>C	R24P	1
132C>A	Y44stop	1
Exon 2		
159G>C	M53I	6
199G>C	G67R	1
247C>A	H83N	1
334C>G	R112G	2
442G>A	A148T (polymorphism)	2

Thirteen of the 48 families (27%) have a *CDKN2A* mutation. Three families have an exon 1α mutation, and ten have an exon 2 mutation. Two additional families have the exon 2 A148T suspected polymorphism. No exon 3 mutations have been identified. Six of the 13 families with identified mutations carry the M53I mutation. In 33 families, families F8-F12, F14-F16, F18, F20-F21, F23-F27, F31-F32, and F34-48, no *CDKN2A* mutation or polymorphism was detected. The new observation in this section of the thesis is the identification of a previously undescribed mutation, H83N.

4.8.1 Results of Family Studies

The mutation data generated on each of the mutation positive families is presented in this section. The family pedigree, dHPLC analysis, sequencing results with the predicted amino acid change and where developed, the restriction enzyme assay are presented for each family. All exons of *CDKN2A* were screened by either dHPLC and/or sequencing for each of the probands. Where a mutation was not found, all other available affected members of each family were also sequenced. Where a mutation was identified, only the exon in which the mutation occurred was amplified and sequenced in available affected and currently unaffected individuals.

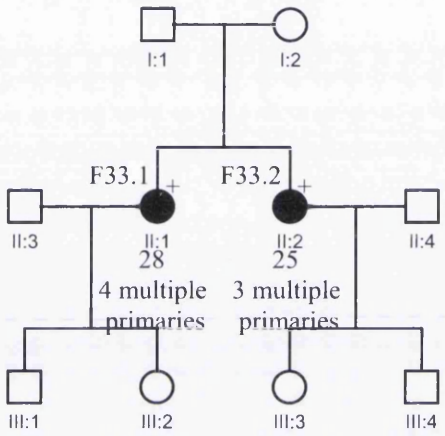
4.9 The H83N Mutation

4.9.1 Mutation Analysis in Family 33 (Figure 4.9)

Family 33 has two affected members with melanoma, both female, who presented with melanoma aged 25 and 28 years. Both individuals have multiple primary melanomas.

FIGURE 4.9

Scottish Melanoma Family 33



4.9.2 Identification of a Chromatogram WAVE Shift

Exons 1α and 2 of the proband, individual F33.1 were amplified and subjected to dHPLC analysis (Figure 4.10). A chromatogram WAVE shift from the normal was identified in exon 2.

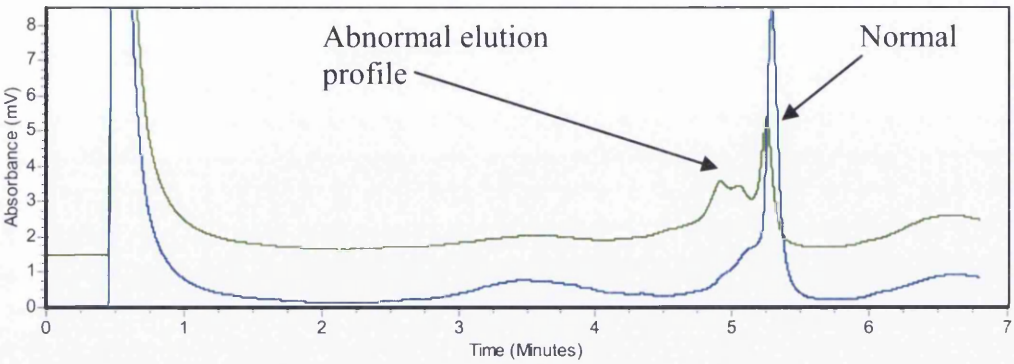
4.9.3 Identification of Mutation H83N

A new exon 2 amplicon was generated for direct sequencing of the proband using both forward and reverse exon 2 primers.

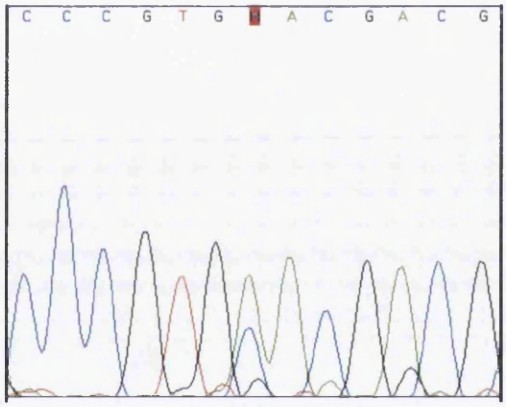
A heterozygous C to A transition at nucleotide position 247C>A was identified (Figure 4.10). This base change is predicted to lead to the substitution of histidine (H) for an asparagine (N) at codon 83 (H83N) (Figure 4.10). This mutation has not been previously reported. Exon 2 from another available family member was screened by direct sequencing. The predicted substitution observed in the proband was also found in the affected family member F33.2. No currently unaffected family members were available for screening.

FIGURE 4.10

Family 33 – Identification of Mutation H83N (247C>A)



dHPLC WAVE analysis of PCR amplified genomic DNA from individual F33.1. Proband DNA was amplified using intronic primer pair *CDKN2A* exon 2 (Appendix 1, 2).



Sequence analysis of PCR amplified genomic DNA from individual F33.1. Proband DNA was amplified and sequenced using intronic primer pair *CDKN2A* exon 2 (Appendix 1, 2).

	P	V	**H**	D	A
Wild Type	CCC	GTG	CAC	GAC	GCT
Mutant	CCC	GTG	**A**AC	GAC	GCT
			N		

(H83N)

Nucleic acid and derived amino acid sequences from the mutated region of *CDKN2A*. The amino acid substitution of a histidine (H) for an asparagine (N) at position 83, H83N is shown.

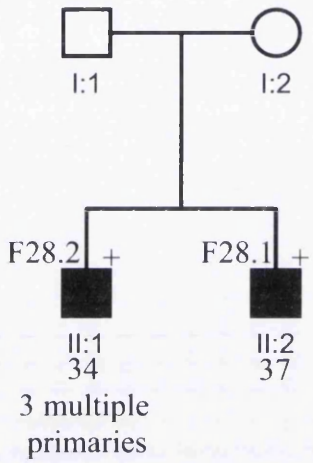
4.10 The 32-33ins9-32 24bp Duplication

4.10.1 Mutation Analysis in Family 28 (Figure 4.11)

Family 28 has two affected members with melanoma, both male, who presented with melanoma aged 34 and 37 years. Individual II:1 (F28.2) has multiple primary melanomas.

FIGURE 4.11

Scottish Melanoma Family 28

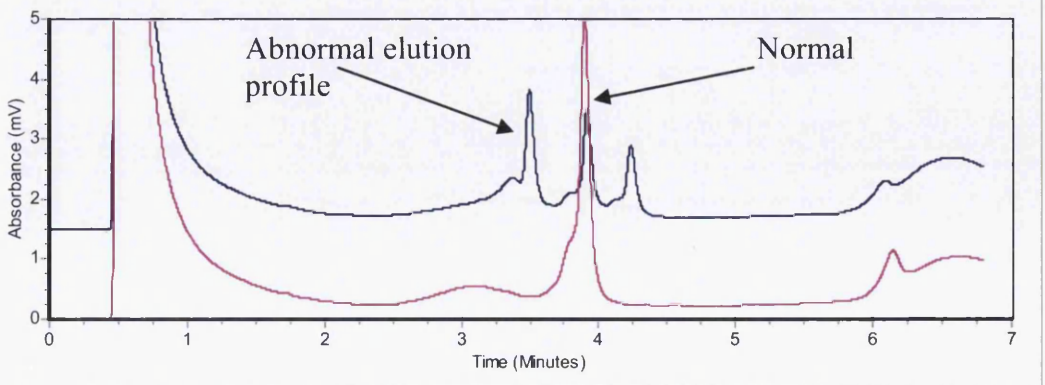


4.10.2 Identification of a Chromatogram WAVE Shift

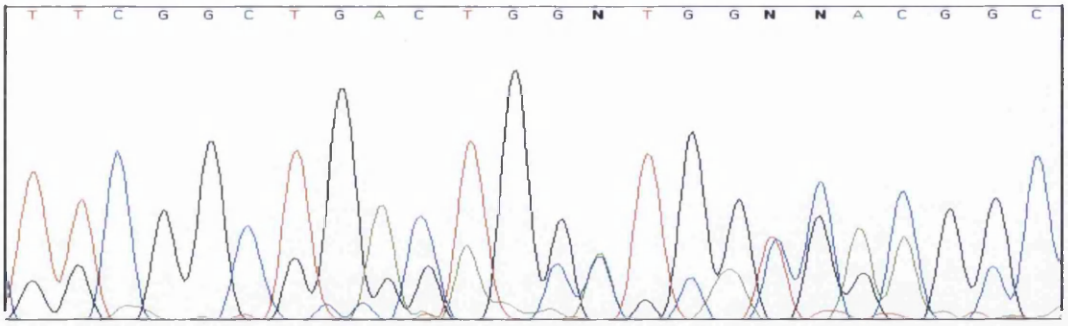
Exons 1α and 2 of the proband, individual F28.1 were amplified and subjected to dHPLC analysis (Figure 4.12). A chromatogram WAVE shift from the normal was identified in exon 1α.

FIGURE 4.12

Family 28 – Identification of Mutation 32-33ins9-32 24bp Duplication



dHPLC WAVE analysis of PCR amplified genomic DNA from individual F28.1. Proband DNA was amplified using intronic primer pair *CDKN2A* exon 1α (Appendix 1, 2).



Sequence analysis of PCR amplified genomic DNA from individual F28.1. Proband DNA was amplified and sequenced using intronic primer pair *CDKN2A* exon 1α (Appendix 1, 2).

MEPAAGSSMEPSADWLATAAARGRVEEVRALLEAGALPNAPNSYGRRP
IQ

atggagcc**ggcggcggggagcagcatggagcc**ggcggcggggagcagcatggagccttcggctgactggc
tggccacggccgcggcccggggtcgggtagaggaggtgcgggcgctgctggaggcgggggcgctgcccaac
gcaccgaatagttacggtcggaggccgatccag

Nucleic acid and derived amino acid sequences from the mutated region of *CDKN2A*. The 24bp fragment of bases 9-32 (black bold underlined) inserted between bases 32-33 (red bold underlined), 32-33ins9-32 24bp duplication is shown.

4.10.3 Identification of Mutation 32-33ins9-32 24bp Duplication

A new exon 1α amplicon was generated for direct sequencing of the proband using both forward and reverse exon 1α primers.

A heterozygous 24bp fragment of bases 9-32 inserted between bases 32-33 was identified (Figure 4.12). This insertion is in frame. This mutation has been previously incorrectly reported as 1-8 24bp insertion (Goldstein et al. 1995; Walker et al. 1995; Flores et al. 1997; Pollock et al. 1998). Exon 1α from the other available family member was screened by direct sequencing. The predicted duplication observed in the proband was also found in the affected family member F28.2.

No currently unaffected family members were available for screening.

4.10.4 Development of a Gel Based Assay

The insertion creates a mutant allele, which, identified by sequencing, is 24bp larger than the normal allele (Figure 4.13). The duplication produces a fragment of 371bp in affected individual F28.1, see lanes 2-4 (Figure 4.14). The normal allele (347bp) is present, see lane 6.

FIGURE 4.13

Development of a Gel Based Assay to Detect Mutation 32-33ins 9-32 24bp Duplication

Diagrammatic representation of the 347bp PCR amplified fragment of exon 1α of *CDKN2A*. The mutation creates a 24bp duplication of base pairs 9-32 and inserts the 24bp at base pair 32 to give rise to a 371bp PCR amplified fragment of exon 1α of *CDKN2A*.

Duplication

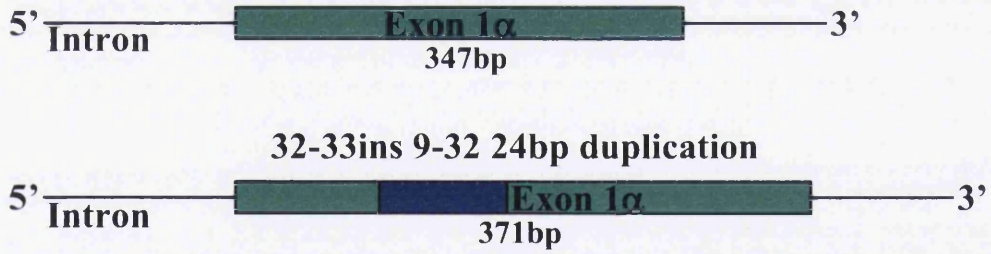
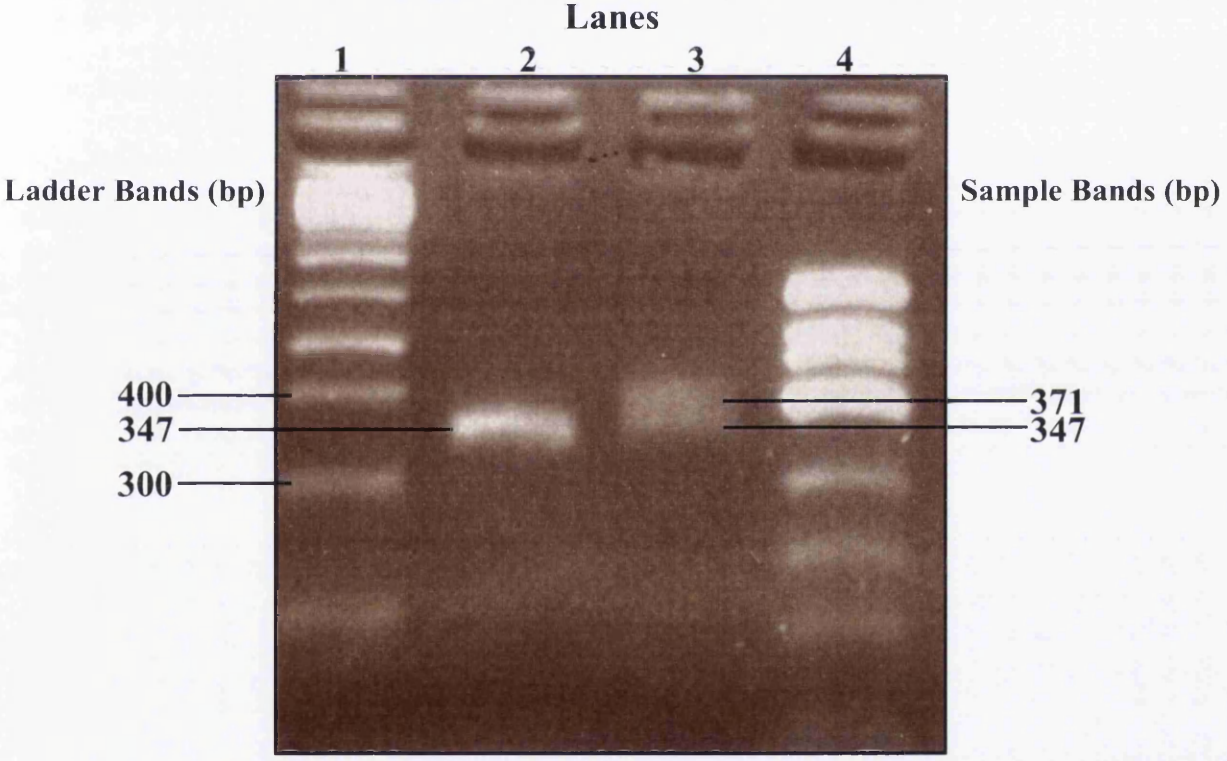


FIGURE 4.14

Development of a Gel Based Assay to Detect Mutation 32-33ins 9-32 24bp Duplication

a) The expected 371bp and 347bp amplicons of the 32-33ins 9-32 24bp duplication were confirmed by resolution on a 4% agarose gel with ethidium bromide. The amplicons were electrophoresed in 1X TAE buffer for 20 minutes. The size of the amplicons which are represented below shown in base pairs were deduced by comparison to the migration of the known size of the DNA ladder fragments in lanes 1 and 4. The amplicons' sizes are consistent with the expected 371bp and 347bp fragments and was sequenced to confirm this.



Electrophoresis Conditions: 4% agarose gel, 1X TAE, 10µl size markers, 5µl PCR product, 2µl loading mix, 100V, 20 minutes, room temperature

Lane 1 – 1kb Plus size marker

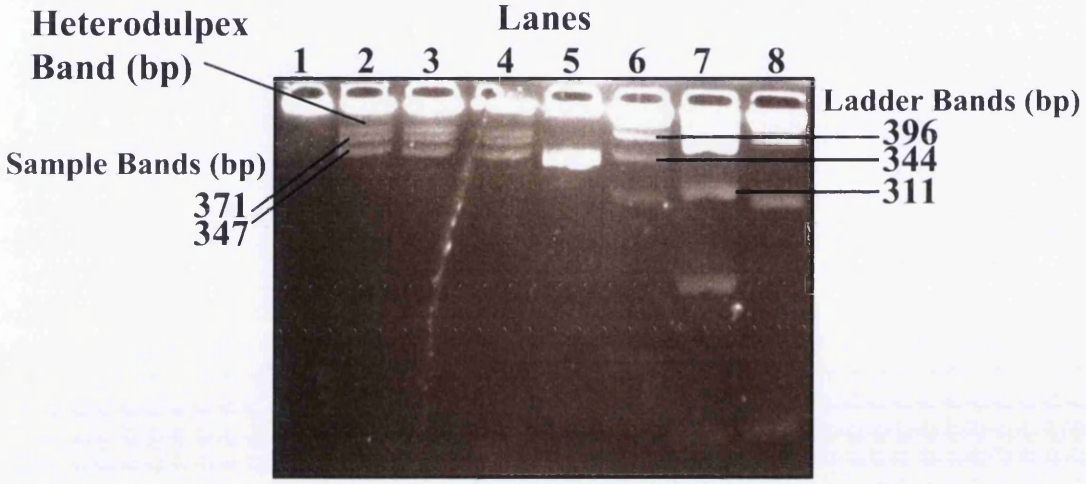
Lane 2 – PCR product from a normal control

Lane 3 – PCR product from individual F28.1

Lane 4 – PhiX174/*Hinf* I size marker

See page 60 for patient details.

b) The expected 371bp and 347bp amplicons of the 32-33ins 9-32 24bp duplication were confirmed by resolution on a Spreadex 600 gel with ethidium bromide. The amplicons were electrophoresed in 30mM TAE buffer for 10 minutes. The size of the amplicons which are represented below shown in base pairs were deduced by comparison to the migration of the known size of the DNA ladder fragments in lanes 1 and 4. The amplicons' sizes are consistent with the expected 371bp and 347bp fragments and was sequenced to confirm this.



Electrophoresis Conditions: Spreadex 600 gel, 30mM TAE, 10µl size markers, 5µl PCR product, 2µl loading mix, 100V, 20 minutes, room temperature

Lane 1 – 1kb size marker

Lane 2 – PCR product from individual F28.1

Lane 3 – PCR product from individual F28.1

Lane 4 – PCR product from individual F28.1

Lane 5 – PCR product from a normal control

Lane 6 – 1kb size marker

Lane 7 – PhiX174/*Hinf* I size marker

Lane 8 – 1kb Plus size marker See page 60 for patient details.

Spreadex gels (Elchrom Scientific) are a novel synthetic matrix of gel polymers arranged to selectively retard migration so that the bands remain sharp but are more spread out relative to each other.

4.10.5 Heteroduplex Analysis

The resolution of the duplication is much clearer in the Spreadex 600 gel compared with the 4% agarose gel. The expected 371bp and 347bp amplicons of individuals F28.1 and F28.2 are resolved, however, there is also detection of an extra slow moving band present. This extra band has thought to be caused by heteroduplex formation, which has less electrophoretic mobility than that of homoduplexes and therefore detected as an extra slow moving band. The formation of this heteroduplex band is likely to have occurred during the annealing step of the PCR programme or when the PCR products from these heterozygous individuals were cooled. This would have allowed the single mutant strands to base pair with the complementary strands from the wild type allele and form heteroduplexes rather than homoduplexes.

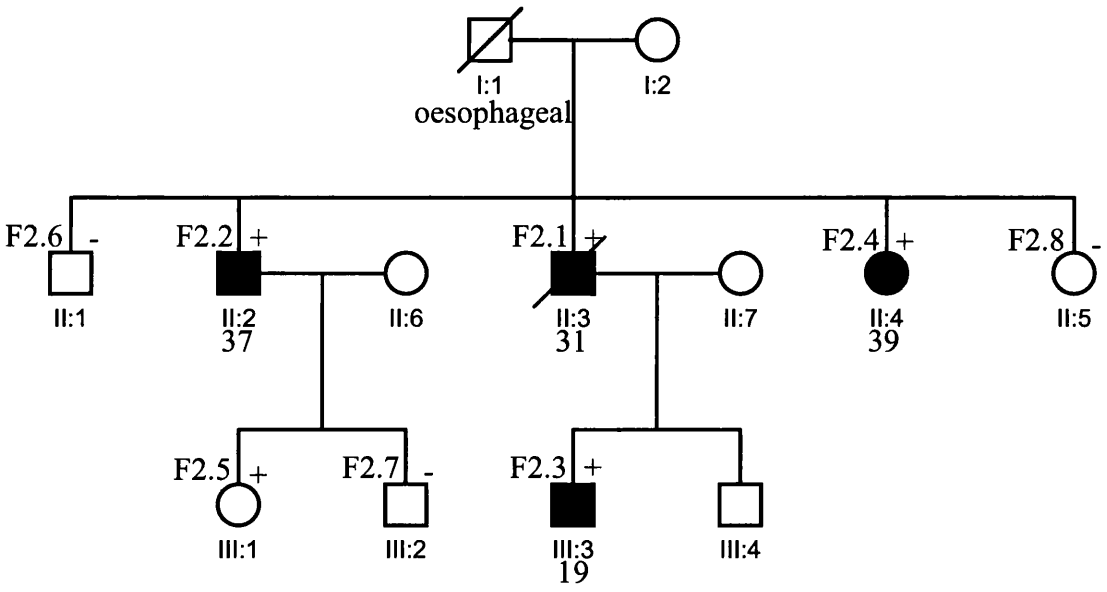
4.11 The R24P Mutation

4.11.1 Mutation Analysis in Family 2 (Figure 4.15)

Family 2 has four affected members with melanoma, three male and one female, who presented with melanoma aged 19, 31, 37 and 39 years. There is also a member of this family with oesophageal cancer.

FIGURE 4.15

Scottish Melanoma Family 2



4.11.2 Identification of a Chromatogram WAVE Shift

Exons 1α and 2 of the proband, individual F2.1 were amplified and subjected to dHPLC analysis (Figure 4.16). A chromatogram WAVE shift from the normal was identified in exon 1α.

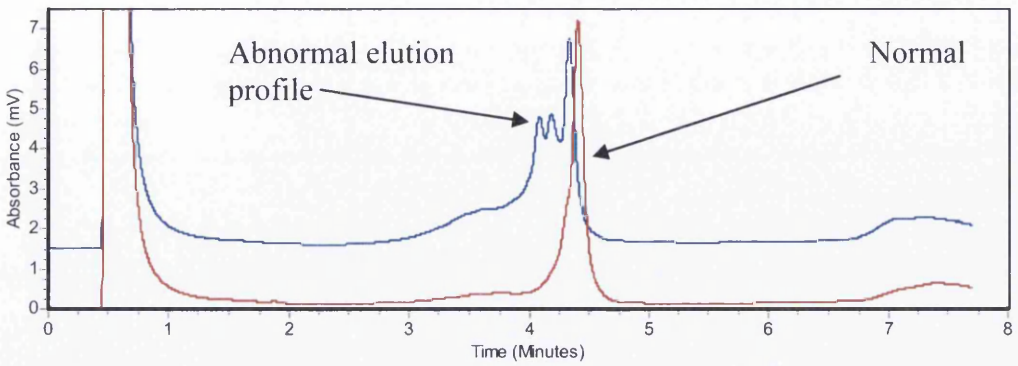
4.11.3 Identification of Mutation R24P

A new exon 1α amplicon was generated for direct sequencing of the proband using both forward and reverse exon 1α primers.

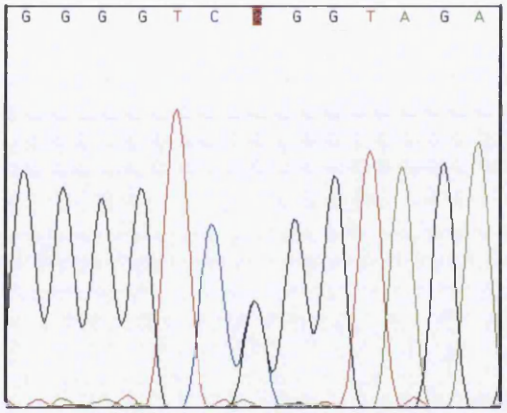
A heterozygous G to C transition at nucleotide position 71G>C was identified (Figure 4.16). This base change is predicted to lead to the substitution of arginine (R) for a proline (P) at codon 24 (R24P) (Figure 4.16). This mutation has been previously reported (Monzon et al. 1998; MacKie et al. 1998). Exon 1α from each of the family members was screened by direct sequencing. The predicted substitution observed in the proband was also found in the affected family members F2.2, F2.3 and F2.4, but no substitution was present in the currently unaffected family members F2.6, F2.7 and F2.8. The presence of the substitution in the affected individuals and not in unaffected individuals increases the chance that this identifies a pathological mutation. The predicted substitution was also identified in currently unaffected individual F2.5.

FIGURE 4.16

Family 2 – Identification of Mutation R24P (71G>C)



dHPLC WAVE analysis of PCR amplified genomic DNA from individual F2.1. Proband DNA was amplified using intronic primer pair *CDKN2A* exon 1α (Appendix 1, 2).



Sequence analysis of PCR amplified genomic DNA from individual F2.1. Proband DNA was amplified and sequenced using intronic primer pair *CDKN2A* exon 1α (Appendix 1, 2).

	R	G	**R**	V	E
Wild Type	CGG	GGT	C**G**G	GTA	GAG
Mutant	CGG	GGT	C**C**G	GTA	GAG
			P		

(R24P)

Nucleic acid and derived amino acid sequences from the mutated region of *CDKN2A*. The amino acid substitution of an arginine (R) for a proline (P) at position 24, R24P is shown.

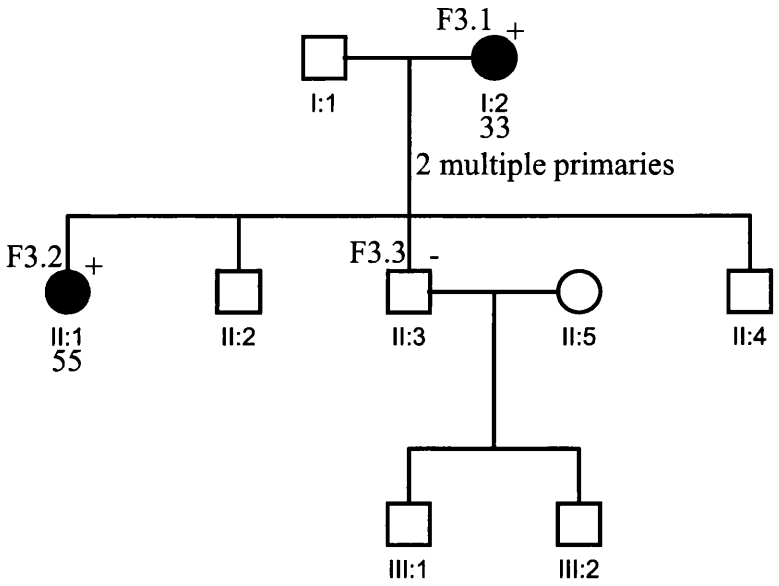
4.12 The Y44stop Mutation

4.12.1 Mutation Analysis in Family 3 (Figure 4.17)

Family 3 has two affected members with melanoma, both female, who presented with melanoma aged 33 and 55 years. Individual F3.1 has multiple primary melanomas.

FIGURE 4.17

Scottish Melanoma Family 3



4.12.2 Identification of a Chromatogram WAVE Shift

Exons 1α and 2 of the proband, individual F3.1 were amplified and subjected to dHPLC analysis (Figure 4.18). A chromatogram WAVE shift from the normal was identified in exon 1α.

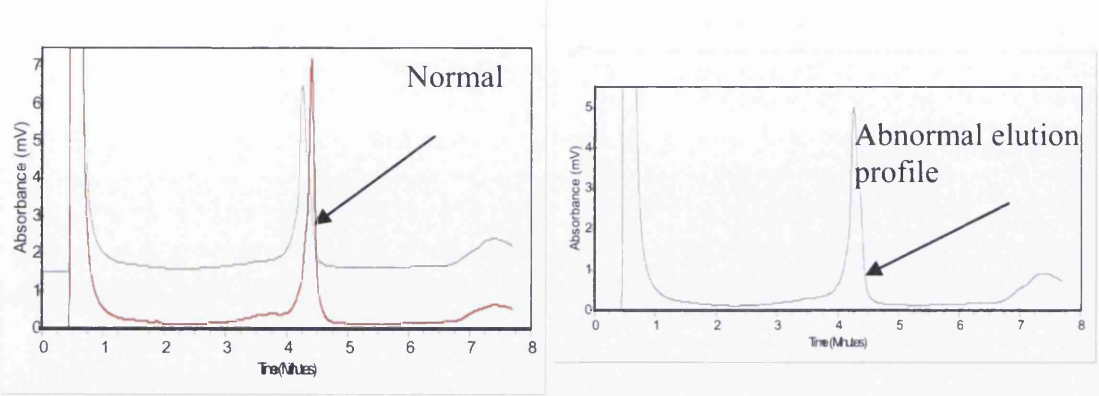
4.12.3 Identification of Mutation Y44stop

A new exon 1α amplicon was generated for direct sequencing of the proband using both forward and reverse exon 1α primers.

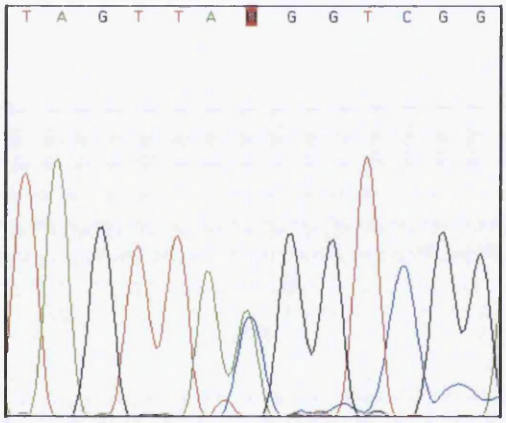
A heterozygous C to A transition at nucleotide position 132C>A was identified (Figure 4.18). This base change is predicted to lead to the substitution of tyrosine (Y) for a stop codon at codon 44 (Y44stop) (Figure 4.18). This mutation has been previously reported (MacKie et al. 1998). Exon 1α from each of the family members was screened by direct sequencing. The predicted substitution observed in the proband was also found in the affected family member F3.2, but no substitution was present in the currently unaffected family member F3.3. The presence of the substitution in the affected individuals and not in unaffected individual increases the chance that this identifies a pathological mutation.

FIGURE 4.18

Family 3 – Identification of Mutation Y44stop (132C>A)



dHPLC WAVE analysis of PCR amplified genomic DNA from individual F3.1. Proband DNA was amplified using intronic primer pair *CDKN2A* exon 1α (Appendix 1, 2).



Sequence analysis of PCR amplified genomic DNA from individual F3.1. Proband DNA was amplified and sequenced using intronic primer pair *CDKN2A* exon 1α (Appendix 1, 2).

	N	S	**Y**	G	R
Wild Type	AAT	AGT	TA**C**	GGT	CGG
Mutant	AAT	AGT	TA**A**	GGT	CGG
			R		

(Y44stop)

Nucleic acid and derived amino acid sequences from the mutated region of *CDKN2A*. The amino acid substitution of a tyrosine (Y) for a stop codon at position 44, Y44stop is shown.

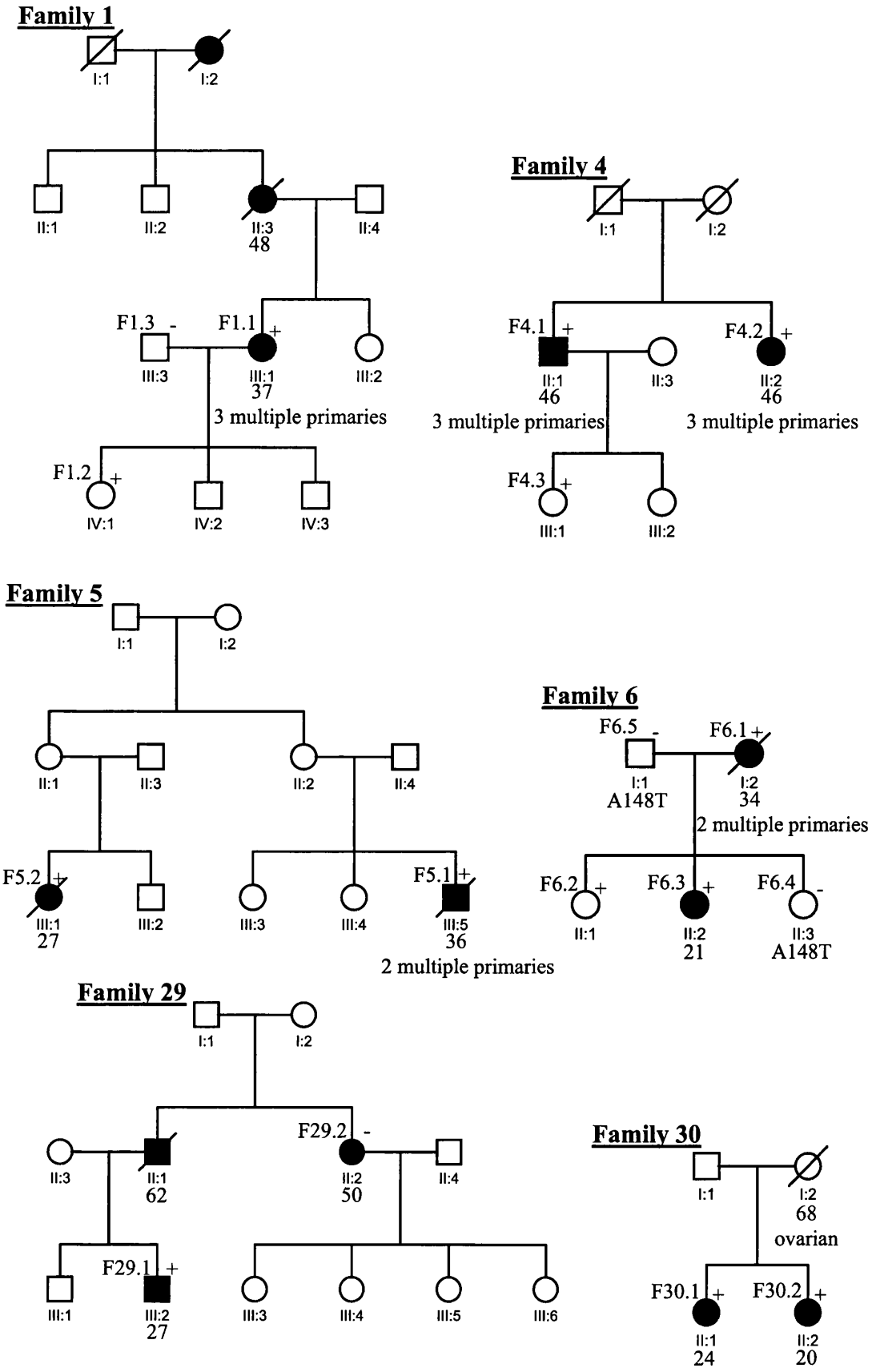
4.13 The M53I Mutation

4.13.1 Mutation Analysis in Families 1, 4, 5, 6, 29 and 30 (Figure 4.19)

Family 1 has three affected members with melanoma, all female, two of whom presented with melanoma at 37 and 48 years of age. Individual III:1 (F1.1) has multiple primary melanomas. Family 4 has two affected members with melanoma, one male and one female, who both presented with melanoma at 46 years of age. Both individuals have multiple primary melanomas. Family 5 has two affected members with melanoma, one male and one female, who presented with melanoma at 27 and 36 years of age. Individual III:5 (F5.1) has multiple primary melanomas. Family 6 has two affected members with melanoma, both female, who presented with melanoma aged 21 and 34 years. Individual I:2 (F6.1) has multiple primary melanomas. Family members F6.4 and F6.5 who do not have melanoma do however carry the polymorphism A148T as described on page 105. Family 29 has three affected members with melanoma, two male and one female, who presented with melanoma aged 27, 50 and 62 years. Family 30 has two affected members with melanoma, both female, who presented with melanoma aged 20 and 24 years. There is also a member of this family with ovarian cancer.

FIGURE 4.19

Scottish Melanoma Families 1, 4, 5, 6, 29 and 30



4.13.2 Identification of a Chromatogram WAVE Shift

Exons 1α and 2 of the probands, individuals F1.1, F4.1, F5.1, F6.1, F29.1 and F30.1 were amplified and subjected to dHPLC analysis (Figure 4.20). Chromatogram WAVE shifts from the normal were identified in exon 2.

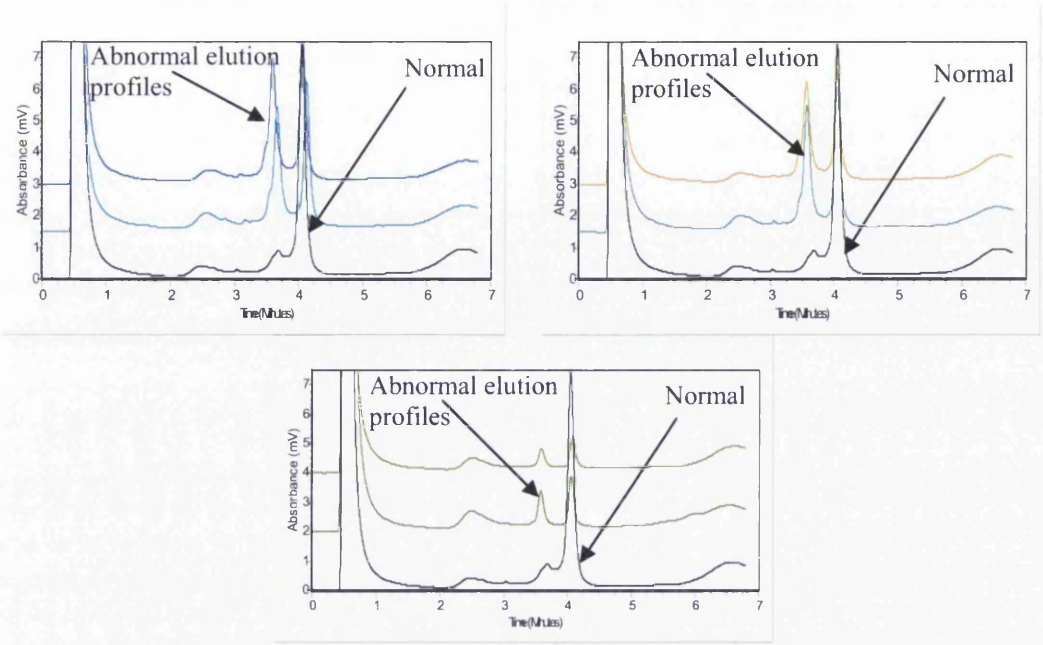
4.13.3 Identification of Mutation M53I

New exon 2 amplicons were generated for direct sequencing of the probands using both forward and reverse exon 2 primers.

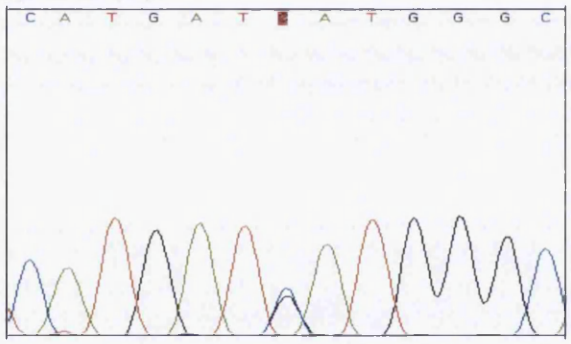
A heterozygous G to C transition at nucleotide position 159G>C was identified (Figure 4.20). This base change is predicted to lead to the substitution of methionine (M) for an isoleucine (I) at codon 53 (M53I) (Figure 4.20). This mutation has been previously reported (Harland et al. 1997; MacKie et al. 1998; Monzon et al. 1998; Pollock et al. 1998). Exon 2 from each of the family members was screened by direct sequencing. The predicted substitution observed in the probands was also found in the affected family members F4.2, F5.2, F6.3 and F30.2, but no substitution was present in the currently unaffected family members F1.3, F6.4 and F6.5. The presence of the substitution in the affected individuals and not in unaffected individuals increases the chance that this identifies a pathological mutation. The predicted substitution was also identified in currently unaffected individuals F1.2, F4.3 and F6.2 but it was not identified in affected individual F29.2 (see cloning method 3.15).

FIGURE 4.20

Families 1, 4, 5, 6, 29 and 30 – Identification of Mutation M53I (159G>C)



dHPLC WAVE analysis of PCR amplified genomic DNA from individuals F1.1, F4.1, F5.1, F6.1, F29.1 and F30.1. Proband DNA was amplified using intronic primer pair *CDKN2A* exon 2 (Appendix 1, 2).



Sequence analysis of PCR amplified genomic DNA from individuals F1.1, F4.1, F5.1, F6.1, F29.1 and F30.1. Proband DNA was amplified and sequenced using intronic primer pair *CDKN2A* exon 2 (Appendix 1, 2).

	V	M	**M**	M	G
Wild Type	GTC	ATG	AT**G**	ATG	GGC
Mutant	GTC	ATG	AT**C**	ATG	GGC
			I		

(M53I)

Nucleic acid and derived amino acid sequences from the mutated region of *CDKN2A*. The amino acid substitution of a methionine (M) for an isoleucine (I) at position 53, M53I is shown.

4.13.4 Cloning Analysis of F29.2

Mutation M53I, which was identified in individual F29.1, was screened for in the other affected family members. No DNA was available however from the deceased II:1. Surprisingly the mutation was not identified in affected family member F29.2.

To rule out sample mix-up, a fresh blood sample was taken from both individuals F29.1 and F29.2, and once again the mutation was present in affected individual F29.1 but not in affected family member F29.2. Unfortunately in the exact place where the mutation should be in individual F29.2 a "dye blob" would appear on the chromatogram. "Dye blobs" are unincorporated dye terminator molecules that have remained in the sample by passing through the cleanup columns with the purified DNA. They make broad peaks at various points in the chromatogram, although "dye blobs" tend to be worst near the beginning of a read. In this case the "dye blob" would always appear over the mutation site no matter what purifying method was used to remove the unincorporated terminators, which meant that it was difficult to fully interpretate the electropherogram. Therefore the PCR amplicons of individual F29.2 were cloned using the TOPO TA Cloning Kit for sequencing (Method 3.15) to determine exactly whether or not affected individual F29.2 carried the familial mutation. TA Cloning was the chosen approach to obtain experience in this technique.

The primers used for the amplification of the fragment containing exon 2 were the same set which were designed for independent amplification of the complete coding region of exon 2 and its splice sites as previously described. The final amplification conditions are presented in Appendix 2. A 2µl aliquot of DNA (extracted as described using method 3.1d) from patient F29.2 was amplified with the set of primers in a 50µl PCR reaction. A fragment size of 458bp for exon 2 was generated. The single discrete band obtained from amplification was resolved on an ethidium bromide stained 1.2% agarose gel to verify the size of the amplified band. In parallel with cloning of the patient's DNA, 1µl of control DNA, which was supplied with the kit, was also amplified. The supplied amplification conditions are presented in Appendix 2. A fragment size of 750bp was generated. The single discrete band obtained from

amplification was resolved on an ethidium bromide stained 0.8% agarose gel to verify the size of the amplified band.

Seven LB plates were prepared, six of which contained 50μg/ml of kanamycin and one that contained 100μg/ml of ampicillin. The kanamycin plates were used for the control transformation of vector only and vector plus insert and also for the transformation of the patient DNA. An ampicillin plate was only used for the pUC19 transformation because the control template is a plasmid that encodes an ampicillin resistance gene and transformants carrying this plasmid will also be ampicillin resistant, therefore upon analysis colonies would not contain the desired construct. The transformation control is a pUC19 plasmid, which checks the transformation efficiency of the competent cells. Sodium chloride and magnesium chloride of a concentration of 200mM NaCl, 10mM MgCl2 was added to the chemically competent *E. coli.* Inclusion of salt allows for longer incubation times because it prevents topoisomerase I from rebinding and potentially nicking the DNA after ligating the PCR product and dissociating from the DNA. The result is more intact molecules leading to higher transformation efficiencies.

While an efficient cloning reaction should produce hundreds of colonies, the cells used were old and few colonies grew well. There were only about 30 colonies on the ampicillin pUC19 transformation plate and therefore the transformation efficiency of the competent cells was quite poor. There were no colonies on the kanamycin vector-only reaction plates. There were about 160 and 150 white colonies on the kanamycin vector plus PCR insert reaction plates, indicating that they contained the 750bp insert. There were about 105 and 60 white colonies on the kanamycin vector plus PCR patient DNA insert reaction plates, indicating that they contained the 458bp exon 2 insert. Ten white colonies, five from each plate, were picked for analysis. DNA was extracted from the colonies using the QIAprep Spin Miniprep Kit and then sequenced in both directions. All ten colonies in both forward and reverse directions did not carry any mutation and therefore conclude that melanoma patient F29.2 must be a sporadic case of melanoma in family 29.

4.13.5 Development of a Restriction Test

The 159G>C mutation creates a *Bcl* I site when the mutant C base is present in amplified product (Figure 4.21a).

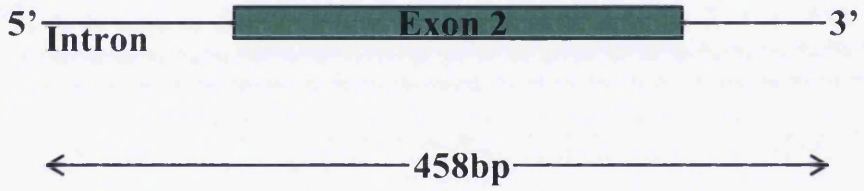
Digestion with *Bcl* I, produces full fragment sizes of 374bp and 84bp in affected individuals F1.1, F4.1, F5.1, F6.1, F29.2 and F30.1, see lanes 2-7 (Figure 4.21b). The normal allele (458bp) and the undigested normal allele (458bp) are both present, see lanes 8-10.

FIGURE 4.21

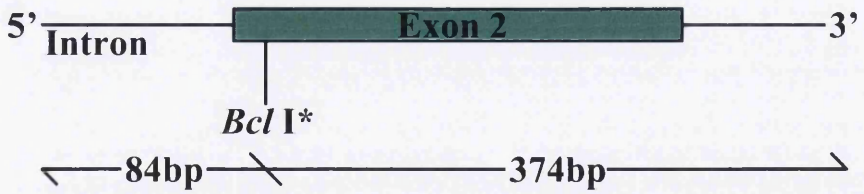
Development of a *Bcl* I Restriction Site Created by Mutation M53I

a) Diagrammatic representation of the 458bp PCR amplified fragment of exon 2 of *CDKN2A*. The mutation creates a *Bcl* I restriction site 84bp into the PCR fragment in the presence of the 159G>C substitution.

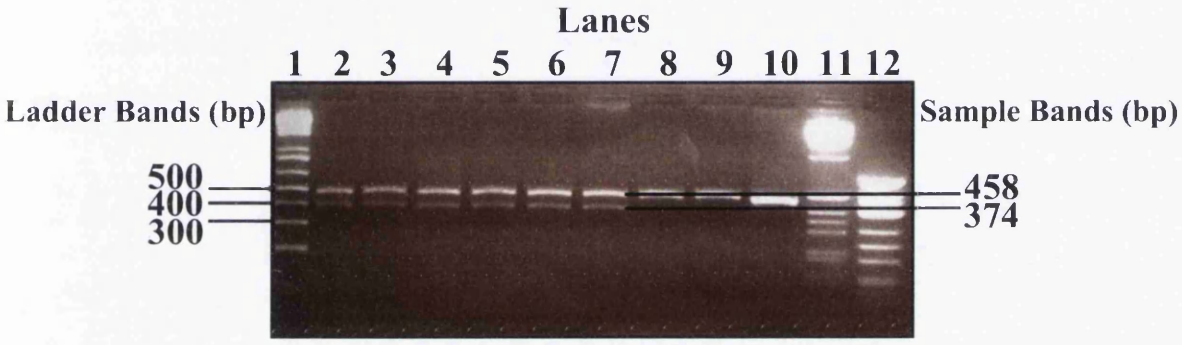
Normal (m53)



Mutant (I53)



b) The expected 458bp and 374bp DNA fragments from the *Bcl* I digested amplicons were confirmed by resolution on a 3% agarose gel with ethidium bromide. The amplicons were electrophoresed in 1X TAE buffer for 20 minutes. The size of the digested amplicons which are represented below shown in base pairs were deduced by comparison to the migration of the known size of the DNA ladder fragments in lanes 1, 11 and 12.



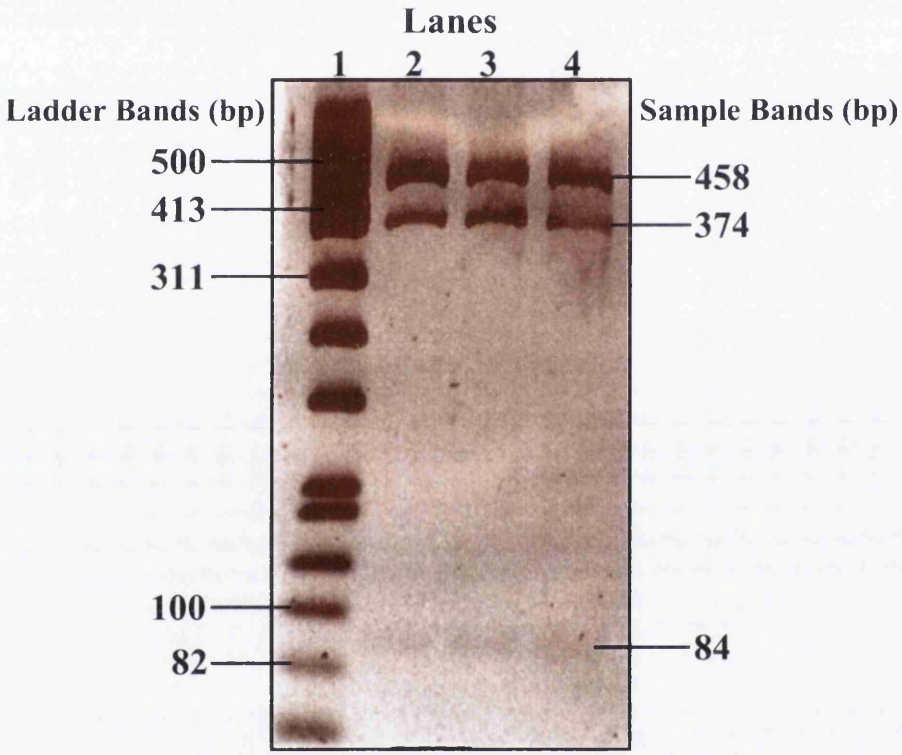
Electrophoresis Conditions: 3% agarose gel, 1X TAE, 10µl size markers, 5µl digest, 2µl loading mix, 100V, 20 minutes, room temperature

Lane 1 – 1kb Plus size marker
Lane 2 – digested PCR product from individual F1.1
Lane 3 – digested PCR product from individual F4.1
Lane 4 – digested PCR product from individual F5.1
Lane 5 – digested PCR product from individual F6.2
Lane 6 – digested PCR product from individual F29.1
Lane 7 – digested PCR product from individual F30.1
Lane 8 – digested PCR product from individual F6.3
Lane 9 – digested PCR product from individual F29.2
Lane 10 – undigested PCR product from a normal control
Lane 11 – 1kb size marker
Lane 12 – PhiX174/*Hinf* I size marker

See page 60 for patient details.

The 84bp fragment is unresolved on the 3% agarose gel; therefore the following 6% polyacrylamide gel was prepared to further resolve the bands.

c) The expected 458bp, 374bp and 84bp DNA fragments from the *Bcl* I digested amplicons were confirmed by resolution on a 6% polyacrylamide gel with ethidium bromide. The amplicons were electrophoresed in 1X TBE buffer for 10 minutes. The size of the digested amplicons which are represented below shown in base pairs were deduced by comparison to the migration of the known size of the DNA ladder fragments in lane 1.



Electrophoresis Conditions: 6% polyacrylamide gel, 1X TBE, 10µl size marker, 5µl digest, 2µl loading mix, 100V, 20 minutes, room temperature

Lane 1 – PhiX174/*Hinf* I size marker

Lane 2 – digested PCR product from individual F1.1

Lane 3 – digested PCR product from individual F4.1

Lane 4 – digested PCR product from individual F5.

See page 60 for patient details.

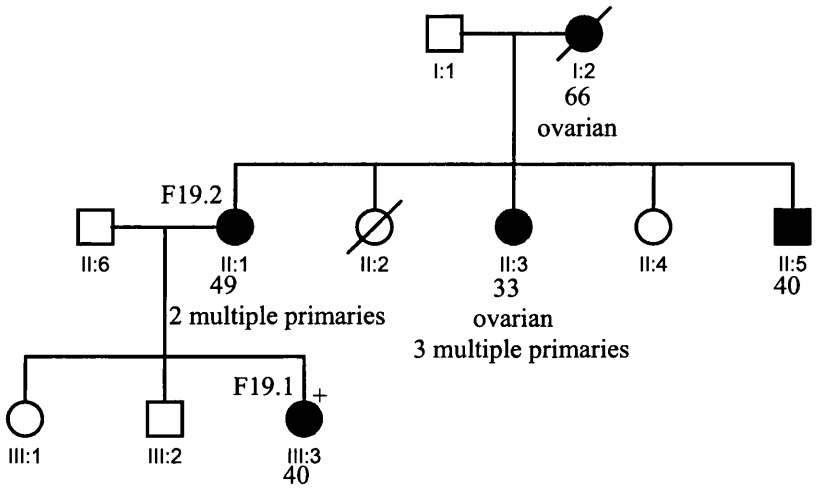
4.14 The G67R Mutation

4.14.1 Mutation Analysis in Family 19 (Figure 4.22)

Family 19 has five affected members with melanoma, four female and one male, who presented with melanoma at 33, 2 x 40, 49 and 66 years of age. Individuals II:1 (F19.2) and II:3 have multiple primary melanomas. There are also two members of this family with ovarian cancer.

FIGURE 4.22

Scottish Melanoma Family 19



4.14.2 Identification of a Chromatogram WAVE Shift

Exons 1α and 2 of the proband, individual F19.1 were amplified and subjected to dHPLC analysis (Figure 4.23). A chromatogram WAVE shift from the normal was identified in exon 2.

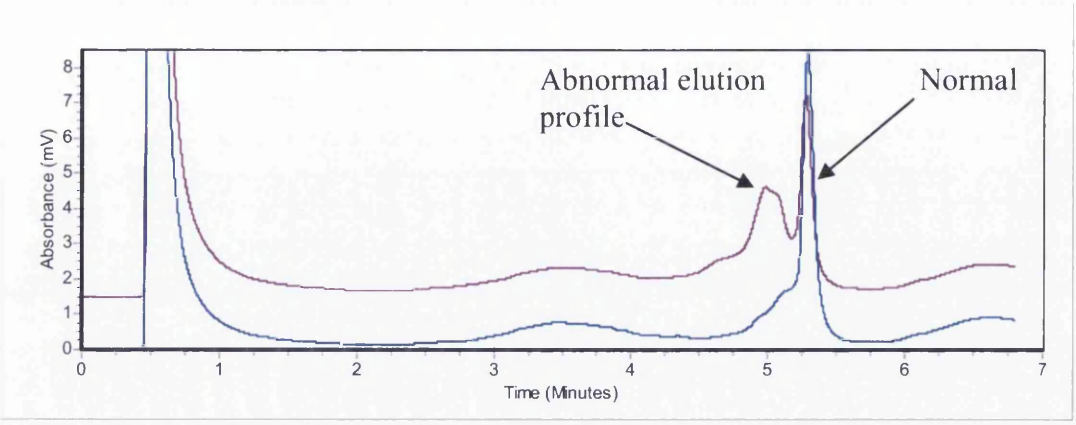
4.14.3 Identification of Mutation G67R

A new exon 2 amplicon was generated for direct sequencing of the proband using both forward and reverse exon 2 primers.

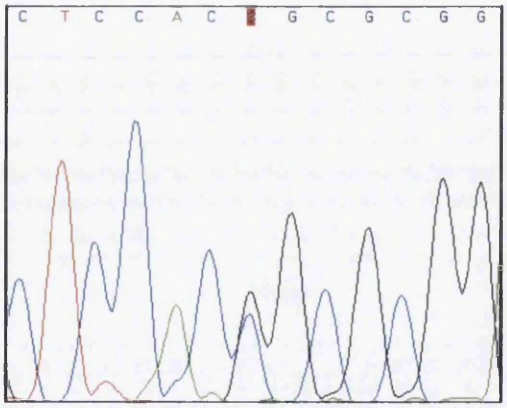
A heterozygous G to C transition at nucleotide position 199G>C was identified (Figure 4.23). This base change is predicted to lead to the substitution of glycine (G) for an arginine (R) at codon 67 (G67R) (Figure 4.23). This mutation has previously been reported in and English family (Newton Bishop et al. 1999). No other samples from family members were available to be screened.

FIGURE 4.23

Family 19 – Identification of Mutation G67R (199G>C)



dHPLC WAVE analysis of PCR amplified genomic DNA from individual F19.1. Proband DNA was amplified using intronic primer pair *CDKN2A* exon 2 (Appendix 1, 2).



Sequence analysis of PCR amplified genomic DNA from individual F19.1. Proband DNA was amplified and sequenced using intronic primer pair *CDKN2A* exon 2 (Appendix 1, 2).

	L	H	G	A	E
Wild Type	CTC	CAC	GGC	GCG	GAG
Mutant	CTC	CAC	CGC	GCG	GAG
			R		

(G67R)

Nucleic acid and derived amino acid sequences from the mutated region of *CDKN2A*. The amino acid substitution of a glycine (G) for an arginine (R) at position 67, G67R is shown.

4.15 The R112G Mutation

4.15.1 Mutation Analysis in Families 17 and 22 (Figure 4.24)

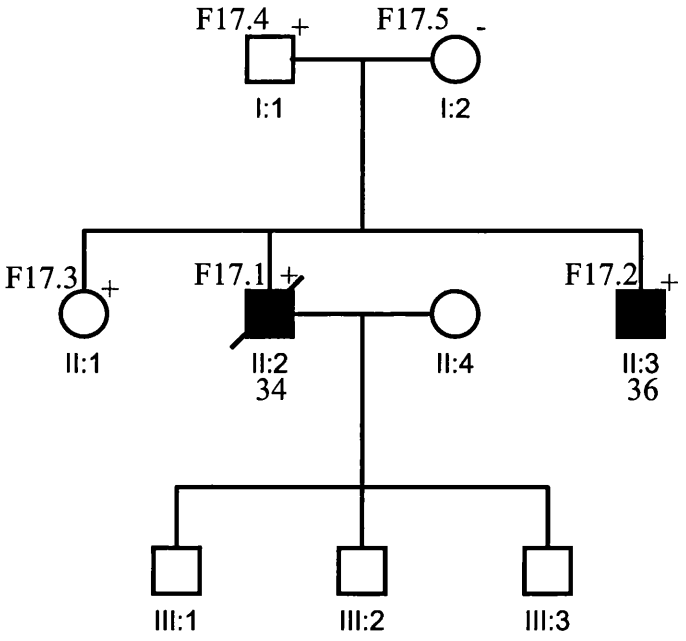
Family 17 has two affected male members with melanoma, who presented with melanoma at 34 and 36 years of age. Family 22 has three affected members with melanoma, two of whom are male and one is female, they presented with melanoma at 29, 33 and 38 years of age.

4.15.2 Identification of a Chromatogram WAVE Shift

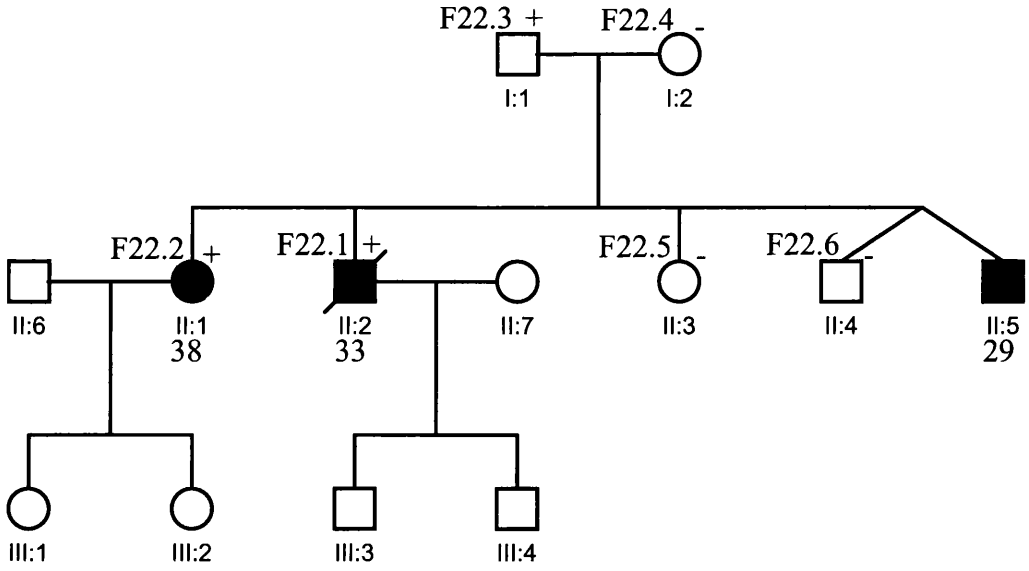
Exons 1α and 2 of the probands, individuals F17.1 and F22.1 were amplified and subjected to dHPLC analysis (Figure 4.25). Chromatogram WAVE shifts from the normal were identified in exon 2.

FIGURE 4.24

Scottish Melanoma Family 17



Scottish Melanoma Family 22



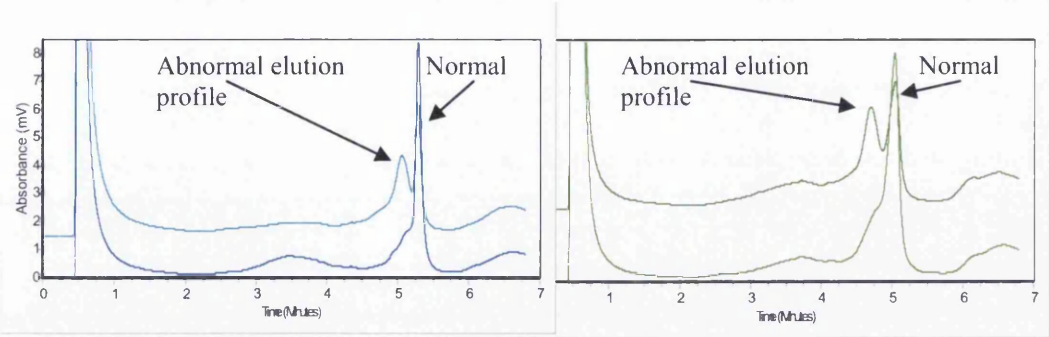
4.15.3 Identification of Mutation R112G

New exon 2 amplicons were generated for direct sequencing of the probands using both forward and reverse exon 2 primers.

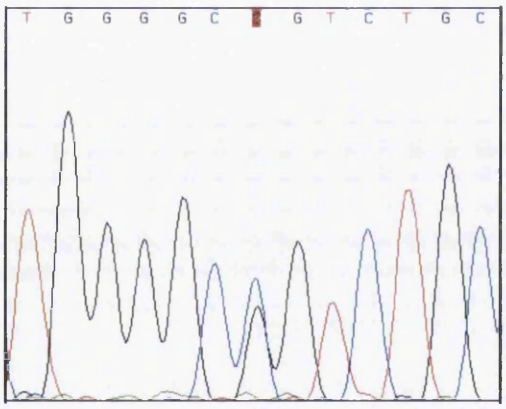
A heterozygous C to G transition at nucleotide position 334C>G was identified (Figure 4.25). This base change is predicted to lead to the substitution of arginine (R) for a glycine (G) at codon 112 (R112G) (Figure 4.25). This mutation has been previously reported (Holland et el. 1999). Exon 2 from each of the family members was screened by direct sequencing. The predicted substitution observed in the probands was also found in the affected family members F17.2 and F22.2, but no substitution was present in the currently unaffected family members F17.5, F22.4, F22.5 and F22.6. The presence of the substitution in the affected individuals and not in unaffected individuals increases the chance that this identifies a pathological mutation. The predicted substitution was also identified in currently unaffected individuals F17.3, F17.4 and F22.3.

FIGURE 4.25

Families 17 and 22 – Identification of Mutation R112G (334C>G)



dHPLC WAVE analysis of PCR amplified genomic DNA from individuals F17.1 and F22.1. Proband DNA was amplified using intronic primer pair *CDKN2A* exon 2 (Appendix 1, 2).



Sequence analysis of PCR amplified genomic DNA from individuals F17.1 and F22.1. Proband DNA was amplified and sequenced using intronic primer pair *CDKN2A* exon 2 (Appendix 1, 2).

	W	G	**R**	L	P
Wild Type	TGG	GGC	**C**GT	CTG	CCC
Mutant	TGG	GGC	**G**GT	CTG	CCC

G

(R112G)

Nucleic acid and derived amino acid sequences from the mutated region of *CDKN2A*. The amino acid substitution of an arginine (R) for a glycine (G) at position 112, R112G is shown.

4.16 The A148T Suspected Polymorphism

4.16.1 Mutation Analysis in Families 7 and 13

Family 7 has two affected members with melanoma, one male and one female, who presented with melanoma aged 43 and 45 years. Family 13 has two male affected members with melanoma, who presented with melanoma aged 34 and 36 years. As reported on page 122, two unaffected members of family 6 (F6.4 and F6.5) also carry this polymorphism.

4.16.2 Identification of a Chromatogram WAVE Shift

Exons 1α and 2 of the probands, individuals F7.1 and F13.1 were amplified and subjected to dHPLC analysis (Figure 4.26). Chromatogram WAVE shifts from the normal were identified in exon 2.

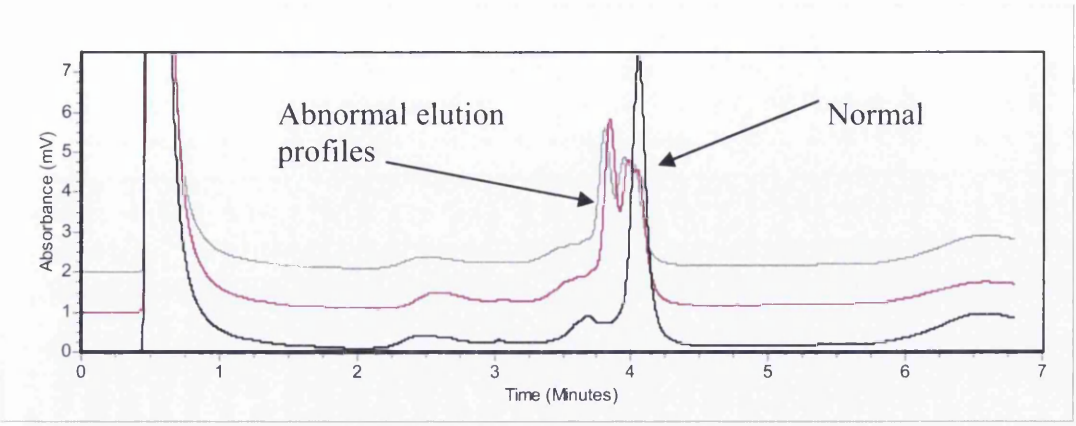
4.16.3 Identification of the Suspected Polymorphism A148T

New exon 2 amplicons were generated for direct sequencing of the probands using both forward and reverse exon 2 primers.

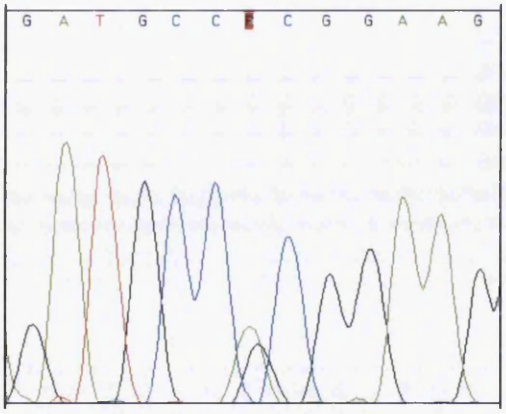
A heterozygous G to A transition at nucleotide position 442G>A was identified (Figure 4.26). This base change is predicted to lead to the substitution of alanine (A) for a threonine (T) at codon 148 (A148T) (Figure 4.26). This mutation has been previously reported as a polymorphism (Hussussian et al. 1994). Exon 2 from each of the family members was screened by direct sequencing. The predicted substitution observed in the probands was not present in the currently unaffected family members F6.2 and F7.2. The presence of the substitution in the affected individuals and not in unaffected individuals increases the chance that this identifies a pathological mutation. However, the predicted substitution was also identified in currently unaffected individuals F6.4, F6.5 and F13.3 but it was not identified in affected individual F6.1, F6.3, and F13.2, which leads us to believe that this predicted substitution is a polymorphism.

FIGURE 4.26

Families 7 and 13 – Identification of Mutation A148T (442G>A)



dHPLC WAVE analysis of PCR amplified genomic DNA from individuals F7.1 and F13.1. Proband DNA was amplified using intronic primer pair *CDKN2A* exon 2 (Appendix 1, 2).



Sequence analysis of PCR amplified genomic DNA from individuals F7.1 and F13.1. Proband DNA was amplified and sequenced using intronic primer pair *CDKN2A* exon 2 (Appendix 1, 2).

	D	A	**A**	E	G
Wild Type	GAT	GCC	**G**CG	GAA	GGT
Mutant	GAT	GCC	**A**CG	GAA	GGT

T

(A148T)

Nucleic acid and derived amino acid sequences from the mutated region of *CDKN2A*. The amino acid substitution of an alanine (A) for a threonine (T) at position 148, A148T is shown.

4.17 Multiple Primary Melanomas

Approximately 5% of all melanoma patients develop additional primary melanomas, which suggests that a genetic predisposition or de novo mutations in predisposing genes may be involved with multiple primary melanomas (MPMs). This study therefore also included the identification of possible *CDKN2A* mutations in 13 cases of MPM with familial melanoma and 11 sporadic melanoma cases (Tables 4.2, 4.3). There was no available DNA for individual F19.2 who has two MPMs.

TABLE 4.2

Multiple Primary Melanomas – Patients with Familial Melanoma

Family Identifier	*CDKN2A* Mutation	Number of Primary Melanomas	Age of Diagnosis
F1.1	M53I	3	37
F3.1	Y44stop	2	33
F4.1	M53I	3	46
F4.2	M53I	3	46
F5.1	M53I	2	36
F6.1	M53I	2	34
F11.2	None detected	2	33
F28.2	24bp Duplication	3	34
F33.1	H83N	4	28
F33.2	H83N	3	25
F42.1	None detected	3	21
F43.1	None detected	3	43
F43.2	None detected	2	19

TABLE 4.3

Multiple Primary Melanomas – Patients with Sporadic Melanoma

Sporadic Identifier	*CDKN2A* Mutation	Number of Primary Melanomas	Age of Diagnosis
SMP1	M53I	5	31
SMP2	None detected	5	38
SMP3	M53I	9	23
SMP4	None detected	3	71
SMP5	None detected	3	33
SMP6	None detected	3	51
SMP7	-30G>T	3	64
SMP8	None detected	4	65
SMP9	None detected	5	62
SMP10	None detected	4	74
SMP11	None detected	4	50

Germline mutations in the *CDKN2A* gene were identified in 12/24 (50%) of MPM cases, nine with a family history of melanoma and three sporadic cases. Four of the ten families with MPM have the M53I mutation, one family has the Y44stop mutation, one family has the exon 1α 32-33ins9-32 24base pair duplication, one family has the H83N mutation, and the remaining three families have no detectable mutation. Three sporadic melanoma patients with MPM have *CDKN2A* mutations. Two individuals have the M53I mutation and one individual has a previously unreported –30G>T mutation. The remaining eight patients with sporadic melanoma and MPM have no detectable mutation.

The most frequently identified mutation in patients with MPM was M53I, found in five familial cases and in two sporadic cases. *CDKN2A* mutations were more frequent in those patients with a family history of melanoma (9/13 69.2%) than in those without a family history (3/11 27.3%). In the patients with a *CDKN2A* mutation the average number of primary melanomas was 3.5 and in those patients without a detected *CDKN2A* mutation it was slightly lower at 3.4. The mean age of diagnosis in the patients with a *CDKN2A* mutation was at 36.4 years and in those patients without a detected *CDKN2A* mutation it was older at 46.7 years.

4.18 The use of Molecular Modelling to Identify Mutation Sites and their Possible Effects on Pathological Function

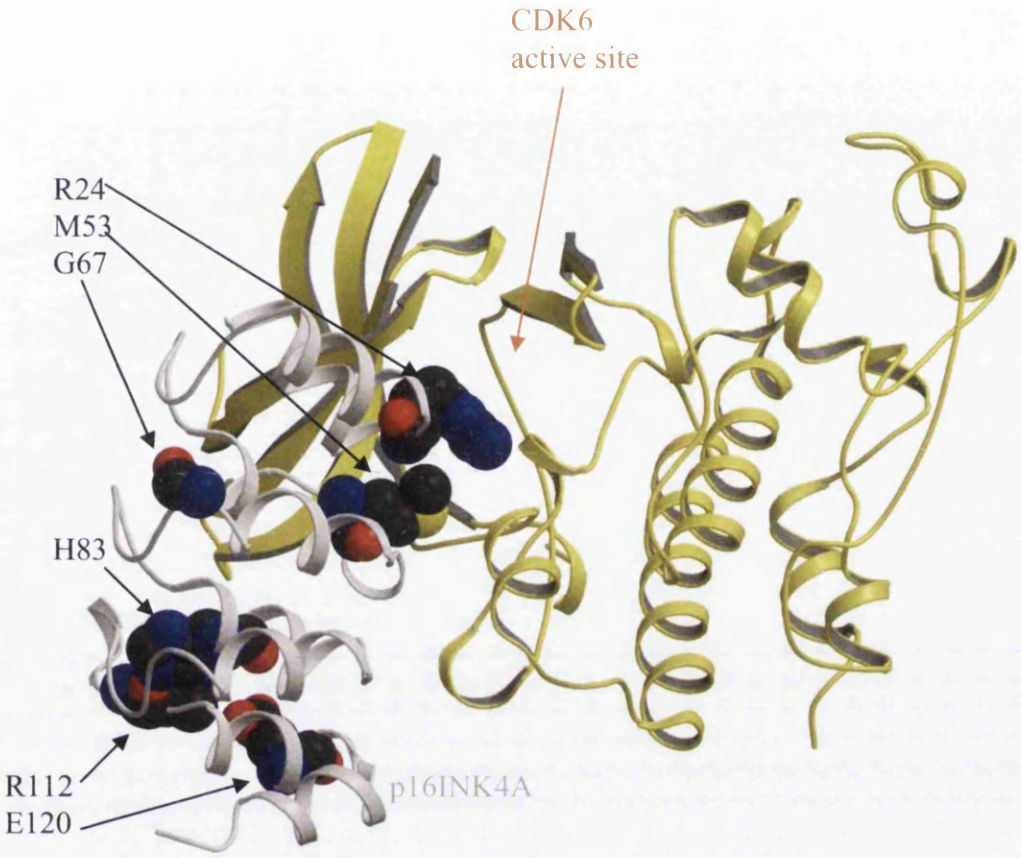
To investigate the potential effect of the newly identified H83N *CDKN2A* mutation and the potential effect of the G67R mutation with unknown function effects, the consequences of these mutations were investigated at the molecular level by consideration of the crystal structure of the CDK6-p16 complex. This work was carried out in collaboration with Professor Hunter, Division of Biological Chemistry and Molecular Microbiology at the School of Life Sciences, University of Dundee (Method 3.16).

The computer graphics program "O" was used to visualize the structure of p16, the interactions formed between this tumour suppressor and the protein kinase CDK6 and how the mutations were predicted to affect the structure and interactions.

The analysis could only be carried out for the CDK6 complexes, as the crystalline structure for CDK4 is not yet available. The structure of CDK6 resembles other ATP-dependent protein kinases and consists of an N-terminal and a C-terminal domain with the active site formed in a crevice between these two lobes (Figure 4.27). The kinase inhibitor p16INK4A consists of four ankyrin repeats, a 33-amino acid segment that folds into a β-hairpin-helix-loop-helix motif (Zhang and Peng 2000) and is a characteristic unit implicated in many protein-protein associations. The tumour suppressor p16INK4A binds adjacent to the ATP-binding active site of CDK6 and interacts with both N and C-terminal domains to distort the kinase structure. Binding of the suppressor prevents the kinase-cyclin associations required for progression through the cell cycle.

FIGURE 4.27

Ribbon Representation of p16INK4A and CDK6



The computer graphics program "O" was used to visualize the p16-CDK6 complex. The kinase is depicted as yellow ribbons, the tumour suppressor as grey. Six residues from p16INK4A, discussed in text, as shown as van der Waals radii coloured according to atom type: C black, N blue, O red and S yellow. The single letter amino acid code is used in labelling.

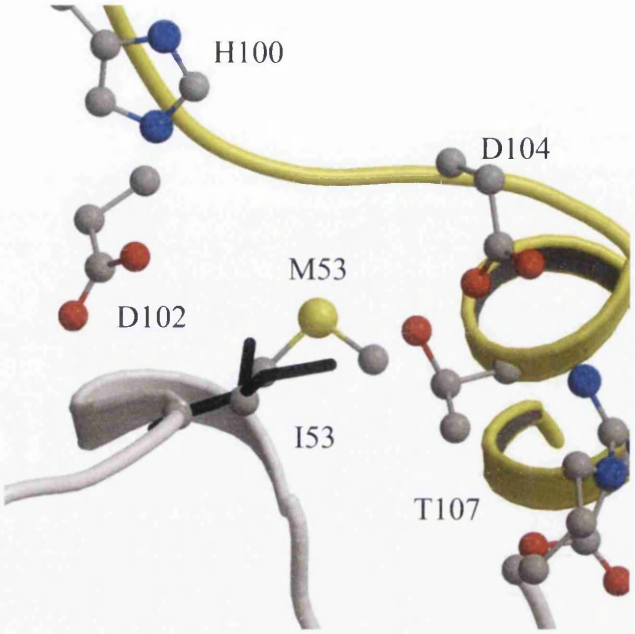
A newly reported mutation H83N is presented in this thesis. H83 is buried within the core of p16INK4A and the side chain of histidine participates in three hydrogen bonds to different parts of the tumour suppressor molecule main chain (the carbonyl groups of V196 and R112, and the amide group of R80) that serve to stabilise the conformation of two loops that interact with residues 31 to 38 of CDK6. Replacing the basic histidine with asparagine would ensure the loss of at least one hydrogen bond within the core of the molecule, which might destabilise the p16INK4A structure.

The side chain of residue R24 of p16INK4A forms hydrogen bond interactions with Q149 and E104 of CDK6. The crystal structure shows the side chain of Q149 placed so that the NE2 group contacts the R24 guanidinium group, however it is more likely that it is the Q149 OE1 group that participates in the association. If R24 was mutated to P then this would remove the strong electrostatic interactions and reduce the affinity for complex formation between the two proteins.

Within 6°A of amino acid R24 is M53 (Figure 4.28). The side chain of this hydrophobic residue makes van der Waals interactions with the main chain atoms of D102, E103 and T107 of CDK6. A mutation to I would likely reduce the area of interaction and therefore be slightly disruptive of complex formation. Mutations to the R and M residues of either p16INK4A or p14ARF would be predicted to have similar effects on binding to either CDK4 or CDK6.

FIGURE 4.28

The Site of M53



Specific residues discussed in text, as shown as ball-and-stick models coloured according to atom type: C grey, N blue, O red and S yellow. The replacement of isoleucine for methionine is highlighted by representing the isoleucine as black sticks.

One mutation reported in this thesis is G67R, which has only been reported once before (Newton Bishop et al. 1999). G67 is situated on the opposite side of the p16INK4A inhibitor kinase interface and is not involved in the protein:protein contacts between the tumour suppressor and kinase. This residue is however an important component of the helix-turn-strand motif of the ankyrin repeats. It is situated at the end of the helix on a tight turn into the β-sheet. A mutation at this point of the protein may affect the secondary structure and perhaps the folding of the protein. Four ankyrin repeat units span exons 1 and 2 of *CDKN2A*, and are essential to p16INK4A binding and function. G67 is a strictly conserved amino acid and the first residue of the second ankyrin repeat and shares secondary structure alignment with G101 (not shown), the latter is a residue of proven significance to p16INK4A function (Hussussian et al. 1994; Kamb et al. 1994; Ciotti et al. 2000). Any mutation at this point would be likely to impair function as replacing glycine with arginine could influence the fold and

alter the position of adjacent helices, which taper down to the binding site with CDK6. This G67R substitution could also change the binding sites to other as yet unidentified proteins. A mutation at the same residue G67S has been observed previously (Holland et al., 1999) and is likely to affect p16INK4A function as shown by site-directed mutagenesis (Tevelev et al. 1996; Zhang and Peng 1996) by the described p16INK4A structure (Byeon et al. 1998) and also reduced binding to CDK4 (Rizos et al., 2001).

R112 makes a salt bridge interaction with E120, which helps to stabilise the structure of the loops that interact with CDK6. A mutation from R to G would disrupt this part of the structure and also complex formation.

While molecular modelling is an important lead, functional studies are still required to confirm the functional consequences of any mutation. We would like to determine whether the mutation inhibits cell cycle progression, if p16INK4A is still able to bind to CDK4 and whether pRB phosphorylation inhibition is reduced. We have therefore agreed collaboration with the University of Toronto who have functional assays to determine cell proliferation and protein-protein interactions and yeast-two-hybrid assays to evaluate functional importance as part of their battery of research techniques. We have supplied them with a sample of the H83N mutant DNA for testing.

4.19 Statistical Analysis of the Correlation between Phenotype and Familial Melanoma Patients with and without a *CDKN2A* Mutation

All 62 familial melanoma patients were assessed for their *CDKN2A* genotype with regard to skin type, eye and hair colour as previously described in 2.4.1. The phenotypic characteristics of all patients are summarised in table 2.1.

Statistical analysis was performed using the methods described in method 3.17.

No association between skin type, eye or hair colour and the presence or absence of a *CDKN2A* mutation within familial melanoma patients was observed in this data set (Tables 4.4-4.6). Due to the large quantity of results, all findings are reported in tabular form in Appendix 5 (Skin Type - tables 15, 18; Eye Colour - tables 14, 17; Hair Colour - tables 13, 16).

TABLE 4.4

Correlation between Skin Type and Familial Melanoma Patients with and without a *CDKN2A* Mutation

	Never Tans Always Burns	Burns Easily Tans Rarely	Burns Rarely Tans Easily	Never Burns Always Tans
***CDKN2A* Mutation Positive**	5	15	0	0
***CDKN2A* Mutation Negative**	10	29	3	0

P-value = 0.957 (not significant)

TABLE 4.5

Correlation between Eye Colour and Familial Melanoma Patients with and without a *CDKN2A* Mutation

	Blue	Green	Brown	Other
***CDKN2A* Mutation Positive**	15	1	1	3
***CDKN2A* Mutation Negative**	28	3	6	5

P-value = 0.713 (not significant)

TABLE 4.6

Correlation between Hair Colour and Familial Melanoma Patients with and without a *CDKN2A* Mutation

	Red	Blonde	Light Brown	Dark Brown/Black
***CDKN2A* Mutation Positive**	2	7	7	4
***CDKN2A* Mutation Negative**	8	8	14	12

P-value = 0.475 (not significant)

CHAPTER 5

STUDIES TO ESTABLISH A COMMON FOUNDER OF THE M53I MUTATION

5.1 Haplotyping of M53I Scottish Melanoma Families

The most common *CDKN2A* mutation identified in this thesis from the cohort of Scottish melanoma families is the M53I mutation. The M53I mutation was originally detected in an Australian melanoma kindred, and has now been reported in three additional Australian families, two American kindreds, two English kindreds and one family each in, Canada (Ontario) and France. Table 5.1 gives details of all currently reported M53I melanoma families worldwide.

TABLE 5.1

Currently Reported Melanoma Families Worldwide with M53I Mutations

Number of M53I Families	Country	Population (millions)
6	Scotland (MacKie et al. 1998; this thesis)	5
4	Australia (Walker et al. 1995; Flores et al. 1997; Holland et al. 1999)	19.5
2	U.S.A (FitzGerald et al. 1996; Tsao et al. 2000)	286.8
2	England (Harland et al. 1997; Newton Bishop et al. 1999)	49.9
1	Canada, Ontario (Monzon et al. 1998)	11
1	France (Soufir et al. 1998)	60.1

Previous examination of five melanoma families from Australia and North America with the M53I mutation suggested the possibility of a common haplotype (Pollock et al., 1998). The high prevalence of reported M53I mutation positive families per head of population in Scotland led this study to investigate the possibility that the M53I mutation is of Scottish origin.

In addition to the six M53I families from Scotland, DNA from five M53I families from Toronto, Canada, four from Brisbane, Australia, three from Boston, U.S.A and two Scottish patients with sporadic melanoma with multiple primaries were studied.

5.2 Families

Details and pedigrees of the six Scottish families have already been given in Chapter 4. Table 5.2 shows details of the Scottish, Canadian, Australian and American M53I melanoma families.

TABLE 5.2

Details of Melanoma Families with M53I Mutations

M53I Family Identifier	Country	Ancestry	Number of Individuals in the Pedigree with Melanoma	Number of M53I Carriers Available for Haplotyping
1	Scotland	Scottish	3	2
4	Scotland	Scottish	2	2
5	Scotland	Scottish	2	2
6	Scotland	Scottish	2	2
29	Scotland	Scottish	3	1
30	Scotland	Scottish	2	2
FAM-1	Canada	Scottish	5	4
FAM-2	Canada	Scottish	2	1
FAM-3	Canada	Scottish	2	1
FAM-4	Canada	Scottish	4	1
FAM-5	Canada	Scottish	3	1
41001	Australia	Scottish	14	2
41031	Australia	Irish/English/Chinese	5	2
60001	Australia	Scottish	15	4
41156	Australia	Irish	5	2
L25	America	Scottish/Irish/French	8	1
637	America	Scottish/French Canadian	3	1
590	America	Irish/English	5	1

The six Scottish families have either two or three melanoma patients per family. In addition, patients from four of the families had multiple primary melanomas. Members of the six Scottish families with M53I mutations have been questioned about their family history, and in no family can a link to any of the other M53I Scottish families be traced, going back to generations born around 1900. All six families give a history of Scottish ancestry. Two individuals from each of the M53I Scottish families were available for haplotyping except family 29 in which only one individual was available.

Of the five Canadian families, two families have two melanoma patients per family, one family has three melanoma patients, one family has four melanoma patients and one family has five melanoma patients. In addition, patients from four of the families had multiple primary melanomas. All five Canadian families are thought to have Scottish ancestries. One individual from each of the M53I Canadian families was available for haplotyping except family FAM-1 in which four carriers and two non-carriers were available.

Two of the four Australian families have five melanoma patients, one family has 14 melanoma patients and one family has 15 melanoma patients. In addition, patients from all four of the families have multiple primary melanomas. Members of the four Australian families with M53I mutations have been questioned about their family history. Two families are thought to have Scottish ancestries, one family is thought to have mixed Irish, English and Chinese ancestry and one family is thought to have Irish ancestry. Two individuals from two of the M53I Australian families (41001 and 41156) were available for haplotyping. Family 41031 had two carriers and two non-carriers available and in family 60001 there were four carriers available.

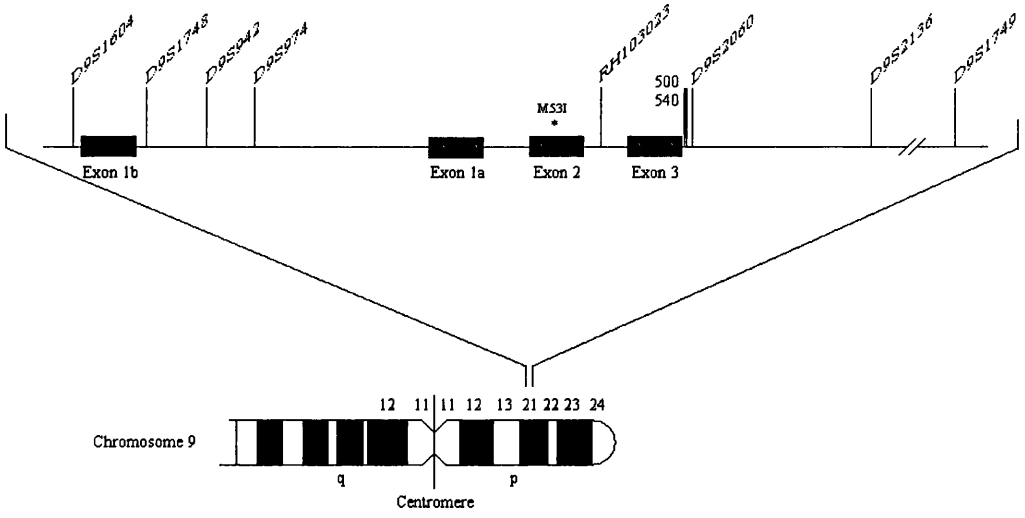
The three American families have three, five and eight melanoma patients per family. In addition, patients from all three of the families have multiple primary melanomas. Members of the three American families with M53I mutations have been questioned about their family history. One family is thought to have Scottish, Irish and French ancestry, one family is thought to have Irish and English ancestry and one family is thought to have Scottish and French Canadian ancestry. One individual from each of the M53I American families was available for haplotyping.

5.3 Determination of Allele Length Employed

Eight markers, D9S1604, D9S1748, D9S942, D9S974, RH103023, D9S2060, D9S2136 and D9S1749, as illustrated in figure 5.1, were genotyped to determine which alleles from loci flanking *CDKN2A* were transmitted with melanoma in each of the 18 M53I families. Ninety chromosomes from 45 Scottish control subjects were also characterised to determine the allele frequency of the markers in the general Scottish population.

FIGURE 5.1

Physical Map of the Eight Markers used in Haplotype Analysis



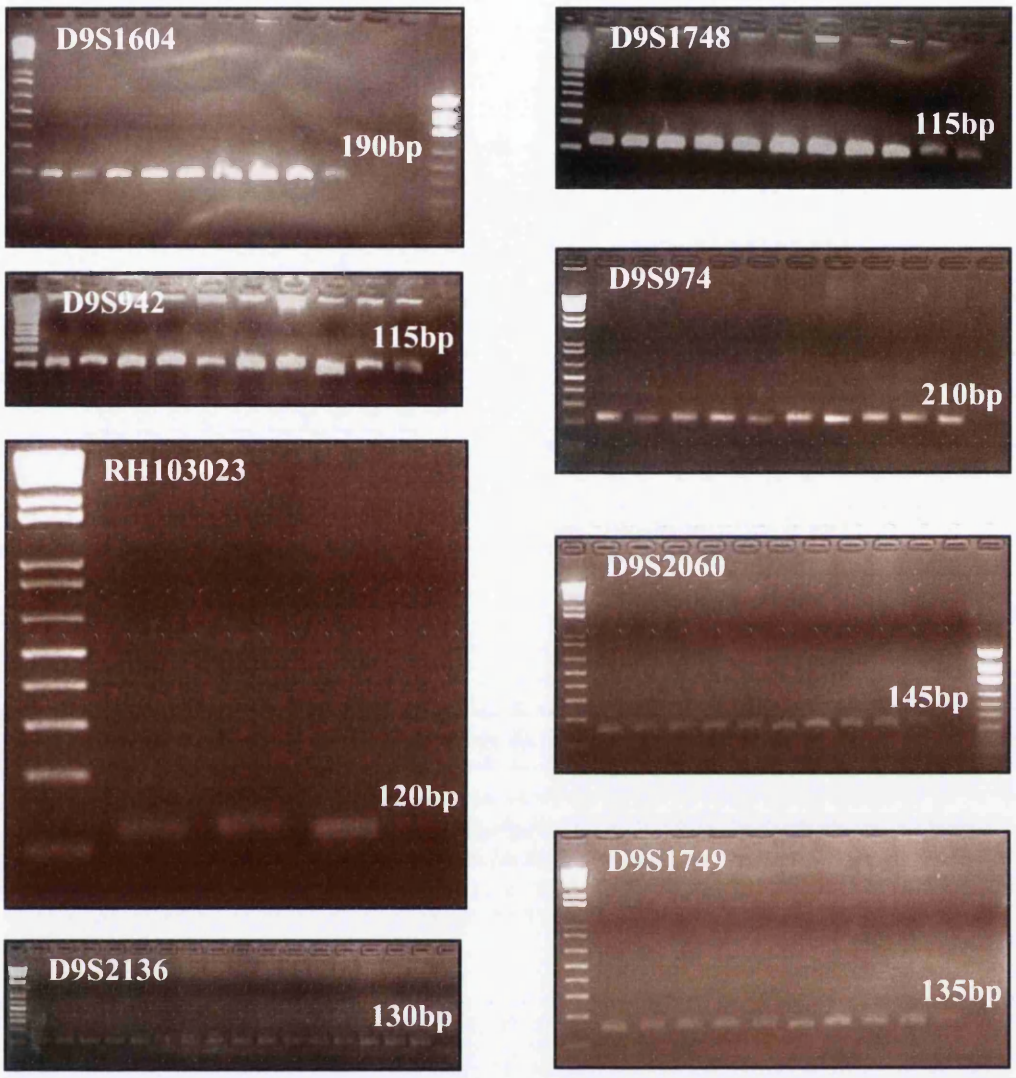
The location of the M53I mutation and *CDKN2A* locus with respect to these markers is shown. Distances are not shown to scale.

Primers were designed for independent amplification of each of the eight markers (Methods 3.4.2-3.4.5). The sequences of the primers used for amplification of the microsatellite markers at 9p21 were from the Genome Database (http://gdbwww.gdb.org/). All forward primers were 5' labelled with fluorescent dye FAM. The final amplification conditions are presented in Appendix 2. Routinely a 1μl aliquot of DNA at 100ng/μl (extracted as described using method 3.1a) from each patient was amplified with each of the sets of primers. DNA samples sent from Canada, Australia and America were diluted to a concentration of 100ng/μl (Method 3.3).

Fragment sizes of approximately 190bp for D9S1604, 115bp for D9S1748 and D9S942, 210bp for D9S974, 120bp RH103023, 145bp for D9S2060, 130bp for D9S2136 and 135bp for D9S1749 were generated. Negative controls (no DNA) were run along with each reaction. Once all the DNA samples had been transferred to PCR tubes, the same reagents were used for the negative control. The absence of amplicons in the negative control meant that there had been no cross-contamination between the samples or into the reagents. The distinct bands obtained from amplification were resolved on ethidium bromide stained agarose gels to verify amplification and estimate the size of the amplified band (Method 3.5). The distinct bands are presented in figure 5.2.

FIGURE 5.2

Amplification of Markers Surrounding the *CDKN2A* Locus



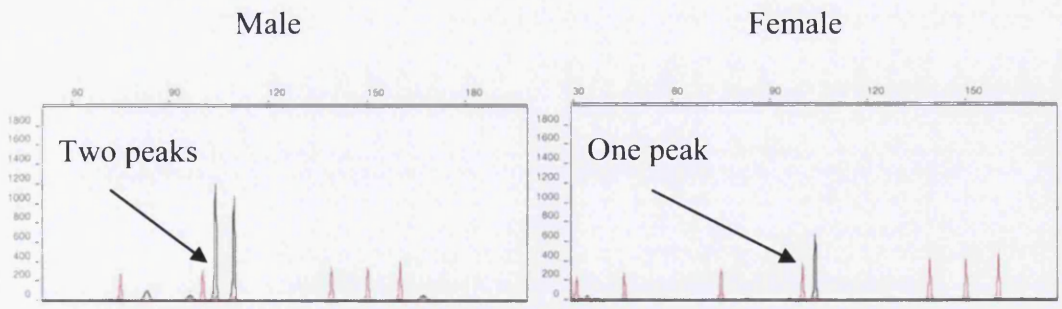
The approximate sizes of the amplicons of each marker were determined by resolution on 2% agarose gels with ethidium bromide staining in 1X TAE buffer for 20 minutes. The size of the amplicons shown in base pairs were deduced by comparison to the migration of the known size of the DNA ladder fragments in lane 1. 1µl of genomic DNA was routinely amplified by conditions specified in Appendix 2 and with primers specified in Appendix 1. The amplicons' sizes are consistent with the approximate sizes of fragments as detailed in the Genome Database.

5.4 Accuracy of Allele Sizing

The ABI ROX 500 size standard was used to accurately calculate the sizes of the alleles. The accuracy of the sizing of alleles using the ABI ROX size standard was checked to ensure that allele sizes quoted in this thesis were comparable to other published work (Method 3.13). The AMXY (amelogenin) marker, located on chromosomes Xp22.1-22.31 and Yp11.2, carries a small deletion of 6bp in the first intron on the X chromosome, facilitating the design of distinct X and Y specific PCR primers. The AMXY fragment identifies an X-specific 104bp fragment and a Y-specific 110bp (Nakahori et al. 1991). Ten male DNAs and ten female DNAs were amplified (Mann et al., 2001) and the fragments of known bp composition were sized by comparison with ROX standards using the same analysis conditions as used in the sizing of the alleles. One fluorescent peak of 110bp (Y) and one fluorescent peak of 104bp (X) were observed when samples from males were tested. Only one fluorescent peak of 104bp (XX) was observed when samples from females were tested (Figure 5.3). Each of the male and female specific fragments was accurately sized using the ABI ROX 500 size standard. This confirms that the methods used in this thesis for allele sizing accurately identify the bp composition of the alleles.

FIGURE 5.3

GeneScan Results of Male and Female AMXY Controls



The ABI ROX 500 size standard, shown here in red, was used to accurately calculate the sizes of the alleles of the AMXY (amelogenin) marker. One fluorescent peak of 110bp (Y) and one fluorescent peak of 104bp (X) were observed when samples were male. Only one fluorescent peak of 104bp (XX) was observed when samples were female.

5.5 Genotyping

Amplicons of each individual marker, from each patient and control, were genotyped to determine each individual allele size (Method 3.12). In total 32 M53I mutation carriers, four non-carrier relatives and 45 control subjects were haplotyped. Ninety chromosomes from the 45 geographically matched control subjects were genotyped to determine the allele frequency of the markers in the general Scottish population. These allele frequencies are given in table 5.3. Figure 5.4 shows the GeneScan electropherograms of PCR amplified genomic DNA for each 9p21 marker from Scottish family member F30.1 as an example of the M53I carriers' results.

TABLE 5.3

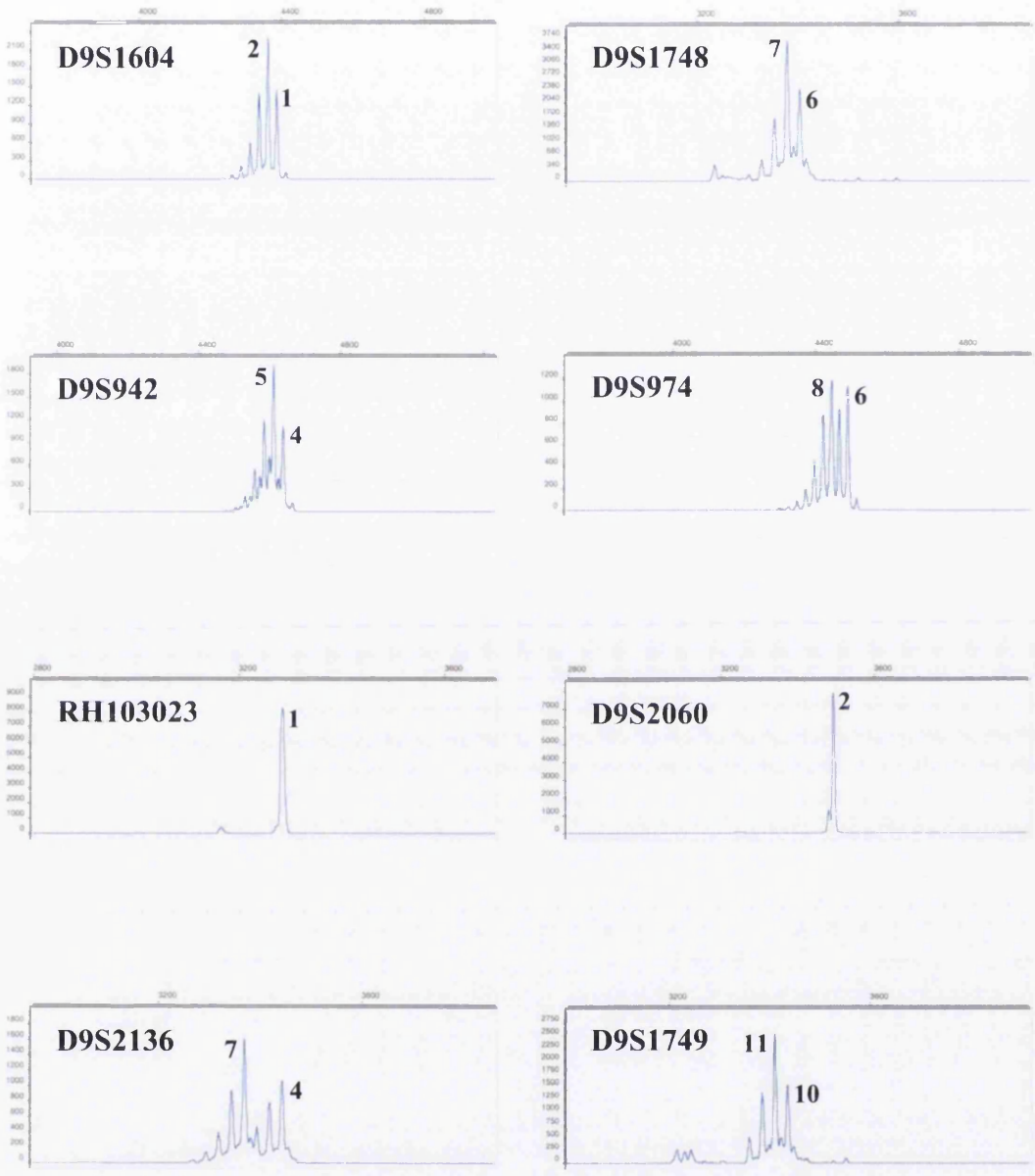
Allele Frequencies in Scottish Control Chromosomes at each Marker

Allele	D9S1604	D9S1748	D9S942	D9S974	D9S2060	D9S2136	D9S1749
1	0.300	0.050	0.056	0.023	0.022	0.012	0.000
2	0.700	0.050	0.111	0.000	0.756	0.000	0.000
3		0.100	0.667	0.104	0.222	0.035	0.000
4		0.223	0.157	0.012		0.163	0.000
5		0.200	0.009	0.105		0.035	0.000
6		0.150	0.000	0.256		0.232	0.000
7		0.113	0.000	0.174		0.070	0.000
8		0.038		0.186		0.372	0.012
9		0.076		0.093		0.081	0.012
10		0.000		0.012			0.036
11				0.000			0.059
12				0.023			0.095
13				0.000			0.143
14				0.000			0.095
15				0.000			0.036
16				0.012			0.083
17							0.107
18							0.048
19							0.024
20							0.036
21							0.012
22							0.071
23							0.024
24							0.048
25							0.012
26							0.036
27							0.000
28							0.012
Size of allele 1	190bp	123bp	121bp	223bp	148bp	141bp	165bp

Allele 1 is the largest allele observed either in M53I family members or in the 90 control chromosomes. The size of allele 1 is given for each marker.

FIGURE 5.4

GeneScan Results of the Eight Markers used in Haplotype Analysis



GeneScan electropherograms of PCR amplified genomic DNA for each 9p21 marker from Scottish family member F30.1. Peaks are labeled with the assigned allele number.

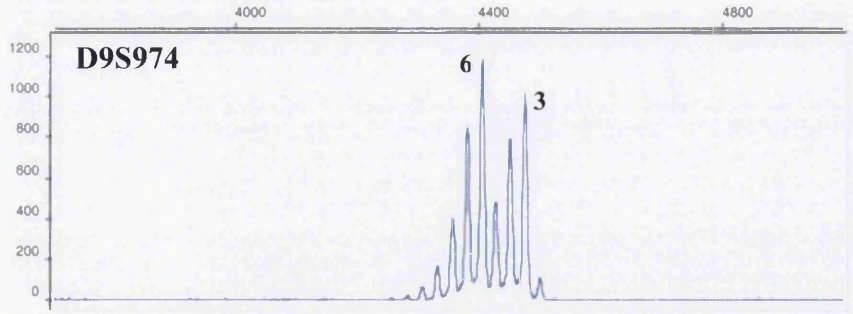
5.6 Stutter Products

During the PCR amplification of the dinucleotide microsatellite loci, minor products that were between one and four repeat units shorter than the main allele were sometimes produced as illustrated in figure 5.5. These "stutter" peaks may have been caused by polymerase slippage during the elongation step of PCR. When evaluating the data with multiple stutter products present, particularly in samples with two alleles close in size, analysis was complicated. For example, peaks in a position one repeat unit smaller than the main allele could have been interpreted either as a stutter band or as an allele. The amount of stutter did not depend on the quantity of DNA amplified during PCR. The largest peak in each sample represents the "true" allele.

Amplification of the dinucleotide repeat markers yielded allele peaks and associated PCR stutter bands within a maximum range of six base pairs from the allele peak. The number of allele peaks depended on whether the individual tested was a heterozygote or homozygote. For example, the GeneScan electropherogram of dinucleotide repeat marker D9S974 from a heterozygous individual (213bp, 219bp) is shown in figure 5.5. Allele sizes differ by 6bp. The 2bp stutter peak to the left of each allele peak was always of lower intensity than the allele peak itself. The larger 219bp allele peak is of lower intensity than the smaller 213bp allele. In heterozygotes, the higher molecular weight allele often produced a fluorescent signal of lower intensity than the lower molecular weight peak perhaps because of a less efficient amplification of the larger fragment.

FIGURE 5.5

GeneScan Electropherogram of a Dinucleotide Repeat Marker from a Heterozygous Individual with Stutter Peaks



The GeneScan electropherogram from dinucleotide repeat marker D9S974 of a heterozygous control individual (213bp, 219bp). Peaks are labeled with the assigned allele number.

Stutter products were not a problem in the microsatellite analysis as they allowed the true allele peaks to be distinguished from non-specific PCR products, as non-specific PCR products are not associated with stutter bands.

5.7 Haplotype Analysis

Haplotype analysis using eight polymorphic markers spanning the *CDKN2A* locus was interpreted by eye on available M53I carriers from each family to determine whether carriers from the 18 families harbored the same mutation identically by descent. Table 5.4 shows the disease haplotypes for the six Scottish M53I families. Both alleles are indicated for each marker with the segregating allele shown in blue type. Results are displayed in the same format as others publishing in this field.

TABLE 5.4

Haplotype Analysis of Scottish M53I Mutation Carriers for 9p Markers

Haplotype for each Marker											
	Family Identifier										
Markers	**F1.1**	**F1.2***	**F4.1**	**F4.3***	**F5.1**	**F5.2**	**F6.2***	**F6.3**	**F29.1**	**F30.1**	**F30.2**
D9S1749	8/9	8/9	8/10	8/9	8/9	8/9	10/11	10/11	12/13	10/11	10/11
D9S2136	4/4	4/9	4/5	5/6	4/6	4/8	4/6	4/6	4/8	4/7	4/7
D9S2060	2/2	2/2	2/2	2/2	2/2	2/2	2/2	2/2	2/2	2/2	2/2
RH103023	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1
M53I	**G/C**	**G/C**	**G/C**	**G/C**	**G/C**	**G/C**	**G/C**	**G/C**	**G/C**	**G/C**	**G/C**
D9S974	3/8	5/8	6/8	8/9	5/8	4/8	1/8	1/8	6/8	6/8	8/9
D9S942	4/5	4/5	4/5	4/5	4/5	4/5	4/5	4/5	4/5	4/5	4/5
D9S1748	6/8	6/9	6/7	6/7	6/6	6/8	6/8	6/8	5/6	6/7	3/6
D9S1604	1/2	1/2	2/2	2/2	2/2	2/2	2/2	2/2	2/2	1/2	2/2

The position of the mutation relative to the markers is shown

F1, F4, F5, F6, F29 and F30 refer to family identifiers

Blue denotes consensus haplotype

Grey shaded area denotes individuals who do not carry the consensus allele for that marker

* denotes M53I carriers who are currently unaffected

Data collected from marker RH103023 was not informative as all families and control subjects share the one allele.

All six Scottish families showed a haplotype consistent with a single genetic origin for the M53I mutation. The D9S2136 - D9S1604 haplotype 4-2-8-4-6-2 appears to be common across all six Scottish families. One currently unaffected carrier of Scottish family F4 (F4.3) had the 5/6 alleles at marker D9S2136, rather than the allele 4 seen in all other families and in affected family member F4.1 of the same family. All but one Scottish family showed co-segregation of either the 9 or 10 allele for marker D9S1749. Family 29 showed alleles 12/13 to co-segregate with the M53I melanoma mutation. D9S1749 has previously been shown to vary in allele size because of replication slippage resulting in the loss or gain of one or more repeat units during meiosis (Pollock et al, 1998).

In addition to the 18 M53I families, two Scottish patients with sporadic melanoma and no family history of melanoma were haplotyped (Table 5.5). Both these individuals have multiple primary melanomas and also have the M53I mutation. Both of the M53I Scottish patients with sporadic melanoma carried the core disease haplotype.

TABLE 5.5

Haplotype Analysis of Scottish M53I Mutation Carriers with Sporadic Melanoma with Multiple Primary Melanomas

Haplotype for each Marker		
	Sporadic Identifier	
Markers	**SMP1**	**SMP3**
D9S1749	8/9	8/9
D9S2136	4/4	4/9
D9S2060	2/2	2/2
RH103023	1/1	1/1
M53I	**G/C**	**G/C**
D9S974	3/8	5/8
D9S942	4/5	4/5
D9S1748	6/8	6/9
D9S1604	1/2	1/2

The position of the mutation relative to the markers is shown

SMP1 and SMP3 refer to patients with sporadic melanoma with multiple primaries and the M53I mutation

Blue denotes consensus haplotype

Grey shaded area denotes individuals who do not carry the consensus allele for that marker

Data collected from marker RH103023 was not informative as all patients and control subjects share the one allele.

Table 5.6 shows the disease haplotypes for the 12 M53I families from Canada, Australia and America. Both alleles are indicated for each marker with the segregating allele shown in blue type. The two non-carriers from Canadian family FAM-1 and the two non-carriers from Australian family 41031 were also haplotyped and alleles are indicated in table 1 in Appendix 4.

TABLE 5.6

Haplotype Analysis of M53I Mutation Carriers from Families from Toronto, Brisbane & Boston for 9p Markers

	Haplotype for each Marker																			
	Family Identifier																			
	Canadian								Australian										American	
	FAM-1				FAM-2	FAM-3	FAM-4	FAM-5	41001		41031		60001				41156		L25	637	5
Markers	**T1**	**T2**	**T3***	**T6***	**T7**	**T8**	**T9**	**T10**	**B1**	**B2**	**B3**	**B6**	**B7**	**B8**	**B9**	**B10**	**B11**	**B12**	**A1**	**A2**	
D9S1749	9/10	9/10	9/10	9/10	10/11	5/6	9/10	8/9	10/11	9/10	10/11	9/10	1/2	1/2	1/2	1/2	10/11	10/11	9/10	4/5	10
D9S2136	4/7	4/8	4/8	4/8	4/6	4/6	7/9	4/4	4/8	4/8	4/6	4/7	4/8	4/9	4/8	4/5	4/6	4/8	3/4	4/5	4
D9S2060	2/2	2/2	2/2	2/2	2/2	2/2	2/2	2/2	2/2	2/2	2/3	2/2	2/3	2/2	2/3	2/2	2/2	2/2	2/2	2/2	2
RH103023	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1
M53I	**G/C**	**G/C**	**G/C**	**G/C**	**G/C**	**G/C**	**G/C**	**G/C**	**G/C**	**G/C**	**G/C**	**G/C**	**G/C**	**G/C**	**G/C**	**G/C**	**G/C**	**G/C**	**G/C**	**G/C**	**G**
D9S974	8/8	8/9	8/8	8/9	8/12	6/8	8/8	3/8	6/8	8/8	7/8	8/8	8/8	4/8	7/8	7/8	7/8	7/8	8/8	8/16	8
D9S942	3/4	4/5	4/5	4/5	4/5	4/5	4/5	4/5	4/5	4/5	4/5	4/5	5/6	6/7	6/7	6/7	4/5	4/5	4/5	3/4	4
D9S1748	6/7	6/10	5/6	6/10	4/6	6/7	4/6	6/8	4/6	5/6	4/4	4/8	4/4	4/6	4/4	4/9	6/9	6/7	5/6	4/5	4
D9S1604	1/2	1/2	2/2	1/2	2/2	1/2	2/2	1/2	2/2	2/2	2/2	2/2	2/2	2/2	2/2	2/2	2/2	1/2	2/2	2/2	2

The position of the mutation relative to the markers is shown

T1-T10 denotes samples from Toronto, **B1-B12** denotes samples from Brisbane, **A1-A3** denotes samples from Boston

Blue denotes consensus haplotype

Grey shaded area denotes individuals who do not carry the consensus allele for that marker

* denotes M53I carriers who are currently unaffected

Data collected from marker RH103023 was not informative as all families and control subjects share the one allele

Eight of the twelve M53I families from DNA samples from Canada, Australia and America showed a haplotype consistent with a single genetic origin for the M53I mutation. The D9S1749 - D9S1604 haplotype 10-4-2-8-4-6-2 appears to be common across eight of these twelve families. One Canadian family FAM-4 (T9) had the 7/9 alleles at D9S2136, rather than the allele 4 seen in all other haplotype sharing families. Three families, Australian families 41031 and 60001 and American family 637, showed co-segregation of allele 4 instead of the consensus allele 6 for marker D9S1748. This could be accounted for by replication slippage of marker D9S1748 gaining one repeat unit during meiosis. All but one of the haplotype sharing families showed co-segregation of the 4 allele for marker D9S942. Australian family 60001 showed allele 6 to co-segregate with the M53I melanoma mutation. Australian families 41031 and 60001 could not be assigned the consensus haplotype, as has been previously reported by Pollock et al, 1998. Their study accounted for these families not sharing the disease haplotype by replication slippage.

Analysis of the 18 melanoma families from around the world carrying the M53I mutation showed that 14 of them share a common haplotype for markers D9S2136 - D9S1604 4-2-8-4-6-2. This haplotype was found consistently in all tested M53I mutation carriers in 13 families (F1, F5, F6, F29, F30, FAM-1, FAM-2, FAM-3, FAM-5, 41001, 41156, L25 and 590).

After allowing for a high mutation rate at D9S1749, the most common allele observed was allele 10. Among the 18 families' haplotypes, 11 families carry the 10 allele and nine families carry the 9 allele. Analysis of the 18 melanoma showed that 10 of them share a common haplotype for markers D9S1749 - D9S1604 10-4-2-8-4-6-2. This haplotype was found consistently in all tested M53I mutation carriers in eight families (F6, F30, FAM-1, FAM-2, 41001, 41156, L25 and 590).

Forty-five controls were typed for the same markers to determine the frequency of alleles at each marker in the Scottish population. The consensus M53I haplotype D9S1749 - D9S1604 10-4-2-8-4-6-2 or D9S2136-D9S1604 4-2-8-4-6-2 was not determined in any of the control subjects and therefore the probability of finding the M53I haplotype in the general Scottish population is extremely low. The frequencies of the M53I common haplotype for markers D9S1749-D9S1604 were 0.036, 0.163, 0.756, 0.186, 0.157, 0.150 and 0.700.

The probability of finding the M53I haplotype in the general population is therefore extremely low (1.4×10^{-5}) and even if the D9S1749 marker was not included in the haplotype, the frequency is still very low (3.8×10^{-4}). These results confirm that all cases are distantly related.

Six of the 18 families all live in Scotland and have a Scottish ancestry. In addition, nine of the families from out-with Scotland has a history of Scottish ancestry. Therefore the mutation that arose on a chromosome carrying a common ancestral haplotype probably occurred in Scotland due to the majority of families having a Scottish ancestry.

CHAPTER 6

MC1R VARIANTS IN FAMILIAL MELANOMA, UNAFFECTED FAMILY MEMBERS, SPORADIC MELANOMA AND CONTROL SUBJECTS

6.1 *MC1R* Sequence Variation Detection

It has been postulated that variants of the *MC1R* gene are common low penetrance melanoma susceptibility alleles in sporadic melanoma (Valverde et al. 1996; Ichii-Jones et al. 1998; Palmer et al. 2000; Kennedy et al. 2001). The study of this thesis therefore sequenced the entire *MC1R* gene for variants in 60 familial melanoma patients, 25 unaffected family members, 28 cases of sporadic melanoma and 67 healthy phenotyped control subjects. All patients, unaffected family members and control subjects were phenotyped. In all groups, the relationship of the *MC1R* variants to other risk factors such as skin type, hair and eye colour was also investigated.

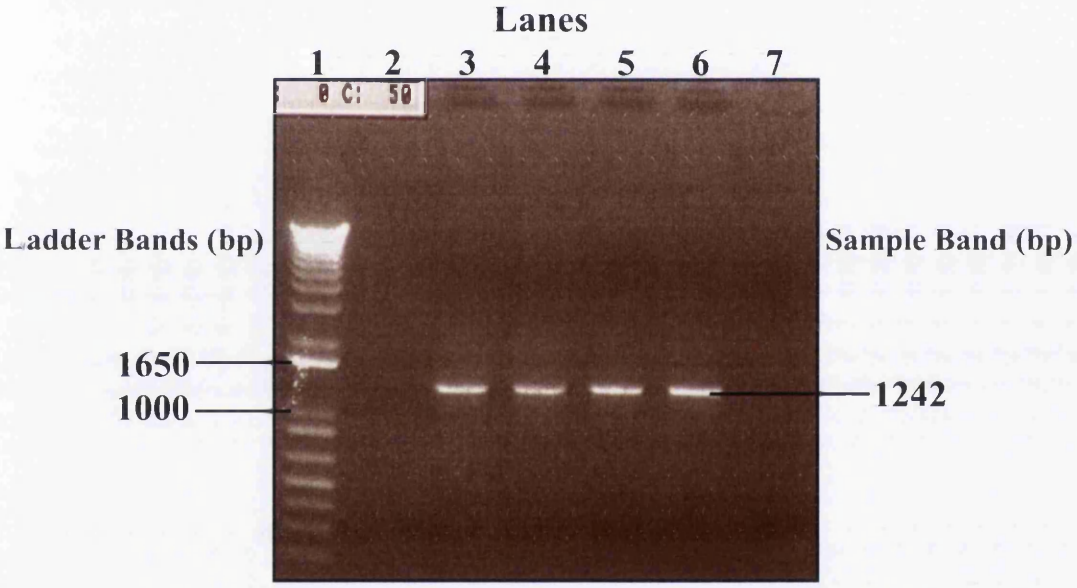
6.2 Mutation Detection Strategy Employed

A set of primers was designed for amplification of the single 951 nucleotide coding region of *MC1R* and its splice sites (Method 3.4). The final amplification conditions are presented in Appendix 2. Routinely a 1µl aliquot of DNA (extracted as described using method 3.1a) from each patient was amplified with the set of primers. Fragment sizes of 1242bp were generated. Negative controls (no DNA) were run along with each reaction. Once all the DNA samples had been transferred to PCR tubes, the same reagents were used for the negative control. The absence of amplicons in the negative control meant that there had been no cross-contamination between the samples or into the reagents. The distinct bands obtained from amplification were resolved on ethidium bromide stained agarose gels to verify the size of the amplified band (Method 3.5) and are presented in figure 6.1.

FIGURE 6.1

Amplification of *MC1R*

The expected 1242bp amplicons of *MC1R* were confirmed by resolution on a 0.6% agarose gel with ethidium bromide. The amplicons were electrophoresed in 1X TAE buffer for 20 minutes. The size of the amplicons which are represented below shown in base pairs were deduced by comparison to the migration of the known size of the DNA ladder fragments in lane 1. 1µl of genomic DNA was routinely amplified by conditions specified in Appendix 2 and with primers specified in Appendix 1. The amplicons' size is consistent with the expected 1242bp fragment.



Electrophoresis Conditions: 0.6% agarose gel, 1X TAE, 10µl size marker, 5µl PCR product, 2µl loading mix, 100V, 20 minutes, room temperature

Lane 1 – 1kb Plus size marker

Lane 3 – PCR product from individual F1.1

Lane 4 – PCR product from individual F2.1

Lane 5 – PCR product from individual F3.1

Lane 6 – PCR product from individual F4.1

Lane 7 – negative control

See page 60 for patient details.

6.3 Direct Sequencing of All Amplicons

MC1R was sequenced directly (Methods 3.7-3.10) rather than performing an initial screen such as dHPLC because the gene is highly polymorphic with over 35 variants in the published literature and therefore the majority of samples would have shown an abnormal chromatogram and would need to be sequenced. Each chromatogram was analysed using SeqScape (Method 3.11). The primers used for sequence analysis included the same amplification primers but also an additional three primers that gave rise to five overlapping sequenced fragments. Briefly, the MC1R-F primer binds nucleotides –135- -116 and sequences in the forward direction, the MC1R-NT-F primer binds nucleotides 377-358 and sequences in the reverse direction, the MC1R-TM-F primer binds nucleotides 202-221 and sequences in the forward direction, the MC1R-CT-F primer binds nucleotides 518-537 and sequences in the forward direction and the MC1R-R primer binds nucleotides 1108-1083 and sequences in the reverse direction. DNA sequence of the complete coding region and flanking DNA were routinely obtained and nucleic acid changes identified in all fragments sequenced.

6.4 Sequence Analysis

In total, 13 *MC1R* variants leading to an amino acid substitution were detected among the 180 samples sequenced (Table 6.1). Three of these (L44I, M128K, A171G) have not been described elsewhere and appear to be very rare. L44I is present in only one sporadic melanoma patient, M128K is present in only one currently unaffected member of a melanoma family and A171G is present in only one familial melanoma patient. Three silent nucleotide changes (G239G, T314T, C315C) were also detected but these have not been included in the statistical analysis, as they do not lead to an amino acid substitution.

Figure 6.2 illustrates each of the *MC1R* variants identified in this Scottish study.

TABLE 6.1

***MC1R* Variants Identified in this Scottish Study**

180 samples were sequenced in total.

Nucleotide Variation	Amino Acid Substitution	No. of Melanoma Patients or Unaffected Family Members in which Variant was Detected	No. of Control Subjects in which Variant was Detected
130C>A	***L44I***	***1***	***0***
178G>T	V60L	35	19
252C>A	D84E	8	1
274G>A	V92M	18	10
284C>T	T95M	1	0
383T>A	***M128K***	***1***	***0***
425G>A	R142H	3	2
451C>T	R151C	32	12
464T>C	I155T	4	4
478C>T	R160W	28	14
488G>A	R163Q	11	7
512C>G	***A171G***	***2***	***0***
717C>T	*G239G	1	0
880G>C	D294H	11	3
942A>G	*T314T	24	6
945C>T	*C315C	1	0

Italics denote variants that have not previously been published.

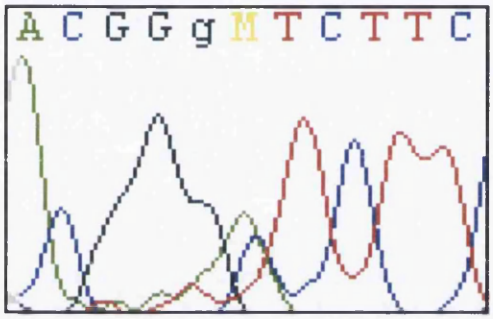
* denotes a silent amino acid substitution and have not been included in the statistical analysis.

FIGURE 6.2

Identification of *MC1R* Variants

Sequence analysis of PCR amplified genomic DNA using intronic primer pair MC1R-F, MC1R-R followed by sequencing using primers MC1R-F, MC1R-NT-F, MC1R-TM-F, MC1R-CT-F and MC1R-R (Appendix 1) are shown in the left hand column. Nucleic acid and derived amino acid sequences from the mutated region of *MC1R* are shown in the right hand column.

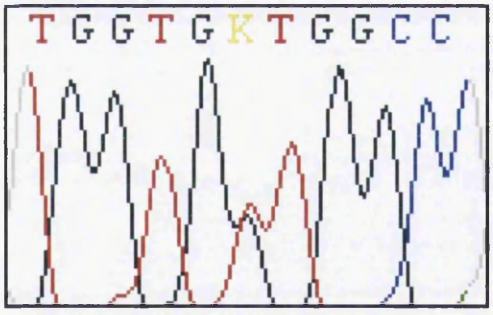
Variant L44I (130C>A)



	D	G	**L**	F	L
Wild Type	GAC	GGG	CTC	TTC	CTC
Variant	GAC	GGG	ATC	TTC	CTC
			I		
			(L44I)		

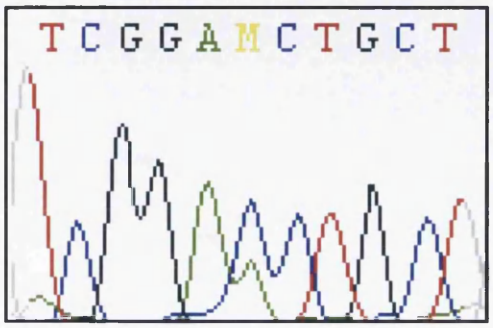
L44I has not previously been published

Variant V60L (178G>T)



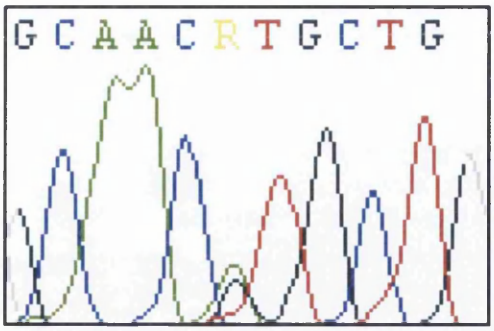
	L	V	**V**	A	T
Wild Type	CTG	GTG	GTG	GCC	ACC
Variant	CTG	GTG	TTG	GCC	ACC
			L		
			(V60L)		

Variant D84E (252C>A)



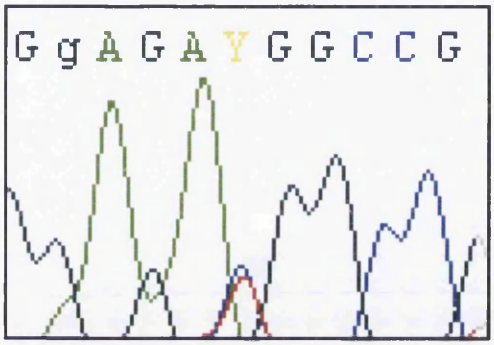
	L	S	**D**	L	L
Wild Type	TTG	TCG	GAC	CTG	CTG
Variant	TTG	TCG	GAA	CTG	CTG
			E		
			(D84E)		

Variant V92M (274G>A)



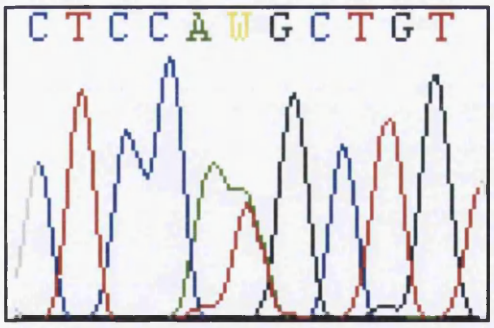
	S	N	V	L	E
Wild Type	AGC	AAC	GTG	CTG	GAG
Variant	AGC	AAC	ATG	CTG	GAG
			M		
			(V92M)		

Variant T95M (284C>T)



	L	E	T	A	V
Wild Type	CTG	GAG	ACG	GCC	GTC
Variant	CTG	GAG	ATG	GCC	GTC
			M		
			(T95M)		

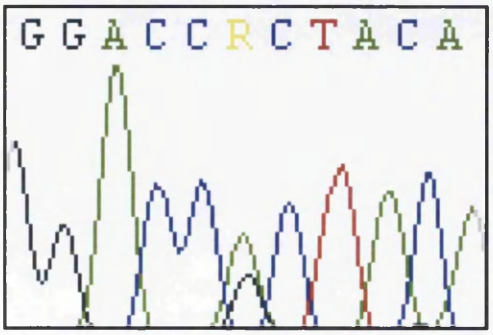
Variant M128K (383T>A)



	S	S	M	L	S
Wild Type	AGC	TCC	ATG	CTG	TCC
Variant	AGC	TCC	AAG	CTG	TCC
			K		
			(M128K)		

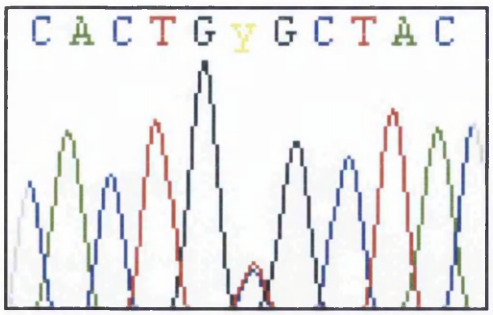
M128K has not previously been published

Variant R142H (425G>A)



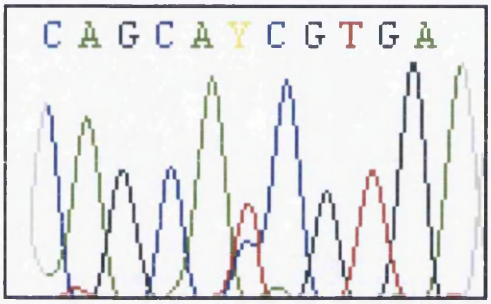
	V	D	R	Y	I
Wild Type	GTG	GAC	CGC	TAC	ATC
Variant	GTG	GAC	CGC	TAC	ATC
			H		
			(R142H)		

Variant R151C (451C>T)



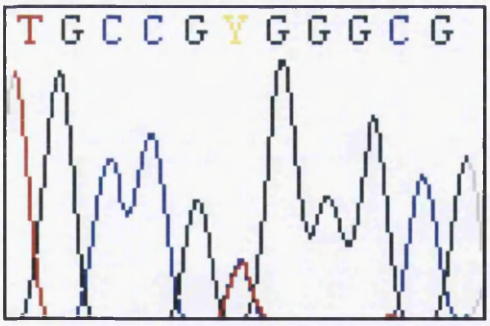
	A	L	**R**	Y	H
Wild Type	GCA	CTG	CGC	TAC	CAC
Variant	GCA	CTG	TGC	TAC	CAC
			C		
			(R151C)		

Variant I155T (464T>C)



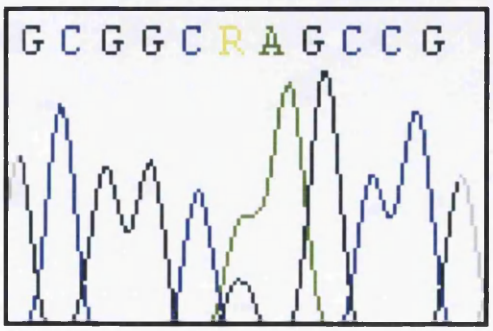
	H	S	**I**	V	T
Wild Type	CAC	AGC	ATC	GTG	ACC
Variant	CAC	AGC	ACC	GTG	ACC
			T		
			(I155T)		

Variant R160W (478C>T)



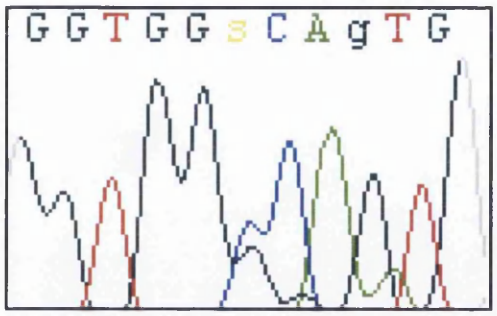
	L	P	**R**	A	R
Wild Type	CTG	CCG	CGG	GCG	CGG
Variant	CTG	CCG	TGG	GCG	CGG
			W		
			(R160W)		

Variant R163Q (488G>A)



	A	R	**R**	A	V
Wild Type	GCG	CGG	CGA	GCC	GTT
Variant	GCG	CGG	CAA	GCC	GTT
			Q		
			(R163Q)		

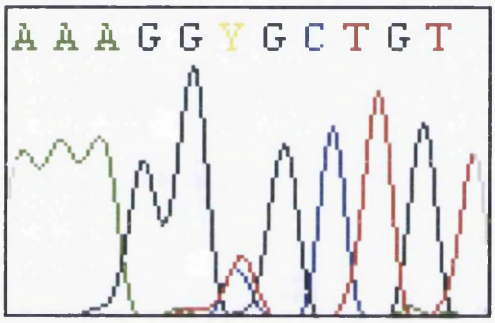
Variant A171G (512C>G)



	W	V	**A**	S	V
Wild Type	TGG	GTG	GCC	AGT	GTC
Variant	TGG	GTG	GGC	AGT	GTC
			G		
			(A171G)		

A171G has not previously been published

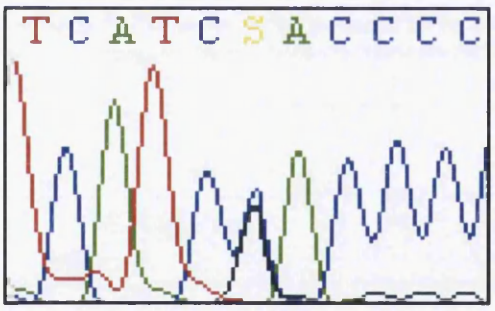
Variant G239G (717C>T)



	L	K	**G**	A	V
Wild Type	CTT	AAA	GGC	GCT	GTC
Variant	CTT	AAA	GGT	GCT	GTC
			G		
			(G239G)		

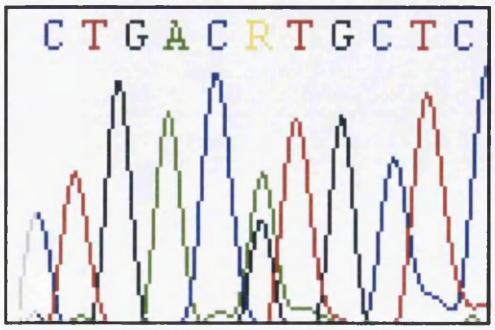
G239G is a silent amino acid substitution, not included in the statistical analysis

Variant D294H (880G>C)



	I	I	**D**	P	L
Wild Type	ATC	ATC	GAC	CCC	CTC
Variant	ATC	ATC	CAC	CCC	CTC
			H		
			(D294H)		

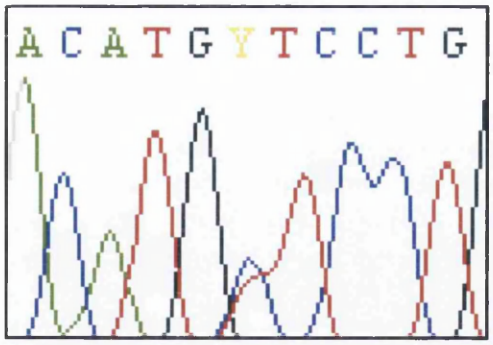
Variant T314T (942A>G)



	V	L	**T**	C	S
Wild Type	GTG	CTG	ACA	TGC	TCC
Variant	GTG	CTG	ACG	TGC	TCC
			T		
			(T314T)		

T314T is a silent amino acid substitution, not included in the statistical analysis

Variant C315C (945C>T)



	L	T	C	S	W
Wild Type	CTG	ACA	TGC	TCC	TGG
Variant	CTG	ACA	TGT	TCC	TGG
			C		
			(C315C)		

C315C is a silent amino acid substitution, not included in the statistical analysis

6.5 Results of Phenotyping & Genotyping of Patient and Control Cohort for *MC1R* Study

Information on the presence of variants of the *MC1R* gene was obtained for 180/202 (89.1%) of the study participants. Tables 6.2, 6.3 and 6.4 gives details of the *MC1R* variants in relation to melanoma status (yes, Y or no, N), skin type, eye and hair colour and *CDKN2A* mutations of all patients with melanoma, unaffected family members and of control subjects.

MC1R variants were recorded by sequencing of the entire coding region of the gene.

Thirteen distinct variants were established for *MC1R* as follows:

a = V60L
b = D84E
c = V92M
d = T95M
e = M128K
f = R142H
g = R151C
h = I155T
i = R160W
j = R163Q
k = A171G
l = D294H
m = L44I
None = no variants identified

CDKN2A variants were recorded by sequencing of the entire coding region of the gene. Nine distinct variants were established for *CDKN2A* as follows:

A = Duplication
B = R24P
C = Y44stop
D = M53I
E = G67R
F = H83N
G = R112G
H = D125H
I = A148T
None = no variants identified

TABLE 6.2

***MC1R* Variants, Skin Type, Eye and Hair Colour and *CDKN2A* Mutations in Familial Melanoma Patients & their Unaffected Relatives**

Identifier	Melanoma	Skin Type	Eye Colour	Hair Colour	*MC1R* Variant(s)	*CDKN2A* Variant
F1.1	Y	1	1	2	a, i	D
F1.2	N	1	1	2	i	D
F1.3	N	2	1	3		None
F2.1	Y	2	1	4	a, g	B
F2.2	Y	2	1	3	a	B
F2.3	Y	2	1	3		B
F2.4	Y	2	1	2	a, g	B
F2.5	N	2	1	3		B
F2.6	N	2	4	2	a, g	None
F2.7	N	2	1	4	j	None
F2.8	N	2	1	3	g, j	None
F3.1	Y	2	4	3	g, l	C
F3.2	Y	2	1	3	g, l	C
F3.3	N	2	4	3	l	None
F4.1	Y	2	4	3	l	D
F4.2	Y	2	1	3		D
F4.3	N	2	1	3	e	D
F5.1	Y	2	3	4	a	D
F5.2	Y	2	4	4	a	D
F6.1	Y	1	1	1		D
F6.2	N	1	1	1	a, c	D
F6.3	Y	2	1	2	a, g	D
F6.4	N	2	1	2	c, i	I
F6.5	N	1	4	1	a, i	I
F7.1	Y	1	4	1	g, i	I
F7.2	N	2	1	2	g, i	H
F8.1	Y	2	3	4	g, j	None
F8.2	Y	2	1	3		None
F9.1	Y	1	1	1	g, i	None
F10.1	Y	2	1	3	a, a	None
F10.2	Y	2	1	2	a, g	None
F10.3	N	2	1	3	a	
F11.1	Y	1-2	1	1	c	None
F11.2	Y	2	1	4	c, j	None
F11.3	Y	2	4	2	None	None
F12.1	Y	1	1	1	i, i	None
F13.1	Y	3	3	4	a, g	I
F13.2	Y	2	3	4	a, a	None
F13.3	N	2	4	2	a	I
F13.4	N	3	3	4	a	

Identifier	Melanoma	Skin Type	Eye Colour	Hair Colour	*MC1R* Variant(s)	*CDKN2A* Variant
F14.1	Y	2	1	3	c, j, j	None
F14.2	Y	2	1	4	c, j, j	None
F15.1	Y	2	1	2	c	None
F15.2	N	2	1	2	a, c	
F16.1	Y	1	1	2	c, g	None
F17.1	Y	2	1	2	a, a	G
F17.2	Y	2	1	2	a, a	G
F17.3	N	1	4	1	a	G
F17.4	N	2	1	2	a, i	G
F17.5	N	2	1	2	a	None
F18.1	Y	2	1	3	a	None
F18.2	Y	2	1	3	a	None
F19.1	Y	1	1	4	a, d	E
F20.1	Y	3	4	4	b	None
F21.1	Y	2	1	4	c, l	None
F21.2	Y	2	3	4	c, l	None
F22.1	Y	1	1	3	i, k	G
F22.2	Y	1	2	1	i	G
F22.3	N	2	1	4	k	G
F22.4	N	2	2	3	c, i	None
F22.5	N	2	1	3	c	None
F22.6	N	2	2	3	i	None
F23.1	Y	2	1	2	j	None
F23.2	N	2	4	2	g	None
F23.3	N	2	1	2	None	None
F23.4	N	2	1	3	g, j	None
F24.1	Y	2	1	3	h, i	None
F24.2	Y	2	1	3	i, i	None
F25.1	Y	2	4	4	c	None
F25.2	Y	1	2	2		None
F26.1	Y	2	1	3	i	None
F26.2	Y					None
F27.1	Y	1	1	1	f	None
F27.2	Y	1	2	1	b, f	None
F28.1	Y	2	1	3	b, g	A
F28.2	Y	2	1	3		A
F29.1	Y	2	1	3	a	D
F29.2	Y	2	4	2	c	None
F30.1	Y				g, l	D
F30.2	Y				g, j	D
F31.1	Y	1	1	1	g, g	None
F32.1	Y	2	1	4	None	None
F32.2	Y	1	1	3	a	None
F33.1	Y	2	1	2	c, g	F
F33.2	Y	2	1	2	g, i	F
F34.1	Y	2	2	3	a, a	None

Identifier	Melanoma	Skin Type	Eye Colour	Hair Colour	*MC1R* Variant(s)	*CDKN2A* Variant
F35.1	Y	2	2	3	h	None
F35.2	Y	2	1	2	g	None
F36.1	Y	2	1	2	h	None
F37.1	Y	2	1	3		None
F37.2	Y	2	1	3		None
F38.1	Y	2	1	3		None
F38.2	Y	2	1	4		None
F39.1	Y	1	1	2		None
F40.1	N	1	1	1	None	None
F41.1	Y	2	3	4		None
F41.2	Y	2	1	2		None
F42.1	Y	2	3	4		None
F42.2	Y					None
F43.1	Y	3	1	4	b, j, j	None
F43.2	Y	1	1	3	b, i	None
F44.1	Y	2	1	3	c, g	None
F45.1	Y	2	3	3	g	None
F46.1	Y					None
F47.1	Y	1	1	1		None
F48.1	N	2	1	3		None

TABLE 6.3

***MC1R* Variants, Skin Type, Eye and Hair Colour and *CDKN2A* Mutations in Sporadic Melanoma Patients**

Identifier	Melanoma	Skin Type	Eye Colour	Hair Colour	*MC1R* Variant(s)	*CDKN2A* Variant
S1	Y				a, h	None
S2	Y	1	1	1	g, i	None
S3	Y	2	2	3	j, j	None
S4	Y	1	1	1	a, i, m	D
S5	Y	1	2	1	i, g	D
S6	Y				l	None
S7	Y	2	1	3	i, l	None
S8	Y	2	1	3	None	None
S9	Y	2	1	2	a, g	None
S10	Y	2	1	3	f, i	None
S11	Y	2	2	1	None	None
S12	Y	1	1	2	i	None
S13	Y	3	1	1	a, b	None
S14	Y	2	1	3	b	None
S15	Y	2	1	2	None	None
S16	Y	2	1	3	i	None
S17	Y	3	1	2	g	None
S18	Y	2	1	1	a	None
S19	Y	3	1	2	i	None
S20	Y	2	1	2	a	None
S21	Y	2	1	2	g	None
S22	Y	2	3	3	i, i	None
S23	Y	2	1	2	l	None
S24	Y	1	3	3	b	None
S25	Y	2	1	3	c, i	None
S26	Y	1	1	2	g	None
S27	Y	2	1	3	g	None
S28	Y	3	3	3	l	None

TABLE 6.4

***MC1R* Variants, Skin Type, Eye and Hair Colour in Control Subjects**

Identifier	Skin Type	Eye Colour	Hair Colour	*MC1R* Variant(s)
C1	2	4	4	None
C2	2	2	2	None
C3	3	3	4	None
C4	2	1	2	h, l
C5	2	1	4	None
C6	2	2	1	None
C7	2	1	4	c, c
C8	3	3	4	g
C9	3	3	4	None
C10	3	3	4	h
C11	2	1	2	h, i
C12	1	4	1	g, i
C13	2	4	4	a, i
C14	3	1	3	a
C15	2	1	3	g
C16	2	4	4	j
C17	2	1	3	a
C18	3	1	3	j
C19	2	1	2	a
C20	3	1	3	i, j
C21	3	1	2	a, l
C22	2	1	4	None
C23	1	1	1	f, j
C24	3	2	3	a
C25	3	1	2	c
C26	2	1	2	i
C27	1	1	2	i
C28	3	1	2	a
C29	3	3	4	i
C30	3	3	4	h
C31	2	1	2	a, i
C32	3	3	2	None
C33	3	3	4	g
C34	2	1	2	g
C35	2	1	3	None
C36	2	1	3	c
C37	2	1	2	c, g
C38	2	1	4	None
C39	1	2	3	a, a
C40	2	4	3	c
C41	3	1	4	f
C42	1	3	1	

Identifier	Skin Type	Eye Colour	Hair Colour	*MC1R* Variant(s)
C43	1	1	2	a, i
C44	2	3	2	c
C45	3	4	4	a, j
C46	2	1	2	g
C47	3	3	3	None
C48	1	1	1	a, i
C49	3	3	4	a, j
C50	1	1	1	a
C51	1	1	2	c, i
C52	3	3	3	c
C53	2	1	3	a, i
C54	3	3	4	a, c
C55	2	1	2	g
C56	3	1	2	a, b
C57	1	2	1	g, i
C58	3	3	4	g
C59	2	3	3	c, g
C60	2	1	3	i
C61	2	2	4	None
C62	3	1	2	l
C63	3	3	4	a
C64	1	1	2	j
C65	4	3	4	None
C66	2	1	2	a
C67	1	1	2	g
C68	4	1	2	None

6.6 *MC1R* Variants and Statistical Analyses

Statistical analysis was performed using the methods described in method 3.17. Due to the large quantity of results, only significant and approaching significant results are presented in this chapter. Results not presented in this chapter are reported in tabular form in Appendix 5.

To investigate whether there is a relationship between an individual having any particular *MC1R* variant and melanoma, a two-tailed Fisher's exact test was performed on cases and on control subjects for each *MC1R* variant identified in the entire data set (Appendix 5, Tables 1-4).

Among the 13 different variants identified in all sequenced individuals, the most frequently occurring variant in the melanoma patients is V60L. V60L was found in 25/88 (28.4%) melanoma patients and 19/67 (28.4%) control subjects. R151C occurred in 27/88 (30.7%) of patients compared with 12/67 (17.9%) of control subjects and R160W in 21/88 (23.9%) compared with 14/67 (20.9%) of control subjects. D84E was found in 8/88 (9.1%) patients compared with 1/67 (1.5%) controls subjects.

Table 6.5 shows the significant and nearly significant observations in familial and sporadic melanoma patients, and in the control subjects. 83/88 (94.3%) of patients and 53/88 (79.1%) of control subjects carry an *MC1R* variant.

TABLE 6.5

***MC1R* Variants Found in Familial and Sporadic Melanoma Patients Compared with Control Subjects**

	No. of Cases	No. of Controls
Any variant	83	53
No variant	5	14
P-value = 0.006 ** significant (not significant after Bonferroni correction)		
OR = 4.385, CI 1.492-12.883		
D84E present	8	1
D84E absent	80	66
P-value = 0.079 (not significant)		
OR = 6.600, CI 0.805-54.128		
R151C present	28	12
R151C absent	61	55
P-value = 0.065 (not significant)		
OR = 2.104, CI 0.976-4.536		

Bonferroni adjusted significance level for multiple tests = 0.004

There is a significant difference between carrying an *MC1R* variant or not between the melanoma cases and control subjects (P = 0.006; OR = 4.385; CI 1.492-12.883.

There is a nearly significant difference between carrying the R151C variant or not between the melanoma cases and control subjects (P = 0.065; OR = 2.104; CI 0.976-4.536).

There is a nearly significant difference between carrying the D84E variant or not between the cases and control subjects (P = 0.079; OR = 6.600; CI 0.805-54.128). However the Bonferroni adjusted significance level for multiple tests in this case is 0.004 and therefore these results do not remain significant.

The most commonly occurring variants in the familial melanoma patients are:

- V60L in 19/60 (31.7%) compared with 19/67 (28.4%) control subjects.
- V92M in 12/60 (20%) compared with 10/67 (14.9%) control subjects.
- R151C in 20/60 (33.3%) compared with 12/67 (17.9%) control subjects.
- R160W in 11/60 (18.3%) compared with 14/67 (20.9%) control subjects.
- 58/60 (96.7%) familial melanoma patients and 53/67 (79.1%) control subjects carry at least one *MC1R* variant.

Table 6.6 shows only the significant observations of *MC1R* variants in familial melanoma patients, compared with the control subjects. There is again a significant difference between carrying any *MC1R* variant or none between the familial cases and control subjects (P = 0.003; OR = 7.660; CI 1.663-35.296). There is a significant difference between carrying the R151C variant or not between the familial cases and control subjects (P = 0.043; OR = 2.406; CI 1.062-5.452). The Bonferroni adjusted significance level for multiple tests in this case is 0.004 and hence carrying any *MC1R* variant remains significant, although carrying the R151C variant loses significance.

TABLE 6.6

Correlation between *MC1R* Variants in Familial Melanoma Patients and Control Subjects

	No. of Cases	**No. of Control**
Any variant	58	53
No variant	2	14
P-value = 0.003 ** significant		
OR = 7.660, CI 1.663-35.296		
R151C present	21	12
R151C absent	40	55
P-value = 0.043 * significant (not significant after Bonferroni correction)		
OR = 2.406, CI 1.062-5.452		

Bonferroni adjusted significance level for multiple tests = 0.004

6.6.1 MC1R Variants in Familial Melanoma Patients with CDKN2A Mutations Compared with those with no Detectable CDKN2A Mutation

The following results show *MC1R* variants in *CDKN2A* positive and negative patients before statistical analysis. The most frequently occurring variants in the *CDKN2A* positive patients are:

- V60L in 12/23 (52.2%) *CDKN2A* positive patients compared with 7/37 (18.9%) *CDKN2A* negative patients.
- R151C in 12/23 (52.2%) *CDKN2A* positive patients compared with 8/37 (21.6%) *CDKN2A* negative patients.
- R160W in 5/23 (21.7%) *CDKN2A* positive patients compared with 6/37 (16.2%) *CDKN2A* negative patients.
- D294H in 4/23 (17.4%) *CDKN2A* positive patients compared with 2/37 (5.4%) *CDKN2A* negative patients.
- The V92M variant is only present in 1/23 (4.3%) *CDKN2A* positive patients compared with 11/37 (29.7%) *CDKN2A* negative patients.
- The R163Q variant is only present in 1/23 (4.3%) *CDKN2A* positive patients compared with 6/37 (16.2%) *CDKN2A* negative patients.
- 23/23 (100%) *CDKN2A* positive patients and 35/37 (94.6%) *CDKN2A* negative patients carry an *MC1R* variant.

It should be noted that relatively small numbers are included in some of these comparisons.

Table 6.7 shows the significant observations in familial melanoma patients with and without a *CDKN2A* mutation, excluding *CDKN2A* polymorphisms. There is a significant difference between carrying the V60L variant or not between the *CDKN2A* positive cases and *CDKN2A* negative cases (P = 0.017; OR = 3.818; CI 1.315-11.084). There is also a significant difference between carrying the R151C variant or not between the *CDKN2A* positive cases and *CDKN2A* negative cases (P = 0.029; OR = 3.515; CI 1.160-10.650). There is a significant difference between carrying the V92M variant or not between the *CDKN2A* negative cases and *CDKN2A* positive cases (P = 0.021; OR = 9.308; CI 1.112-77.888), although the CI is wide. This is perhaps unexpected in that there are more patients without a *CDKN2A* mutation with the V92M variant than in those patients with a *CDKN2A* mutation. There is a nearly significant difference between carrying the R163Q variant or not between the *CDKN2A* positive cases and *CDKN2A* negative cases (P = 0.078; OR = 0.157; CI 0.018-1.327).

Unfortunately the Bonferroni adjusted significance level for multiple tests in this case is 0.004 and therefore none of these results remain significant after correction.

TABLE 6.7

Correlation between *MC1R* Variants in Familial Melanoma Patients with and without a *CDKN2A* Mutation

	***CDKN2A* Mutation Positive**	***CDKN2A* Mutation Negative**
V60L present	14	10
V60L absent	11	30
P-value = 0.017 * significant (not significant after Bonferroni correction)		
OR = 3.818, CI 1.315-11.084		
V92M present	1	11
V92M absent	22	26
P-value = 0.021 * significant (not significant after Bonferroni correction)		
OR = 0.107, CI 0.013-0.899		
R151C present	12	9
R151C absent	11	29
P-value = 0.029 * significant (not significant after Bonferroni correction)		
OR = 3.515, CI 1.160-10.650		
R163Q present	1	9
R163Q absent	22	31
P-value = 0.078 (not significant)		
OR = 0.157, CI 0.018-1.327		

Bonferroni adjusted significance level for multiple tests = 0.004

6.6.2 MC1R Variants in Non- Familial Melanoma Cases

The most frequently occurring variants in the 28 sporadic melanoma patients are:

- V60L in 6/28 (21.4%) compared with 19/67 (28.4%) control subjects.
- R151C in 7/28 (25%) compared with 12/67 (17.9%) control subjects.
- R160W in 10/28 (35.7%) compared with 14/67 (20.9%) control subjects.
- 25/28 (89.3%) patients and 53/67 (79.1%) control subjects carry at least one *MC1R* variant.

Table 6.8 below shows the nearly significant finding in sporadic melanoma patients, compared with in the control subjects. D84E was found in 3/28 (10.7%) compared with 1/67 (1.5%) control subjects (P = 0.075; OR = 7.920; CI 0.787-79.753).

TABLE 6.8

Correlation between *MC1R* Variants in Sporadic Melanoma Patients and Control Subjects

	No. of Cases	No. of Controls
D84E present	3	1
D84E absent	25	66

P-value = 0.075 (not significant)
OR = 7.920, CI 0.787-79.753

6.7 Comparison to the Number of *MC1R* variants in Melanoma Patients and Control Subjects

The number of *MC1R* variants was compared between all melanoma patients, both familial and sporadic, and in the control subjects by performing a two-tailed Fisher's exact test. Table 6.9 below shows that there is a significant relationship between melanoma cases and having two or more *MC1R* variants with 48/87 (55.2%) cases with two or more variants compared with 21/67 (31.3%) control subjects (P = 0.003; OR 2.696; CI 1.384-5.253).

All variants were heterozygous, except for V60L which was detected in five homozygous familial melanoma patients and one control, V92M which was detected in one homozygous control, R151C which was detected in one homozygous familial melanoma patient, R160W which was detected in two homozygous familial melanoma patients and one sporadic patient and R163Q which was detected in three homozygous familial melanoma patients and one sporadic patient.

TABLE 6.9

Correlation between the Number of *MC1R* Variants and Melanoma (Familial and Sporadic Patients) and Control Subjects

	≤ One *MC1R* Variant	≥ Two *MC1R* Variants
No. of Cases	39	48
No. of Controls	46	21
P-value = 0.003 ** significant OR = 2.696, CI 1.384-5.253		

The number of *MC1R* variants was then compared in melanoma families studying both affected and unaffected family members, and in the control subjects by performing a two-tailed Fisher's exact test. Table 6.10 below shows that there is a significant relationship between melanoma family members and having two or more *MC1R* variants with 47/84 (56%) members with two or more variants compared with 21/67 (31.3%) control subjects (P = 0.003; OR 2.782; CI 1.421-5.450).

TABLE 6.10

Correlation between the Number of *MC1R* Variants in Familial Melanoma (Affected and Unaffected) and Control Subjects

	≤ One *MC1R* Variant	≥ Two *MC1R* Variants
Affected and Unaffected Family Members	37	47
No. of Controls	46	21
P-value = 0.003 ** significant OR = 2.782, CI 1.421-5.450		

The number of *MC1R* variants was then compared between melanoma patients from affected families and the control subjects by performing a two-tailed Fisher's exact test. Table 6.11 below shows that there is a very highly significant relationship between familial melanoma cases and having two or more *MC1R* variants with 37/59 (62.7%) cases with two or more variants compared with 21/67 (31.3%) control subjects (P = 0.001; OR 3.684; CI 1.761-7.707).

TABLE 6.11

Correlation between the Number of *MC1R* Variants in Familial Melanoma Patients and Control Subjects

	≤ One *MC1R* Variant	≥ Two *MC1R* Variants
No. of Cases	22	37
No. of Controls	46	21
P-value = 0.001 *** significant OR = 3.684, CI 1.761-7.707		

The number of *MC1R* variants was compared between unaffected family members of familial melanoma patients and the control subjects by performing a two-tailed Fisher's exact test (Appendix 5, Table 19). There is no significant difference between unaffected family members and control subjects and how many *MC1R* variants they carry.

6.8 Comparison of the Number of *MC1R* variants in *CDKN2A* Positive Melanoma Patients and Control Subjects

The number of *MC1R* variants was compared between familial melanoma patients with a *CDKN2A* mutation and in the control subjects by performing a two-tailed Fisher's exact test. Table 6.12 below shows that there is a significant relationship between *CDKN2A* positive melanoma cases and having two or more *MC1R* variants with 15/21 (71.4%) cases with two or more variants compared with 21/67 (31.3%) control subjects (P = 0.002, OR 5.476, CI 1.863-16.099).

TABLE 6.12

Correlation between the Number of *MC1R* Variants in Familial Melanoma Patients with a *CDKN2A* Mutation and Control Subjects

	≤ One *MC1R* Variant	≤ Two *MC1R* Variants
No. of Cases	6	15
No. of Controls	46	21
P-value = 0.002 ** significant OR = 5.476, CI 1.863-16.099		

6.9 Comparison of the Number of *MC1R* variants in *CDKN2A* Negative Melanoma Patients and Control Subjects

The number of *MC1R* variants was compared between familial melanoma patients without a *CDKN2A* mutation and in the control subjects by performing a two-tailed Fisher's exact test. Table 6.13 below shows that there is a significant relationship between *CDKN2A* negative melanoma cases and having two or more *MC1R* variants with 22/38 (57.9%) cases with two or more variants compared with 21/67 (31.3%) control subjects (P = 0.013; OR 3.012; CI 1.319-6.875).

TABLE 6.13

Correlation between the Number of *MC1R* Variants in Familial Melanoma Patients without a *CDKN2A* Mutation and Control Subjects

	≤ One *MC1R* Variant	≥ Two *MC1R* Variants
No. of Cases	16	22
No. of Controls	46	21
P-value = 0.013 * significant OR = 3.012, CI 1.319-6.875		

The number of *MC1R* variants was compared between familial melanoma patients with a *CDKN2A* mutation and familial melanoma patients without a *CDKN2A* mutation by performing a two-tailed Fisher's exact test (Appendix 5, Table 20).

There is no significant association between *CDKN2A* status in melanoma cases and having two or more *MC1R* variants.

The number of *MC1R* variants was compared between familial melanoma patients and currently unaffected family members by performing a two-tailed Fisher's exact test. Table 6.14 below shows that there is almost a significant difference between melanoma cases and having two or more *MC1R* variants with 37/59 (62.7%) patients with two or more variants compared with 10/25 (40%) unaffected family members (P = 0.091; OR 2.523; CI 0.967-6.579).

TABLE 6.14

Number of *MC1R* Variants in Familial Melanoma Patients Compared with their Unaffected Relatives

	≤ One *MC1R* Variant	≥ Two *MC1R* Variants
No. of Familial Melanoma Patients	22	37
No. of Unaffected Relatives	15	10
P-value = 0.091 (not significant) OR = 2.523, CI 0.967-6.579		

The number of *MC1R* variants was compared between sporadic melanoma patients and the control subjects by performing a two-tailed Fisher's exact test. There is no significant difference between sporadic melanoma patients and control subjects and how many *MC1R* variants they carry in this small data set (Table 6.15).

TABLE 6.15

Correlation between the Number of *MC1R* Variants in Sporadic Melanoma Patients and Control Subjects

	≤ One *MC1R* Variant	≥ Two *MC1R* Variants
No. of Cases	17	11
No. of Control Subjects	46	21
P-value = 0.482 (not significant) OR = 1.417, CI 0.566-3.547		

The number of *MC1R* variants was compared between familial melanoma patients and sporadic melanoma patients by performing a two-tailed Fisher's exact test. Table 6.16 below shows that there is almost a significant difference between familial melanoma cases and having two or more *MC1R* variants with 37/59 (62.7%) familial melanoma patients with two or more variants compared with 11/28 (39.3%) sporadic cases (P = 0.064; OR 2.599; CI 1.032-6.548).

TABLE 6.16

Correlation between the Number of *MC1R* Variants in Familial and Sporadic Melanoma Patients

	≤ One *MC1R* Variant	≥ Two *MC1R* Variants
No. of Familial Cases	22	37
No. of Sporadic Cases	17	11
P-value = 0.064 (not significant)		
OR = 2.599, CI 1.032-6.548		

6.10 Correlation Between *MC1R* Variants and Phenotype

The following sections look at phenotype in relation to *MC1R* variants.

6.10.1 Skin Type

In order to determine whether there is any significant relationship between having a particular *MC1R* variant and a particular skin type, 2 × 4 tables were constructed to include the four skin types and the *MC1R* variants detected in the control subjects. Statistical analysis was performed on phenotyped control subjects only (Appendix 5, Table 7). This table only shows data for which a P-value could be calculated. The following table 6.17 shows the significant observation in the phenotyped control subjects. Among the variants identified the only one to show a significant finding is R160W (P = 0.007). The Bonferroni adjusted significance level for multiple tests here is 0.0071 and therefore R160W remains significantly correlated with skin type after correction.

TABLE 6.17

Correlation between Skin Type and *MC1R* variants in Control Subjects

Chi-squared Test

	Never Tans Always Burns	Burns Easily Tans Rarely	Burns Rarely Tans Easily	Never Burns Always Tans
R160W present	6	6	2	0
R160W absent	5	23	23	2

P-value = 0.007 ** significant

Bonferroni adjusted significance level for multiple tests = 0.0071

Therefore it can be seen that the presence of the R160W variant in normal Scottish individuals is significantly associated with paler skin type.

To investigate in more detail as to which *MC1R* variant correlates with skin type 1, a two-tailed Fisher's exact test was performed for each variant on all cases, relatives and control subjects, with skin type 1 (Appendix 5, Table 31).

The following table 6.18 shows the significant and nearly significant observations. Among the variants identified the only one to show a significant finding with skin type 1 is R160W ($P < 0.001$). The Bonferroni adjusted significance level for multiple tests here is 0.005 and therefore R160W remains significantly correlated with skin type 1 after correction. R142H ($P = 0.052$) is nearly significant.

TABLE 6.18

Correlation between *MC1R* Variants and Skin Type 1

	Skin Type 1	Other Skin Type
R160W present	18	24
R160W absent	16	116
P-value < 0.001 *** significant		
OR = 5.438, CI 2.433-12.152		
R142H present	3	2
R142H absent	31	138
P-value = 0.052 (not significant)		
OR = 6.677, CI 1.070-41.674		

Bonferroni adjusted significance level for multiple tests = 0.005

6.10.2 Eye Colour

With the intention of determining whether there is any significant relationship between having a particular *MC1R* variant and a particular eye colour, 2 × 4 tables were constructed to include the four eye colours and the *MC1R* variants detected in the control subjects. Statistical analysis was performed on phenotyped control subjects (Appendix 5, Table 6) and all blue-eyed individuals (Appendix 5, Table 32). No significant relationship could be identified between eye colour and *MC1R* variants in this data set.

6.10.3 Hair Colour

In order to determine whether there is any significant relationship between having a particular *MC1R* variant and a particular colour of hair, 2 × 4 tables were constructed to include the four hair colours and the *MC1R* variants detected in the control subjects. Statistical analysis was performed on phenotyped control subjects only (Appendix 5, Table 5). This table only shows data for which a P-value could be calculated. No significant relationship could be identified between hair colour and *MC1R* variants in this data set.

6.11 Correlation between the *MC1R* Variants, Red Hair and Melanoma

To investigate in more detail as to which *MC1R* variant correlates with red hair in these Scottish individuals, a two-tailed Fisher's exact test was performed for each variant on all individuals, cases, relatives and control subjects, with red hair (Appendix 5, Table 33).

The following table 6.19 shows the significant observations. Among the variants identified, both R142H (P = 0.019) and R160W (P = 0.018) show a significant positive association with red hair. Unfortunately the Bonferroni adjusted significance level for multiple tests in this case is 0.006 and therefore these two observations lose significance.

TABLE 6.19

Correlation between *MC1R* Variants and Red Hair in all DNA Samples Available

	Red Hair	Other than Red Hair
R142H present	3	2
R142H absent	21	148
P-value = 0.019 * significant (not significant after Bonferroni correction)		
OR = 10.571, CI 1.668-67.009		
R160W present	11	31
R160W absent	13	119
P-value = 0.018 * significant (not significant after Bonferroni correction)		
OR = 3.248, CI 1.327-7.949		

Bonferroni adjusted significance level for multiple tests = 0.006

Some previous studies have associated the R142H, R151C, R160W and D294H variants with red hair (Valverde et al. 1995; Smith et al. 1998; Flanagan et al. 2000). Numbers are small and statistical analysis has not always been rigorous. This is discussed further on page 274. In this thesis, a significant association with melanoma and R151C and a significant association with skin type 1 and R160W have been found.

Among the 67 control subjects tested for *MC1R* variants, two individuals are compound heterozygotes for R151C/R160W. Both individuals have red hair and skin type 1. Among the 88 melanoma cases, four have red hair and also are compound heterozygotes R151C/R160W. Two are homozygous; one for R151C and one for R160W, both of whom have red hair.

6.11.1 Correlation Between Having Melanoma and Carrying a Postulated Red Hair Variant

In order to identify whether or not there is a correlation between having melanoma and carrying a postulated red hair variant of *MC1R*, a two-tailed Fisher's exact test was performed on all red haired melanoma patients, both familial and sporadic, and in the red haired control subjects (Appendix 5, Table 21). No significant relationship could be determined in this small data set.

6.11.2 Correlation Between Having Red Hair and Carrying a Postulated Red Hair Variant

To establish if there is an association between the postulated red hair variants and having red hair, a two-tailed Fisher's exact test was performed on all red haired individuals whether they are melanoma patients, family members or control subjects (Appendix 5, Table 22). Of the seven red headed control subjects, four were found to be compound heterozygotes for at least one of the postulated red hair variants (R142H, R151C, R160W). DNA from one of the red haired control subjects failed on sequence analysis. The D294H variant was not seen in any red haired control or melanoma patient. From the sample of 27 red haired individuals (seven control subjects, six sporadic melanoma patients, ten familial melanoma patients and four currently unaffected family members) a significant association between red hair and the presence of the high-penetrance *MC1R* alleles R142H, R151C, R160W and D294H could not be demonstrated with this data. The control red haired individuals were tested, but again no significant relationship could be determined between carrying these variants and having red hair (Appendix 5, Table 23).

The possible correlation between carrying any of the variants R142H, R151C, R160W and D294H and having melanoma was considered by performing a two-tailed Fisher's exact test on all melanoma patients, both familial and sporadic, and the control subjects. Table 6.20 below shows a nearly significant association as 51/88 (58%) patients one or more of these variants compared with 29/67 (43.3%) control subjects (P = 0.077; OR = 1.806; CI 0.950-3.434).

TABLE 6.20

Correlation between the Presence of *MC1R* R142H, R151C, R160W or D294H Variant in Melanoma (Familial and Sporadic Patients) and Control Subjects

	No. of Cases	No. of Controls
R142H, R151C, R160W or D294H Present	51	29
R142H, R151C, R160W and D294H Absent	37	38
P-value = 0.077 (not significant)		
OR = 1.806, CI 0.950-3.434		

To determine if there is a possible correlation between carrying any of these variants and having familial melanoma a two-tailed Fisher's exact test was performed on all familial melanoma patients compared to the control subjects (Appendix 5, Table 24). No significant differences were identified between the two groups.

The possible correlation between carrying a R142H, R151C, R160W or D294H variant and having sporadic melanoma was considered by performing a two-tailed Fisher's exact test on sporadic melanoma patients compared to the control subjects. Table 6.21 below shows the nearly significant connection as 18/28 (64.3%) patients carry one or more of these variants compared with 29/67 (43.3%) control subjects (P = 0.074; OR = 2.359; CI 0.948-5.869).

TABLE 6.21

Correlation between *MC1R* R142H, R151C, R160W or D294H Variant and Sporadic Melanoma Patients and Control Subjects

	No. of Cases	No. of Controls
R142H, R151C, R160W or D294H Present	18	29
R142H, R151C, R160W and D294H Absent	10	38
P-value = 0.074 (not significant)		
OR = 2.359, CI 0.948-5.869		

To determine if there is a possible correlation between carrying a R142H, R151C, R160W or D294H variant and having familial melanoma with a *CDKN2A* mutation, a two-tailed Fisher's exact test was performed on all familial melanoma patients with a *CDKN2A* mutation and the control subjects. Table 6.22 below shows the nearly significant association as 14/21 (66.7%) patients carry a R142H, R151C, R160W or D294H variant compared with 29/67 (43.3%) control subjects (P = 0.081; OR = 2.621; CI 0.937-7.326).

TABLE 6.22

Correlation between *MC1R* R142H, R151C, R160W or D294H Variant and Familial Melanoma Patients with a *CDKN2A* Mutation and Control Subjects

	No. of Cases	No. of Controls
R142H, R151C, R160W or D294H Present	14	29
R142H, R151C, R160W and D294H Absent	7	38
P-value = 0.081 (not significant)		
OR = 2.621, CI 0.937-7.326		

The possible correlation between carrying a R142H, R151C, R160W or D294H variant and having familial melanoma without a *CDKN2A* mutation was considered by performing a two-tailed Fisher's exact test on all familial

melanoma patients without a *CDKN2A* mutation and the control subjects (Appendix 5, Table 25). No significant differences were identified between the two groups.

To determine if there is a possible correlation between carrying a R142H, R151C, R160W or D294H variant and having familial melanoma compared with sporadic melanoma, a two-tailed Fisher's exact test was performed on all familial melanoma patients and on all sporadic melanoma patients (Appendix 5, Table 26). No significant differences were identified between the two groups.

In order to determine if there is a significant difference between familial melanoma patients and their currently unaffected family members, and carrying a R142H, R151C, R160W or D294H variant, a two-tailed Fisher's exact test was carried out on all family members, affected and unaffected (Appendix 5, Table 27). No significant differences were identified between the two groups.

To investigate whether there is a difference between carrying a *CDKN2A* mutation and a R142H, R151C, R160W or D294H variant or not, a two-tailed Fisher's exact test was carried out on both *CDKN2A* positive and negative familial melanoma patients (Appendix 5, Table 28). No significant differences were identified between the two groups.

To determine if there is a possible correlation between carrying a R142H, R151C, R160W or D294H variant and being an unaffected family member, a two-tailed Fisher's exact test was performed on all currently unaffected family members and in the control subjects (Appendix 5, Table 29). No significant differences were identified between the two groups.

In order to determine if there is a significant difference between familial melanoma patients taken together with their currently unaffected family members and carrying a R142H, R151C, R160W or D294H variant, and control subjects, a two-tailed Fisher's exact test was carried out on all family members, affected and unaffected and control subjects (Appendix 5, Table 30). No significant differences were identified between the two groups.

6.12 Summary of Statistically Significant Findings

This section of work has given rise to many observations. The following points summarise the statistically significant findings.

- Collectively, both familial and sporadic melanoma patients are more likely to carry a *MC1R* variant than normal control subjects (P = 0.006; OR = 4.485; CI 1.492-12.883).
- Melanoma patients carry significantly more *MC1R* variants than control subjects (P = 0.003; OR = 2.696; CI 1.384-5.253).
- Familial melanoma patients are more likely to carry the R151C variant than normal control subjects (P = 0.043; OR = 2.406; CI 1.062-5.452) and are more likely to carry any *MC1R* variant than normal control subjects (P = 0.003; OR = 7.660; CI 1.663-35.296).
- *CDKN2A* positive familial melanoma patients are more likely to carry the V60L and R151C variants than *CDKN2A* negative familial melanoma patients (P = 0.017; OR = 3.818; CI 1.315-11.084 and P = 0.029; OR = 3.515; CI 1.160-10.650, respectively). Conversely, *CDKN2A* negative familial melanoma patients are more likely to carry the V92M variant than *CDKN2A* positive familial melanoma patients (P = 0.021; OR = 9.308; CI 1.112-77.888).
- Variant R160W is significantly correlated with skin type 1 (P < 0.001).
- Variants R142H (P = 0.019) and R160W (P = 0.018) are significantly correlated with red hair, although this significance is lost after the Bonferroni correction.

It should however be noted that this study has used greater statistical rigor than any other previously published paper, and that numbers are relatively small. Larger confirmatory studies using the same degree of statistical rigor are needed.

CHAPTER 7

MUTATION SCREENING OF EXON 15 OF THE *BRAF* GENE

7.1 Exon 15 *BRAF* Mutation Detection

Recent studies have identified that the exon 15 V599E *BRAF* mutation is common in primary melanoma tissue (Davies et al. 2002). At the time Davies published in Nature, no data was available on *BRAF* mutations in germline DNA from familial melanoma patients, and the number of melanoma tissue samples investigated for *BRAF* mutations was low. I therefore searched for exon 15 *BRAF* mutations in germline DNA from patients with familial melanoma, both with and without identified *CDKN2A* mutations. I then investigated frozen melanoma tissue from secondary tumours for exon 15 *BRAF* mutations. Finally, I looked for exon 15 BRAF mutations in a large panel of primary melanomas of which there was details of body site and histogenetic type.

7.2 Exon 15 *BRAF* Studies on Germline DNA from Patients with Familial Melanoma

7.2.1 Source of DNA

Blood samples were obtained from 42 patients with familial melanoma. DNA was extracted, stored and used (Methods 3.1a, 3.2). DNA was also available from fresh secondary melanoma tissue from two familial probands F4.1 and F10.1.

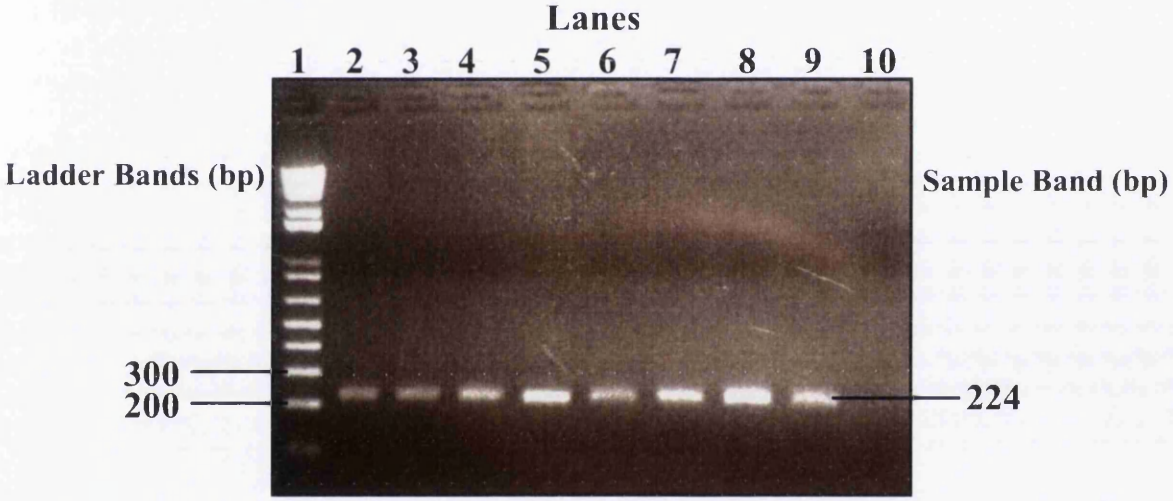
7.2.2 Mutation Strategy Employed

The exon 15 set of primers taken from Davies et al were designed to amplify the complete coding region of exon 15 and its splice sites. The final amplification conditions are presented in Appendix 2. Routinely a 1μl aliquot of genomic DNA (extracted as described using method 3.1a) from each patient was amplified with the set of primers (Method 3.4). Fragment sizes of 224bp for exon 15 were generated. Negative controls (no DNA) were run along with each reaction. Once all the DNA samples had been transferred to PCR tubes, the same reagents were used for the negative control. The absence of amplicons in the negative control meant that there had been no cross-contamination between the samples or into the reagents. The distinct bands obtained from amplification were resolved on ethidium bromide stained agarose gels to verify the size of the amplified band (Method 3.5) and are presented in figure 7.1.

FIGURE 7.1

Amplification of Exon 15 of *BRAF*

The expected 224bp amplicons of exon 15 of *BRAF* were confirmed by resolution on a 1.5% agarose gel with ethidium bromide. The amplicons were electrophoresed in 1X TAE buffer for 20 minutes. The size of the amplicons which are represented below shown in base pairs were deduced by comparison to the migration of the known size of the DNA ladder fragments in lane 1. 1µl of genomic DNA or 2µl of somatic DNA was routinely amplified by conditions specified in Appendix 2 and with primers specified in Appendix 1. The amplicons' size is consistent with the expected 224bp fragment and was sequenced to confirm this.



Electrophoresis Conditions: 1.5% agarose gel, 1X TAE, 10µl size marker, 5µl PCR product, 2µl loading mix, 100V, 20 minutes, room temperature

Lane 1 – 1kb Plus size marker

Lane 2 – PCR product from individual F1.1

Lane 3 – PCR product from individual F2.1

Lane 4 – PCR product from individual F3.1

Lane 5 – PCR product from individual F4.1

Lane 6 – PCR product from individual F5.1

Lane 7 – PCR product from individual F6.2

Lane 8 – PCR product from individual F7.1

Lane 9 – PCR product from individual F8.1

Lane 10 – negative control

See page 60 for patient details.

7.2.3 Direct Sequencing of all Amplicons

Exon 15 of *BRAF* was subjected to direct sequencing (Methods 3.7-3.11). The primers used for sequence analysis were the same as the amplification primers. DNA sequence of the complete exon and at least 20 base pairs of flanking DNA were routinely obtained. In total, only one nucleic acid change was found during sequencing of the samples. Any changes were confirmed by reamplification and sequencing of the mutated exon and the resulting amino acid change identified.

7.2.4 Sequence Analysis of Amplicons

Genomic DNA from familial melanoma cases

DNA from the peripheral blood of the 42 familial melanoma cases contained no exon 15 *BRAF* mutations. This result was irrespective of the number of affected family members. Secondly, this result was irrespective of whether the families are *CDKN2A* mutation positive or *CDKN2A* mutation negative.

Somatic DNA from familial melanoma cases

The DNA from the two samples of secondary melanoma from individuals F4.1 and F10.1 with a family history of melanoma also failed to show exon 15 *BRAF* mutations.

7.3 Detection of *BRAF* Mutations in Frozen Melanoma Tissue from Secondary Tumours from Patients with Sporadic Melanoma

7.3.1 Source of Material

DNA was available from 24 tumour samples from 22 cases of sporadic melanoma (Methods 3.1c, 3.2). All samples were from secondary tumour. Twenty-two of these cases were from lymph nodes and two from soft tissue metastases. Table 7.1 details the age at diagnosis, sex of the patient, DNA source, histology of the primary melanoma, body site and thickness of the frozen secondary melanoma tumours.

Blood samples were obtained from two patients with sporadic melanoma. DNA was extracted, stored and used (Methods 3.1a, 3.2).

TABLE 7.1

Details of Secondary Melanoma Samples Obtained from Sporadic Melanoma Patients Assessed for Exon 15 *BRAF* mutations

Sample Identifier	Age	Sex	DNA Source	Primary Type Histology	Body Site	Thickness (mm)
*96037	50	F	2 x Soft Tissue	SSM	Calf	4.3
87004	52	F	Nodal	SSM	Calf	1.6
92017	47	M	Nodal	SSM	Back	0.2
88137	45	M	Nodal	SSM	Trunk	1.0
94330	55	M	Nodal	SSM	Arm	4.0
*91054	52	F	Nodal	SSM	Thigh	2.0
94126	54	F	Nodal	SSM	Leg	0.6
99067	78	F	Nodal	SSM	Calf	4.3
97165	76	F	Nodal	Nodular	Leg	7.0
96150	48	M	Nodal	Nodular	Leg	1.0
96250	48	F	Nodal	Nodular	Thigh	5.0
91065	68	F	Nodal	Nodular	Leg	3.7
996156	49	M	Nodal	Nodular	Arm	2.8
85207	52	F	Nodal	Nodular	Leg	2.5
97263	78	M	Nodal	Nodular	Leg	13.0
94098	68	F	Nodal	Nodular	Knee	12.0
93087	66	F	Nodal	Nodular	Arm	5.0
96161	65	F	Nodal	Nodular	Sole	8.0
94186	84	F	Nodal	Nodular	Leg	5.0
86240	72	F	Nodal	Nodular	Leg	5.3
97260	79	F	Nodal	Acral	Heel	3.0
95015	56	F	2 x Nodal	Acral	Toe	6.0

Abbreviations for melanoma type are:

SSM = Superficial Spreading Melanoma

Nodular = Nodular Melanoma

Acral = Acral Lentiginous Melanoma

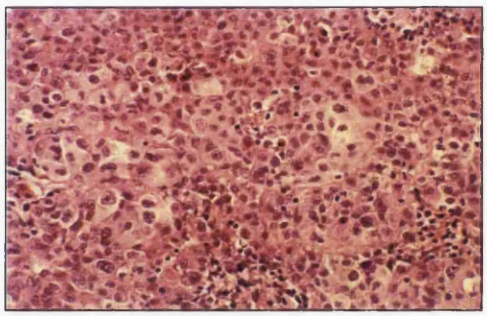
* = Blood samples were also obtained

See pages 36-38 for a description of the melanoma type.

A typical secondary melanoma is illustrated in figure 7.2.

FIGURE 7.2

Secondary Melanoma Cells in a Lymph Node



Example of the pathology of invasive secondary melanoma cells in a lymph node.

7.3.2 Mutation Strategy Employed

Primers and amplification conditions were used as previously described in the familial section (page 204). Routinely a 2µl aliquot of somatic DNA (extracted as described using method 3.1c) was amplified.

7.3.3 Sequence Analysis of Amplicons

Genomic DNA – Two Samples

Blood samples from patient 96037, taken at the same time as tumour excision, were negative for the V599E mutation, which was identified in the corresponding two tumour samples (see following section). A second patient 91054, where both tumour and blood were available, did not carry the V599E mutation or any other exon 15 mutations in either of the samples.

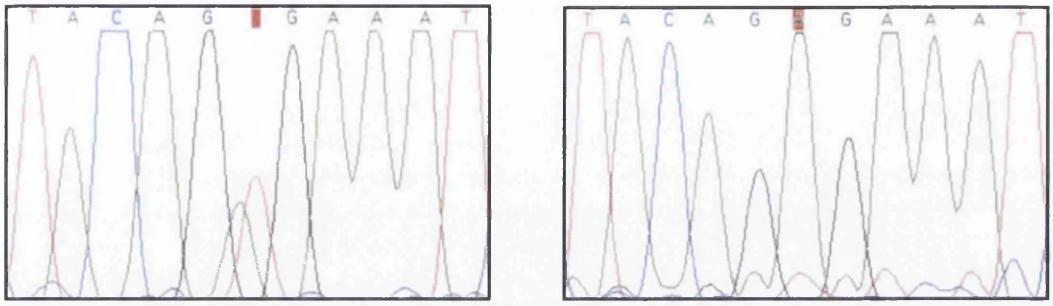
Somatic Secondary Melanoma Tissue DNA – 24 Samples

Identification of Mutation V599E

A heterozygous T to A transition at nucleotide position 1796T>A was identified (Figure 7.3). This base change is predicted to lead to the substitution of a valine (V) for a glutamic acid (E) at codon 599 (V599E) (Figure 7.3). This mutation has been previously reported (Davies et al).

FIGURE 7.3

Identification of Mutation V599E (1796T>A) in Melanoma Tumour Tissue



Sequence analysis of PCR amplified somatic DNA from tumour samples Braf30 and 96037. Tumour DNA was amplified and sequenced using intronic primer pair *BRAF* exon 15 (Appendix 1, 2). Tumour DNA that has lost 1 allele, the wild type allele, will amplify only the remaining allele, the mutant allele, as shown in the right hand side panel.

	A	T	**V**	K	S
Wild Type	GCT	ACA	**GTG**	AAA	TCT
Mutant	GCT	ACA	**GAG**	AAA	TCT
			E		
			(V599E)		

Nucleic acid and derived amino acid sequences from the mutated region of *BRAF*. The amino acid substitution of a valine (V) for a glutamic acid (E) at position 599, V599E is shown.

The V599E substitution was detected in secondary melanoma DNA from 6/22 cases (27%) of secondary melanoma as shown in table 7.2. Four of the samples of secondary melanoma which contained this *BRAF* mutation were from DNA extracted from metastatic deposits in lymph nodes, all from different individuals and 2 were from soft tissue metastatic nodules both from the same individual 96037, taken approximately 9 months apart. The average age at primary melanoma diagnosis of patients with these secondary tumours is 61 years, and those that carry the V599E mutation have an average age at diagnosis of 58 years. Six of the samples came from male patients with one being mutation positive and 16 came from female patients with four being mutation positive. The average thickness of these primary tumours from which these secondary tumours came from is 4.5mm and those with the V599E mutation have an average thickness of 4.2mm. These results are not significant.

TABLE 7.2

Summary of Sequence Analysis of Frozen Secondary Melanoma Tumours

Sample Identifier	Age	Sex	DNA Source	Primary Type Histology	Body Site	Thickness (mm)	Sequence Result 1	Sequence Result 2
96037	50	F	2 x Soft Tissue	SSM	Calf	4.3	V599E	V599E
87004	52	F	Nodal	SSM	Calf	1.6	WT	WT
92017	47	M	Nodal	SSM	Back	0.2	WT	WT
88137	45	M	Nodal	SSM	Trunk	1.0	WT	WT
94330	55	M	Nodal	SSM	Arm	4.0	WT	WT
91054	52	F	Nodal	SSM	Thigh	2.0	WT	WT
94126	54	F	Nodal	SSM	Leg	0.6	WT	WT
99067	78	F	Nodal	SSM	Calf	4.3	WT	WT
97165	76	F	Nodal	Nodular	Leg	7.0	V599E	V599E
96150	48	M	Nodal	Nodular	Leg	1.0	V599E	V599E
96250	48	F	Nodal	Nodular	Thigh	5.0	V599E	V599E
91065	68	F	Nodal	Nodular	Leg	3.7	V599E	V599E
996156	49	M	Nodal	Nodular	Arm	2.8	WT	WT
85207	52	F	Nodal	Nodular	Leg	2.5	WT	WT
97263	78	M	Nodal	Nodular	Leg	13.0	WT	WT
94098	68	F	Nodal	Nodular	Knee	12.0	WT	WT
93087	66	F	Nodal	Nodular	Arm	5.0	WT	WT
96161	65	F	Nodal	Nodular	Sole	8.0	WT	WT
94186	84	F	Nodal	Nodular	Leg	5.0	WT	WT
86240	72	F	Nodal	Nodular	Leg	5.3	WT	WT
97260	79	F	Nodal	Acral	Heel	3.0	WT	WT
95015	56	F	Nodalx2	Acral	Toe	6.0	WT	WT

Abbreviations for melanoma type are:

SSM = Superficial Spreading Melanoma

Nodular = Nodular Melanoma

Acral = Acral Lentiginous Melanoma

See pages 36-38 for a description of the melanoma type.

WT denotes a wild type sequence

F denotes female

M denotes male

7.4 Confirmation of Mutation V599E in Tumour Tissue by Restriction Digestion

A restriction assay was developed to test for the V599E mutation (Method 3.14). Details of this assay are presented in Appendix 3.

The exonic sequence of both wild type and the heterozygous mutant was analysed to identify restriction enzyme sites. For mutation V599E the sequence was altered such that a restriction site was destroyed. This assay allowed confirmation of the presence of the mutation in the secondary tumours and was also used as a screen for the large number of paraffin embedded primary melanoma tissues.

7.4.1 Development of a Restriction Test

The 1796T>A mutation removes a *Tsp*R I site when the mutant A base is present in amplified product (Figure 7.4a).

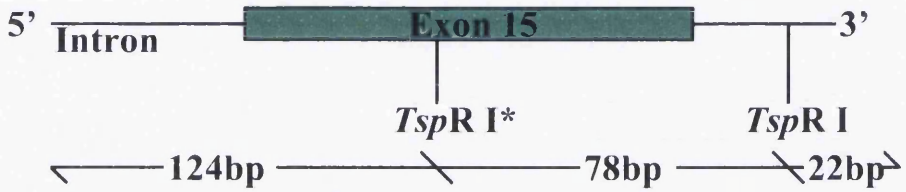
Digestion with *Tsp*R I produces fragments of 202bp and 22bp in affected secondary tumour samples 96250 and 97165, see lanes 2 and 3 (Figure 7.4b). The normal allele (124bp, 78bp and 22bp) and the undigested normal allele (224bp) are both present, see lanes 2-5.

FIGURE 7.4

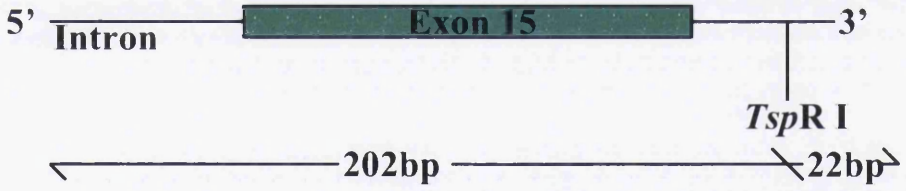
Development of a *Tsp*R I Restriction Site Created by Mutation V599E for Secondary Melanoma Tumour Samples

a) Diagrammatic representation of the 224bp PCR amplified fragment of exon 15 of *BRAF*. The mutation deletes a *Tsp*R I restriction site 124bp into the PCR fragment that is present in the wild type in the absence of the 1796T>A substitution.

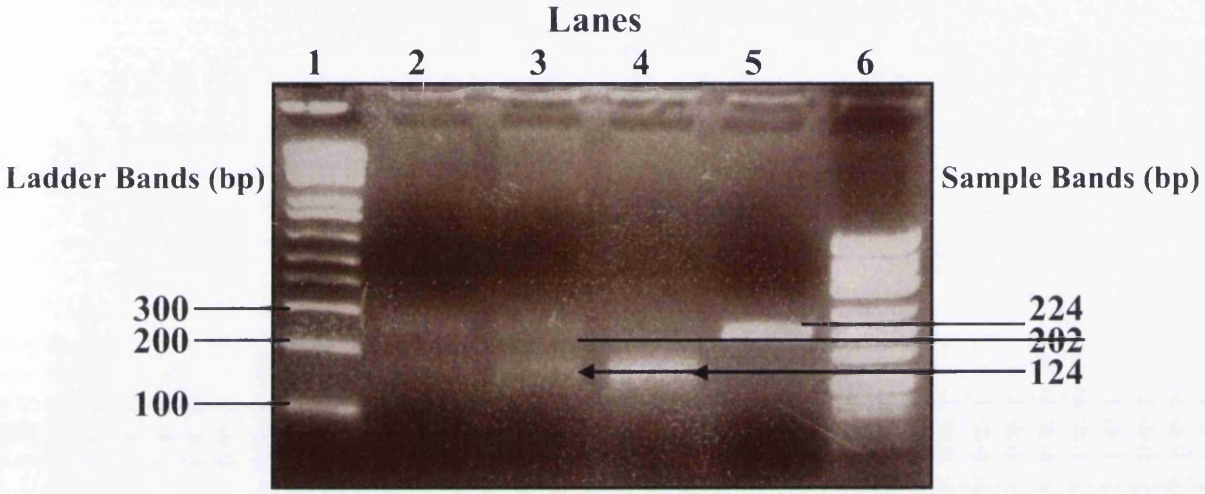
Normal (v599)



Mutant (E599)



b) The expected 202bp and 124bp DNA fragments from the *Tsp*R I digested amplicons of secondary tumour were confirmed by resolution on a 3% agarose gel with ethidium bromide. The fragments were electrophoresed in 1X TAE buffer for 20 minutes. The size of the digested amplicons which are represented below shown in base pairs were deduced by comparison to the migration of the known size of the DNA ladder fragments in lanes 1 and 6.



Electrophoresis Conditions: 3% agarose gel, 1X TAE, 10µl size markers, 5µl digest, 2µl loading mix, 100V, 20 minutes, room temperature

Lane 1 – 1kb Plus size marker

Lane 2 – digested PCR product from tissue 96250

Lane 3 – digested PCR product from tissue 97165

Lane 4 – digested PCR product from a normal control

Lane 5 – undigested PCR product from a normal control

Lane 6 – PhiX174/*Hin*f I size marker

See page 208 for tissue details.

7.5 Detection of *BRAF* Mutations in Formalin Fixed and Paraffin Processed Tissue from Primary Melanomas from Patients with Sporadic Melanoma

7.5.1 Source of Material

DNA was extracted, stored and used from 52 paraffin embedded primary melanoma samples (Methods 3.1b, 3.2). The samples are described in table 7.3, which gives details of the age at which diagnosis was first given, sex of the patient, pathological type, body site and thickness of the tumour.

TABLE 7.3

Primary Tumours from Sporadic Melanoma Patients Assessed for Exon 15 *BRAF* Mutations

Sample Identifier	Age	Sex	Histology	Body Site	Thickness (mm)
Braf1	53	M	SSM	Scalp	6.8
Braf2	70	M	SSM	Trunk	6.0
Braf3	58	M	SSM	Trunk	3.8
Braf4	48	M	SSM	Neck	5.7
Braf5	66	M	SSM	Back	3.5
Braf6	69	F	SSM	Leg	6.3
Braf7	76	F	SSM	Arm	2.0
Braf8	65	F	SSM	Leg	2.9
Braf9	75	F	SSM	Leg	18.0
Braf10	53	M	SSM	Back	2.6
Braf11	76	M	SSM	Face	5.4
Braf12	80	M	SSM	Ear	5.8
Braf13	76	F	SSM	Face	15.2
Braf14	45	M	SSM	Cheek	2.5
Braf15	52	F	SSM	Leg	11.8
Braf16	62	M	SSM	Leg	9.9
Braf17	54	F	SSM	Leg	4.9
Braf18	71	F	Nodular	Leg	10.0
Braf19	69	M	Nodular	Upper Arm	6.0
Braf20	70	F	Nodular	Thigh	4.9
Braf21	76	F	Nodular	Face	3.5
Braf22	86	F	Nodular	Leg	14.6
Braf23	87	F	Nodular	Buttock	5.0
Braf24	82	F	Nodular	Back	6.0
Braf25	66	M	Nodular	Foot	22.0
Braf26	82	M	Nodular	Scalp	6.0
Braf27	51	F	Nodular	Ear	3.0

Sample Identifier	Age	Sex	Histology	Body Site	Thickness (mm)
Braf28	71	M	Nodular	Scalp	9.0
Braf29	72	M	Acral	Toe	3.5
Braf30	65	F	Acral	Leg	4.2
Braf31	61	M	Acral	Ankle	5.8
Braf32	77	M	Acral	Leg	10.0
Braf33	69	F	Acral	Sole	11.5
Braf34	66	M	Acral	Toe	3.3
Braf35	45	F	Acral	Leg	4.2
Braf36	87	F	Acral	Sole	6.1
Braf37	74	M	Acral	Sole	9.3
Braf38	78	F	Acral	Leg	0.6
Braf39	63	F	Acral	Leg	7.5
Braf40	80	M	Acral	Sole	5.5
Braf41	74	M	Acral	Leg	4.2
Braf42	72	M	Mucosal	Lip	7.5
Braf43	40	F	LMM	Face	1.0
Braf44	79	M	LMM	Foot	1.2
Braf45	76	M	LMM	Face	6.1
Braf46	61	M	LMM	Face	9.5
Braf47	88	M	LMM	Face	4.3
Braf48	74	M	LMM	Face	8.4
Braf49	73	F	LMM	Face	2.7
Braf50	58	F	LMM	Neck	1.0
Braf51	73	M	LMM	Ear	1.1
Braf52	72	M	LMM	Face	3.9

Abbreviations for melanoma type are:
SSM = Superficial Spreading Melanoma
Nodular = Nodular Melanoma
Acral = Acral Lentiginous Melanoma
Mucosal = Mucosal Melanoma
LMM = Lentigo Maligna Melanoma

In addition to the above 52 samples, tissue from five further primary melanomas from five individuals were available, but the DNA from these failed to amplify on repeated occasions. These were one 3.5mm Superficial Spreading Melanoma from a male back aged 72 years and four Acral Lentiginous Melanomas melanomas, three from females and one from a male, all from the foot. Thicknesses ranged from 1.6mm-7.0mm. The reason for this failure to amplify is not clear. All samples selected were less than two years old but it is possible that these five samples remained in formalin for longer than the others before processing. Unfortunately the details in the pathology records do not allow confirmation or refute of this hypothesis.

7.5.2 Mutation Strategy Employed

The same set of primers and amplification conditions as previously described in the familial section were used. Routinely a 2μl aliquot of somatic DNA (extracted as described using method 3.1b) was amplified with the set of primers. When paraffin embedded DNA was amplified and was not present on the check gel, a 2μl aliquot of PCR product was reamplifed using the same set of primers and amplification conditions.

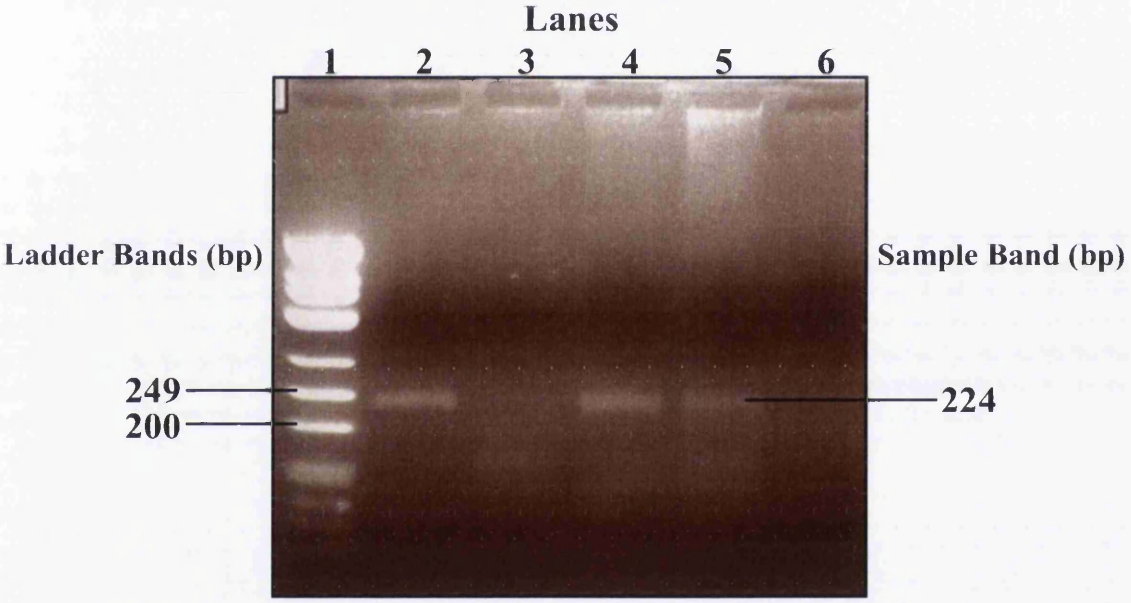
7.5.3 Paraffin Embedded Primary Melanoma Tissue – 52 Samples

The *Tsp*R I digest, as described in section 7.4 above, did not work well for the paraffin embedded primary melanoma tissues. Unfortunately the 202bp and the 124bp fragments were unresolved for the paraffin embedded primary melanoma tissues (Figure 7.5). All samples were therefore sequenced directly (Methods 3.7-3.11).

FIGURE 7.5

***Tsp*R I Restriction Assay for Paraffin Embedded Primary Melanoma Tissues**

The expected 202bp and 124bp DNA fragments from the *Tsp*R I digested paraffin embedded primary melanoma tissue 2nd round PCR products were unable to be resolved on a 3% (2% agarose / 1% Nesieve) gel with ethidium bromide. The fragments were electrophoresed in 1X TAE buffer for 20 minutes. The size of the digested amplicons which are represented below shown in base pairs were deduced by comparison to the migration of the known size of the DNA ladder fragments in lane 1.



Electrophoresis Conditions: 3% agarose gel, 1X TAE, 10µl size markers, 5µl digest, 2µl loading mix, 100V, 20 minutes, room temperature

Lane 1 – PhiX174/*Hin*f I size marker

Lane 2 – undigested PCR product from a normal control

Lane 3 – digested PCR product from a normal control

Lane 4 – digested PCR product from tissue Braf1

Lane 5 – digested PCR product from tissue Braf42

Lane 6 – negative control

See pages 216-217 for tissue details.

7.5.4 Sequence Analysis of Amplicons

Identification of Mutation V599E

Five samples failed to amplify possibly because of degradation. The V599E substitution was detected in tumour DNA from 13 of the remaining 52 samples (25%) of paraffin embedded primary melanoma. A new exon 15 amplicon was generated for direct sequencing of the mutation positive samples using both forward and reverse exon 15 primers to confirm the presence of the mutation. When sequencing for the second time, two of the mutation positive paraffin embedded primary melanoma samples, Braf4 and Braf42, showed a wild type sequence. All samples, whether positive or negative, were therefore sequenced again. A sample, Braf5, which had shown to be wild type during the first sequence analysis, was mutation positive on the second sequence analysis. All samples were sequenced until two concurring mutation positive results were obtained as shown in table 7.4. While samples Braf4, Braf5 and Braf42 all gave two wild type and two mutated sequence results, in all three cases the results using the two additional techniques described below showed the DNA to be mutated, illustrating the value of using additional confirmatory techniques.

TABLE 7.4

Summary of Sequence Analysis of Paraffin Embedded Primary Melanoma Tumours

Sample Identifier	Sequence Result 1	Sequence Result 2	Sequence Result 3	Sequence Result 4
Braf1	V599E	V599E		
Braf2	V599E	V599E		
Braf3	V599E	V599E		
*Braf4	V599E	WT	V599E	WT
*Braf5	WT	V599E	WT	V599E
Braf6	WT	WT		
Braf7	WT	WT		
Braf8	WT	WT		
Braf9	WT	WT		
Braf10	WT	WT		
Braf11	WT	WT		
Braf12	WT	WT		
Braf13	WT	WT		
Braf14	WT	WT		
Braf15	WT	WT		

Sample Identifier	Sequence Result 1	Sequence Result 2	Sequence Result 3	Sequence Result 4
Braf16	WT	WT		
Braf17	WT	WT		
Braf18	V599E	V599E		
Braf19	V599E	V599E		
Braf20	V599E	V599E		
Braf21	WT	WT		
Braf22	WT	WT		
Braf23	WT	WT		
Braf24	WT	WT		
Braf25	WT	WT		
Braf26	WT	WT		
Braf27	WT	WT		
Braf28	WT	WT		
Braf29	V599E	V599E		
Braf30	V599E	V599E		
Braf31	WT	WT		
Braf32	WT	WT		
Braf33	WT	WT		
Braf34	WT	WT		
Braf35	WT	WT		
Braf36	WT	WT		
Braf37	WT	WT		
Braf38	WT	WT		
Braf39	WT	WT		
Braf40	WT	WT		
Braf41	WT	WT		
*Braf42	V599E	WT	WT	V599E
Braf43	V599E	V599E		
Braf44	V599E	V599E		
Braf45	WT	WT		
Braf46	WT	WT		
Braf47	WT	WT		
Braf48	WT	WT		
Braf49	WT	WT		
Braf50	WT	WT		
Braf51	WT	WT		
Braf52	WT	WT		

WT denotes wild type

* denotes samples which produced two WT and two mutated sequence results

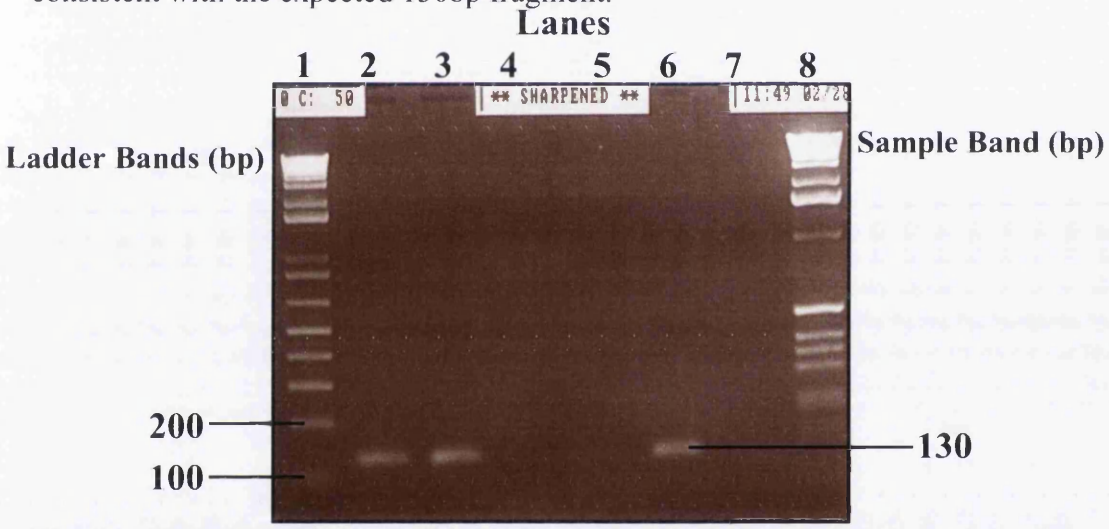
7.5.5 Allele Specific PCR for the Detection of Mutation V599E in Paraffin Embedded Primary Melanoma Tissue

In order to validate the above sequencing results by only amplifying mutant DNA and to be able to perform a rapid screen of paraffin embedded primary melanoma tissues, an allele specific PCR was designed. For this study, the same reverse primer as used previously and taken from Davies et al was used along with an allele specific forward primer designed to only amplify DNA where the mutant V599E allele was present. To confirm that amplification has been performed, a second reaction was set up in parallel using the same reverse primer along with an allele specific forward primer designed to amplify DNA where the normal allele was present. This would therefore amplify all samples, however the reaction using the mutant primer, which has an A instead of a T as its end base, would only anneal to DNA containing the V599E mutation. The final developed amplification conditions are presented in Appendix 2. A 2µl aliquot of somatic DNA from each sample was amplified with each set of primers. A 2µl aliquot of PCR product was then reamplifed using the same sets of primers and amplification conditions. The distinct bands obtained from amplification are presented in figure 7.6.

FIGURE 7.6

Development of an Allele Specific PCR for the Detection of Mutation V599E in Paraffin Embedded Primary Melanoma Tissue

The expected 130bp amplicons of the V599E allele specific PCR were confirmed by resolution on a 2% agarose gel with ethidium bromide. The amplicons were electrophoresed in 1X TAE buffer for 20 minutes. The size of the amplicons which are represented below shown in base pairs were deduced by comparison to the migration of the known size of the DNA ladder fragments in lanes 1 and 8. 2µl of somatic DNA was routinely amplified by conditions specified in Appendix 2 and with primers specified in Appendix 1. The amplicons' size is consistent with the expected 130bp fragment.



Electrophoresis Conditions: 2% agarose gel, 1X TAE, 10µl size marker, 5µl PCR product, 2µl loading mix, 100V, 20 minutes, room temperature

Lane 1 – 1kb Plus size marker

Lane 2 – PCR product from normal Braf12 tissue for v599

Lane 3 – PCR product from mutation positive tissue Braf4 for v599

Lane 4 – negative control for v599

Lane 5 – PCR product from normal Braf12 tissue for E599

Lane 6 – PCR product from mutation positive tissue Braf4 for E599

Lane 7 – negative control for E599

Lane 8 – 1kb size marker

See page 216-217 for tissue details.

For V599E mutation negative samples the reactions amplified with the normal forward primer, however no product amplified with the mutant forward primer. Samples in which the mutation was present, both normal and mutant forward primers amplified the DNA. The results of the allele specific PCR are detailed in table 7.5. All previously sequenced mutation positive and negative samples were confirmed with the allele specific PCR

TABLE 7.5

Summary of V599E Allele Specific PCR on Paraffin Embedded Primary Melanoma Tumours

Sample Identifier	Allele Specific PCR Result
Braf1	V599E
Braf2	V599E
Braf3	V599E
*Braf4	V599E
*Braf5	V599E
Braf6	WT
Braf7	WT
Braf8	WT
Braf9	WT
Braf10	WT
Braf11	WT
Braf12	WT
Braf13	WT
Braf14	WT
Braf15	WT
Braf16	WT
Braf17	WT
Braf18	V599E
Braf19	V599E
Braf20	V599E
Braf21	WT
Braf22	WT
Braf23	WT
Braf24	WT
Braf25	WT
Braf26	WT
Braf27	WT
Braf28	WT
Braf29	V599E
Braf30	V599E

Sample Identifier	Allele Specific PCR Result
Braf31	WT
Braf32	WT
Braf33	WT
Braf34	WT
Braf35	WT
Braf36	WT
Braf37	WT
Braf38	WT
Braf39	WT
Braf40	WT
Braf41	WT
*Braf42	V599E
Braf43	V599E
Braf44	V599E
Braf45	WT
Braf46	WT
Braf47	WT
Braf48	WT
Braf49	WT
Braf50	WT
Braf51	WT
Braf52	WT

See table 7.3 for pathological details.

WT denotes wild type

* denotes samples which produced two WT and two mutated sequence results

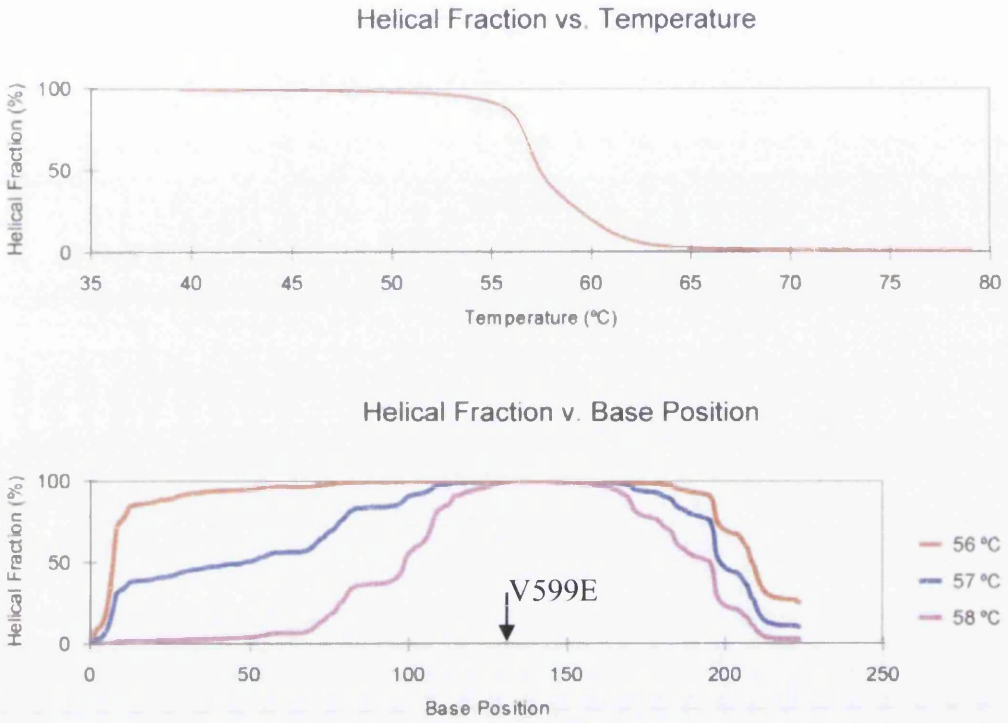
7.5.6 dHPLC Analysis of Paraffin Embedded Primary Melanoma Tissue Samples

As a second method to resolve the somatic mutations detected in the sequencing results, and to be able to perform a rapid screen and most importantly to identify low-level mutant heteroduplex peaks in the paraffin embedded primary melanoma tissues dHPLC analysis was performed.

Optimum dHPLC temperatures were determined by a temperature scan, using the WAVEMaker 4.0 melting profile as a starting point for this 37.5% GC rich fragment. Three mutation positive samples were originally run at four temperatures to determine the profile of the mutated samples. The two best temperatures were chosen (Figure 7.7). For exon 15 of *BRAF*, samples were run at 56°C and 58°C, although all mutations were detected at 56°C.

FIGURE 7.7

Optimum dHPLC Temperatures



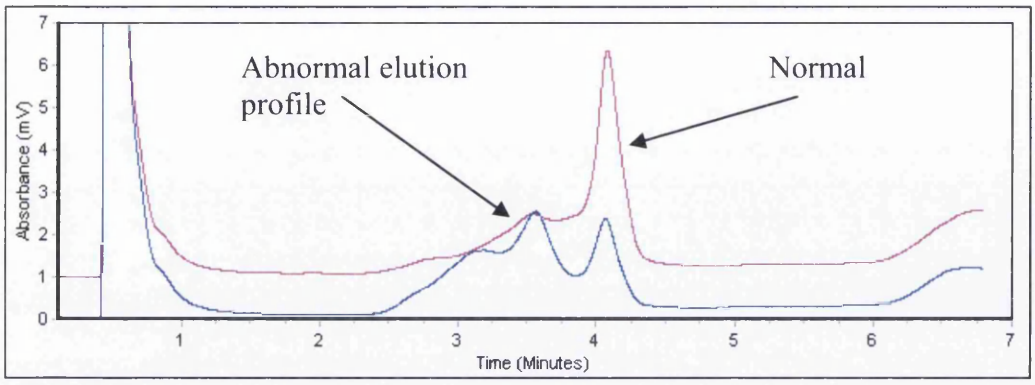
DNA from all 52 paraffin embedded primary melanoma tissue was amplified using the Davies' primers as previously described and 50µl PCR products were then subjected to dHPLC analysis. Each elution profile was compared with the profiles associated with homozygous wild type and mutated sequence controls as shown previously in figure 4.4.

7.5.7 Identification of a Chromatogram WAVE Shift

Thirteen abnormal chromatogram patterns were identified for exon 15 and its flanking sequences (Figure 7.8). The more sensitive dHPLC WAVE analysis returned a mutation positive finding in the three cases that had shown both positive and negative mutation status on sequencing, table 7.6. All sequenced mutation negative samples were also dHPLC mutation negative.

FIGURE 7.8

Identification of Mutation V599E (1796T>A) by dHPLC



dHPLC WAVE analysis of PCR amplified somatic DNA from tumour sample Braf19. Tumour DNA was amplified using intronic primer pair *BRAF* exon 15 (Appendix 1). All elution profiles which were abnormal were identical in pattern to each other.

TABLE 7.6

Summary of dHPLC Results of Paraffin Embedded Primary Melanoma Tumours

Sample Identifier	dHPLC Result
Braf1	Abnormal
Braf2	Abnormal
Braf3	Abnormal
*Braf4	Abnormal
*Braf5	Abnormal
Braf6	WT
Braf7	WT
Braf8	WT
Braf9	WT
Braf10	WT
Braf11	WT
Braf12	WT
Braf13	WT
Braf14	WT
Braf15	WT
Braf16	WT
Braf17	WT
Braf18	Abnormal
Braf19	Abnormal

Sample Identifier	dHPLC Result
Braf20	Abnormal
Braf21	WT
Braf22	WT
Braf23	WT
Braf24	WT
Braf25	WT
Braf26	WT
Braf27	WT
Braf28	WT
Braf29	Abnormal
Braf30	Abnormal
Braf31	WT
Braf32	WT
Braf33	WT
Braf34	WT
Braf35	WT
Braf36	WT
Braf37	WT
Braf38	WT
Braf39	WT
Braf40	WT
Braf41	WT
*Braf42	Abnormal
Braf43	Abnormal
Braf44	Abnormal
Braf45	WT
Braf46	WT
Braf47	WT
Braf48	WT
Braf49	WT
Braf50	WT
Braf51	WT
Braf52	WT

WT denotes wild type

* denotes samples which produced two WT and two mutated sequence results

7.5.8 Contamination

At one point during the analysis of the paraffin embedded primary melanoma tissues there was an incident of PCR product carryover. The PCR product carryover contaminated the second round of amplification. This was obviously a concern because the amplified PCR product served as an ideal template for the subsequent amplifications of that same target. The transfer of aerosol of the amplified product meant on one occasion significant contamination, which resulted in false-positives, and the detection and amplification of the contaminating sequence at the expense of the target sequence in every tissue sample. To minimize PCR product carryover in the subsequent reactions, reactions were physically separated prior to and following amplification, new reagents were used and separate sets of pipettes, filter-plugged pipette tips, microcentrifuge tubes and gloves were used when handling pre- and post-PCR solutions.

7.5.9 Summary of Paraffin Embedded Primary Melanoma Tissue Studies

The complete set of mutation data generated on each of the paraffin embedded primary melanoma tissue is presented in table 7.7. The sequencing results, allele specific PCR and dHPLC analysis are presented for each tissue sample. All paraffin embedded primary melanoma tissue DNAs were screened for mutations in exon 15 of *BRAF* firstly by direct sequencing. Where a mutation was identified, the sample was reamplified and sequenced again to confirm the mutation. In the majority of cases the mutation was confirmed, however in two cases the mutation was not confirmed and therefore the sample was reamplified and sequenced again for a third time. In three samples the pick up rate for the mutation was 50%, therefore all samples, positive or negative for the mutation were reamplified and sequenced until a positive or negative result was obtained twice. Due to this pick up rate in paraffin embedded primary melanoma tissue other screening methods, dHPLC and allele specific PCR, were investigated.

The V599E substitution was detected in primary tumour DNA from 13/52 cases (25%). Five of the samples of primary melanoma which contained this *BRAF* mutation were from DNA extracted from 17 Superficial Spreading Melanomas, three from 11 Nodular Melanomas, two from 13 Acral Lentiginous

Melanomas, one from one Mucosal Melanoma and two from ten Lentigo Maligna Melanomas. The average patient's age at diagnosis of these primary tumours is 68.6 years, and those that carry the V599E mutation have an average age at diagnosis of 64.1 years (NS). Twenty-nine of the samples came from male patients with nine (31%) being mutation positive and 23 came from female patients with four (17.4) being mutation positive. The average thickness of these primary tumours is 6.3mm and those with the V599E mutation have an average thickness of 4.9mm.

TABLE 7.7

Summary of Paraffin Embedded Primary Melanoma Tissue Studies

Sample Identifier	Age	Sex	Histology	Body Site	Thickness	Sequence Result 1	Sequence Result 2	Sequence Result 3	Sequence Result 4	Allele Specific PCR Result	dHPLC Result
Braf1	53	M	SSM	Scalp	6.8	V599E	V599E			Mutation	Abnormal
Braf2	70	M	SSM	Trunk	6.0	V599E	V599E			Mutation	Abnormal
Braf3	58	M	SSM	Trunk	3.8	V599E	V599E			Mutation	Abnormal
Braf4	48	M	SSM	Neck	5.7	V599E	WT	V599E	WT	Mutation	Abnormal
Braf5	66	M	SSM	Back	3.5	WT	V599E	WT	V599E	Mutation	Abnormal
Braf6	69	F	SSM	Leg	6.3	WT	WT			WT	WT
Braf7	76	F	SSM	Arm	2.0	WT	WT			WT	WT
Braf8	65	F	SSM	Leg	2.9	WT	WT			WT	WT
Braf9	75	F	SSM	Leg	18.0	WT	WT			WT	WT
Braf10	53	M	SSM	Back	2.6	WT	WT			WT	WT
Braf11	76	M	SSM	Face	5.4	WT	WT			WT	WT
Braf12	80	M	SSM	Ear	5.8	WT	WT			WT	WT
Braf13	76	F	SSM	Face	15.2	WT	WT			WT	WT
Braf14	45	M	SSM	Cheek	2.5	WT	WT			WT	WT
Braf15	52	F	SSM	Leg	11.8	WT	WT			WT	WT
Braf16	62	M	SSM	Leg	9.9	WT	WT			WT	WT
Braf17	54	F	SSM	Leg	4.9	WT	WT			WT	WT
Braf18	71	F	Nodular	Leg	10.0	V599E	V599E			Mutation	Abnormal
Braf19	69	M	Nodular	Upper Arm	6.0	V599E	V599E			Mutation	Abnormal

223

Sample Identifier	Age	Sex	Histology	Body Site	Thickness	Sequence Result 1	Sequence Result 2	Sequence Result 3	Sequence Result 4	Allele Specific PCR Result	dHPLC Result
Braf20	70	F	Nodular	Thigh	4.9	V599E	V599E			Mutation	Abnormal
Braf21	76	F	Nodular	Face	3.5	WT	WT			WT	WT
Braf22	86	F	Nodular	Leg	14.6	WT	WT			WT	WT
Braf23	87	F	Nodular	Buttock	5.0	WT	WT			WT	WT
Braf24	82	F	Nodular	Back	6.0	WT	WT			WT	WT
Braf25	66	M	Nodular	Foot	22.0	WT	WT			WT	WT
Braf26	82	M	Nodular	Scalp	6.0	WT	WT			WT	WT
Braf27	51	F	Nodular	Ear	3.0	WT	WT			WT	WT
Braf28	71	M	Nodular	Scalp	9.0	WT	WT			WT	WT
Braf29	72	M	Acral	Toe	3.5	V599E	V599E			Mutation	Abnormal
Braf30	65	F	Acral	Leg	4.2	V599E	V599E			Mutation	Abnormal
Braf31	61	M	Acral	Ankle	5.8	WT	WT			WT	WT
Braf32	77	M	Acral	Leg	10.0	WT	WT			WT	WT
Braf33	69	F	Acral	Sole	11.5	WT	WT			WT	WT
Braf34	66	M	Acral	Toe	3.3	WT	WT			WT	WT
Braf35	45	F	Acral	Leg	4.2	WT	WT			WT	WT
Braf36	87	F	Acral	Sole	6.1	WT	WT			WT	WT
Braf37	74	M	Acral	Sole	9.3	WT	WT			WT	WT
Braf38	78	F	Acral	Leg	0.6	WT	WT			WT	WT
Braf39	63	F	Acral	Leg	7.5	WT	WT			WT	WT
Braf40	80	M	Acral	Sole	5.5	WT	WT			WT	WT
Braf41	74	M	Acral	Leg	4.2	WT	WT			WT	WT
Braf42	72	M	Mucosal	Lip	7.5	V599E	WT	WT	V599E	Mutation	Abnormal

224

Sample Identifier	Age	Sex	Histology	Body Site	Thickness	Sequence Result 1	Sequence Result 2	Sequence Result 3	Sequence Result 4	Allele Specific PCR Result	dHPLC Result
Braf43	40	F	LMM	Face	1.0	V599E	V599E			Mutation	Abnormal
Braf44	79	M	LMM	Foot	1.2	V599E	V599E			Mutation	Abnormal
Braf45	76	M	LMM	Face	6.1	WT	WT			WT	WT
Braf46	61	M	LMM	Face	9.5	WT	WT			WT	WT
Braf47	88	M	LMM	Face	4.3	WT	WT			WT	WT
Braf48	74	M	LMM	Face	8.4	WT	WT			WT	WT
Braf49	73	F	LMM	Face	2.7	WT	WT			WT	WT
Braf50	58	F	LMM	Neck	1.0	WT	WT			WT	WT
Braf51	73	M	LMM	Ear	1.1	WT	WT			WT	WT
Braf52	72	M	LMM	Face	3.9	WT	WT			WT	WT

Abbreviations for melanoma type are:

SSM = Superficial Spreading Melanoma

Nodular = Nodular Melanoma

Acral = Acral Lentiginous Melanoma

Mucosal = Mucosal Melanoma

LMM = Lentigo Maligna Melanoma

225

7.5.10 Summary of all Exon 15 BRAF Frozen and Paraffin Embedded Melanoma Tissue Studies

In summary, the exon 15 1796T>A *BRAF* mutation has been detected in 13/52 (25%) of primary melanoma samples and 6/24 (25%) of secondary melanoma samples, giving an overall mutation pickup rate for somatic mutations of 19/76 (25%). The percentage of Superficial Spreading Melanomas showing the exon 15 *BRAF* mutation was 7/26 (26.9%), in 7/23 (30.4%) Nodular Melanomas, in 2/16 (12.5%) Acral Lentiginous Melanomas, in 1/1 (100%) Mucosal Melanoma and 2/10 (20%) Lentigo Maligna Melanomas.

The average age at diagnosis of these tumours is 66.3 years, and those that carry the V599E mutation have an average age at diagnosis of 62.4 years. Thirty-five of the samples came from male patients with ten being mutation positive and 39 came from female patients with eight being mutation positive. The average thickness of these tumours is 5.7mm and those with the V599E mutation have an average thickness of 4.7mm. The tissue samples analyzed in this study were chosen for their thickness and do not represent the normal thickness of tissue samples for the Scottish population. The body site of the tissues of the *BRAF* mutation positive samples do not differ from those of the *BRAF* negative population sampled (Table 7.8).

TABLE 7.8a

Summary of Mutation Positive Paraffin Embedded Primary Melanoma Tissue Studies

Sample Identifier	Age	Sex	Histology	Body Site	Thickness	Mutation Analysis
Braf1	53	M	SSM	Scalp	6.8	V599E
Braf2	70	M	SSM	Trunk	6.0	V599E
Braf3	58	M	SSM	Trunk	3.8	V599E
Braf4	48	M	SSM	Neck	5.7	V599E
Braf5	66	M	SSM	Back	3.5	V599E
Braf18	71	F	Nodular	Leg	10.0	V599E
Braf19	69	M	Nodular	Upper Arm	6.0	V599E
Braf20	70	F	Nodular	Thigh	4.9	V599E
Braf29	72	M	Acral	Toe	3.5	V599E
Braf30	65	F	Acral	Leg	4.2	V599E
Braf42	72	M	Mucosal	Lip	7.5	V599E
Braf43	40	F	LMM	Face	1.0	V599E
Braf44	79	M	LMM	Foot	1.2	V599E

TABLE 7.8b

Summary of Mutation Positive Frozen Secondary Melanoma Tumour Studies

Sample Identifier	Age	Sex	DNA Source	Primary Type Histology	Body Site	Thickness	Mutation Analysis
96037	50	F	2 x Soft Tissue	SSM	Calf	4.3	V599E
97165	76	F	Nodal	Nodular	Leg	7.0	V599E
96150	48	M	Nodal	Nodular	Leg	1.0	V599E
96250	48	F	Nodal	Nodular	Thigh	5.0	V599E
91065	68	F	Nodal	Nodular	Leg	3.7	V599E

CHAPTER 8

DISCUSSION OF MUTATION SCREENING OF THE *CDKN2A* GENE

8.1 *CDKN2A* Discussion

The work presented in this thesis adds 32 new melanoma families to the 16 already reported from Scotland (MacKie et al. 1998) (population 5 million). In total, 13/48 (27%) Scottish families have detectable *CDKN2A* mutations. One of the mutations detected (H83N) have not previously been reported in melanoma, and molecular modelling suggests the likely functional result of this mutation. Function results are awaited from an active collaboration with the University of Toronto. Of the 48 Scottish families, one family, F19, has five affected family members and carries a *CDKN2A* mutation. One family, F2, has four affected members and is mutation positive. Five families, F1, F8, F20, F21 and F29 have three affected members in each family and three of these families (60%) carry a *CDKN2A* mutation. The remaining 41 families have only two affected members, with eight (20%) of these families mutation positive.

8.2 Detection of *CDKN2A* mutations by dHPLC

Fifteen abnormal chromatogram patterns were identified for exons 1α and 2 of the *CDKN2A* gene using dHPLC analysis. To validate dHPLC, all probands from each family were sequenced whether or not the chromatogram was abnormal or wild type. No false negatives or false positives were obtained by dHPLC in any sample. All of the 15 amplicons that showed a non wild type profile were sequenced and all 15 amplicons contained a nucleic acid change.

Orlow et al. (2001) has also validated dHPLC as a rapid detection method for the identification of *CDKN2A* mutations. They screened exons 1α, 2, and 3 from 129 samples and 13 known mutants, yielding 347 products that were examined at different temperatures. Forty-two of the amplicons showed a distinct non-wild-type chromatogram. Sequencing of all 129 samples identified 16 different nucleotide variations. Most importantly, as in this thesis, no false negatives or false positives were obtained by dHPLC in samples with mutations or polymorphisms.

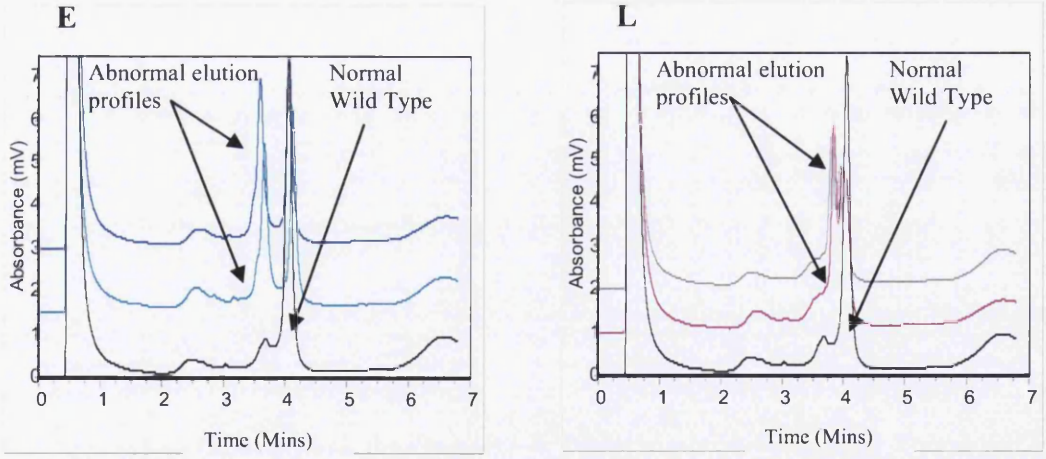
Thus the work presented in this thesis confirms that of Orlow et al. dHPLC is a reliable method for the screening of a large number of samples for *CDKN2A* mutations and polymorphisms and is fast, sensitive, and cost-efficient.

Another useful application of dHPLC rather than direct sequencing is that individual profiles from amplicons containing different mutations are generated. For example, in elution profile E (Figure 8.1) there are amplicons from two melanoma patients and one from a control subject. Both patients have identical elution profiles and both carry the same mutation. These identical profiles could be used when screening additional family members for an already identified familial mutation.

The results from the dHPLC analysis gave rise to 15 abnormal elution profiles. It was found that each different profile was the result of amplicons each with a different mutation. For example, elution profiles E and L (Figure 8.1) show a different elution profile from each other. Profile E has two spaced out tall peaks of almost equal height in comparison to profile L that has two peaks very close together and of different heights. The different profiles of each different mutation could be used as a guide to which mutation will be present upon sequencing.

FIGURE 8.1

Applications of dHPLC analysis



Elution profiles obtained in melanoma patients as compared with a wild-type elution profile are shown.

8.3 Determining the Nature of the Nucleic Acid Change

Eight different nucleic acid changes were identified in samples from 15 probands. Three families carry an exon 1α mutation and ten families carry an exon 2 mutation. Two additional families have the exon 2 A148T suspected polymorphism. No exon 3 mutations were identified in this study. Nine probands showed a change at SNP 500 and five probands showed a change at SNP 540.

8.3.1 The H83N Mutation

A previously unreported mutation was identified within the Scottish melanoma families presented in this thesis. A heterozygous C to A transition at nucleotide position 247C>A was identified in family 33. This base change is predicted to lead to the substitution of histidine (H) for an asparagine (N) at codon 83 (H83N). Family 33 has two affected members with melanoma. Both family members have multiple primary melanomas. Both affected family members carry the H83N mutation. No currently unaffected family members were available for screening.

Molecular modelling was used to investigate the potential effects on the pathological function of the H83N mutation. H83 is buried within the core of p16INK4A and the side chain of histidine participates in three hydrogen bonds to different parts of the tumour suppressor molecule main chain that serve to stabilise the conformation of two loops that interact with residues 31 to 38 of CDK6. Replacing the basic histidine with asparagine would ensure the loss of at least one hydrogen bond within the core of the molecule, which might destabilise the p16INK4A structure. This molecular modelling and the fact that the mutation was identified in both affected members of the one family would suggest that the H83N mutation has a pathological effect on p16INK4A.

8.3.2 The 32-33ins9-32 24bp Duplication

A heterozygous 24bp fragment of bases 9-32 inserted between bases 32-33 was identified in a family with two affected members, one of whom has multiple primary melanomas. This insertion is in frame. This mutation has been previously incorrectly reported as a 1-8 24bp insertion by Goldstein et al. (1995),

Walker et al. (1995), and Flores et al. (1997). Pollock et al. (1998) identified two new melanoma kindreds that carried the duplication. This brought the total number of melanoma families described with this mutation to five, from three continents: Europe, North America, and Australasia. This suggested to Pollock et al. (1998) that there had been at least three independent 24bp duplication events. The duplication may have arisen due to an unequal crossing-over between the two 24bp repeats naturally present in the wild-type sequence, possibly through polymerase slippage during replication. Further evidence that this repeat region is unstable and prone to slippage was provided by the identification of a somatic 24bp deletion of one of the normally occurring repeats in a prostate tumour (Komiya et al. 1995)

8.3.3 The R24P Mutation

A heterozygous G to C transition at nucleotide position 71G>C was identified in family 2. This base change is predicted to lead to the substitution of arginine (R) for a proline (P) at codon 24 (R24P). Family 2 is the second largest family in the Scottish cohort with four affected members with melanoma. There is also a member of this family with oesophageal cancer. The predicted substitution was also identified in a currently unaffected family member.

In a patient with multiple primary melanomas, Monzon et al. (1998) identified a R24P mutation in the *CDKN2A* gene. This mutation had previously been reported in an Australian melanoma family and was found to cosegregate with cases of melanoma (Holland et al. 1995). This mutation has also been identified in an English melanoma family (Harland et al. 1997).

Molecular modelling was used to investigate the potential effects on the pathological function of the R24P mutation. The side chain of R24 of p16INK4A forms hydrogen bond interactions with CDK6. If R24 mutated to R24P this would remove strong electrostatic interactions and reduce the affinity for complex formation between the two proteins.

Functional studies have shown the R24P mutant to behave normally in a CDK4 binding assay, but the cell cycle-inhibitory activity of the R24P mutant was reduced. Therefore this mutation may be defective in interaction with proteins other than CDKs (Becker et al. 2001).

8.3.4 The Y44X Mutation

A heterozygous C to A transition at nucleotide position 132C>A was identified in family 3. This base change is predicted to lead to the substitution of tyrosine (Y) for a stop codon at codon 44 (Y44X). Both affected members in this family carry the mutation.

8.3.5 The G67R Mutation

A heterozygous G to C transition at nucleotide position 199G>C was identified in family 19. This base change is predicted to lead to the substitution of glycine (G) for an arginine (R) at codon 67 (G67R). Family 19 is the largest family in the Scottish cohort with five affected members with melanoma. Family members with multiple primary melanomas are present in this G67R family and there is also a member of this family with ovarian cancer.

Molecular modelling was used to investigate the potential effects on the pathological function of the G67R mutation. The G67 residue is situated at the end of the helix-turn-strand motif on a tight turn into the β-sheet of the ankyrin repeats. Glycine 67 is highly conserved, lying within the second ankyrin repeat and shares secondary structure alignment with glycine 101, a residue of proven significance to p16INK4A function. Glycine 67 is the first residue of loop 2 of p16INK4A (Byeon et al. 1998). A mutation at this point of the protein may affect the secondary structure and perhaps the folding of the protein. Any mutation at this point would be likely to impair function and replacing glycine with arginine could influence the fold and alter the position of adjacent helices, which taper down to the binding site with CDK6. This G67R substitution could also change the binding sites to other as yet unidentified proteins.

Newton Bishop et al. (1999) screened 42 English families for CDKN2A mutations. They identified the single base pair substitution G67R in one melanoma family.

Holland et al. (1999) analyzed 131 Australian melanoma probands. They identified a mutation at the same glycine 67 residue to serine (G67S). G67S is likely to affect p16INK4A function as shown by site-directed mutagenesis (Tevelev et al. 1996; Zhang and Peng 1996) and also by reduced binding to CDK4 (Rizos et al., 2001). The fact that there are two mutations at the G67

residue in melanoma families shows that this residue is important and that a mutation at this amino acid may have an effect on the pathological function of p16INK4A.

8.3.6 The R112G Mutation

A heterozygous C to G transition at nucleotide position 334C>G was identified in two Scottish families, families 17 and 22. This base change is predicted to lead to the substitution of arginine (R) for a glycine (G) at codon 112 (R112G). The predicted substitution observed in the probands was also found in other affected family members and also in three currently unaffected family members.

Molecular modelling was used to investigate the potential effects on the pathological function of the R112G mutation. R112 makes a salt bridge interaction with residue E120, which helps to stabilise the structure of the loops that interact with CDK6. A mutation from R to G would disrupt this part of the structure and also complex formation.

In 1999 Holland et al. analysed 131 Australian melanoma probands. They identified an R112G mutation. Arginine 112, is not conserved but is immediately flanked by several highly conserved residues. This mutation has also been observed as a somatic event in a sporadic metastatic melanoma (Platz et al. 1996).

8.3.7 The A148T Suspected Polymorphism

A heterozygous G to A transition at nucleotide position 442G>A was identified in three families. This base change is predicted to lead to the substitution of alanine (A) for a threonine (T) at codon 148 (A148T). The presence of this substitution in affected family members and not in unaffected family members increases the chance that this is a pathological mutation. However, A148T was also identified in currently unaffected family members and most importantly was not identified in a number of affected family members from the same families. This would lead us to believe that this predicted substitution is a polymorphism.

Bertram et al. (2002) investigated the role of A148T as a low penetrance melanoma or nevus susceptibility variant in 488 adults from 179 English families

of patients with the atypical nevus phenotype and/or a family history of melanoma, and a population-based sample of 599 women. They found a very similar prevalence of A148T (4.9% and 5.2%) in both the atypical nevus phenotype and/or a family history of melanoma patients, and the population-based control samples. In addition, they showed no association between the A148T variant and nevus number or history of melanoma. Therefore this data does not suggest that the A148T variant is a low penetrance melanoma allele or nevus susceptibility allele.

8.3.8 SNP 500 and SNP 540

No exon 3 mutations were identified in this study, but nine probands showed a change at SNP 500 C>G and five probands showed a change at SNP 540 C>T in the 3'UTR of the *CDKN2A* gene. The number of individuals typed for these two polymorphisms is too small to draw any definite conclusions about these two polymorphisms. However, it is possible that these variants could either be in linkage disequilibrium with an unidentified variant directly responsible for the increased melanoma risk or alter either the stability of the *CDKN2A* transcript or the level of *CDKN2A* transcription.

Kumar et al. (2001) genotyped the 500 C>G and 540 C>T polymorphisms in 44 Swedish melanoma cases, 187 Norwegian melanoma cases and 235 Nordic population controls. The T-allele frequency (for 540 C>T polymorphism) in melanomas was significantly higher than in controls (0.14 vs. 0.08; $p = 0.01$; OR = 1.71, 95% CI = 1.11-2.66). The heterozygote frequency for this polymorphism was 0.26 (59/229) in melanomas compared to 0.13 (30/235) in healthy controls ($p = 0.0007$; OR = 2.34, 95% CI = 1.40-3.92). The frequency of the 500 C>G polymorphism was not significantly higher in melanomas compared to healthy controls. The 500 C>G polymorphism, however, was in linkage disequilibrium with approximately 50 kb from the C>A intronic polymorphism in the *CDKN2B* gene (determined in 44 melanomas and 90 controls; Fisher exact test, $p<0.0001$).

Straume et al. (2002) investigated the impact of the 540 C>T polymorphism of *CDKN2A* in vertical growth phase melanomas from Norwegian patients. Forty-seven cases (25%) were heterozygous for the 500 C>G polymorphism. The 540 C>T polymorphism was found in 42/185 (23%) of

melanomas and was also associated with improved survival (P = 0.03). There was no association with the clinicopathological variables studied and these polymorphisms. These results suggest a role for the 540 C>T polymorphism of *CDKN2A* in the initiation or early progression of a subset of cutaneous melanomas with less aggressive behaviour.

A summary of all *CDKN2A* mutations currently identified in Scotland is listed below in table 8.1.

TABLE 8.1

Mutations Detected in the *CDKN2A* Gene in 48 Scottish Melanoma Families

***CDKN2A* Mutation**		**Number of Scottish Melanoma Families with Mutation**
Exon 1α		
32-33ins9-32	24bp dup	1
71G>C	R24P	1
132C>A	Y44X	1
Exon 2		
159G>C	M53I	6
199G>C	G67R	1
247C>A	H83N	1
334C>G	R112G	2
442G>A	A148T (polymorphism)	2

The overall mutation rate of 27% is relatively high for small families by comparison with other parts of the world. For example, in the Australian states of Victoria and New South Wales, the mutation rate in melanoma families is reported as 11/131 or 8.4% (Holland et al., 1999) and in Israel it is 2/30 or 7% (Yakobson et al., 2000).

Mantelli et al. (2002) screened for *CDKN2A* mutations in Italian families with two melanoma patients, one of whom was younger than 50 years at onset, the other either being a first-degree relative, or having an additional relative with pancreatic cancer, or having multiple primary melanomas. 62/67 (80%) of families met this criteria. Four *CDKN2A* mutations (G101W, R24P, V126D, and N71S) were found in 21/62 (34%) families. The percentage of families with two melanoma cases/family carrying a mutation was low (7%, 2/27), but rose to 45%

(9/20) if one of the melanoma patients carried multiple melanomas or if pancreatic cancer was present in that family. In the 15 families with three melanoma cases the presence of a mutation was higher (67%, 10/15) and reached 100% in the 4 families with four or more melanoma cases.

The results in this thesis suggest that *CDKN2A* screening is worthwhile even for families with only two melanoma cases, in research settings, irrespective of the presence of multiple primary melanomas or of pancreatic cancer.

8.4 *CDKN2A* Mutations and Age at Melanoma Diagnosis

In the Scottish families with *CDKN2A* mutations, the mean age of melanoma diagnosis is 35 years, and in families with no detected mutations it is 46 years. This difference is significant (P = 0.008) and there is also a highly significant difference between both of these ages at diagnosis and the mean age of 56.7 years at diagnosis for the geographically matched sporadic cases (MacKie et al., 2002). As questioning of the familial melanoma cases (page 57) does not indicate excessive sun exposure by comparison with age matched sporadic cases, it would appear that there may be an interaction between *CDKN2A* mutations and environmental factors including sun exposure and no doubt other genes which accelerates the pathway to melanoma development.

8.5 *CDKN2A* and Other Cancers

In families F19, F30 and F31 there are five cases of ovarian cancer, in families F11 and F21 there are two cases of pancreatic cancer, in family F11 there is also a case of breast cancer, in family F14 there is a case of lymphoma and in family F2 there is a case of oesophageal cancer. It has been previously reported that melanoma families with *CDKN2A* mutations have a higher than expected incidence of pancreatic cancer (Goldstein et al. 1995; Bergman et al. 1996; Ghiorzo et al. 1999; Borg et al. 2000; Vasen et al. 2000). The relationship between the *CDKN2A* gene and pancreatic cancer is however unknown.

8.5.1 Pancreatic Cancer

The Scottish families presented in this thesis do not confirm the association between familial melanoma, *CDKN2A* mutations and pancreatic

cancer, as only two melanoma families also have members with pancreatic cancer (families 11 and 21), neither of whom have detectable *CDKN2A* mutations. It may be of significance that a Scandinavian report (Whelan et al., 1995) finds coexistent pancreatic cancer only in families with the *CDKN2A* 113insR mutation, a mutation that has not been detected in Scottish families.

Borg et al. (2000) analyzed an affected member from each of 52 families from southern Sweden with two or more cases of melanoma for germline mutations in *CDKN2A* and *CDK4* to reveal the contribution of these gene mutations to familial melanoma and to the occurrence of other cancer types. They found *CDKN2A* mutations in 10/52 (19%) families. Nine families carried the ins113R mutation and one family carried a V115G missense mutation. Six of the nine 113insR families had at least one member with multiple primary melanomas; the 113insR families also had a high frequency of other cancer types, in particular, breast cancer (a total of eight cases compared with the expected 2.1; $P = 0.0014$) and pancreatic cancer (a total of six cases compared with the expected 0.16; $P < 0.0001$). These findings show that families with the *CDKN2A* 113insR mutation have an increased risk of multiple primary melanomas, pancreatic cancer and breast cancer.

Bartsch et al. (2002) evaluated the prevalence of mutations in the *CDKN2A* gene in 18 German familial pancreatic cancer families and five German families with at least one patient with pancreatic cancer and another first-degree relative with malignant melanoma. None of 18 familial pancreatic cancer families carried a *CDKN2A* mutation. Two of the five families with pancreatic cancer and melanoma carried a truncating *CDKN2A* mutation (Q50X and E119X). Neither of these mutations were detected in the Scottish families.

Ghiorzo et al. (2004) investigated the association between *CDKN2A* mutations and the occurrence of pancreatic cancer in 49 Italian familial melanoma patients, 66 sporadic pancreatic cancer patients and 54 control subjects. As compared with the general population, the risk of pancreatic cancer was increased 9.4-fold (95% CI 2.7-33.4) in G101W familial melanoma patients and 2.2-fold (95% CI 0.8-5.7) in G101W negative familial melanoma patients. One G101W familial melanoma patient also had a germline deletion of the second allele, including exon 1β. This deletion could mean that p14ARF is

involved in the development of pancreatic cancer and a mutation in both p16INK4A and p14ARF may result in an increased risk of pancreatic cancer in a familial melanoma setting. The G101W mutation was not identified in the Scottish melanoma families.

Goldstein (2004) examined the relationships between familial melanoma, pancreatic cancer and germline *CDKN2A* mutations using published data. She identified 67 different *CDKN2A* mutations in 189 melanoma families. Forty-two melanoma families also had cases of pancreatic cancer in the family. Eighteen mutations were identified in these 42 melanoma families with pancreatic cancer. Comparison of the 147 melanoma families without pancreatic cancer with the 42 families that had pancreatic cancer showed no significant differences in the types or locations of mutations identified. There was however, a significant difference ($p=0.002$) in the distribution of the mutations from each group across the four ankyrin repeats of p16INK4A. The third ankyrin repeat is where most mutations occur in mutation positive melanoma families with pancreatic cancer. Nine of 22 (41%) G101W melanoma families also had pancreatic cancer. Three of ten (30%) V126D melanoma families also had pancreatic cancer. No M53I melanoma families also had pancreatic cancer. The M53I mutation is Scotland's most common melanoma *CDKN2A* mutation.

8.5.2 Breast Cancer

Epidemiologic studies have provided suggestive evidence of a link between melanoma and breast cancer. Goggins et al. (2004) found a significant increased risk of melanoma among female breast cancer survivors and vice versa by following U.S. female breast cancer patients registered in the 1973-1999 Surveillance, Epidemiology and End Result (SEER) database. Among breast cancer patients aged 50 years or under, they also observed a 46% elevated risk of a subsequent melanoma, suggesting that mutations identified in high-risk melanoma groups may also be present at a much lower level in the general breast cancer population. One Scottish melanoma family also has a case of breast cancer. No significant increased risk of melanoma with breast cancer has been observed in Scottish melanoma patients (MacKie and Hole, personal communication).

8.5.3 Ovarian Cancer

In the Scottish families, the commonest additional malignancy is ovarian cancer with three families affected. One family has a G67R *CDKN2A* mutation, one an M53I mutation and one no detectable *CDKN2A* mutation. Clearly further correlation is required with all melanoma families worldwide with and without *CDKN2A* mutations currently recorded to evaluate any significant coexistence of malignancies in addition to melanoma in these families.

Ovarian cancer is one of the most common cancers among women. Arcellana-Panlilio et al. (2002) studied the role of p16INK4 in ovarian granulosa cell tumours. Granulosa cell tumours of the ovary are relatively rare ovarian cancers. Seven of twelve (58%) adult ovarian granulosa cell tumours lacked expression of p16INK4A. In one of these cases, there was a homozygous deletion of the *CDKN2A* locus, and in the other tumours they found hypermethylation of the promoter region, which can lead to gene inactivation.

Kudoh et al. (2002) identified homozygous deletions of the *CDKN2A* gene in 8/45 (18%) cases of primary epithelial ovarian cancer.

8.5.4 Lymphoma

There is a case of lymphoma in one Scottish melanoma family F14. This family has two affected members but no *CDKN2A* mutation identified.

8.6 Multiple Primary Melanomas

Many melanoma families also have members with multiple primary melanomas, and in Scotland there is now a record of 10 such families (1, 3, 4, 5, 6, 11, 28, 33, 42 and 43) with 13 family members affected with MPMs for which there was DNA available. In total, 24 patients with MPM were screened for *CDKN2A* mutations, 13 are classed as familial melanoma and 11 as sporadic melanoma.

Germline mutations in the *CDKN2A* gene were identified in 12/24 (50%) of MPM cases, nine with a family history of melanoma and three sporadic cases. Seven of these families have *CDKN2A* mutations.

The most frequently identified mutation in patients with MPM was M53I, found in five familial cases and in two sporadic cases. *CDKN2A* mutations were

more frequent in those patients with a family history of melanoma (9/13 69.2%) than in those without a family history (3/11 27.3%).

Hashemi et al. (2000) screened 80 Swedish individuals with at least two primary melanomas, for germ-line *CDKN2A* mutations. In 15 patients, at least one additional family member with melanoma was identified. These 15 individuals belonged to 13 separate kindreds with melanoma as two kindreds each had two additional affected relatives. Nine (11%) patients carried a *CDKN2A* mutation, seven of which had a family history of melanoma. Two patients without a family history of melanoma had *CDKN2A* mutations. One had a mutation in the 5' noncoding sequence (-14C/T) and the other had an insertion of an extra T in codon 28, which results in a stop signal in codon 43.

Blackwood et al. (2002) in Pennsylvania identified germline mutations in the *CDKN2A* gene in 9/96 (9%) MPM cases. Six of the mutation positive individuals with MPMs had a family history of melanoma.

8.7 Future Studies

Future studies would include screening for germline deletions of the region on 9p21 containing the *CDKN2A* gene and exon1β. Mutation screening of the non-coding region of the *CDKN2A* gene suggests that mutations in the promoter region are unlikely to play a significant role in linked 9p21 families without *CDKN2A* exonic mutations (Pollock et al. 2001). It is however possible that mutations in noncoding regions may be responsible for predisposition to melanoma. Harland et al. (2000) screened the promoter region of *CDKN2A* in 107 melanoma families. They identified four variants. Two of these (A-191G and A-493T) did not segregate with disease and were present in a control population, indicating that they are unlikely to predispose to melanoma. The A-493T variant appeared to be in linkage disequilibrium with the previously described *CDKN2A* polymorphism A148T. The variant G-735A was detected in the control population, but segregation of this variant with melanoma within families could not be discounted. The fourth variant (G-34T), located in the 5' UTR, creates an aberrant initiation codon. This variant appeared to segregate with melanoma and was not detected in a control population. G-34T has recently been identified in a subset of Canadian melanoma families and was concluded to be associated with predisposition to melanoma.

The creation of an aberrant initiation site in the 5' UTR may have an important role in carcinogenesis in a small percentage of families; however, mutations in the *CDKN2A* promoter appear to have a limited role in predisposition to melanoma.

8.7.1 IVS2, A-G, -105

Harland et al. (2001) reported that affected individuals in six of 90 English melanoma pedigrees screened carried a transition (IVS2-105 A-G) deep in intron 2 of the *CDKN2A* gene. The mutation creates a false GT splice donor site 105 bases 5-prime of exon 3 and results in aberrant splicing of the mRNA. The authors proposed that this mutation and others similar to it might account for a significant proportion of 9p21-linked melanoma pedigrees with no detectable mutations in the coding region of *CDKN2A*.

The intronic sequence of *CDKN2A* has not been investigated for mutations in Scottish melanoma families; however, in the light of the IVS2-105 deep intronic mutation being the most common *CDKN2A* mutation identified in English melanoma families this mutation should now be screened for in those Scottish melanoma pedigrees where coding mutations of *CDKN2A* cannot be identified.

The *CDKN2A* 500 and 540 3' UTR variants and any other SNPs adjacent to *CDKN2A* require further investigation not only in the familial melanoma patients but in sporadic cases too and of course a general population study of their frequency would need to be obtained. It would be useful to obtain the genotype of these SNPs in families with *CDKN2A* mutations to determine the carrier haplotype of any ancestral mutations and also in families without *CDKN2A* mutations to look for haplotypes suggestive of carrying a common, yet currently unrecognised mutation.

8.8 International Collaboration

Mutations in the *CDKN2A* gene are found in 27% of all Scottish melanoma families. Clearly, additional melanoma predisposition genes must exist. Melanoma is not a common tumour, and families are even rarer, so future progress will need worldwide collaboration. The International Melanoma Genetics Consortium is currently carrying out genetic and epidemiological studies and genome-wide scans in melanoma families to localize genes predisposing to melanoma. When new melanoma genes are discovered, interactions with *CDKN2A* and gene-environmental interactions can be investigated more closely. The identification of other genes contributing to melanoma predisposition will increase the ability to identify who might be a target for early detection and prevention campaigns.

8.9 *CDKN2A* Screening

Routine screening for *CDKN2A* mutations in melanoma families is currently not recommended in Scottish clinical practice outside of research settings. This is because a patient's mutation status does not affect the lifetime follow-up strategies. Undetected mutations, other undetected genes, and incomplete penetrance of *CDKN2A* means that predictive DNA testing for melanoma outside of the defined research protocols would be premature. There is a possibility of sporadic melanoma in non-mutation carriers in melanoma families, of which we have one family. Programs of sun protection and skin surveillance should be followed by all high risk individuals irrespective of their mutation status. Although the frequency of *CDKN2A* mutations in Scottish patients with melanoma who have two or more family members with melanoma is 27%, the frequency among all melanoma patients is low, estimated at about 0·2–2·0% (Hansen et al. 2004). Therefore genetic testing would only be appropriate only in well-defined categories of patients with melanoma.

Myriad Genetic Laboratories launched a predictive test, MELARIS™, for malignant melanoma in 2001. This test detects inherited mutations in the *CDKN2A* gene by sequencing. This test aims at providing an assessment of risk in family members who have not so far shown any sign or symptom of the disease. However, a negative result in an unaffected family member could mean

that they are a non-carrier in a *CDKN2A*-mutation family or that they are a carrier of a mutation of another yet unidentified gene in a melanoma family that does not have a *CDKN2A* mutation or that they are a non-carrier in a family that does not have a *CDKN2A* mutation. In families in whom no *CDKN2A* mutation is identified, there should be careful interpretation of false-negative results as the family is still at increased risk of melanoma on the basis of the family history. A negative result in a family member who has melanoma indicates that their family is unlikely to have a *CDKN2A* mutation that is identifiable by the current screening techniques. They may however have a mutation in another melanoma-predisposition gene that has not yet been identified.

CHAPTER 9

DISCUSSION OF THE M53I FOUNDER MUTATION

9.1 Founder Mutation Discussion

The most common *CDKN2A* mutation identified in this thesis from the Scottish melanoma families studied is a heterozygous G to C transition at nucleotide position 159G>C. This base change is predicted to lead to the substitution of methionine (M) for an isoleucine (I) at codon 53 (M53I). It was identified in six Scottish families, families 1, 4, 5, 6, 29 and 30. All affected family members from these six families carry the M53I mutation except affected individual F29.1 (see cloning method 3.15). No *CDKN2A* mutation could be identified in this individual and therefore is thought to be a sporadic melanoma patient within a familial setting.

Reports from a number of countries suggest that apparent founder mutations explain a substantial fraction of hereditary melanoma. These mutations and their suggested country of origin are indicated in table 9.1.

TABLE 9.1

Currently Reported Melanoma Founder Mutations

Study	Mutation	Main Population	Ancestry
Gruis et al. (1995)	'p16-Leiden'	The Netherlands	Dutch
Borg et al. (1996)	insR113	Southern Sweden	Swedish
MacKie et al. (1998)	M53I	Scotland	Scottish
Liu et al. (1999)	G-34T	Canada	British
Ciotti et al. (2000)	G101W	Liguria, Italy	'Celtic'
Goldstein et al. (2001)	V126D	North America	German/English
Yakobson et al. (2003)	V59G	European	Israeli

9.1.1 19bp Deletion 'p16-Leiden'

Most Dutch melanoma families appear to originate from a unique founder mutation (p16-Leiden). Gruis et al. (1995) analyzed 15 Dutch FAMMM syndrome pedigrees in the Netherlands, and identified a 19bp deletion in exon 2 *CDKN2A* germline deletion in 13 of them with Dutch ancestry. The 19bp deletion has also been documented outside the Netherlands, in an Australian family, however the proband has distant Dutch ancestors (Holland et al. 1999).

The deletion causes a reading-frame shift, which encodes a truncated non-functional p16INK4A protein consisting of the N-terminal of p16INK4A and the C-terminal of p14ARF (van der Velden et al. 1999).

9.1.2 3bp Duplication, 113insR

In ten melanoma kindreds from southern Sweden, Borg et al. (1996) identified a novel germline mutation in two families, constituting an inframe 3bp duplication at nucleotide 332 in exon 2. The mutation resulted in an insertion of an extra arginine at codon 113, which interrupts the last of the four ankyrin repeats of the p16INK4A protein, motifs which have been demonstrated as important in binding and inhibiting the activity of CDK4 and CDK6 in cell cycle G1 phase regulation. Analysis of microsatellite markers adjacent to the *CDKN2A* gene at chromosomal region 9p21 in the two families with the mutation showed that they shared a common haplotype, in keeping with a common ancestor. 113insR has been identified in 17 Swedish melanoma families (Hashemi et al, 2001).

9.1.3 -34G-T

CDKN2A coding mutations cosegregate with melanoma in 20-30% of families with melanoma but there are also a number of *CDKN2A* mutation-negative families that demonstrate linkage of inherited melanoma to 9p21 markers (Hayward 1996). Liu et al. (1999) showed that 4/59 (7%) Canadian melanoma kindreds possess a G to T transversion at nucleotide -34 of *CDKN2A*, designated -34G-T. The mutation gives rise to a novel AUG translation initiation codon that decreases translation from the wild-type AUG. The -34G-T mutation was not seen in controls but segregated with melanoma in families. From haplotyping studies, it appeared to have arisen from a common founder in the U.K. Liu et al. (1999) suggested that screening for mutations in the promoter region of the *CDKN2A* gene should be useful in populations in which a low incidence of germline coding mutations of *CDKN2A* has been found in familial melanoma cases.

9.1.4 G101W

In three families with melanoma, Hussussian et al. (1994) identified a glycine 101 to tryptophan mutation in the *CDKN2A* gene. Ciotti et al. (1996) detected the G101W mutation in seven unrelated families from the eastern coast of Liguria, Italy. Ciotti et al. (2000) reported G101W to be the most common *CDKN2A* missense mutation, having been reported in numerous families, with a particularly high prevalence in France and Italy. Soufir et al. 1998 reported the Gly101Trp mutation in five apparently unrelated French families. In all families studied, the mutation appeared to have been derived from a single ancestral haplotype.

Gl01W was found to be temperature sensitive for binding to CDK4 and CDK6 in vitro, for inhibiting cyclin D1-CDK4 in a reconstituted pRb-kinase assay, and for increasing the proportion of G1-phase cells following transfection (Parry and Peters 1996). Ruas et al. (1999) considered the effects G101W might have on the three dimensional structure of the protein. G101 is one of several glycine residues that are critical for the formation of the ankyrin repeat structure. Substitution with any other residue would be energetically unfavourable.

9.1.5 V126D

One of the most common melanoma-related *CDKN2A* mutations reported in North America is valine 126 to aspartic acid (V126D) (Hussussian et 1994). Goldstein et al. (2001) examined nine markers surrounding the *CDKN2A* gene in three American and four Canadian families carrying this mutation. All seven families had a haplotype consistent with a common ancestor/founder. Six of the seven families were shown to have German/English ancestries.

V126D was found to be temperature sensitive for binding to CDK4 and CDK6 in vitro, for inhibiting cyclin D1-CDK4 in a reconstituted pRb-kinase assay, and for increasing the proportion of G1-phase cells following transfection (Parry and Peters 1996). The CDK4-binding ability of the V126D mutant decreases with increasing temperature. In a study by Becker et al. (2001), CDK4 binding of the V126D mutation was reduced to only 20–25%.

9.1.6 V59G

A valine 59 to glycine mutation in the *CDKN2A* gene was found in four families segregating with melanoma. These were an Israeli family of Moroccan Jewish ancestry (Yakobson et al. 2001), two French families (one of Tunisian Jewish ancestry and another without known Jewish roots) (Soufir et al. 1998), and a Spanish family (Ruiz et al. 1999). Yakobson et al. (2003) found that all but one of those affected in these families were heterozygous for the mutation; one affected member of the Israeli family was homozygous. Haplotype analysis indicated a single ancestral founder. The mutation, which occurs in a hydrophobic region with the second ankyrin repeat, impairs p16INK4A function, as shown by studies of protein-protein interactions and cell proliferation assays.

No Ashkenazi melanoma family has been reported to have a mutation in either *CDKN2A* or *CDK4* (Yakobson et al. 1998).

9.2 The M53I Mutation

The M53I mutation was originally detected in an Australian melanoma kindred (Walker et al. 1995). It has now been reported in three additional Australian families (Flores et al. 1997; Holland et al. 1999), two US kindreds (FitzGerald et al. 1996; Tsao et al. 2000) two English kindreds (Harland et al. 1997; Newton Bishop et al. 1999) and one family each in Canada (Ontario) (Monzon et al. 1998) and France (Soufir et al. 1998) (table 9.2). MacKie et al. (1998) identified this mutation in four Scottish melanoma families and also in one patient with multiple primary melanomas with a negative family history (Burden et al. 1999).

TABLE 9.2

Currently Reported Melanoma Families Worldwide with M53I Mutations

Number of M53I Families	Country	Population (millions)
6	Scotland (MacKie et al. 1998; this thesis)	5
4	Australia (Walker et al. 1995; Flores et al. 1997; Holland et al. 1999)	19.5
2	U.S.A (FitzGerald et al. 1996; Tsao et al. 2000)	286.8
2	England (Harland et al. 1997; Newton Bishop et al. 1999)	49.9
1	Canada, Ontario (Monzon et al. 1998)	11
1	France (Soufir et al. 1998)	60.1

Pollock et al. (1998) noted that the M53I mutation had been described in four melanoma families from Australia and one from Canada. Previous examination of these five M53I melanoma families suggested the possibility of a common haplotype (Pollock et al., 1998). The work in this thesis has extended this examination with the investigation of 18 families, six from Scotland, five from Canada, four from Australia (including the previously reported 41001, 41031 and 60001 families) and three from America, all carrying an identical M53I *CDKN2A* mutation. This thesis also includes two additional informative markers close to the *CDKN2A* locus, D9S2136 and D9S2060.

In total, 32 M53I mutation carriers, four non-carrier relatives and 45 control subjects were haplotyped. Ninety chromosomes from the 45 geographically matched control subjects were genotyped to determine the allele frequency of the markers in the general Scottish population. These Scottish control subjects are appropriate in view of the fact that the majority of the families studied are Scottish. The consensus M53I haplotype was not determined in any of the control subjects and therefore the probability of finding the M53I haplotype in the general Scottish population is extremely low.

Haplotype analysis, with seven informative markers surrounding *CDKN2A* at 9p21, of 18 geographically diverse melanoma families carrying the M53I mutation clearly indicates that 14 of them share a common haplotype for markers D9S2136 - D9S1604 4-2-8-4-6-2.

One Canadian family FAM-4 had the 7/9 alleles at D9S2136, rather than the allele 4 seen in all other haplotype sharing families. Three families, Australian families 41031 and 60001 and American family 637, showed co-segregation of allele 4 instead of the consensus allele 6 for marker D9S1748. Family 41031 has a Chinese/English/Irish ancestry so perhaps the M53I mutation has arisen independently in this family. It is more likely however that the difference in allele size could be accounted for by replication slippage of marker D9S1748 gaining two repeat units during meiosis. All but one of the haplotype sharing families showed co-segregation of the 4 allele for marker D9S942. Australian family 60001 showed allele 6 to co-segregate with the M53I melanoma mutation. Australian families 41031 and 60001 could therefore not be assigned the consensus haplotype, as previously shown by Pollock et al, 1998. Their study accounted for these families not sharing the disease haplotype by replication slippage. Gain or loss of one repeat unit is much more likely than gain or loss of two repeat units as shown in these families. Larger mutations can however occur (Primmer et al. 1996). The loss of two repeat units is a rare event and therefore it is possible that the M53I mutation has arisen independently in these families and the haplotype sharing observed around *CDKN2A* occurred by chance. However, the low frequency of this haplotype in the control individuals leads to the conclusion that it is not chance and that these families do not share the disease haplotype because of replication slippage.

9.3 Functional Studies of M53I

Molecular modelling was used to investigate the potential effects of the M53I mutation. The side chain of this highly conserved hydrophobic residue makes van der Waals interactions with main chain atoms of CDK6. A mutation M to I would likely reduce the area of interaction and therefore be slightly disruptive of complex formation.

Functional studies have shown that the protein expressed from the M53I mutation has loss of function and does not bind to CDK4/CDK6, confirming its role as a causal mutation in melanoma (Harland et al. 1997). Sun et al. (1997) also revealed that M53I CDKN2A mutant protein was functionally deleterious as it was unable to bind effectively to CDK4, confirming that this mutation is of pathological significance.

Becker et al. (2001) examined the relationship between the loss of CDK binding by the M53I mutant protein in melanoma cells. The cell cycle-inhibitory activity of the M53I mutant was reduced, and may also be defective in interaction with cellular targets other than CDKs.

Recently Yang et al. (2004) reported a novel germline M53V mutation in *CDKN2A* from a large U.S. melanoma family. The M53I affects *ARF* at D68H but the M53V mutation affects *ARF* at D67G. The occurrence of a second, independent *CDKN2A* M53 alteration highlights M53I as an important causal mutation in melanoma.

In conclusion, this study provides further evidence that the M53I mutation appears to have originated from a common founder and together with work quoted above, gives further evidence demonstrating common founders for most of the recurrent mutations in the *CDKN2A* gene. This suggests that the *CDKN2A* gene is relatively stable and mutation hotspots in the *CDKN2A* gene are uncommon.

The *CDKN2A* M53I mutation appears to play a significant role in melanoma families with a Scottish ancestry. Six of the 18 families studied reside in Scotland and at least 15/18 families have Scottish ancestry. It is therefore reasonable to infer that the M53I mutation arose on a chromosome carrying a common ancestral haplotype and occurred in Scotland. It is likely that the M53I mutation will be found in other populations with ancestral connections to Scotland. In the meantime the 18 known distantly related M53I families are a valuable resource for in depth studies on age of onset, ultraviolet exposure, multiple primary tumours and other details.

CHAPTER 10

DICSUSSION OF *MC1R* VARIANTS IN PHENOTYPED FAMILIAL MELANOMA PATIENTS, UNAFFECTED FAMILY MEMBERS, SPORADIC MELANOMA AND CONTROL SUBJECTS

10.1 Correlation between Phenotype and Melanoma

In chapter 2 the phenotypic characteristics of the melanoma patients and control subjects presented in this thesis were compared with the large epidemiological studies (Palmer et al. 2000; Box et al. 2001) and confirmed that the characteristics of both patients and control subjects were reasonably representative. This allowed both patients and controls to be analysed for their *MC1R* status in chapter 6 and compared to published data.

10.1.1 Correlation between Skin Type and Melanoma

Among the four subsets of different skin type the most frequent phenotype observed in the melanoma patients is to burn easily and tan rarely 60/88 (68.2%) and also in 29/68 (42.7%) controls. Only 7/88 (7.9%) cases compared with 25/68 (36.8%) controls have a burns rarely and tans easily skin type, which is a very highly significant difference between skin types in cases and controls ($P < 0.001$). Valverde et al. (1996) studied 43 unrelated patients with cutaneous melanoma and of 44 unrelated control. They found a weak association between melanoma and skin type.

When similar skin types were grouped together, and compared against all melanoma patients, both affected familial and sporadic, and the control panel 81/88 (92%) melanoma cases have skin type 1 or 2 compared with 41/68 (60.3%) controls ($P < 0.001$; OR = 7.620; CI 3.060-18.974). Palmer et al., (2000) also found that individuals with melanoma were significantly more likely to have a skin colour lighter than that seen in controls. Therefore the individuals presented in this thesis correlate with that of the larger epidemiological studies.

The work from this thesis has shown that there is no association of skin type and the presence or absence of a *CDKN2A* mutation within affected familial melanoma patients. This is in agreement with Box et al. (2001) who showed that *CDKN2A* mutations showed no association with skin type in their data set.

10.1.2 Correlation between Eye Colour and Melanoma

Risk of melanoma with a specific eye colour was investigated by grouping similar eye colours together and comparing against all melanoma patients, both affected familial and sporadic, and the control panel. 70/88 (79.5%) cases have lighter coloured eyes compared with 44/68 (64.7%) controls (P = 0.046; OR = 2.212; CI 1.034-4.350). Palmer et al., (2000) found individuals with melanoma were significantly more likely to have blue/grey eyes (OR = 1.8; CI 1.2-2.7) or hazel/green (OR = 1.6; CI 1.1-2.3). Thus the individuals presented in this thesis correlate with that of the larger epidemiological studies.

10.1.3 Correlation between Hair Colour and Melanoma

Among the four subsets of different hair colour the most frequent colour observed in the melanoma patients is light brown 32/88 (36.4%) compared with 15/68 (22.1%) controls (P = 0.056; OR = 2.019; CI 0.984-4.145). Similarly Palmer et al. (2000) found that individuals with melanoma were significantly more likely to have fair hair (OR = 2.0; CI 1.5-2.8).

The work presented in this study shows that no significant difference was observed between patients who are *CDKN2A* positive or negative and having a particular hair colour. Box et al. (2001) also showed that *CDKN2A* mutations showed no association with hair colour in their data set.

The phenotyping carried out for the work in this thesis can therefore reassure the reader that the melanoma population presented in this thesis is not atypical with regard to skin type, eye or hair colour.

10.2 *MC1R* Discussion

The aim of the analysis of the *MC1R* gene was to try to examine any underlying genetic relationship between *MC1R* variants and familial melanoma. Variants of *MC1R* may confer a low melanoma risk in both familial and sporadic cases but could also modify the risk of melanoma in individuals carrying a *CDKN2A* mutation. The frequencies of the *MC1R* variants identified in Scottish familial melanoma patients, both *CDKN2A* mutation positive and *CDKN2A* mutation negative and also a small number of cases of sporadic melanomas were examined and compared with phenotyped Scottish controls.

In total, 13 *MC1R* variants leading to an amino acid substitution were detected among the 180 individuals available for sequence analysis, three of which have not been described elsewhere (L44I, M128K, A171G) and appear to be very rare. L44I is present in only one sporadic melanoma patient. Both leucine and isoleucine are hydrophobic residues very similar in size and weight and so it is difficult to determine if this substitution would have a functional effect. M128K is present in only one currently unaffected family member. Methionine is a hydrophobic residue whereas lysine is a basic residue of very similar shape to methionine, however they both have very different side chains and so it is probable that this rare substitution has some functional effect. A171G is present in only one familial melanoma patient. Alanine is a small amino acid as is glycine. Although they are very similar in size and weight, they do have different side chains and it is therefore possible this substitution would have a functional effect. Three silent nucleotide changes were also detected (G239G, T314T, C315C).

The sequencing of the entire coding region compared with screening only the most frequently occurring variants has allowed for the detection of unreported *MC1R* variants and also the calculation of frequencies of all variants identified in the Scottish melanoma and general population.

10.2.1 MC1R Variants in Familial and Sporadic Melanoma Patients Combined

This study has identified that 83/88 (94.3%) patients and 53/67 (79.1%) controls carry at least one *MC1R* variant, which is a significant difference between the two groups (P = 0.006; OR = 4.385; CI 1.492-12.883).

Among the 13 different variants identified in the complete set of sequenced individuals, the most frequently occurring variant in the melanoma patients is V60L 25/88 (28.4%). V60L was also found in 19/67 (28.4%) of controls and therefore is thought to be a common polymorphism in the Scottish general population and not contribute to melanoma risk. R151C occurred in 27/88 (30.7%) patients compared with 12/67 (17.9%) controls, which is nearly significant (P = 0.065; OR = 2.104; CI 0.976-4.536). The Australian study of Dwyer et al. (2004) has shown however that carriers of the V60L, R151C and D294H variants were not at a substantially increased risk.

The work of this thesis shows R160W was identified in 21/88 (23.9%) patients and in 14/67 (20.9%) controls (NS). In contrast, Dwyer et al. (2004) showed in an Australian population that carriers of the R160W and the rare D84E variant were at an increased risk of melanoma.

Ichii-Jones et al. (1998) looked at the three variants (D84E, V92M and D294H) in a larger data set of 306 sporadic melanoma cases and 190 basal cell papilloma patients as controls. The allele frequencies were not significantly different between patients and controls. Other alleles were not included in this study. Our data confirms this observation, as does the English report of Hearle et al. 2003.

Palmer et al. (2000) have also reported no significant difference between D84E in melanoma and controls in an Australian population, which may be the same population as the report by Healy et al. (2000), which consisted of 460 melanoma patients and 399 control individuals. Their controls consisted of 218 parents of twins involved in a genetic study of mole formation and 181 controls who were involved in an alcohol use study. *MC1R* variants were present in 72% of melanoma cases and in 56% of control individuals (P < 0.01), compared with 94.3% and 79.1% in this Scottish study. These differences could be because some *MC1R* variants, in particular V60L, are much more common in the Scottish

population overall and therefore would increase variant frequency in both controls and patients.

10.2.2 MC1R Variants in Familial Melanoma Patients

When looking at the familial melanoma patients separately from sporadic melanoma patients, 58/60 (96.7%) affected melanoma patients compared with 53/67 (79.1%) controls carry an *MC1R* variant (P = 0.003; OR = 7.660; CI 1.663-35.296). Not all *MC1R* alleles however conferred the same familial melanoma risk. The most commonly occurring variants in the familial melanoma patients are V60L in 19/60 (31.7%) compared with 19/67 (28.4%) controls (NS). The V92M variant was identified in 12/60 (20%) familial cases compared with 10/67 (14.9%) controls, R151C in 20/60 (33.3%) patients compared with 12/67 (17.9%) controls (P = 0.043; OR = 2.406; CI 1.062-5.452), and R160W in 11/60 (18.3%) cases compared with 14/67 (20.9%) controls. The Bonferroni adjusted significance level for multiple tests in this case is 0.004 and hence carrying any *MC1R* variant remains significant, although carrying the R151C variant loses significance.

For comparison, Box et al. (2001) identified the R151C variant in 26% of family members from 15 melanoma pedigrees (67 affected and 69 unaffected individuals) but in only 10% (2017 individuals) of the southeastern Queensland, Australia general population. This thesis shows the R151C variant to be present in 33.3% of familial melanoma cases in Scotland compared with 17.9% of controls. These frequencies may be higher in both melanoma cases and controls because the Scottish population has a higher degree of Celtic ancestry than Australia.

10.2.3 MC1R Variants in CDKN2A Positive Familial Melanoma Patients

All 23/23 (100%) *CDKN2A* positive familial melanoma patients and 35/37 (94.6%) *CDKN2A* negative familial melanoma patients carry an *MC1R* variant. Individual allele frequencies differ between the two groups. The most frequently occurring variants in the *CDKN2A* positive patients are V60L in 12/23 (52.2%) compared with 7/37 (18.9%) *CDKN2A* negative patients (P = 0.017; OR = 3.818; CI 1.315-11.084). There is also a significant difference between the two groups with carrying the R151C variant in 12/23 (52.2%) *CDKN2A* positive cases compared with 8/37 (21.6%) *CDKN2A* negative patients (P = 0.029; OR = 3.515; CI 1.160-10.650).

In this study, the V92M variant is only present in 1/23 (4.3%) *CDKN2A* positive patients compared with 11/37 (29.7%) *CDKN2A* negative patients (P = 0.021; OR = 9.308; CI 1.112-77.888). Thus, there are more patients without a *CDKN2A* mutation with the V92M variant than there are patients with a *CDKN2A* mutation. The CI in this case however is wide and therefore does not allow definite conclusions to be drawn about the magnitude of risk of carrying or not carrying the V92M variant. Although maybe the V92M variant does play a part in increasing melanoma risk because the *CDKN2A* mutation negative patients still have melanoma. V92M could however just be a neutral polymorphism.

In Australia, Box et al. (2001) screened the *MC1R* gene of 15 *CDKN2A* mutation-carrying melanoma pedigrees in which nine different *CDKN2A* mutations were present. Melanoma risk was increased in individuals carrying both a *CDKN2A* mutation and a *MC1R* variant. The R151C, R160W, and D294H *MC1R* variants were largely responsible for increased risk of melanoma.

van der Velden et al. (2001) carried out a similar study looking at the frequencies of *MC1R* variants in melanoma and non-melanoma p16-Leiden carriers. The p16-Leiden mutation is the most common mutation of the *CDKN2A* gene in Dutch familial melanoma pedigrees. It is a 19-bp germline deletion causing a reading-frame shift, predicted to result in a severely truncated p16 protein as described previously on page 254. van der Velden et al. (2001) compared the frequencies of *MC1R* variants in 101 p16-Leiden carriers, who consisted of 38 melanoma patients and 63 individuals without melanoma with 385 opthalmology patient controls. The only significant difference was the

frequency of the R151C variant, which was found at an increased frequency in 14% of the p16-Leiden carriers, compared with the control group of 5%. They stated that the excess of the R151C allele in the p16-Leiden carriers was mostly due to a high frequency in the melanoma patients (21%). Carriers without melanoma also presented a higher frequency (10%) of the R151C variant than in the control group (5%). They found that within the families, the R151C allele was transmitted from heterozygous parents carrying p16-Leiden to affected offspring more often than by chance.

In this Scottish study and studies from other countries, there are no p16-Leiden mutation families and therefore no comparison can be made.

10.2.4 MC1R Variants in Sporadic Melanoma Patients

The English study of Valverde et al. (1996) was the first to report certain variants of *MC1R* to increase the risk of melanoma. They compared the *MC1R* genotype of the second and seventh transmembrane domains in 43 sporadic melanoma cases and 44 psoriasis patients as controls. They found that *MC1R* variants were significantly more common in cases than controls. 20/43 (46.5%) melanoma patients, but only 8/44 (18.2%) controls were carriers of variant alleles ($P = 0.0094$).

A recent report by Matichard et al. (2004) studying the French population, show *MC1R* variants to be present in 73/108 (67.6%) melanoma patients compared with 33/105 (31.4%) controls ($p<0.0001$), again confirming the association of *MC1R* variants with melanoma.

In contrast, this thesis shows that carrying an *MC1R* variant is high both in 25/28 (89.3%) sporadic melanoma patients and in 53/67 (79.1%) controls (NS). This could be because of the high frequency of the V60L variant in the Scottish population increasing the control frequency.

Matichard et al. (2004) found three frequent variants to be significantly associated with melanoma risk, V60L, R151C and R160W with the strongest risk being for R151C (OR = 6.26). The V60L variant, which was weakly associated with melanoma risk in Australia (Palmer et al. 2000) and The Netherlands (Kennedy et al. 2001), was one of the most frequent variants to be associated with melanoma risk (OR = 3.99, CI 2.03–7.82) in the French study. They did not find D84E, R142H, I155T or D294H to be associated with melanoma.

Likewise, the most frequently occurring variants in the sporadic melanoma patients in this thesis are R160W in 10/28 (35.7%) compared with 14/67 (20.9%) controls, R151C in 7/28 (25%) compared with 12/67 (17.9%) controls and V60L in 6/28 (21.4%) compared with 19/67 (28.4%) controls. None of the differences are significant in this small data set.

Valverde et al. (1996) identified the D84E variant in 10/43 (23%) sporadic melanoma cases with two of these individuals being homozygous for this change, whereas no control subject showed this alteration.

Kennedy et al. (2001) investigated the relationship between *MC1R* gene variants and cutaneous melanoma in a cohort of 123 Dutch sporadic melanoma patients, using 385 opthalmology patients as control subjects. They found the D84E variant in 3/123 (2.4%) melanoma patients compared with 3/385 (0.8%) controls. They identified the D84E variant to be the most significantly associated variant with melanoma OR = 10.4; CI 1.2-68.5 but the wide CI does not allow definite conclusions to be drawn about the magnitude of risk of melanoma. They also showed that while carrying one variant allele was found to give an increased risk of melanoma, two variants doubled this risk.

In this thesis, only 3/28 (10.7%) sporadic melanoma cases carry the D84E variant, which was also identified in 1/67 (1.5%) control individual (P = 0.075; OR = 7.920; CI 0.787-79.753).

Aspartate at codon 84 is highly conserved throughout the melanocortin receptor family and also other G protein coupled receptors making it a probable candidate as to play a direct causal role in melanoma A further possibility is that D84 could be in linkage disequilibrium with another variant outside the *MC1R* coding region.

Valverde et al. (1996) also identified the D294H variant in three sporadic melanoma cases but in none of their control population. In contrast, this thesis has found the D294H variant in four sporadic melanoma patients but also in three controls. These results are similar to those from France (Matichard et al. 2004) who were also unable to significantly associate the D294H variant with melanoma. Both Matichard's data set of 108 cases and 105 controls, and the numbers in this thesis are greater than that of the small Valverde study, giving increased statistical power.

Both the work presented in this thesis and by others (Valverde et al. 1996; Matichard et al. 2004) identify the V92M variant at a similar frequency in sporadic melanoma cases and also in the control group showing that there is no association with melanoma risk for the variant V92M.

10.3 Correlation between the Number of *MC1R* variants and Melanoma Status

In this study all variants were heterozygous, except for V60L which was detected in five homozygous familial melanoma patients and one control, V92M which was detected in one homozygous control, R151C which was detected in one homozygous familial melanoma patient, R160W which was detected in two homozygous familial melanoma patients and one sporadic patient and R163Q which was detected in three homozygous familial melanoma patients and one sporadic patient.

In a study of 123 patients with sporadic melanoma and 385 controls in The Netherlands, compound heterozygote and homozygote carriers of the V60L, V92M, R142H, R151C, R160W and R163Q variants had OR = 4 to develop melanoma, whereas heterozygotes for these variants had half the risk (Kennedy et al. 2001).

There is a significant relationship between both familial and sporadic melanoma and having two or more *MC1R* variants in this thesis, with 48/87 (55.2%) cases carrying two or more variants compared with 21/67 (31.3%) controls (P = 0.003; OR 2.696; CI 1.384-5.253). Kennedy et al. (2001) found that carriers of two variant alleles had a relative risk for melanoma of 4.8 (95% CI 2.5-9.4) compared with carriers of two wild-type alleles, and carriers of one variant allele had again half the risk.

There is a very highly significant relationship between affected familial melanoma cases whether they are *CDKN2A* mutation positive or not, and having two or more *MC1R* variants with 37/59 (62.7%) cases with two or more variants compared with 21/67 (31.3%) controls (P = 0.001; OR 3.684; CI 1.761-7.707).

There is no significant difference between unaffected family members of familial melanoma patients and controls and how many *MC1R* variants they carry.

No significant difference between sporadic melanoma patients and controls and how many *MC1R* variants they carry was identified from this data set.

10.4 Correlation Between *MC1R* Variants and Phenotype

10.4.1 Correlation Between MC1R Variants and Skin Type

Mutation screening of a variety of control groups who do not have melanoma has shown that *MC1R* variants are common among all skin types, although some are particularly associated with fair skin and melanoma, for example, R151C, R160W and D294H (Valverde et al. 1995; Smith et al. 1998; Flanagan et al. 2000; Healy et al. 2000; Palmer et al. 2000). Valverde et al. (1995) reported 76.5% of individuals with skin type 1 carried a variant *MC1R* allele, whereas no individuals with skin type 4 carried a variant. The frequencies were intermediate for individuals with skin types 2 (46.5%) and 3 (5%).

In 1997 Box et al. reported an association between the V60L variant of the *MC1R* gene and fair skin. They also showed that variants R151C, R160W and D294H were most significantly associated with fair skin.

In a study by Smith et al. (1998), 75% of an Irish population with a fair skin type carried an *MC1R* variant and 30% carried two variants.

Healy et al. (2000) investigated the number of variants carried in the *MC1R* gene in individuals from the U.K and Ireland. Individuals with one variant allele had an intermediate skin type 2 or 3, whereas individuals two variant alleles tended to have skin type 1 and those with no variants most often had a shin type 4.

Among the variants identified in the phenotyped controls in this thesis, the only one to show a significant finding is R160W ($P = 0.007$), which still remains significant after the Bonferroni correction. No other variant showed a significant correlation.

Kennedy et al. (2001) found that the D84E, R142H, R151C, R160W and D294H variants were strongly associated with fair skin whereas like this study, the V60L, V92M and R163Q variant alleles, were much weaker or not at all associated with fair skin type. Ichii-Jones et al. (1998) also show no difference of frequencies of the V92M, D294H, or D84E variants in controls with skin types 1, 2, 3 or 4, and therefore suggest that a polymorphism in *MC1R* does not appear to be a major determinant of skin type.

10.4.2 Correlation Between MC1R Variants and Eye Colour

No significant relationship could be identified between eye colour and *MC1R* variants from the data in this thesis, confirming the lack of an association between eye colour and *MC1R* genotype reported by Palmer et al. (2000).

10.4.3 Correlation Between MC1R Variants and Hair Colour

No significant relationship could be identified between hair colour and *MC1R* variants in this thesis. Ichii-Jones et al. (1998) also showed no significant association between *MC1R* variants and hair colour, although the D84E variant was more common in subjects with blonde hair. Palmer et al. (2000) reported a strong relationship between *MC1R* variants and hair colour and Kennedy et al. (2001) found that the D84E, R142H, R151C, R160W and D294H variants were strongly associated with red hair whereas the V60L, V92M and R163Q variant alleles, were much weaker or not at all associated with red hair.

10.5 Correlation between the *MC1R* Variants, Red Hair and Melanoma

10.5.1 Correlation between the MC1R Variants and Red Hair

Among the 67 controls tested for *MC1R* variants, two individuals are compound heterozygotes for variants R151C/R160W. Both individuals have red hair and a skin type that never tans and always burns. Among the 88 melanoma cases, 12 are compound heterozygotes carrying two postulated red hair variants; of these only four (R151C/R160W) have red hair. Two are homozygous for variants; one R151C and one R160W, both of whom have red hair. One currently unaffected family member is a compound heterozygote for postulated red hair variants (R151C/R160W) but does not have red hair.

In a study by Smith et al. (1998), they associated red hair with the R151C, R160W and D294H variants. All individuals carrying two of these variants had red hair, but some red-haired individuals carried only one of these variants. Transfection and binding studies have been carried out to establish the functional significance of the R151C, R160W and D294H *MC1R* variants (Schioth et al. 1999). The R151C, R160W and D294H variants caused reduced binding of the MC1R to MSH, which reduced cAMP production compared with the wildtype MC1R.

The R151C, R160W, D294H, R142H, 86insA, and 537insC variants were found to be associated with red hair from a study involving 174 individuals from 11 red hair pedigrees and 99 unrelated red haired individuals (Flanagan et al. 2000). The V60L variant was associated with red hair but did not reach significance. Flanagan et al. (2000) also identified an overrepresentation of red hair together with R151C in the families studied.

Palmer et al. (2000) found three variants (R151C, R160W and D294H) to each give a 2.2-fold increased risk of melanoma (CI 1.6-3.0) in a study comprising of 460 melanoma patients and 399 controls.

Duffy et al., 2004 concluded that the D84E, R151C, R160W and D294H variants are strong red hair colour alleles and the V60L, V92M and R163Q variants are relatively weak red hair colour alleles, although each of these alleles can occur with red hair. At least one of these three alleles R151C, R160W or D294H was found in 93% of individuals with red hair. They show that significant numbers of red-haired individuals are only seen in homozygous and heterozygous strong/weak red hair colour genotypes with 67.1 and 10.8%, respectively. They report the frequency of red hair in those with a heterozygous strong/other variant genotype is only 1.5%, and less than 1% in those with a homozygous weak red hair colour genotype or a heterozygous weak/other variant genotype, while no redhead was observed with a homozygous other variant or consensus genotype. The only allele that was not seen to be highly associated with red hair in their study was R142H, although neither this nor I155T were found in sufficient numbers in their sample to determine their effect on hair colour.

Among the 67 controls in this thesis, seven have red hair, one of which failed on sequencing. Of the six sequenced red headed control subjects, four were found to be compound heterozygotes for at least one of the postulated red hair variants (R142H, R151C, R160W). The D294H postulated red hair variant was not seen in any red haired control or melanoma patient.

In the sample of 27 red haired individuals in this thesis (seven controls, six sporadic melanoma patients, ten affected familial melanoma patients and four currently unaffected family members) a significant association of red hair with the common high-penetrance *MC1R* alleles (R142H, R151C, R160W, D294H) could not be demonstrated. However, there is a nearly significant association

between carrying a so called red hair variant and having melanoma as 51/88 (58%) patients carry one of these variants compared with 29/67 (43.3%) controls (P = 0.077; OR = 1.806; CI 0.950-3.434).

10.5.2 Correlation between the MC1R Variants and Melanoma

There is a nearly significant correlation between carrying a red hair variant and having sporadic melanoma as 18/28 (64.3%) patients carry a red hair variant compared with 29/67 (43.3%) controls (P = 0.074; OR = 2.359; CI 0.948-5.869). This significance could not be demonstrated in familial melanoma patients, but those patients with a *CDKN2A* mutation show a nearly significant association as 14/21 (66.7%) patients carry a red hair variant compared with 29/67 (43.3%) controls (P = 0.081; OR = 2.621; CI 0.937-7.326). This association could not be identified in those familial melanoma patients without a *CDKN2A* mutation.

In conclusion, the presence of one or more *MC1R* variant appears to increase the risk of melanoma, although the mechanisms underlying this risk are unknown. To understand the significance *MC1R* variants have on melanoma risk, future studies will have to investigate the functional effects these variants have on the melanocyte and also investigate other genes involved in pigmentation. It is possible that in the future, if routine melanoma susceptibility screening is introduced for selected subjects, that a combination of phenotypic characteristics and *MC1R* polymorphism information could be part of the evaluation.

While *CDKN2A* mutations to date appear to be associated with a proportion of familial melanoma cases, it may well be that *MC1R* variants, although a weaker predisposing factor than *CDKN2A* mutations, play a part in melanoma susceptibility in a very much higher number of melanoma patients both with the familial and sporadic forms of the disease.

CHAPTER 11

DISCUSSION OF MUTATION SCREENING OF EXON 15 OF THE *BRAF* GENE

11.1 Exon 15 *BRAF* Mutation Discussion

During the time of this study Davies et al. (2002) reported that the most frequently targeted gene in melanoma is *BRAF*. Davies found *BRAF* gene mutations in 20/34 (59%) melanoma cell lines, 12/15 (80%) short-term cultures and 6/9 (67%) primary melanomas. All mutations were within the kinase domain, with a single substitution (V599E) accounting for 80% of mutations. *BRAF* mutations in germline DNA from familial melanoma patients had not been investigated, and the number of melanoma tissue samples investigated for *BRAF* mutations was low. Only nine primary melanoma samples had been screened and no secondary melanoma samples were reported. One of the aims of this thesis therefore was to screen exon 15 of *BRAF* to determine if the V599E mutation would contribute to melanoma predisposition in familial melanoma as a germline mutation. The study of this thesis also investigated primary and secondary melanomas for exon 15 *BRAF* mutations.

11.2 V600E

In all publications on mutations in the *BRAF* gene, the nucleotide and codon numbers have been based on the NCBI gene bank accession number NM_004333. However, according to NCBI gene bank sequence with accession number NT_007914, there is a discrepancy of one codon (three nucleotides) in exon 1 in the sequence with accession number NM_004333. The sequence analysis of exon 1 of the *BRAF* gene by Kumar et al. (2003) has shown that the sequence derived from NT_007914 is correct. Due to this the sequence numbering of mutation V599E should now be changed to V600E.

11.3 Exon 15 *BRAF* Studies on Germline DNA from Patients with Familial Melanoma

DNA from the peripheral blood of 42 familial melanoma cases contained no exon 15 *BRAF* mutations, in families with any number of affected family members and regardless of whether the families are *CDKN2A* mutation positive or *CDKN2A* mutation negative.

The DNA from the two samples of secondary melanoma from individuals F4.1 and F10.1 with a family history of melanoma also failed to show exon 15 *BRAF* mutations.

Meyer et al. (2003) confirmed the work presented in this thesis as they found no V599E mutations in 172 melanoma patients comprising 46 familial cases, 21 multiple melanoma patients and 106 cases with at least one first-degree relative suffering from other cancers.

Laud et al. (2003) have now also screened the *BRAF* gene for germline mutations in 80 patients either from melanoma families or from patients with multiple primary melanomas without a familial history to determine whether *BRAF* mutations could be an earlier event occurring at the germline level. They identified 13 *BRAF* variants, 4 of which were silent mutations in coding regions and 9 nucleotide substitutions in introns. Importantly none of the variants segregated with melanoma in the 11 melanoma families studied.

Most recently, Casula et al. (2004) investigated the contribution of *BRAF* to melanoma susceptibility in 569 melanoma patients from Italy by dHPLC and sequencing. Three *BRAF* germline sequence variants (M116R in exon 3, V599E, and 2 x G608H, exon 15) were identified in 4/569 (0.7%) patients. The V599E mutation was detected in one germline DNA sample only. No *BRAF* variant (either mutation or polymorphism) was found in individuals with *CDKN2A* mutations.

The results of this thesis and that of those described therefore suggest that exon 15 *BRAF* mutations are not causative germline mutations in melanoma. The investigation in this thesis did not include a study of the entire *BRAF* gene therefore mutations in the *BRAF* gene cannot be ruled out in melanoma patients.

11.4 Exon 15 *BRAF* Studies on Formalin Fixed and Paraffin Processed Tissue from Primary Melanomas from Patients with Sporadic Melanoma

A heterozygous T to A transition at nucleotide position 1796T>A was identified. This base change is predicted to lead to the substitution of a valine (V) for a glutamic acid (E) at codon 599 (V599E). The V599E substitution was detected in primary tumour DNA from 13/52 cases (25%). Five of the samples of primary melanoma which contained this *BRAF* mutation were from DNA extracted from 17 SSM, three from 11 Nodular, two from 13 Acral, one from one Mucosal and two from ten LMM (Table 11.1).

TABLE 11.1

Mutation Positive Primary Tumours from Sporadic Melanoma Patients

Sample Identifier	Age	Sex	Histology	Body Site	Thickness (mm)	Exon 15 Mutation
Braf1	53	M	SSM	Scalp	6.8	V599E
Braf2	70	M	SSM	Trunk	6.0	V599E
Braf3	58	M	SSM	Trunk	3.8	V599E
Braf4	48	M	SSM	Neck	5.7	V599E
Braf5	66	M	SSM	Back	3.5	V599E
Braf18	71	F	Nodular	Leg	10.0	V599E
Braf19	69	M	Nodular	Upper Arm	6.0	V599E
Braf20	70	F	Nodular	Thigh	4.9	V599E
Braf29	72	M	Acral	Toe	3.5	V599E
Braf30	65	F	Acral	Leg	4.2	V599E
Braf42	72	M	Mucosal	Lip	7.5	V599E
Braf43	40	F	LMM	Face	1.0	V599E
Braf44	79	M	LMM	Foot	1.2	V599E

11.5 Exon 15 *BRAF* Studies on Frozen Melanoma Tissue from Secondary Tumours from Patients with Sporadic Melanoma

The V599E substitution was detected in secondary tumour DNA from 6/22 cases (27%) of secondary melanoma (Table 11.2). Four of the samples of secondary melanoma which contained this *BRAF* mutation were from DNA extracted from metastatic deposits in lymph nodes, all from different individuals and 2 were from soft tissue metastatic nodules both from the same individual 96037, taken approximately 9 months apart.

TABLE 11.2

Mutation Positive Secondary Melanoma Tumours from Sporadic Melanoma Patients

Sample Identifier	Age	Sex	DNA Source	Primary Type Histology	Body Site	Thickness (mm)	Exon 15 Mutation
96037	50	F	2 x Soft Tissue	SSM	Calf	4.3	V599E
97165	76	F	Nodal	Nodular	Leg	7.0	V599E
96150	48	M	Nodal	Nodular	Leg	1.0	V599E
96250	48	F	Nodal	Nodular	Thigh	5.0	V599E
91065	68	F	Nodal	Nodular	Leg	3.7	V599E

Laud et al. (2003) also detected T1796A mutations in 6/24 (25%) secondary melanoma samples from sporadic cases. This frequency of mutation detection in the *BRAF* gene is similar to the observations in this thesis (27%).

Table 11.3 indicates the number of primary melanomas studied to date and the proportion which contain a *BRAF* mutation.

TABLE 11.3

Primary Melanomas Analysed for *BRAF* Mutations

Primary Melanomas Studied	Number with *BRAF* Mutation	Reference
9	6 (67%)	Davies et al. (2002)
5	4 (80%)	Pollock et al. (2003)
10	2 (20%)	Cruz et al. (2003)
28	7 (25%)	Dong et al. (2003)
25	13 (52%)	Uribe et al. (2003)
71	42 (59%)	Omholt et al. (2003)
97	28 (29%)	Yazdi et al. (2003)
15	8 (53%)	Reifenberger et al. (2004)
59	18 (30%)	Shinozaki et al. (2004)
35	9 (26%)	Sasaki et al. (2004)
354	**137 (39%)**	**TOTAL**
52	13 (25%)	Lang (this thesis)

Pollock et al. (2003) carried out mutation analysis on microdissected melanoma. They reported the V599E mutation in 4/5 (80%) primary melanomas and 41/60 (68%) melanoma metastases.

Cruz et al. (2003) has examined exons 11 and 15 of the *BRAF* gene for mutations in ten primary melanomas and 34 metastatic melanomas. Mutations in *BRAF* exon 15 were detected in 2/10 (20%) primary melanomas and 14/34 (41%) metastatic melanomas. The most common mutation identified was the V599E mutation, but a novel point mutation (L596Q) was identified in two cases and an in-frame deletion/insertion (VKSRWK599-604D) was discovered in one case. The presence of exon 15 mutations is at a lower percentage than in this thesis for the primary melanomas but at a higher percentage for secondary melanomas.

Omholt et al. (2003) screened 71 primary melanomas and 88 corresponding metastases from 71 patients for *BRAF* exon 11 and exon 15 mutations. They identified mutations in 42/71 patients (59%). Thirty-seven patients had the V599E mutation, and mutations resulting in G468S, V599R, V599K, and K600E changes were detected in one patient each. Furthermore, one patient had a 6-bp insertion between codons 598 and 599, encoding two

threonine residues. In most cases, mutations present in the primary tumours were also found in the corresponding metastases, and mutations did not arise at the metastatic stage if they were not present in the primary lesion.

Reifenberger et al. (2004) investigated 37 sporadic malignant melanomas (15 primary cutaneous melanomas and 22 melanoma metastases) and six melanoma cell lines for mutations in the *BRAF* gene. Eight (53%) primary melanomas, six (27%) melanoma metastases and four (67%) melanoma cell lines carried the V599E *BRAF* mutation. This is a much higher percentage of mutations in their primary melanomas compared with this thesis but the percentage of mutations in the sporadic melanomas is identical.

Shinozaki et al. (2004) assessed *BRAF* mutation frequency in exons 11 and 15 in 59 primary and 68 metastatic melanomas. They found 18/59 (31%) primary melanomas to have a *BRAF* mutation in exon 15 with a significantly higher frequency in patients < 60 years old (P = 0.001). The incidence of *BRAF* mutations did not correlate with Breslow thickness. The population of tumours sampled for this thesis came from older patients than average for the Scottish patient database (MacKie et al., 2002), and represent a thick subset of primary melanomas because of the need to obtain an adequate melanoma cell volume for DNA extraction from paraffin blocks.

11.6 Melanoma Subtypes

This thesis shows a prevalence of *BRAF* mutations in 13/52 (25%) melanomas overall comprising of 5/17 (29%) Superficial Spreading Melanomas, 3/11 (27%) Nodular Melanomas, 2/13 (15%) Acral Lentiginous Melanomas, 1/1 (100%) Mucosal Melanoma and 2/10 (20%) Lentigo Maligna Melanomas.

Sasaki et al. (2004) searched for mutations of *BRAF* in 35 primary sporadic melanomas from 35 Japanese patients and detected the V599E *BRAF* point mutation in nine (26%), which is very similar to this thesis (25%). They found differences in mutation frequency among the four histological subtypes; 4/8 (50%) Superficial Spreading Melanomas and 5/15 (33%) Acral Lentiginous Melanomas had the mutation, but none of six Nodular Melanomas, five Lentigo Maligna Melanomas, or one Mucosal Melanoma.

Further larger studies are required to further investigate the relationship between the V599E mutation in specific melanoma subtypes.

11.7 *BRAF* and UV Radiation

The T1796A mutation is not a common UV-induced base change. The V599E mutation is not a C-to-T or CC-to-TT mutation, which are usually associated with UV exposure. Mutational mechanisms other than UV induced damage must therefore be important for the aetiology of the mutation.

Maldonado et al. (2003) reported that *BRAF* mutations were present in 23/43 melanomas occurring on intermittent sun-exposed skin. *BRAF* mutations in melanomas on chronically sun-damaged skin (1/12 patients) and melanomas on skin relatively unexposed to sun, such as palms, soles, subungual sites (6/39 patients), and mucosal membranes (2/21 patients) were rare.

Edwards et al. (2004) determined the *BRAF* mutation frequency in 13 mucosal melanomas and compared their findings with the published data. None of the 13 mucosal melanomas carried an exon 15 *BRAF* mutation, as compared to 54/165 (33%) primary cutaneous melanomas with *BRAF* mutations in published reports. The absence of *BRAF* mutations identified in mucosal sites suggests that the prevalence of *BRAF* mutations in melanoma may vary depending upon the extent of sun exposure for the tissue of origin.

Cohen et al. (2004) tested 17 malignant mucosal melanomas of the head and neck, 21 cutaneous melanomas, including 13 arising from sun-exposed sites and eight from vulvar skin, for the V599E mutation. They detected the mutation in one (6%) sinonasal melanoma, eight (62%) sun-exposed melanomas but in none of the vulvar melanomas. They concluded that in contrast to cutaneous melanomas arising in sun-exposed sites, mucosal melanomas of the head and neck do not frequently harbour the V599E *BRAF* mutation.

The higher mutation frequency in melanomas arising on intermittently sun-exposed skin suggests a complex causative role of UV radiation requiring further investigation.

It could be that UV radiation causes a greater proliferative response in melanocytes with the V599E *BRAF* mutation than in surrounding cells with wild-type *BRAF*, further promoting melanocytic tumour progression in sun-exposed sites.

11.8 The Presence of *BRAF* Mutations in Benign Melanocytic Nevi

There are a very large number of melanocytic nevi in the general population compared with the relatively low incidence of melanoma. Pollock et al. (2003) therefore investigated exon 15 *BRAF* mutations on microdissected melanoma and nevi samples to evaluate the timing of mutations in *BRAF* during melanocyte development. They found the V599E mutation in 41/60 (68%) melanoma metastases, 4/5 (80%) primary melanomas, and, unexpectedly, in 63/77 (82%) nevi. Many of those nevi were of types not thought to be potential precursors to melanoma. The mutation was not present in normal melanocytes in eight cases for which adjacent normal skin to the nevi was available. This suggests that the V599E mutation occurs during the development of melanocytic nevi but is insufficient for progression to melanoma.

Dong et al. (2003) screened *BRAF* mutations in 65 melanocytic lesions and 25 melanoma cell lines. *BRAF* mutations were identified in 17/24 (71%) benign melanocytic nevi, 5/8 (63%) vertical growth phase melanomas, 8/13 (61%) metastatic melanomas and 18/25 (72%) melanoma cell lines. In contrast, they found *BRAF* mutations in only 2/20 (10%) of early stage or radial growth phase melanomas.

Uribe et al. (2003) investigated the stage in which *BRAF* mutations occur in the malignant transformation of melanocytes. They examined DNA extracted from microdissected formalin-fixed and paraffin-embedded tissues from 22 benign melanocytic nevi, 23 atypical melanocytic nevi, and 25 primary cutaneous melanomas from 63 different patients for *BRAF* mutations. Sixteen benign nevi (73%), 11 atypical melanocytic nevi (48%), and 13 melanomas (52%) had the V599E mutation. No mutations were found in microdissected epidermal keratinocytes and melanocytes adjacent to melanocytic lesions having *BRAF* mutations. No correlation was detected between *BRAF* mutational status and age, sun exposure, or Clark levels in malignant melanoma. Again these findings of a high frequency of *BRAF* mutations in benign melanocytic lesions indicate that the V599E mutation is not sufficient by itself for malignant transformation.

Yazdi et al. (2003) screened primary melanomas, nevi and lesions where a melanoma developed in an underlying nevus. They detected the mutation in

28/97 (29%) melanomas and in 39/187 (21%) nevi. Four of 14 of these melanomas developed in a nevus.

Loewe et al. (2004) analysed 49 melanocytic lesions, which were not considered melanoma at first examination, but showed growth or structural changes within 12 months. The V599E mutation was detected in 16/36 (11 melanomas and five nevi) growing lesions and 4/13 (three melanomas and one nevus) lesions with structural changes. Statistics revealed odds for the presence of the V599E mutation being 13 times higher in growing nevi as compared with nevi without changes and seven times higher in nevi with structural changes.

These five studies all indicate that in some situations *BRAF* mutations are found in stable benign melanocytic lesions and that *BRAF* mutations are neither necessary nor sufficient for melanoma development.

11.9 Possible Function of the V599E Mutation

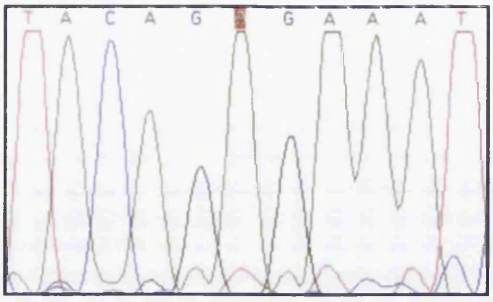
The V599E substitution is an activating mutation within the kinase domain. Mutated *BRAF* proteins have elevated kinase activity. Wan et al. (2004) analyzed 22 *BRAF* mutants and found that 18 had elevated kinase activity and signalled to ERK in vivo, whereas three mutants had reduced kinase activity towards MEK in vitro. Perhaps the V599E mutations could accelerate tumour growth.

11.10 Loss of Heterozygosity in Tumour Cells

Loss of heterozygosity (LOH) in tumour cells can reveal recessive mutations, which predispose to tumourgenesis. One allele is mutated and the other is lost by a large chromosomal deletion. LOH represents the second mutational hit in a particular cell. Allele loss in a tumour must be that of the wild type allele therefore allowing the mutant allele to be dominant and cause the tumour. If the mutant allele was lost then there would be no tumour. LOH or perhaps homozygosity for the mutation occurred in one secondary melanoma in this study (Figure 11.1).

FIGURE 11.1

Possible LOH in Melanoma Tumour Tissue



Sequence analysis of PCR amplified somatic DNA from a secondary melanoma tumour sample 96037. Tumour DNA that has lost one allele, the wild type allele, will amplify only the remaining allele, the mutant allele.

dHPLC was used as a second screen in the analysis of the primary melanoma tissues. If there was a single mutant allele (i.e. wild type loss) no heteroduplex would be formed and the chromatogram profile would be identical to the wild type profile. Therefore, a mutation could have been missed and mistaken for being wild type. However, all the samples presented in this thesis were sequenced prior to dHPLC and therefore any mutant cells present would have been identified. The only reason a mutation would not have been identified would have been if the proportion of wild type cells greatly outnumbered the number of mutant cells in the tumour sample. There probably were a small number of normal cells in the tissue sample allowing for a mixture of wild type and mutant cells, therefore allowing heteroduplex formation to occur in dHPLC. Any mutation would then have been identified with dHPLC even if there had been loss of the normal allele.

CHAPTER 12

SYNTHESIS AND CONCLUSIONS

Prevention and possibly treatment of all cancers depends on a clear understanding of their aetiology. In the majority of solid tumours in adults, this appears to involve complex interactions between environmental agents and individual susceptibility governed by genetic factors. This appears to be true, even in tumours such as lung cancer where although there is a very strong case for tobacco as the environmental carcinogen, there are individual variations in the response to excessive exposure. These individual variations are likely to be under genetic control.

In the case of cutaneous melanoma, the environmental agent most strongly incriminated is excessive exposure to ultraviolet radiation, mainly in natural sunlight. However this association is complex, as short episodes of intense UV overexposure appear to be more damaging than lower level long-term exposure. In addition, Armstrong et al. (1997) has calculated that in up to one third of all cutaneous melanoma cases worldwide, the evidence for ultraviolet exposure being a major aetiological agent is lacking. This clearly suggests either a second environmental agent, or genetic factors. No second environmental agent has yet been incriminated, and attention has therefore moved to genetic factors.

In other tumour types such as colon cancer, study of familial cases has helped identify genes involved in their development and then led to study of the role of these genes in the more common sporadic cases. 2-5% of all melanoma cases have a positive family history, and the gene identified to date, which appears mutated in 25-30% of familial cases, is the *CDKN2A* gene. However, extensive studies on large numbers of sporadic cases of melanoma have so far failed to identify *CDKN2A* mutations in the great majority of cases.

Studies of *MC1R* polymorphisms in sporadic melanoma cases appear more promising in identifying a possible genetic variant affecting the likelihood of melanoma development in the majority of cases. In addition, the known function of the *MSH* gene in determining pigment response to ultraviolet radiation provides an attractive link between environmental and individual genetic aetiological factors. Two previous studies (van der Velden et al. 2001; Box et al. 2001) and the work described in this thesis suggest a possible interaction between some *CDKN2A* mutations and *MC1R* polymorphisms in

familial melanoma, but it is not clear whether or not this has any relevance to the larger body of sporadic cases.

The normal function of the *BRAF* gene suggests that mutations are highly likely to increase cancer risk, and *BRAF* mutations have been reported in a variety of malignancies to date. The first report of *BRAF* mutations in sporadic melanoma cases suggested that the major melanoma susceptibility gene had been identified, but subsequent studies on larger numbers of melanoma cases and also on benign melanocytic lesions indicate that the situation is more complex, and that a *BRAF* mutation is neither necessary nor sufficient for melanoma development. Despite this, studies are currently in progress evaluating Raf kinase inhibitors such as BAY 43-9006 in the management of melanoma. Early results suggest minimal activity as a single agent but better responses when used in combination with chemotherapy.

The aim of many working in melanoma genetics worldwide is to show a pathway to melanoma development linking the currently known abnormalities in *CDKN2A*, *MC1R*, *BRAF* and other genes including the DNA repair family. To date neither the work in this thesis nor in of any other laboratory worldwide has achieved this, but it remains a very worthwhile objective.

There clearly still remains much to learn about familial melanoma as genetic analysis of these families shows germline mutations of *CDKN2A* in 20-30% of patients. Therefore, in at least 70% of familial melanoma cases a pathological mutation still needs to be identified. Familial melanoma cases, although rare in comparison to sporadic melanoma cases, provide an opportunity to study the aetiology of melanoma, and may therefore help in devising new strategies for the early detection and effective treatment. They will also allow us to isolate other genes thought to be involved in the development of melanoma, both of the familial and sporadic form.

Future research will require the use of new high-throughput technologies, such as dHPLC as used in this thesis, to enable rapid large-scale screening of newly identified genes.

In conclusion, due to the relatively small numbers of familial melanoma families worldwide, international collaboration is necessary to pool data sets together that perhaps individually would not reach any statistical significance. For example, a collaborative approach is required to obtain the sample numbers

required to achieve statistical power for linkage to a common disease region and localisation of new candidate genes. This is the primary aim of the International Melanoma Genetics Consortium, Genomel.

References

Aitken, J., Welch, J., Duffy, D., Milligan, A., Green, A., Martin, N., & Hayward, N. 1999, "CDKN2A variants in a population-based sample of Queensland families with melanoma", *J.Natl.Cancer Inst.*, vol. 91, no. 5, pp. 446-452.

Anderson, D. E., Smith, J. L., Jr., & McBride, C. M. 1967, "Hereditary aspects of malignant melanoma", *JAMA*, vol. 200, no. 9, pp. 741-746.

Anderson, D. E. 1971, "Clinical characteristics of the genetic variety of cutaneous melanoma in man", *Cancer*, vol. 28, no. 3, pp. 721-725.

Andrews, J. C. 1968, "Malignant melanoma in siblings", *Arch.Dermatol.*, vol. 98, no. 3, pp. 282-283.

Arcellana-Panlilio, M. Y., Egeler, R. M., Ujack, E., Magliocco, A., Stuart, G. C., Robbins, S. M., & Coppes, M. J. 2002, "Evidence of a role for the INK4 family of cyclin-dependent kinase inhibitors in ovarian granulosa cell tumors", *Genes Chromosomes.Cancer*, vol. 35, no. 2, pp. 176-181.

Armstrong, B. K., Kricker, A., & English, D. R. 1997, "Sun exposure and skin cancer. ", *Australas.J.Dermatol.*, vol. 38, Suppl 1:S1-6.

Bale, S. J., Dracopoli, N. C., Tucker, M. A., Clark, W. H., Jr., Fraser, M. C., Stanger, B. Z., Green, P., Donis-Keller, H., Housman, D. E., & Greene, M. H. 1989, "Mapping the gene for hereditary cutaneous malignant melanoma-dysplastic nevus to chromosome 1p", *N.Engl.J.Med.*, vol. 320, no. 21, pp. 1367-1372.

Bartsch, D. K., Sina-Frey, M., Lang, S., Wild, A., Gerdes, B., Barth, P., Kress, R., Grutzmann, R., Colombo-Benkmann, M., Ziegler, A., Hahn, S. A., Rothmund, M., & Rieder, H. 2002, "CDKN2A germline mutations in familial pancreatic cancer", *Ann.Surg.*, vol. 236, no. 6, pp. 730-737.

Becher, R., Gibas, Z., & Sandberg, A. A. 1983, "Chromosome 6 in malignant melanoma", *Cancer Genet.Cytogenet.*, vol. 9, no. 2, pp. 173-175.

Becker, T. M., Rizos, H., Kefford, R. F., & Mann, G. J. 2001, "Functional impairment of melanoma-associated p16(INK4a) mutants in melanoma cells despite retention of cyclin-dependent kinase 4 binding", *Clin.Cancer Res.*, vol. 7, no. 10, pp. 3282-3288.

Bergman, W., Palan, A., & Went, L. N. 1986, "Clinical and genetic studies in six Dutch kindreds with the dysplastic naevus syndrome", *Ann.Hum.Genet.*, vol. 50 (Pt 3), pp. 249-258.

Bergman, W., Gruis, N. A., Sandkuijl, L. A., & Frants, R. R. 1994, "Genetics of seven Dutch familial atypical multiple mole-melanoma syndrome families: a review of linkage results including chromosomes 1 and 9", *J.Invest Dermatol.*, vol. 103, no. 5 Suppl, pp. 122S-125S.

Bergman, W. & Gruis, N. 1996, "Familial melanoma and pancreatic cancer", *N.Engl.J.Med.*, vol. 334, no. 7, pp. 471-472.

Bertram, C. G., Gaut, R. M., Barrett, J. H., Pinney, E., Whitaker, L., Turner, F., Bataille, V., Dos, S. S., I, Swerdlow, J., Bishop, D. T., & Newton Bishop, J. A. 2002, "An assessment of the CDKN2A variant Ala148Thr as a nevus/melanoma susceptibility allele", *J.Invest Dermatol.*, vol. 119, no. 4, pp. 961-965.

Bishop, D. T., Demenais, F., Goldstein, A. M., Bergman, W., Bishop, J. N., Bressac-de Paillerets, B., Chompret, A., Ghiorzo, P., Gruis, N., Hansson, J., Harland, M., Hayward, N., Holland, E. A., Mann, G. J., Mantelli, M., Nancarrow, D., Platz, A., & Tucker, M. A. 2002, "Geographical variation in the penetrance of CDKN2A mutations for melanoma", *J.Natl.Cancer Inst.*, vol. 94, no. 12, pp. 894-903.

Blackwood, M. A., Holmes, R., Synnestvedt, M., Young, M., George, C., Yang, H., Elder, D. E., Schuchter, L. M., Guerry, D., & Ganguly, A. 2002, "Multiple primary melanoma revisited", *Cancer*, vol. 94, no. 8, pp. 2248-2255.

Borg, A., Johannsson, U., Johannsson, O., Hakansson, S., Westerdahl, J., Masback, A., Olsson, H., & Ingvar, C. 1996, "Novel germline p16 mutation in familial malignant melanoma in southern Sweden", *Cancer Res.*, vol. 56, no. 11, pp. 2497-2500.

Borg, A., Sandberg, T., Nilsson, K., Johannsson, O., Klinker, M., Masback, A., Westerdahl, J., Olsson, H., & Ingvar, C. 2000, "High frequency of multiple melanomas and breast and pancreas carcinomas in CDKN2A mutation-positive melanoma families", *J.Natl.Cancer Inst.*, vol. 92, no. 15, pp. 1260-1266.

Box, N. F., Wyeth, J. R., O'Gorman, L. E., Martin, N. G., & Sturm, R. A. 1997, "Characterization of melanocyte stimulating hormone receptor variant alleles in twins with red hair", *Hum.Mol.Genet.*, vol. 6, no. 11, pp. 1891-1897.

Box, N. F., Duffy, D. L., Chen, W., Stark, M., Martin, N. G., Sturm, R. A., & Hayward, N. K. 2001, "MC1R genotype modifies risk of melanoma in families segregating CDKN2A mutations", *Am.J.Hum.Genet.*, vol. 69, no. 4, pp. 765-773.

Breslow, A. 1970, "Thickness, cross-sectional areas and depth of invasion in the prognosis of cutaneous melanoma", *Ann.Surg.*, vol. 172, no. 5, pp. 902-908.

Burchill, S. A., Ito, S., & Thody, A. J. 1993, "Effects of melanocyte-stimulating hormone on tyrosinase expression and melanin synthesis in hair follicular melanocytes of the mouse", *J.Endocrinol.*, vol. 137, no. 2, pp. 189-195.

Burden, A. D., Newell, J., Andrew, N., Kavanagh, G., Connor, J. M., & MacKie, R. M. 1999, "Genetic and environmental influences in the development of multiple primary melanoma", *Arch.Dermatol.*, vol. 135, no. 3, pp. 261-265.

Byeon, I. J., Li, J., Ericson, K., Selby, T. L., Tevelev, A., Kim, H. J., O'Maille, P., & Tsai, M. D. 1998, "Tumor suppressor p16INK4A: determination of solution structure and analyses of its interaction with cyclin-dependent kinase 4", *Mol.Cell*, vol. 1, no. 3, pp. 421-431.

Casula, M., Colombino, M., Satta, M. P., Cossu, A., Ascierto, P. A., Bianchi-Scarra, G., Castiglia, D., Budroni, M., Rozzo, C., Manca, A., Lissia, A., Carboni, A., Petretto, E., Satriano, S. M., Botti, G., Mantelli, M., Ghiorzo, P., Stratton, M. R., Tanda, F., & Palmieri, G. 2004, "BRAF gene is somatically mutated but does not make a major contribution to malignant melanoma susceptibility: the Italian Melanoma Intergroup Study", *J.Clin.Oncol.*, vol. 22, no. 2, pp. 286-292.

Cawley, E. P., Kruse, W. T., & Pinkus, H. K. 1952, "Genetic aspects of malignant melanoma", *AMA.Arch.Derm.Syphilol.*, vol. 65, no. 4, pp. 440-450.

Chhajlani, V. & Wikberg, J. E. 1992, "Molecular cloning and expression of the human melanocyte stimulating hormone receptor cDNA", *FEBS Lett.*, vol. 309, no. 3, pp. 417-420.

Ciotti, P., Strigini, P., & Bianchi-Scarra, G. 1996, "Familial melanoma and pancreatic cancer. Ligurian Skin Tumor Study Group", *N.Engl.J.Med.*, vol. 334, no. 7, pp. 469-470.

Ciotti, P., Struewing, J. P., Mantelli, M., Chompret, A., Avril, M. F., Santi, P. L., Tucker, M. A., Bianchi-Scarra, G., Bressac-de Paillerets, B., & Goldstein, A. M. 2000, "A single genetic origin for the G101W CDKN2A mutation in 20 melanoma-prone families", *Am.J.Hum.Genet.*, vol. 67, no. 2, pp. 311-319.

Clark, W. H., Jr., From, L., Bernardino, E. A., & Mihm, M. C. 1969, "The histogenesis and biologic behavior of primary human malignant melanomas of the skin", *Cancer Res.*, vol. 29, no. 3, pp. 705-727.

Clark, W. H., Jr., Reimer, R. R., Greene, M., Ainsworth, A. M., & Mastrangelo, M. J. 1978, "Origin of familial malignant melanomas from heritable melanocytic lesions. 'The B-K mole syndrome'", *Arch.Dermatol.*, vol. 114, no. 5, pp. 732-738.

Cohen, Y., Rosenbaum, E., Begum, S., Goldenberg, D., Esche, C., Lavie, O., Sidransky, D., & Westra, W. H. 2004, "Exon 15 BRAF mutations are uncommon in melanomas arising in nonsun-exposed sites", *Clin.Cancer Res.*, vol. 10, no. 10, pp. 3444-3447.

Cruz, F., III, Rubin, B. P., Wilson, D., Town, A., Schroeder, A., Haley, A., Bainbridge, T., Heinrich, M. C., & Corless, C. L. 2003, "Absence of BRAF and NRAS mutations in uveal melanoma", *Cancer Res.*, vol. 63, no. 18, pp. 5761-5766.

Davies, H., Bignell, G. R., Cox, C., Stephens, P., Edkins, S., Clegg, S., Teague, J., Woffendin, H., Garnett, M. J., Bottomley, W., Davis, N., Dicks, E., Ewing, R., Floyd, Y., Gray, K., Hall, S., Hawes, R., Hughes, J., Kosmidou, V., Menzies, A., Mould, C., Parker, A., Stevens, C., Watt, S., Hooper, S., Wilson, R., Jayatilake, H., Gusterson, B. A., Cooper, C., Shipley, J., Hargrave, D., Pritchard-Jones, K., Maitland, N., Chenevix-Trench, G., Riggins, G. J., Bigner, D. D., Palmieri, G., Cossu, A., Flanagan, A., Nicholson, A., Ho, J. W., Leung, S. Y., Yuen, S. T., Weber, B. L., Seigler, H. F., Darrow, T. L., Paterson, H., Marais, R., Marshall, C. J., Wooster, R., Stratton, M. R., & Futreal, P. A. 2002, "Mutations of the BRAF gene in human cancer", *Nature*, vol. 417, no. 6892, pp. 949-954.

Dong, J., Phelps, R. G., Qiao, R., Yao, S., Benard, O., Ronai, Z., & Aaronson, S. A. 2003, "BRAF oncogenic mutations correlate with progression rather than initiation of human melanoma", *Cancer Res.*, vol. 63, no. 14, pp. 3883-3885.

Dracopoli, N. C., Harnett, P., Bale, S. J., Stanger, B. Z., Tucker, M. A., Housman, D. E., & Kefford, R. F. 1989, "Loss of alleles from the distal short arm of chromosome 1 occurs late in melanoma tumor progression", *Proc.Natl.Acad.Sci.U.S.A*, vol. 86, no. 12, pp. 4614-4618.

Duffy, D. L., Box, N. F., Chen, W., Palmer, J. S., Montgomery, G. W., James, M. R., Hayward, N. K., Martin, N. G., & Sturm, R. A. 2004, "Interactive effects of MC1R and OCA2 on melanoma risk phenotypes", *Hum.Mol.Genet.*, vol. 13, no. 4, pp. 447-461.

Dwyer, T., Stankovich, J. M., Blizzard, L., FitzGerald, L. M., Dickinson, J. L., Reilly, A., Williamson, J., Ashbolt, R., Berwick, M., & Sale, M. M. 2004, "Does the addition of information on genotype improve prediction of the risk of melanoma and nonmelanoma skin cancer beyond that obtained from skin phenotype?", *Am.J.Epidemiol.*, vol. 159, no. 9, pp. 826-833.

Edwards, R. H., Ward, M. R., Wu, H., Medina, C. A., Brose, M. S., Volpe, P., Nussen-Lee, S., Haupt, H. M., Martin, A. M., Herlyn, M., Lessin, S. R., & Weber, B. L. 2004, "Absence of BRAF mutations in UV-protected mucosal melanomas", *J.Med.Genet.*, vol. 41, no. 4, pp. 270-272.

Elwood, J. M. & Jopson, J. 1997, "Melanoma and sun exposure: an overview of published studies", *Int.J.Cancer*, vol. 73, no. 2, pp. 198-203.

Eychene, A., Barnier, J. V., Apiou, F., Dutrillaux, B., & Calothy, G. 1992, "Chromosomal assignment of two human B-raf(Rmil) proto-oncogene loci: B-raf-1 encoding the p94Braf/Rmil and B-raf-2, a processed pseudogene", *Oncogene*, vol. 7, no. 8, pp. 1657-1660.

FitzGerald, M. G., Harkin, D. P., Silva-Arrieta, S., MacDonald, D. J., Lucchina, L. C., Unsal, H., O'Neill, E., Koh, J., Finkelstein, D. M., Isselbacher, K. J., Sober, A. J., & Haber, D. A. 1996, "Prevalence of germ-line mutations in p16, p19ARF, and CDK4 in familial melanoma: analysis of a clinic-based population", *Proc.Natl.Acad.Sci.U.S.A*, vol. 93, no. 16, pp. 8541-8545.

Fitzpatrick, T. B. 1988, "The validity and practicality of sun-reactive skin types I through VI", *Arch.Dermatol.*, vol. 124, no. 6, pp. 869-871.

Flanagan, N., Healy, E., Ray, A., Philips, S., Todd, C., Jackson, I. J., Birch-Machin, M. A., & Rees, J. L. 2000, "Pleiotropic effects of the melanocortin 1 receptor (MC1R) gene on human pigmentation", *Hum.Mol.Genet.*, vol. 9, no. 17, pp. 2531-2537.

Flores, J. F., Pollock, P. M., Walker, G. J., Glendening, J. M., Lin, A. H., Palmer, J. M., Walters, M. K., Hayward, N. K., & Fountain, J. W. 1997, "Analysis of the CDKN2A, CDKN2B and CDK4 genes in 48 Australian melanoma kindreds", *Oncogene*, vol. 15, no. 24, pp. 2999-3005.

Foulkes, W. D., Flanders, T. Y., Pollock, P. M., & Hayward, N. K. 1997, "The CDKN2A (p16) gene and human cancer", *Mol.Med.*, vol. 3, no. 1, pp. 5-20.

Fung, D. C., Holland, E. A., Becker, T. M., Hayward, N. K., Bressac-de Paillerets, B., & Mann, G. J. 2003, "eMelanoBase: an online locus-specific variant database for familial melanoma", *Hum.Mutat.*, vol. 21, no. 1, pp. 2-7.

Gantz, I., Yamada, T., Tashiro, T., Konda, Y., Shimoto, Y., Miwa, H., & Trent, J. M. 1994, "Mapping of the gene encoding the melanocortin-1 (alpha-melanocyte stimulating hormone) receptor (MC1R) to human chromosome 16q24.3 by Fluorescence in situ hybridization", *Genomics*, vol. 19, no. 2, pp. 394-395.

Ghiorzo, P., Ciotti, P., Mantelli, M., Heouaine, A., Queirolo, P., Rainero, M. L., Ferrari, C., Santi, P. L., De Marchi, R., Farris, A., Ajmar, F., Bruzzi, P., & Bianchi-Scarra, G. 1999, "Characterization of ligurian melanoma families and risk of occurrence of other neoplasia", *Int.J.Cancer*, vol. 83, no. 4, pp. 441-448.

Ghiorzo, P., Pastorino, L., Bonelli, L., Cusano, R., Nicora, A., Zupo, S., Queirolo, P., Sertoli, M., Pugliese, V., & Bianchi-Scarra, G. 2004, "INK4/ARF germline alterations in pancreatic cancer patients", *Ann.Oncol.*, vol. 15, no. 1, pp. 70-78.

Gilchrest, B. A., Eller, M. S., Geller, A. C., & Yaar, M. 1999, "The pathogenesis of melanoma induced by ultraviolet radiation", *N.Engl.J.Med.*, vol. 340, no. 17, pp. 1341-1348.

Gillanders, E., Juo, S. H., Holland, E. A., Jones, M., Nancarrow, D., Freas-Lutz, D., Sood, R., Park, N., Faruque, M., Markey, C., Kefford, R. F., Palmer, J., Bergman, W., Bishop, D. T., Tucker, M. A., Bressac-de Paillerets, B., Hansson, J., Stark, M., Gruis, N., Bishop, J. N., Goldstein, A. M., Bailey-Wilson, J. E., Mann, G. J., Hayward, N., & Trent, J. 2003, "Localization of a novel melanoma susceptibility locus to 1p22", *Am.J.Hum.Genet.*, vol. 73, no. 2, pp. 301-313.

Goggins, W., Gao, W., & Tsao, H. 2004, "Association between female breast cancer and cutaneous melanoma", *Int.J.Cancer*, vol. 111, no. 5, pp. 792-794.

Goldstein, A. M., Dracopoli, N. C., Ho, E. C., Fraser, M. C., Kearns, K. S., Bale, S. J., McBride, O. W., Clark, W. H., Jr., & Tucker, M. A. 1993, "Further evidence for a locus for cutaneous malignant melanoma-dysplastic nevus (CMM/DN) on chromosome 1p, and evidence for genetic heterogeneity", *Am.J.Hum.Genet.*, vol. 52, no. 3, pp. 537-550.

Goldstein, A. M., Fraser, M. C., Struewing, J. P., Hussussian, C. J., Ranade, K., Zametkin, D. P., Fontaine, L. S., Organic, S. M., Dracopoli, N. C., & Clark, W. H., Jr. 1995, "Increased risk of pancreatic cancer in melanoma-prone kindreds with p16INK4 mutations", *N.Engl.J.Med.*, vol. 333, no. 15, pp. 970-974.

Goldstein, A. M., Liu, L., Shennan, M. G., Hogg, D., Tucker, M. A., & Struewing, J. P. 2001, "A common founder for the V126D CDKN2A mutation in seven North American melanoma-prone families", *Br.J.Cancer*, vol. 85, no. 4, pp. 527-530.

Goldstein, A. M. 2004, "Familial melanoma, pancreatic cancer and germline CDKN2A mutations", *Hum.Mutat.*, vol. 23, no. 6, p. 630.

Gruis, N. A., van der Velden, P. A., Sandkuijl, L. A., Prins, D. E., Weaver-Feldhaus, J., Kamb, A., Bergman, W., & Frants, R. R. 1995, "Homozygotes for CDKN2 (p16) germline mutation in Dutch familial melanoma kindreds", *Nat.Genet.*, vol. 10, no. 3, pp. 351-353.

Hansen, C. B., Wadge, L. M., Lowstuter, K., Boucher, K., & Leachman, S. A. 2004, "Clinical germline genetic testing for melanoma", *Lancet Oncol.*, vol. 5, no. 5, pp. 314-319.

Harding, R. M., Healy, E., Ray, A. J., Ellis, N. S., Flanagan, N., Todd, C., Dixon, C., Sajantila, A., Jackson, I. J., Birch-Machin, M. A., & Rees, J. L. 2000, "Evidence for variable selective pressures at MC1R", *Am.J.Hum.Genet.*, vol. 66, no. 4, pp. 1351-1361.

Harland, M., Meloni, R., Gruis, N., Pinney, E., Brookes, S., Spurr, N. K., Frischauf, A. M., Bataille, V., Peters, G., Cuzick, J., Selby, P., Bishop, D. T., & Bishop, J. N. 1997, "Germline mutations of the CDKN2 gene in UK melanoma families", *Hum.Mol.Genet.*, vol. 6, no. 12, pp. 2061-2067.

Harland, M., Holland, E. A., Ghiorzo, P., Mantelli, M., Bianchi-Scarra, G., Goldstein, A. M., Tucker, M. A., Ponder, B. A., Mann, G. J., Bishop, D. T., & Newton, B. J. 2000, "Mutation screening of the CDKN2A promoter in melanoma families", *Genes Chromosomes.Cancer*, vol. 28, no. 1, pp. 45-57.

Harland, M., Mistry, S., Bishop, D. T., & Bishop, J. A. 2001, "A deep intronic mutation in CDKN2A is associated with disease in a subset of melanoma pedigrees", *Hum.Mol.Genet.*, vol. 10, no. 23, pp. 2679-2686.

Hashemi, J., Platz, A., Ueno, T., Stierner, U., Ringborg, U., & Hansson, J. 2000, "CDKN2A germ-line mutations in individuals with multiple cutaneous melanomas", *Cancer Res.*, vol. 60, no. 24, pp. 6864-6867.

Hashemi, J., Bendahl, P. O., Sandberg, T., Platz, A., Linder, S., Stierner, U., Olsson, H., Ingvar, C., Hansson, J., & Borg, A. 2001, "Haplotype analysis and age estimation of the 113insR CDKN2A founder mutation in Swedish melanoma families", *Genes Chromosomes.Cancer*, vol. 31, no. 2, pp. 107-116.

Hayward, N. K. 1996, "The current situation with regard to human melanoma and genetic inferences", *Curr.Opin.Oncol.*, vol. 8, no. 2, pp. 136-142.

Hayward, N. 2000, "New developments in melanoma genetics", *Curr.Oncol.Rep.*, vol. 2, no. 4, pp. 300-306.

Hayward, N. K. 2003, "Genetics of melanoma predisposition", *Oncogene*, vol. 22, no. 20, pp. 3053-3062.

Healy, E., Flannagan, N., Ray, A., Todd, C., Jackson, I. J., Matthews, J. N., Birch-Machin, M. A., & Rees, J. L. 2000, "Melanocortin-1-receptor gene and sun sensitivity in individuals without red hair", *Lancet*, vol. 355, no. 9209, pp. 1072-1073.

Hearle, N., Humphreys, J., Damato, B. E., Wort, R., Talaban, R., Wixey, J., Green, H., Easton, D. F., & Houlston, R. S. 2003, "Role of MC1R variants in uveal melanoma", *Br.J.Cancer*, vol. 89, no. 10, pp. 1961-1965.

Hecht, F. 1989, "The annals of cancer genetics. The description by Norris of hereditary malignant melanoma of the skin in 1820", *Cancer Genet.Cytogenet.*, vol. 42, no. 1, pp. 153-156.

Hewitt, C., Lee, W. C., Evans, G., Howell, A., Elles, R. G., Jordan, R., Sloan, P., Read, A. P., & Thakker, N. 2002, "Germline mutation of ARF in a melanoma kindred", *Hum.Mol.Genet.*, vol. 11, no. 11, pp. 1273-1279.

Holland, E. A., Beaton, S. C., Becker, T. M., Grulet, O. M., Peters, B. A., Rizos, H., Kefford, R. F., & Mann, G. J. 1995, "Analysis of the p16 gene, CDKN2, in 17 Australian melanoma kindreds", *Oncogene*, vol. 11, no. 11, pp. 2289-2294.

Holland, E. A., Schmid, H., Kefford, R. F., & Mann, G. J. 1999, "CDKN2A (P16(INK4a)) and CDK4 mutation analysis in 131 Australian melanoma probands: effect of family history and multiple primary melanomas", *Genes Chromosomes.Cancer*, vol. 25, no. 4, pp. 339-348.

Hunt, G., & Thody, A. J., 1995, "Agouti protein can act independently of melanocyte-stimulating hormone to inhibit melanogenesis", *J.Endocrinol.*, vol. 147, no. 2, pp. 1-4.

Hussussian, C. J., Struewing, J. P., Goldstein, A. M., Higgins, P. A., Ally, D. S., Sheahan, M. D., Clark, W. H., Jr., Tucker, M. A., & Dracopoli, N. C. 1994, "Germline p16 mutations in familial melanoma", *Nat.Genet.*, vol. 8, no. 1, pp. 15-21.

Ichii-Jones, F., Lear, J. T., Heagerty, A. H., Smith, A. G., Hutchinson, P. E., Osborne, J., Bowers, B., Jones, P. W., Davies, E., Ollier, W. E., Thomson, W., Yengi, L., Bath, J., Fryer, A. A., & Strange, R. C. 1998, "Susceptibility to melanoma: influence of skin type and polymorphism in the melanocyte stimulating hormone receptor gene", *J.Invest Dermatol.*, vol. 111, no. 2, pp. 218-221.

Jemal, A., Devesa, S. S., Hartge, P., & Tucker, M. A. 2001, "Recent trends in cutaneous melanoma incidence among whites in the United States", *J.Natl.Cancer Inst.*, vol. 93, no. 9, pp. 678-683.

Jones, T. A., Zou, J. Y., Cowan, S. W., & Kjeldgaard. 1991, "Improved methods for building protein models in electron density maps and the location of errors in these models", *Acta.Crystallogr.A.*, vol. 1, no. 47, pp. 110-119.

Kamb, A., Gruis, N. A., Weaver-Feldhaus, J., Liu, Q., Harshman, K., Tavtigian, S. V., Stockert, E., Day, R. S., III, Johnson, B. E., & Skolnick, M. H. 1994, "A cell cycle regulator potentially involved in genesis of many tumor types", *Science*, vol. 264, no. 5157, pp. 436-440.

Kamb, A., Shattuck-Eidens, D., Eeles, R., Liu, Q., Gruis, N. A., Ding, W., Hussey, C., Tran, T., Miki, Y., & Weaver-Feldhaus, J. 1994, "Analysis of the p16 gene (CDKN2) as a candidate for the chromosome 9p melanoma susceptibility locus", *Nat.Genet.*, vol. 8, no. 1, pp. 23-26.

Kefford, R. F., Salmon, J., Shaw, H. M., Donald, J. A., & McCarthy, W. H. 1991, "Hereditary melanoma in Australia. Variable association with dysplastic nevi and absence of genetic linkage to chromosome 1p", *Cancer Genet.Cytogenet.*, vol. 51, no. 1, pp. 45-55.

Kefford, R. F., Newton Bishop, J. A., Bergman, W., & Tucker, M. A. 1999, "Counseling and DNA testing for individuals perceived to be genetically predisposed to melanoma: A consensus statement of the Melanoma Genetics Consortium", *J.Clin.Oncol.*, vol. 17, no. 10, pp. 3245-3251.

Kennedy, C., ter Huurne, J., Berkhout, M., Gruis, N., Bastiaens, M., Bergman, W., Willemze, R., & Bavinck, J. N. 2001, "Melanocortin 1 receptor (MC1R) gene variants are associated with an increased risk for cutaneous melanoma which is largely independent of skin type and hair color", *J.Invest Dermatol.*, vol. 117, no. 2, pp. 294-300.

Komiya, A., Suzuki, H., Aida, S., Yatani, R., & Shimazaki, J. 1995, "Mutational analysis of CDKN2 (CDK4I/MTS1) gene in tissues and cell lines of human prostate cancer", *Jpn.J.Cancer Res.*, vol. 86, no. 7, pp. 622-625.

Kudoh, K., Ichikawa, Y., Yoshida, S., Hirai, M., Kikuchi, Y., Nagata, I., Miwa, M., & Uchida, K. 2002, "Inactivation of p16/CDKN2 and p15/MTS2 is associated with prognosis and response to chemotherapy in ovarian cancer", *Int.J.Cancer*, vol. 99, no. 4, pp. 579-582.

Kumar, R., Smeds, J., Berggren, P., Straume, O., Rozell, B. L., Akslen, L. A., & Hemminki, K. 2001, "A single nucleotide polymorphism in the 3'untranslated region of the CDKN2A gene is common in sporadic primary melanomas but mutations in the CDKN2B, CDKN2C, CDK4 and p53 genes are rare", *Int.J.Cancer*, vol. 95, no. 6, pp. 388-393.

Kumar, R., Angelini, S., Czene, K., Sauroja, I., Hahka-Kemppinen, M., Pyrhonen, S., & Hemminki, K. 2003, "BRAF mutations in metastatic melanoma: a possible association with clinical outcome", *Clin.Cancer Res.*, vol. 9, no. 9, pp. 3362-3368.

Lang, J., Boxer, M., & MacKie, R. 2003, "Absence of exon 15 BRAF germline mutations in familial melanoma", *Hum.Mutat.*, vol. 21, no. 3, pp. 327-330.

Laud, K., Kannengiesser, C., Avril, M. F., Chompret, A., Stoppa-Lyonnet, D., Desjardins, L., Eychene, A., Demenais, F., Lenoir, G. M., & Bressac-de Paillerets, B. 2003, "BRAF as a melanoma susceptibility candidate gene?", *Cancer Res.*, vol. 63, no. 12, pp. 3061-3065.

Liu, L., Dilworth, D., Gao, L., Monzon, J., Summers, A., Lassam, N., & Hogg, D. 1999, "Mutation of the CDKN2A 5' UTR creates an aberrant initiation codon and predisposes to melanoma", *Nat.Genet.*, vol. 21, no. 1, pp. 128-132.

Liu, Q., Yan, Y. X., McClure, M., Nakagawa, H., Fujimura, F., & Rustgi, A. K. 1995, "MTS-1 (CDKN2) tumor suppressor gene deletions are a frequent event in esophagus squamous cancer and pancreatic adenocarcinoma cell lines", *Oncogene*, vol. 10, no. 3, pp. 619-622.

Loewe, R., Kittler, H., Fischer, G., Fae, I., Wolff, K., & Petzelbauer, P. 2004, "BRAF kinase gene V599E mutation in growing melanocytic lesions", *J.Invest Dermatol.*, vol. 123, no. 4, pp. 733-736.

Lynch, H. T., Frichot, B. C., III, & Lynch, J. F. 1978, "Familial atypical multiple mole-melanoma syndrome", *J.Med.Genet.*, vol. 15, no. 5, pp. 352-356.

Lynch, H. T., Fusaro, R. M., Pester, J., & Lynch, J. F. 1980, "Familial atypical multiple mole melanoma (FAMMM) syndrome: genetic heterogeneity and malignant melanoma", *Br.J.Cancer*, vol. 42, no. 1, pp. 58-70.

Lynch, H. T., Brand, R. E., Hogg, D., Deters, C. A., Fusaro, R. M., Lynch, J. F., Liu, L., Knezetic, J., Lassam, N. J., Goggins, M., & Kern, S. 2002, "Phenotypic variation in eight extended CDKN2A germline mutation familial atypical multiple mole melanoma-pancreatic carcinoma-prone families: the familial atypical mole melanoma-pancreatic carcinoma syndrome", *Cancer*, vol. 94, no. 1, pp. 84-96.

MacKie, R. M., Andrew, N., Lanyon, W. G., & Connor, J. M. 1998, "CDKN2A germline mutations in U.K. patients with familial melanoma and multiple primary melanomas", *J.Invest Dermatol.*, vol. 111, no. 2, pp. 269-272.

MacKie, R. M., Bray, C. A., Hole, D. J., Morris, A., Nicolson, M., Evans, A., Doherty, V., & Vestey, J. 2002, "Incidence of and survival from malignant melanoma in Scotland: an epidemiological study", *Lancet*, vol. 360, no. 9333, pp. 587-591.

Maldonado, J. L., Fridlyand, J., Patel, H., Jain, A. N., Busam, K., Kageshita, T., Ono, T., Albertson, D. G., Pinkel, D., & Bastian, B. C. 2003, "Determinants of BRAF mutations in primary melanomas", *J.Natl.Cancer Inst.*, vol. 95, no. 24, pp. 1878-1890.

Mann, K., Fox, S. P., Abbs, S. J., Yau, S. C., Scriven, P. N., Docherty, Z., & Ogilvie, C. M. 2001, "Development and implementation of a new rapid aneuploidy diagnostic service within the UK National Health Service and implications for the future of prenatal diagnosis", *Lancet*, vol. 358, no. 9287, pp. 1057-1061.

Mantelli, M., Barile, M., Ciotti, P., Ghiorzo, P., Lantieri, F., Pastorino, L., Catricala, C., Torre, G. D., Folco, U., Grammatico, P., Padovani, L., Pasini, B., Rovini, D., Queirolo, P., Rainero, M. L., Santi, P. L., Sertoli, R. M., Goldstein, A. M., & Bianchi-Scarra, G. 2002, "High prevalence of the G101W germline mutation in the CDKN2A (P16(ink4a)) gene in 62 Italian malignant melanoma families", *Am.J.Med.Genet.*, vol. 107, no. 3, pp. 214-221.

Marrett, L. D., Nguyen, H. L., & Armstrong, B. K. 2001, "Trends in the incidence of cutaneous malignant melanoma in New South Wales, 1983-1996", *Int.J.Cancer*, vol. 92, no. 3, pp. 457-462.

Matichard, E., Verpillat, P., Meziani, R., Gerard, B., Descamps, V., Legroux, E., Burnouf, M., Bertrand, G., Bouscarat, F., Archimbaud, A., Picard, C., Ollivaud, L., Basset-Seguin, N., Kerob, D., Lanternier, G., Lebbe, C., Crickx, B., Grandchamp, B., & Soufir, N. 2004, "Melanocortin 1 receptor (MC1R) gene variants may increase the risk of melanoma in France independently of clinical risk factors and UV exposure", *J.Med.Genet.*, vol. 41, no. 2, p. e13.

Meyer, P., Klaes, R., Schmitt, C., Boettger, M. B., & Garbe, C. 2003, "Exclusion of BRAFV599E as a melanoma susceptibility mutation", *Int.J.Cancer*, vol. 106, no. 1, pp. 78-80.

Monzon, J., Liu, L., Brill, H., Goldstein, A. M., Tucker, M. A., From, L., McLaughlin, J., Hogg, D., & Lassam, N. J. 1998, "CDKN2A mutations in multiple primary melanomas", *N.Engl.J.Med.*, vol. 338, no. 13, pp. 879-887.

Murphy, J. A., Barrantes-Reynolds, R., Kocherlakota, R., Bond, J. P., & Greenblatt, M. S. 2004, "The CDKN2A database: Integrating allelic variants with evolution, structure, function, and disease association", *Hum.Mutat.*, vol. 24, no. 4, pp. 296-304.

Nakahori, Y., Hamano, K., Iwaya, M., & Nakagome, Y. 1991, "Sex identification by polymerase chain reaction using X-Y homologous primer", *Am.J.Med.Genet.*, vol. 39, no. 4, pp. 472-473.

Newton Bishop, J. A., Harland, M., Bennett, D. C., Bataille, V., Goldstein, A. M., Tucker, M. A., Ponder, B. A., Cuzick, J., Selby, P., & Bishop, D. T. 1999, "Mutation testing in melanoma families: INK4A, CDK4 and INK4D", *Br.J.Cancer*, vol. 80, no. 1-2, pp. 295-300.

Nobori, T., Miura, K., Wu, D. J., Lois, A., Takabayashi, K., & Carson, D. A. 1994, "Deletions of the cyclin-dependent kinase-4 inhibitor gene in multiple human cancers", *Nature*, vol. 368, no. 6473, pp. 753-756.

Olsen, R., Overbo, K., & Myrnes, B. 1991, "Alkaline phosphatase from the hepatopancreas of Shrimp *(Pandalus borealis)*: A dimeric enzyme with catalytically active subunits.", *Comp.Biochem.Physiol.*, vol. 99, no. B, pp. 755-761.

Omholt, K., Platz, A., Kanter, L., Ringborg, U., & Hansson, J. 2003, "NRAS and BRAF mutations arise early during melanoma pathogenesis and are preserved throughout tumor progression", *Clin.Cancer Res.*, vol. 9, no. 17, pp. 6483-6488.

Orlow, I., Roy, P., Barz, A., Canchola, R., Song, Y., & Berwick, M. 2001, "Validation of denaturing high performance liquid chromatography as a rapid detection method for the identification of human INK4A gene mutations", *J.Mol.Diagn.*, vol. 3, no. 4, pp. 158-163.

Osterlind, A., Tucker, M. A., Stone, B. J., & Jensen, O. M. 1988, "The Danish case-control study of cutaneous malignant melanoma. II. Importance of UV-light exposure", *Int.J.Cancer*, vol. 42, no. 3, pp. 319-324.

Palmer, J. S., Duffy, D. L., Box, N. F., Aitken, J. F., O'Gorman, L. E., Green, A. C., Hayward, N. K., Martin, N. G., & Sturm, R. A. 2000, "Melanocortin-1 receptor polymorphisms and risk of melanoma: is the association explained solely by pigmentation phenotype?", *Am.J.Hum.Genet.*, vol. 66, no. 1, pp. 176-186.

Parry, D. & Peters, G. 1996, "Temperature-sensitive mutants of p16CDKN2 associated with familial melanoma", *Mol.Cell Biol.*, vol. 16, no. 7, pp. 3844-3852.

Pavey, S., Conroy, S., Russell, T., & Gabrielli, B. 1999, "Ultraviolet radiation induces p16CDKN2A expression in human skin", *Cancer Res.*, vol. 59, no. 17, pp. 4185-4189.

Pavey, S., Russell, T., & Gabrielli, B. 2001, "G2 phase cell cycle arrest in human skin following UV irradiation", *Oncogene*, vol. 20, no. 43, pp. 6103-6110.

Pavey, S. & Gabrielli, B. 2002, "Alpha-melanocyte stimulating hormone potentiates p16/CDKN2A expression in human skin after ultraviolet irradiation", *Cancer Res.*, vol. 62, no. 3, pp. 875-880.

Platz, A., Ringborg, U., Lagerlof, B., Lundqvist, E., Sevigny, P., & Inganas, M. 1996, "Mutational analysis of the CDKN2 gene in metastases from patients with cutaneous malignant melanoma", *Br.J.Cancer*, vol. 73, no. 3, pp. 344-348.

Platz, A., Hansson, J., Mansson-Brahme, E., Lagerlof, B., Linder, S., Lundqvist, E., Sevigny, P., Inganas, M., & Ringborg, U. 1997, "Screening of germline mutations in the CDKN2A and CDKN2B genes in Swedish families with hereditary cutaneous melanoma", *J.Natl.Cancer Inst.*, vol. 89, no. 10, pp. 697-702.

Pollock, P. M., Spurr, N., Bishop, T., Newton-Bishop, J., Gruis, N., van der Velden, P. A., Goldstein, A. M., Tucker, M. A., Foulkes, W. D., Barnhill, R., Haber, D., Fountain, J., & Hayward, N. K. 1998, "Haplotype analysis of two recurrent CDKN2A mutations in 10 melanoma families: evidence for common founders and independent mutations", *Hum.Mutat.*, vol. 11, no. 6, pp. 424-431.

Pollock, P. M., Stark, M. S., Palmer, J. M., Walters, M. K., Aitken, J. F., Martin, N. G., & Hayward, N. K. 2001, "Mutation analysis of the CDKN2A promoter in Australian melanoma families", *Genes Chromosomes.Cancer*, vol. 32, no. 1, pp. 89-94.

Pollock, P. M. & Meltzer, P. S. 2002, "A genome-based strategy uncovers frequent BRAF mutations in melanoma", *Cancer Cell*, vol. 2, no. 1, pp. 5-7.

Pollock, P. M., Harper, U. L., Hansen, K. S., Yudt, L. M., Stark, M., Robbins, C. M., Moses, T. Y., Hostetter, G., Wagner, U., Kakareka, J., Salem, G., Pohida, T., Heenan, P., Duray, P., Kallioniemi, O., Hayward, N. K., Trent, J. M., & Meltzer, P. S. 2003, "High frequency of BRAF mutations in nevi", *Nat.Genet.*, vol. 33, no. 1, pp. 19-20.

Primmer, C. R., Saino, N., Moller, A. P., & Ellegren, H. 1996, "Directional evolution in germline microsatellite mutations", *Nat.Genet.*, vol. 13, no. 4, pp. 391-393.

Ranade, K., Hussussian, C. J., Sikorski, R. S., Varmus, H. E., Goldstein, A. M., Tucker, M. A., Serrano, M., Hannon, G. J., Beach, D., & Dracopoli, N. C. 1995, "Mutations associated with familial melanoma impair p16INK4 function", *Nat.Genet.*, vol. 10, no. 1, pp. 114-116.

Randerson-Moor, J. A., Harland, M., Williams, S., Cuthbert-Heavens, D., Sheridan, E., Aveyard, J., Sibley, K., Whitaker, L., Knowles, M., Bishop, J. N., & Bishop, D. T. 2001, "A germline deletion of p14(ARF) but not CDKN2A in a melanoma-neural system tumour syndrome family", *Hum.Mol.Genet.*, vol. 10, no. 1, pp. 55-62.

Reifenberger, J., Knobbe, C. B., Sterzinger, A. A., Blaschke, B., Schulte, K. W., Ruzicka, T., & Reifenberger, G. 2004, "Frequent alterations of Ras signaling pathway genes in sporadic malignant melanomas", *Int.J.Cancer*, vol. 109, no. 3, pp. 377-384.

Reymond, A. & Brent, R. 1995, "p16 proteins from melanoma-prone families are deficient in binding to Cdk4", *Oncogene*, vol. 11, no. 6, pp. 1173-1178.

Rizos, H., Puig, S., Badenas, C., Malvehy, J., Darmanian, A. P., Jimenez, L., Mila, M., & Kefford, R. F. 2001, "A melanoma-associated germline mutation in exon 1beta inactivates p14ARF", *Oncogene*, vol. 20, no. 39, pp. 5543-5547.

Ruas, M. & Peters, G. 1998, "The p16INK4a/CDKN2A tumor suppressor and its relatives", *Biochim.Biophys.Acta*, vol. 1378, no. 2, p. F115-F177.

Ruas, M., Brookes, S., McDonald, N. Q., & Peters, G. 1999, "Functional evaluation of tumour-specific variants of p16INK4a/CDKN2A: correlation with protein structure information", *Oncogene*, vol. 18, no. 39, pp. 5423-5434.

Ruiz, A., Puig, S., Malvehy, J., Lazaro, C., Lynch, M., Gimenez-Arnau, A. M., Puig, L., Sanchez-Conejo, J., Estivill, X., & Castel, T. 1999, "CDKN2A mutations in Spanish cutaneous malignant melanoma families and patients with multiple melanomas and other neoplasia", *J.Med.Genet.*, vol. 36, no. 6, pp. 490-493.

Sasaki, Y., Niu, C., Makino, R., Kudo, C., Sun, C., Watanabe, H., Matsunaga, J., Takahashi, K., Tagami, H., Aiba, S., & Horii, A. 2004, "BRAF point mutations in primary melanoma show different prevalences by subtype", *J.Invest Dermatol.*, vol. 123, no. 1, pp. 177-183.

Schioth, H. B., Phillips, S. R., Rudzish, R., Birch-Machin, M. A., Wikberg, J. E., & Rees, J. L. 1999, "Loss of function mutations of the human melanocortin 1 receptor are common and are associated with red hair", *Biochem.Biophys.Res.Commun.*, vol. 260, no. 2, pp. 488-491.

Serrano, M., Hannon, G. J., & Beach, D. 1993, "A new regulatory motif in cell-cycle control causing specific inhibition of cyclin D/CDK4", *Nature*, vol. 366, no. 6456, pp. 704-707.

Shinozaki, M., Fujimoto, A., Morton, D. L., & Hoon, D. S. 2004, "Incidence of BRAF oncogene mutation and clinical relevance for primary cutaneous melanomas", *Clin.Cancer Res.*, vol. 10, no. 5, pp. 1753-1757.

Smith-Sorensen, B. & Hovig, E. 1996, "CDKN2A (p16INK4A) somatic and germline mutations", *Hum.Mutat.*, vol. 7, no. 4, pp. 294-303.

Smith, F. E., Henly, W. S., Knox, J. M., & Lane, M. 1966, "Familial melanoma", *Arch.Intern.Med.*, vol. 117, no. 6, pp. 820-823.

Smith, R., Healy, E., Siddiqui, S., Flanagan, N., Steijlen, P. M., Rosdahl, I., Jacques, J. P., Rogers, S., Turner, R., Jackson, I. J., Birch-Machin, M. A., & Rees, J. L. 1998, "Melanocortin 1 receptor variants in an Irish population", *J.Invest Dermatol.*, vol. 111, no. 1, pp. 119-122.

Soufir, N., Avril, M. F., Chompret, A., Demenais, F., Bombled, J., Spatz, A., Stoppa-Lyonnet, D., Benard, J., & Bressac-de Paillerets, B. 1998, "Prevalence of p16 and CDK4 germline mutations in 48 melanoma-prone families in France. The French Familial Melanoma Study Group", *Hum.Mol.Genet.*, vol. 7, no. 2, pp. 209-216.

Stone, S., Jiang, P., Dayananth, P., Tavtigian, S. V., Katcher, H., Parry, D., Peters, G., & Kamb, A. 1995, "Complex structure and regulation of the P16 (MTS1) locus", *Cancer Res.*, vol. 55, no. 14, pp. 2988-2994.

Straume, O., Smeds, J., Kumar, R., Hemminki, K., & Akslen, L. A. 2002, "Significant impact of promoter hypermethylation and the 540 C>T polymorphism of CDKN2A in cutaneous melanoma of the vertical growth phase", *Am.J.Pathol.*, vol. 161, no. 1, pp. 229-237.

Sun, S., Pollock, P. M., Liu, L., Karimi, S., Jothy, S., Milner, B. J., Renwick, A., Lassam, N. J., Hayward, N. K., Hogg, D., Narod, S. A., & Foulkes, W. D. 1997, "CDKN2A mutation in a non-FAMMM kindred with cancers at multiple sites results in a functionally abnormal protein", *Int.J.Cancer*, vol. 73, no. 4, pp. 531-536.

Tevelev, A., Byeon, I. J., Selby, T., Ericson, K., Kim, H. J., Kraynov, V., & Tsai, M. D. 1996, "Tumor suppressor p16INK4A: structural characterization of wild-type and mutant proteins by NMR and circular dichroism", *Biochemistry*, vol. 35, no. 29, pp. 9475-9487.

Thody, A. J., Higgins, E. M., Wakamatsu, K., Ito, S., Burchill, S. A., & Marks, J. M. 1991, "Pheomelanin as well as eumelanin is present in human epidermis", *J.Invest.Dermatol.*, vol. 97, no. 2, pp. 340-344.

Trent, J. M., Rosenfeld, S. B., & Meyskens, F. L. 1983, "Chromosome 6q involvement in human malignant melanoma", *Cancer Genet.Cytogenet.*, vol. 9, no. 2, pp. 177-180.

Tsao, H., Zhang, X., Kwitkiwski, K., Finkelstein, D. M., Sober, A. J., & Haluska, F. G. 2000, "Low prevalence of germline CDKN2A and CDK4 mutations in patients with early-onset melanoma", *Arch.Dermatol.*, vol. 136, no. 9, pp. 1118-1122.

Uribe, P., Wistuba, I. I., & Gonzalez, S. 2003, "BRAF mutation: a frequent event in benign, atypical, and malignant melanocytic lesions of the skin", *Am.J.Dermatopathol.*, vol. 25, no. 5, pp. 365-370.

Valverde, P., Healy, E., Jackson, I., Rees, J. L., & Thody, A. J. 1995, "Variants of the melanocyte-stimulating hormone receptor gene are associated with red hair and fair skin in humans", *Nat.Genet.*, vol. 11, no. 3, pp. 328-330.

Valverde, P., Healy, E., Sikkink, S., Haldane, F., Thody, A. J., Carothers, A., Jackson, I. J., & Rees, J. L. 1996, "The Asp84Glu variant of the melanocortin 1 receptor (MC1R) is associated with melanoma", *Hum.Mol.Genet.*, vol. 5, no. 10, pp. 1663-1666.

van der Velden, P. A., Sandkuijl, L. A., Bergman, W., Hille, E. T., Frants, R. R., & Gruis, N. A. 1999, "A locus linked to p16 modifies melanoma risk in Dutch familial atypical multiple mole melanoma (FAMMM) syndrome families", *Genome Res.*, vol. 9, no. 6, pp. 575-580.

van der Velden, P. A., Sandkuijl, L. A., Bergman, W., Pavel, S., van Mourik, L., Frants, R. R., & Gruis, N. A. 2001, "Melanocortin-1 receptor variant R151C modifies melanoma risk in Dutch families with melanoma", *Am.J.Hum.Genet.*, vol. 69, no. 4, pp. 774-779.

Vasen, H. F., Gruis, N. A., Frants, R. R., Der Velden, P. A., Hille, E. T., & Bergman, W. 2000, "Risk of developing pancreatic cancer in families with familial atypical multiple mole melanoma associated with a specific 19 deletion of p16 (p16-Leiden)", *Int.J.Cancer*, vol. 87, no. 6, pp. 809-811.

Walker, G. J., Hussussian, C. J., Flores, J. F., Glendening, J. M., Haluska, F. G., Dracopoli, N. C., Hayward, N. K., & Fountain, J. W. 1995, "Mutations of the CDKN2/p16INK4 gene in Australian melanoma kindreds", *Hum.Mol.Genet.*, vol. 4, no. 10, pp. 1845-1852.

Walker, G. J., Indsto, J. O., Sood, R., Faruque, M. U., Hu, P., Pollock, P. M., Duray, P., Holland, E. A., Brown, K., Kefford, R. F., Trent, J. M., Mann, G. J., & Hayward, N. K. 2004, "Deletion mapping suggests that the 1p22 melanoma susceptibility gene is a tumor suppressor localized to a 9-Mb interval", *Genes Chromosomes.Cancer*, vol. 41, no. 1, pp. 56-64.

Walter, S. D., King, W. D., & Marrett, L. D. 1999, "Association of cutaneous malignant melanoma with intermittent exposure to ultraviolet radiation: results of a case-control study in Ontario, Canada", *Int.J.Epidemiol.*, vol. 28, no. 3, pp. 418-427.

Wan, P. T., Garnett, M. J., Roe, S. M., Lee, S., Niculescu-Duvaz, D., Good, V. M., Jones, C. M., Marshall, C. J., Springer, C. J., Barford, D., & Marais, R. 2004, "Mechanism of activation of the RAF-ERK signaling pathway by oncogenic mutations of B-RAF", *Cell*, vol. 116, no. 6, pp. 855-867.

Weinstock, M. A. 1998, "Issues in the epidemiology of melanoma", *Hematol.Oncol.Clin.North Am.*, vol. 12, no. 4, pp. 681-698.

Whelan, A. J., Bartsch, D., & Goodfellow, P. J. 1995, "Brief report: a familial syndrome of pancreatic cancer and melanoma with a mutation in the CDKN2 tumor-suppressor gene", *N.Engl.J.Med.*, vol. 333, no. 15, pp. 975-977.

Yakobson, E. A., Zlotogorski, A., Shafir, R., Cohen, M., Icekson, M., Landau, M., Brenner, S., Usher, S., & Peretz, H. 1998, "Screening for tumour suppressor p16(CDKN2A) germline mutations in Israeli melanoma families", *Clin.Chem.Lab Med.*, vol. 36, no. 8, pp. 645-648.

Yakobson, E., Shemesh, P., Azizi, E., Winkler, E., Lassam, N., Hogg, D., Brookes, S., Peters, G., Lotem, M., Zlotogorski, A., Landau, M., Safro, M., Shafir, R., Friedman, E., & Peretz, H. 2000, "Two p16 (CDKN2A) germline mutations in 30 Israeli melanoma families", *Eur.J.Hum.Genet.*, vol. 8, no. 8, pp. 590-596.

Yakobson, E., Eisenberg, S., Isacson, R., Halle, D., Levy-Lahad, E., Catane, R., Safro, M., Sobolev, V., Huot, T., Peters, G., Ruiz, A., Malvehy, J., Puig, S., Chompret, A., Avril, M. F., Shafir, R., Peretz, H., & Bressac-de Paillerets, B. 2003, "A single Mediterranean, possibly Jewish, origin for the Val59Gly CDKN2A mutation in four melanoma-prone families", *Eur.J.Hum.Genet.*, vol. 11, no. 4, pp. 288-296.

Yang, G., Niendorf, K. B., & Tsao, H. 2004, "A novel methionine-53-valine mutation of p16 in a hereditary melanoma kindred", *J.Invest Dermatol.*, vol. 123, no. 3, pp. 574-575.

Yazdi, A. S., Palmedo, G., Flaig, M. J., Puchta, U., Reckwerth, A., Rutten, A., Mentzel, T., Hugel, H., Hantschke, M., Schmid-Wendtner, M. H., Kutzner, H., & Sander, C. A. 2003, "Mutations of the BRAF gene in benign and malignant melanocytic lesions", *J.Invest Dermatol.*, vol. 121, no. 5, pp. 1160-1162.

Zhang, B. & Peng, Z. 1996, "Defective folding of mutant p16(INK4) proteins encoded by tumor-derived alleles", *J.Biol.Chem.*, vol. 271, no. 46, pp. 28734-28737.

Zhang, B. & Peng, Z. 2000, "A minimum folding unit in the ankyrin repeat protein p16(INK4)", *J.Mol.Biol.*, vol. 299, no. 4, pp. 1121-1132.

Zhang, Y., Xiong, Y., & Yarbrough, W. G. 1998, "ARF promotes MDM2 degradation and stabilizes p53: ARF-INK4a locus deletion impairs both the Rb and p53 tumor suppression pathways", *Cell*, vol. 92, no. 6, pp. 725-734.

Zuo, L., Weger, J., Yang, Q., Goldstein, A. M., Tucker, M. A., Walker, G. J., Hayward, N., & Dracopoli, N. C. 1996, "Germline mutations in the p16INK4a binding domain of CDK4 in familial melanoma", *Nat.Genet.*, vol. 12, no. 1, pp. 97-99.

APPENDIX 1

PRIMER SEQUENCES

Primer Sequences

Gene:

Name
CDKN2A exon 1α

Primers:

Primer Name	Primer Sequence
CDKN2A 1F	ACCGGAGGAAGAAAGAGGAG
CDKN2A 1R	GCGCTACCTGATTCCAATTC

Gene:

Name
CDKN2A exon 2

Primers:

Primer Name	Primer Sequence
CDKN2A 2F	GTGAGGGGGCTCTACACAAG
CDKN2A 2R	CTGTGCTGGAAAATGAATGC

Gene:

Name
CDKN2A exon 3

Primers:

Primer Name	Primer Sequence
CDKN2A 3F	TGCCACACATCTTTGACCTC
CDKN2A 3R	TTTACGGTAGTGGGGGAAGG

Gene:

Name
MC1R

Primers:

Primer Name	Primer Sequence
MC1R F	AGATGGAAGGAGGCAGGCAT
MC1R R	CCGCGCTTCAACACTTTCAGAGATCA

Gene:

Name
MC1R sequencing

Primers:

Primer Name	Primer Sequence
MC1R NT F	CTGCAGGTGATCACGTCAAT
MC1R TM F	AACCTGCACTCACCCATGTA
MC1R CT F	TCGTCTTCAGCACGCTCTTC

Gene:

Name
AMXY

Primers:

Primer Name	Primer Sequence
AMXY F	*CCCTGGGCTCTGTAAAGAATAGTG
AMXY R	ATCAGAGCTTAAACTGGGAAGCTG

Gene:

Name
BRAF exon 15

Primers:

Primer Name	Primer Sequence
BRAF 15F	TCATAATGCTTGCTCTGATAGGA
BRAF 15R	GGCCAAAAATTTAATCAGTGG

Gene:

Name
BRAF V599E

Primers:

Primer Name	Primer Sequence
V599E F	AATAGGTGATTTTGGTCTAGCTACAGT
V599E R	AATAGGTGATTTTGGTCTAGCTACAGA

Marker:

Name
D9S1749

Primers:

Primer Name	Primer Sequence
PkY6.F	*AGGAGAGGGTACGCTTGCAA
PkY6.R	TACAGGGTGCGGGTGCAGATAA

Marker:

Name
D9S2136

Primers:

Primer Name	Primer Sequence
1069.1F	*ATTCAACGAGTGGGATGAAG
1069.1R	TCCAGGTTGCTGCAAATGCC

Marker:

Name
D9S2060

Primers:

Primer Name	Primer Sequence
SHGC-9726.PrG-19451	*CATCAGTCACCGAAGGTCCT
SHGC-9726.PrG-19452	AATGGACATTTACGGTAGTGGG

Marker:

Name
RH103023

Primers:

Primer Name	Primer Sequence
stSG54862.A	*TTTATTCATTTGCTTGTGGCC
stSG54862.B	GGTCCCGATTTAGAAGGAGC

Marker:

Name
D9S974

Primers:

Primer Name	Primer Sequence
B4CA	*GAGCCTGGTCTGGATCATAA
B4TG	AAGCTTACAGAACCAGACAG

Marker:

Name
D9S942

Primers:

Primer Name	Primer Sequence
CT.29.F	*GCAAGATTCCAAACAGTA
CT.29.R	CTCATCCTGCGGAAACCATT

Marker:

Name
D9S1748

Primers:

Primer Name	Primer Sequence
PkY3.F	*CACCTCAGAAGTCAGTGAGT
PkY3.R	GTGCTTGAAATACACCTTTCC

Marker:

Name
D9S1604

Primers:

Primer Name	Primer Sequence
CT18-F	*CCTGGGTCTCCAATTTGTCA
CT18-R	AGCACATGACACTGTGTGTG

* denotes fluorescently labelled primer

APPENDIX 2

THERMOCYCLING CONDITIONS

Thermocycling Conditions

CDKN2A	Exon 1α		
	94°C	5 minutes	1 cycle
	94°C 61°C 72°C	1 minute 1 minute 1 minute	30 cycles
	72°C	10 minutes	1 cycle
	4°C	soak	

CDKN2A	Exon 2		
	94°C	5 minutes	1 cycle
	94°C 58°C 72°C	1 minute 1 minute 1 minute	30 cycles
	72°C	10 minutes	1 cycle
	4°C	soak	

CDKN2A	Exon 3		
	94°C	5 minutes	1 cycle
	94°C 60°C 72°C	1 minute 1 minute 1 minute	30 cycles
	72°C	10 minutes	1 cycle
	4°C	soak	

MC1R	Entire Coding Region		
	94°C	5 minutes	1 cycle
	94°C 65°C 72°C	1 minute 1 minute 90 seconds	35 cycles
	72°C	10 minutes	1 cycle
	4°C	soak	

AMXY	Entire Coding Region		
	95°C	15 minutes	1 cycle
	94°C 56.5°C 72°C	30 seconds 1 minute 90 seconds	30 cycles
	72°C	10 minutes	1 cycle
	8°C	soak	

BRAF	Exon 15		
	94°C	5 minutes	1 cycle
	94°C 60°C 72°C	1 minute 1 minute 1 minute	30 cycles
	72°C	10 minutes	1 cycle
	4°C	soak	

BRAF	V599E Allele Specific		
	94°C	5 minutes	1 cycle
	94°C 57°C 72°C	1 minute 1 minute 1 minute	30 cycles
	72°C	10 minutes	1 cycle
	4°C	soak	

CDKN2A	D9S1749		
	94°C	3 minutes	1 cycle
	94°C 55°C 72°C	45 seconds 45 seconds 45 seconds	30 cycles
	4°C	soak	

CDKN2A	D9S2136		
	94°C	5 minutes	1 cycle
	94°C 60°C 72°C	30 seconds 1 minute 30 seconds	35 cycles
	72°C	5 minutes	1 cycle
	4°C	soak	

CDKN2A	D9S2060		
	94°C	3 minutes	1 cycle
	94°C 62°C 72°C	1 minute 1 minute 1 minute	30 cycles
	4°C	soak	

CDKN2A	RH103023		
	94°C	5 minutes	1 cycle
	94°C 61°C 72°C	1 minute 1 minute 1 minute	35 cycles
	72°C	10 minutes	1 cycle
	4°C	soak	

CDKN2A	D9S974		
	94°C	5 minutes	1 cycle
	94°C 55°C 72°C	1 minute 1 minute 1 minute	30 cycles
	4°C	soak	

CDKN2A	D9S942		
	94°C	5 minutes	1 cycle
	94°C 53°C 72°C	1 minute 1 minute 1 minute	35 cycles
	4°C	soak	

CDKN2A	D9S1748		
	94°C	5 minutes	1 cycle
	94°C 53°C 72°C	1 minute 1 minute 1 minute	35 cycles
	4°C	soak	

CDKN2A	D9S1604		
	94°C	5 minutes	1 cycle
	94°C 55°C 72°C	1 minute 1 minute 1 minute	30 cycles
	4°C	soak	

PCR clean up	SHRIMP		
	37°C	30 minutes	1 cycle
	72°C	15 minutes	1 cycle
	8°C	5 minutes	1 cycle

Sequencing	BigDye 3		
	96°C 50°C 60°C	10 seconds 5 seconds 4 minutes	25 cycles
	4°C	soak	

TOPO Cloning	Control PCR Product		
	94°C	2 minutes	1 cycle
	94°C 55°C 72°C	1 minute 1 minute 1 minute	25 cycles
	72°C	7 minutes	1 cycle

Denature			
	95°C	10 minutes	1 cycle

Renature			
	95°C	5 minutes	1 cycle
	95°C – 0.7°C	25 seconds	90 cycles
	25°C	soak	

APPENDIX 3

RESTRICTION ENZYME DIGESTS

Restriction Enzyme Digests

Primers:

Primer Name	Primer Sequence
CDKN2A 2F	GTGAGGGGGCTCTACACAAG
CDKN2A 2R	CTGTGCTGGAAAATGAATGC

CDKN2A	**Exon 2**
	M53I
	***Bcl* I 50°C**
	15µl PCR Product 1.5µl *Bcl* I 3µl Buffer C 1.5µl Spermidine 9µl dH_2O
	30µl total volume per digest

Expected fragment sizes: 84bp, 374bp

Primers:

Primer Name	Primer Sequence
BRAF 15F	TCATAATGCTTGCTCTGATAGGA
BRAF 15R	GGCCAAAAATTTAATCAGTGG

BRAF	**Exon 15**
	V599E
	***Tsp* RI 65°C**
	30µl PCR Product 3.5µl *Tsp* RI 5µl Buffer 4 5µl BSA 6.5µl dH_2O
	50µl total volume per digest

Expected fragment sizes: 22bp, 202bp

APPENDIX 4

HAPLOTYPE ANALYSIS OF M53I MUTATION NON-CARRIERS

TABLE 1

Haplotype Analysis of M53I Mutation Non-Carriers from a Family from Toronto and a Family from Australia for 9p Markers

	Haplotype for each Marker Family Identifier			
	Canadian FAM-1		Australian 41031	
Markers	**T4**	**T5**	**B4**	**B5**
D9S1749	13/14	11/12	15/16	14/15
D9S2136	6/8	7/8	4/9	4/9
D9S2060	2/2	2/2	2/3	2/2
RH103023	1/1	1/1	1/1	1/1
M53I	G/G	G/G	G/G	G/G
D9S974	6/9	8/8	6/7	6/7
D9S942	4/5	3/4	4/5	4/5
D9S1748	9/10	4/7	7/9	6/9
D9S1604	1/2	1/2	1/1	1/2

The position of the mutation relative to the markers is shown.

T4 and **T5** denotes individuals not carrying the M53I mutation from a M53I mutation carrying Toronto family and **B4** and **B5** denotes individuals not carrying the M53I mutation from a M53I mutation carrying Brisbane family

Blue denotes most common allele for that marker as seen in the M53I carriers

Grey shaded area denotes individuals who do not carry the most common allele for that marker

Data collected from marker RH103023 was not informative as the families all shared the common population alleles.

Family members T4 and B4 both have melanoma but do not carry the M53I mutation that is carried in their family. They are therefore family members with sporadic melanoma in a familial setting. Family members T5 and B5 do not carry the M53I mutation and do not have melanoma.

APPENDIX 5

STATISTICAL ANALYSIS

Statistical analysis was performed using the methods described in method 3.17. For each Test of Independence a Two-tailed Fisher's Exact Test was performed, unless stated otherwise. Odds ratios (OR) are indicated with 95% confidence interval (CI) in all statistical analysis. Alleles were counted twice in homozygous individuals which increases the N value of subjects for certain variants.

TABLE 1

***MC1R* Variants Found in Familial and Sporadic Melanoma Patients Compared with Control Subjects**

	No. of Cases	No. of Controls
Any variant	83	53
No variant	5	14
P-value = 0.006 ** significant (not significant after Bonferroni correction)		
OR = 4.385, CI 1.492-12.883		
L44I present	1	0
L44I absent	87	67
P-value = 1.000 (not significant)		
V60L present	30	20
V60L absent	63	48
P-value = 0.733 (not significant)		
OR = 1.143, CI 0.580-2.254		
D84E present	8	1
D84E absent	80	66
P-value = 0.079 (not significant)		
OR = 6.600, CI 0.805-54.128		
V92M present	13	11
V92M absent	75	57
P-value = 0.826 (not significant)		
OR = 0.898, CI 0.375-2.152		
T95M present	1	0
T95M absent	87	67
P-value = 1.000 (not significant)		
R142H present	3	2
R142H absent	85	65
P-value = 1.000 (not significant)		
OR = 1.147, CI 0.186-7.066		
R151C present	28	12
R151C absent	61	55
P-value = 0.065 (not significant)		
OR = 2.104, CI 0.976-4.536		
I155T present	4	4
I155T absent	84	63
P-value = 0.727 (not significant)		
OR = 0.750, CI 0.181-3.115		

	No. of Cases	No. of Controls
R160W present	24	14
R160W absent	67	53
P-value = 0.457 (not significant)		
OR = 1.356, CI 0.640-2.874		
R163Q present	12	7
R163Q absent	80	60
P-value = 0.805 (not significant)		
OR = 1.286, CI 0.478-3.462		
A171G present	1	0
A171G absent	87	67
P-value = 1.000 (not significant)		
D294H present	10	3
D294H absent	78	64
P-value = 0.152 (not significant)		
OR = 2.735, CI 0.722-10.361		

Bonferroni adjusted significance level for multiple tests = 0.004

TABLE 2

Correlation between *MC1R* Variants in Familial Melanoma Patients and Control Subjects

	No. of Cases	No. of Controls
Any variant	58	53
No variant	2	14
P-value = 0.003 ** significant		
OR = 7.660, CI 1.663-35.296		
V60L present	24	20
V60L absent	41	48
P-value = 0.461 (not significant)		
OR = 1.405, CI 0.680-2.901		
D84E present	5	1
D84E absent	55	66
P-value = 0.100 (not significant)		
OR = 6.000, CI 0.680-52.902		
V92M present	12	11
V92M absent	48	57
P-value = 0.648 (not significant)		
OR = 1.295, CI 0.525-3.198		
T95M present	1	0
T95M absent	59	67
P-value = 0.472 (not significant)		
R142H present	2	2
R142H absent	58	65
P-value = 1.000 (not significant)		
OR = 1.121, CI 0.153-8.213		

	No. of Cases	No. of Controls
R151C present	21	12
R151C absent	40	55
P-value = 0.043 * significant (not significant after Bonferroni correction)		
OR = 2.406, CI 1.062-5.452		
I155T present	3	4
I155T absent	57	63
P-value = 1.000 (not significant)		
OR = 0.829, CI 0.178-3.864		
R160W present	13	14
R160W absent	49	53
P-value = 1.000 (not significant)		
OR = 1.004, CI 0.430-2.348		
R163Q present	10	7
R163Q absent	53	60
P-value = 0.439 (not significant)		
OR = 1.617, CI 0.575-4.549		
A171G present	1	0
A171G absent	59	67
P-value = 0.472 (not significant)		
D294H present	6	3
D294H absent	54	64
P-value = 0.305 (not significant)		
OR = 2.370, CI 0.566-9.930		

Bonferroni adjusted significance level for multiple tests = 0.004

TABLE 3
Correlation between *MC1R* Variants in Familial Melanoma Patients with and without a *CDKN2A* Mutation

	CDKN2A Mutation Positive	*CDKN2A* Mutation Negative
Any variant	23	35
No variant	0	2
P-value = 0.519 (not significant)		
V60L present	14	10
V60L absent	11	30
P-value = 0.017 * significant (not significant after Bonferroni correction)		
OR = 3.818, CI 1.315-11.084		
D84E present	1	4
D84E absent	22	33
P-value = 0.640 (not significant)		
OR = 0.375, CI 0.039-3.582		
V92M present	1	11
V92M absent	22	26
P-value = 0.021 * significant (not significant after Bonferroni correction)		
OR = 0.107, CI 0.013-0.899		
[OR = 9.308, CI 1.112-77.888]		
T95M present	1	0
T95M absent	22	37
P-value = 0.383 (not significant)		
R142H present	0	2
R142H absent	23	35
P-value = 0.519 (not significant)		
R151C present	12	9
R151C absent	11	29
P-value = 0.029 * significant (not significant after Bonferroni correction)		
OR = 3.515, CI 1.160-10.650		
I155T present	0	3
I155T absent	23	34
P-value = 0.279 (not significant)		
R160W present	5	8
R160W absent	18	31
P-value = 1.000 (not significant)		
OR = 1.076, CI 0.306-3.792		
R163Q present	1	9
R163Q absent	22	31
P-value = 0.078 (not significant)		
OR = 0.157, CI 0.018-1.327		
A171G present	1	0
A171G absent	22	37
P-value = 0.383 (not significant)		
D294H present	4	2
D294H absent	19	35
P-value = 0.191 (not significant)		
OR = 3.684, CI 0.617-21.999		
Bonferroni adjusted significance level for multiple tests = 0.004		

TABLE 4

Correlation between *MC1R* Variants in Sporadic Melanoma Patients and Control Subjects

	No. of Cases	No. of Controls
Any variant	25	53
No variant	3	14
P-value = 0.379 (not significant)		
OR = 0.454, CI 0.120-1.726		
L44I present	1	0
L44I absent	27	67
P-value = 0.295 (not significant)		
V60L present	6	20
V60L absent	22	48
P-value = 0.463 (not significant)		
OR = 0.655, CI 0.231-1.857		
D84E present	3	1
D84E absent	25	66
P-value = 0.075 (not significant)		
OR = 7.920, CI 0.787-79.753		
V92M present	1	11
V92M absent	27	57
P-value = 0.105 (not significant)		
OR = 0.192, CI 0.024-1.564		
R142H present	1	2
R142H absent	27	65
P-value = 1.000 (not significant)		
OR = 1.204, CI 0.105-13.839		
R151C present	7	12
R151C absent	21	55
P-value = 0.574 (not significant)		
OR = 1.528, CI 0.530-4.406		
I155T present	1	4
I155T absent	27	63
P-value = 1.000 (not significant)		
OR = 0.583, CI 0.062-5.464		
R160W present	11	14
R160W absent	18	53
P-value = 0.127 (not significant)		
OR = 2.313, CI 0.891-6.004		
R163Q present	2	7
R163Q absent	27	60
P-value = 0.719 (not significant)		
OR = 0.635, CI 0.124-3.259		
D294H present	4	3
D294H absent	24	64
P-value = 0.190 (not significant)		
OR = 3.556, CI 0.741-17.069		

The following three tables are 2 x 4 tables on which Chi-Squared Tests were performed.

TABLE 5

Correlation between Hair Colour and *MC1R* Variants in Control Subjects

	Red	Blonde	Light Brown	Dark Brown/Black
Any variant	5	21	13	14
No variant	1	3	2	8
P-value = 0.188 (not significant)				
V60L present	2	7	6	5
V60L absent	4	17	10	17
P-value = 0.796 (not significant)				
V92M present	0	4	4	3
V92M absent	6	20	11	20
P-value = 0.553 (not significant)				
R151C present	2	5	2	3
R151C absent	4	19	13	19
P-value = 0.660 (not significant)				
R160W present	3	6	3	2
R160W absent	3	18	12	20
P-value = 0.159 (not significant)				

TABLE 6

Correlation between Eye Colour and *MC1R* Variants in Control Subjects

	Blue	Green	Brown	Other
Any variant	33	3	12	5
No variant	5	3	5	1
P-value = 0.154 (not significant)				
V60L present	12	3	3	2
V60L absent	26	4	14	4
P-value = 0.599 (not significant)				
R151C present	6	1	4	1
R151C absent	32	5	13	5
P-value = 0.920 (not significant)				
I155T present	2	0	2	0
I155T absent	36	6	15	6
P-value = 0.391 (not significant)				
R160W present	10	1	1	2
R160W absent	28	5	16	4
P-value = 0.306 (not significant)				

TABLE 7

Correlation between Skin Type and *MC1R* variants in Control Subjects

	Never Tans Always Burns	Burns Easily Tans Rarely	Burns Rarely Tans Easily	Never Burns Always Tans
V60L present	5	6	9	0
V60L absent	7	23	16	2
P-value = 0.303 (not significant)				
V92M present	1	7	3	0
V92M absent	10	23	22	2
P-value = 0.405 (not significant)				
R151C present	3	6	3	0
R151C absent	8	23	22	2
P-value = 0.508 (not significant)				
I155T present	0	2	2	0
I155T absent	11	27	23	2
P-value = 0.877 (not significant)				
R160W present	6	6	2	0
R160W absent	5	23	23	2
P-value = 0.007 ** significant				
R163Q present	2	1	4	0
R163Q absent	9	28	21	2
P-value = 0.228 (not significant)				
D294H present	0	1	2	0
D294H absent	11	28	23	2
P-value = 0.467 (not significant)				

Bonferroni adjusted significance level for multiple tests = 0.0071

TABLE 8

Correlation between Hair Colour and Melanoma Patients (Familial and Sporadic) and Control Subjects

	No. of Cases	No. of Controls
Red Hair	16	7
Other Hair Colour	72	61
P-value = 0.182 (not significant) OR = 1.937, CI 0.748-5.014		
Blonde Hair	24	24
Other Hair Colour	64	44
P-value = 0.299 (not significant) OR = 0.688, CI 0.347-1.362		
Light Brown Hair	32	15
Other Hair Colour	56	53
P-value = 0.056 (not significant) OR = 2.019, CI 0.984-4.145		
Dark Brown/Black Hair	16	22
Other Hair Colour	72	46
P-value = 0.059 (not significant) OR = 0.465, CI 0.221-0.976 [OR = 2.152, CI 1.024-4.523]		

TABLE 9

Eye Colour in Melanoma Patients (Familial and Sporadic) Compared with Control Subjects

	No. of Cases	No. of Controls
Blue Eyes	63	38
Other Eye Colour	25	30
P-value = 0.045 * significant OR = 1.989, CI 1.022-3.873		
Green Eyes	7	6
Other Eye Colour	81	62
P-value = 1.000 (not significant) OR = 0.893, CI 0.286-2.791		
Brown Eyes	10	18
Other Eye Colour	78	50
P-value = 0.020 * significant OR = 0.356, CI 0.152-0.832 [OR = 2.808, CI 1.199-6.574]		
Eye Colour other than Blue, Green or Brown	8	6
Blue, Green or Brown Eyes	80	62
P-value = 1.000 (not significant) OR = 1.033, CI 0.341-3.133		

TABLE 10

Correlation between Skin Type and Melanoma Patients (Familial and Sporadic) and Control Subjects

	No. of Cases	No. of Controls
Never Tans, Always Burns	21	12
Other Skin Type	67	56
P-value = 0.430 (not significant) OR = 1.463, CI 0.662-3.233		
Burns Easily, Tans Rarely	60	29
Other Skin Type	28	39
P-value = 0.002 ** significant OR = 2.882, CI 1.493-5.561		
Burns Rarely, Tans Easily	7	25
Other Skin Type	81	43
P-value < 0.001 *** significant OR = 0.149, CI 0.059-0.372 [OR = 6.728, CI 2.692-16.816]		
Never Burns, Always Tans	0	2
Other Skin Type	88	66
P-value = 0.188 (not significant)		

Bonferroni adjusted significance level for multiple tests = 0.013

TABLE 11

Correlation between Lighter and Darker Hair Colour and Melanoma Patients (Familial and Sporadic) and Control Subjects

	No. of Cases	No. of Controls
Red or Blonde Hair	40	31
Brown or Black Hair	48	37
P-value = 1.000 (not significant) OR = 0.995, CI 0.527-1.875		

TABLE 12

Correlation between Lighter and Darker Eye Colour and Melanoma Patients (Familial and Sporadic) and Control Subjects

	No. of Cases	No. of Controls
Blue or Green Eyes	70	44
Brown or Other Eye Colour	18	24
P-value = 0.046 * significant OR = 2.212, CI 1.034-4.350		

TABLE 13

Correlation between Hair Colour and Familial Melanoma Patients with and without a *CDKN2A* Mutation

	***CDKN2A* Mutation Positive**	***CDKN2A* Mutation Negative**
Red Hair	2	8
Other Hair Colour	18	34
P-value = 0.478 (not significant) OR = 0.472, CI 0.091-2.463		
Blonde Hair	7	8
Other Hair Colour	13	34
P-value = 0.211 (not significant) OR = 2.288, CI 0.690-7.590		
Light Brown Hair	7	14
Other Hair Colour	13	28
P-value = 1.000 (not significant) OR = 1.077, CI 0.351-3.303		
Dark Brown/Black Hair	4	12
Other Hair Colour	16	30
P-value = 0.549 (not significant) OR = 0.625, CI 0.173-2.257		

TABLE 14

Correlation between Eye Colour and Familial Melanoma Patients with and without a *CDKN2A* Mutation

	***CDKN2A* Mutation Positive**	***CDKN2A* Mutation Negative**
Blue Eyes	15	28
Other Eye Colour	5	14
P-value = 0.569 (not significant) OR = 1.500, CI 0.453-4.972		
Green Eyes	1	3
Other Eye Colour	19	39
P-value = 1.000 (not significant) OR = 0.684, CI 0.067-7.023		
Brown Eyes	1	6
Other Eye Colour	19	36
P-value = 0.412 (not significant) OR = 0.316, CI 0.035-2.818		
Eye Colour other than Blue, Green or Brown	3	5
Blue, Green or Brown Eyes	17	37
P-value = 1.000 (not significant) OR = 1.306, CI 0.279-6.105		

TABLE 15

Correlation between Skin Type and Familial Melanoma Patients with and without a *CDKN2A* Mutation

	CDKN2A Mutation Positive	*CDKN2A* Mutation Negative
Never Tans, Always Burns	5	10
Other Skin Type	15	32
P-value = 1.000 (not significant) OR = 1.067, CI 0.310-3.673		
Burns Easily, Tans Rarely	15	29
Other Skin Type	5	13
P-value = 0.768 (not significant) OR = 1.345, CI 0.403		
Burns Rarely, Tans Easily	0	3
Other Skin Type	20	39
P-value = 0.545 (not significant)		

TABLE 16

Correlation between Lighter and Darker Hair Colour and Familial Melanoma Patients with and without a *CDKN2A* Mutation

	CDKN2A Mutation Positive	*CDKN2A* Mutation Negative
Red or Blonde Hair	9	16
Brown or Black Hair	11	26
P-value = 0.782 (not significant) OR = 1.330, CI 0.452-3.911		

TABLE 17

Correlation between Lighter and Darker Eye Colour and Familial Melanoma Patients with and without a *CDKN2A* Mutation

	CDKN2A Mutation Positive	*CDKN2A* Mutation Negative
Blue or Green Eyes	16	31
Brown or Other Eye Colour	4	11
P-value = 0.755 (not significant) OR = 1.419, CI 0.389-5.175		

TABLE 18

Correlation between Skin Type 1 or 2 and Skin Type 3 or 4 and Familial Melanoma Patients with and without a *CDKN2A* Mutation

	CDKN2A Mutation Positive	*CDKN2A* Mutation Negative
Skin Type 1or 2	20	39
Skin Type 3 or 4	0	3
P-value = 0.545 (not significant)		

TABLE 19

Correlation between the Number of *MC1R* Variants in Unaffected Familial Melanoma and Control Subjects

	≤ One *MC1R* Variant	≥ Two *MC1R* Variants
No. of Unaffected Familial Melanoma Individuals	15	10
No. of Controls	46	21
P-value = 0.465 (not significant) OR = 1.460, CI 0.564-3.784		

TABLE 20

Correlation between the Number of *MC1R* Variants in Familial Melanoma Patients with and without a *CDKN2A* Mutation

	≤ One *MC1R* Variant	≥ Two *MC1R* Variants
No. of *CDKN2A* Mutation Positive Cases	6	15
No. of *CDKN2A* Mutation Negative Cases	16	22
P-value = 0.402 (not significant) OR = 1.818, CI 0.579-5.714		

TABLE 21

Correlation between Postulated Red Hair *MC1R* Variants in Melanoma Patients (Familial and Sporadic) and Control Subjects

	Postulated Red Hair Variant Present	Postulated Red Hair Variant Absent
No. of Cases	10	4
No. of Controls	5	5
P-value = 0.403 (not significant) OR = 2.500, CI 0.458-13.649		

TABLE 22

Correlation between Postulated Red Hair *MC1R* Variants in All Red Haired Individuals (Affected and Unaffected Familial and Sporadic Melanoma and Control Subjects) and All Individuals without Red Hair (Affected and Unaffected Familial and Sporadic Melanoma and Control Subjects)

	Postulated Red Hair Variant Present	Postulated Red Hair Variant Absent
Red Hair	15	9
Other Hair Colour	73	77
P-value = 0.272 (not significant) OR = 1.758, CI 0.725-4.265		

TABLE 23

Correlation between Postulated Red Hair *MC1R* Variants in Control Subjects with Red Hair and Control Subjects without Red Hair

	Postulated Red Hair Variant Present	Postulated Red Hair Variant Absent
Red Hair	4	2
Other Hair Colour	25	35
P-value = 0.392 (not significant) OR = 2.800, CI 0.475-16.493		

TABLE 24

Correlation between *MC1R* R142H, R151C, R160W or D294H Variant in Familial Melanoma Patients and Control Subjects

	No. of Cases	No. of Controls
R142H, R151C, R160W or D294H Present	33	29
R142H, R151C, R160W and D294H Absent	27	38
P-value = 0.216 (not significant) OR = 1.602, CI 0.794-3.230		

TABLE 25

Correlation between *MC1R* R142H, R151C, R160W or D294H Variant in Familial Melanoma Patients without a *CDKN2A* Mutation and Control Subjects

	No. of Cases	No. of Controls
R142H, R151C, R160W or D294H Present	19	29
R142H, R151C, R160W and D294H Absent	20	38
P-value = 0.687 (not significant) OR = 1.245, CI 0.564-2.749		

TABLE 26

Correlation between *MC1R* R142H, R151C, R160W or D294H Variant in Familial and Sporadic Melanoma Patients

	No. of Familial Cases	No. of Sporadic Cases
R142H, R151C, R160W or D294H Present	33	18
R142H, R151C, R160W and D294H Absent	27	10
P-value = 0.490 (not significant) OR = 0.679, CI 0.269-1.713		

TABLE 27

Correlation between *MC1R* R142H, R151C, R160W or D294H Variant in Affected and Unaffected Familial Melanoma

	No. of Familial Melanoma Individuals	No. of Unaffected Familial Melanoma Individuals
R142H, R151C, R160W or D294H Present	33	12
R142H, R151C, R160W and D294H Absent	27	13
P-value = 0.636 (not significant)		
OR = 1.324, CI 0.520-3.373		

TABLE 28

Correlation between *MC1R* R142H, R151C, R160W or D294H Variant in Familial Melanoma Patients with and without a *CDKN2A* Mutation

	CDKN2A Mutation Positive	*CDKN2A* Mutation Negative
R142H, R151C, R160W or D294H Present	14	19
R142H, R151C, R160W and D294H Absent	7	20
P-value = 0.277 (not significant)		
OR = 2.105, CI 0.698-6.346		

TABLE 29

Correlation between *MC1R* R142H, R151C, R160W or D294H Variant in Unaffected Familial Melanoma and Control Subjects

	No. of Unaffected Familial Melanoma Individuals	No. of Controls
R142H, R151C, R160W or D294H Present	12	29
R142H, R151C, R160W and D294H Absent	13	38
P-value = 0.814 (not significant)		
OR = 1.210, CI 0.481-3.040		

TABLE 30

Correlation between *MC1R* R142H, R151C, R160W or D294H Variant in Affected and Unaffected Familial Melanoma and Control Subjects

	No. of Affected & Unaffected Familial Melanoma Individuals	No. of Controls
R142H, R151C, R160W or D294H Present	45	29
R142H, R151C, R160W and D294H Absent	40	38
P-value = 0.256 (not significant)		
OR = 1.474, CI 0.774-2.807		

TABLE 31

Correlation between *MC1R* Variants and Skin Type 1

	Skin Type 1	Other Skin Type
L44I present	1	0
L44I absent	33	140
P-value = 0.195 (not significant)		
V60L present	11	42
V60L absent	23	98
P-value = 0.836 (not significant)		
OR = 1.116, CI 0.499-2.494		
D84E present	3	5
D84E absent	31	135
P-value = 0.358 (not significant)		
OR = 2.613, CI 0.593-11.520		
V92M present	2	25
V92M absent	32	115
P-value = 0.113 (not significant)		
OR = 0.288, CI 0.065-1.279		
T95M present	1	0
T95M absent	33	140
P-value = 0.195 (not significant)		
R142H present	3	2
R142H absent	31	138
P-value = 0.052 (not significant)		
OR = 6.677, CI 1.070-41.674		
R151C present	9	31
R151C absent	25	109
P-value = 0.650 (not significant)		
OR = 1.266, CI 0.536-2.991		
R160W present	18	24
R160W absent	16	116
P-value < 0.001 *** significant		
OR = 5.438, CI 2.433-12.152		
R163Q present	2	15
R163Q absent	32	125
P-value = 0.531 (not significant)		
OR = 0.521, CI 0.113-2.395		
A171G present	1	1
A171G absent	33	139
P-value = 0.354 (not significant)		
OR = 4.212, CI 0.257-69.109		

Bonferroni adjusted significance level for multiple tests = 0.005

TABLE 32

Correlation between *MC1R* Variants and Blue Eyes

	Blue Eyes	Other than Blue Eyes
L44I present	1	0
L44I absent	111	62
P-value = 1.000 (not significant)		
V60L present	36	17
V60L absent	76	45
P-value = 0.607 (not significant)		
OR = 1.254, CI 0.632-2.486		
D84E present	5	3
D84E absent	107	59
P-value = 1.000 (not significant)		
OR = 0.919, CI 0.212-3.982		
V92M present	18	9
V92M absent	94	53
P-value = 0.831 (not significant)		
OR = 1.128, CI 0.473-2.687		
T95M present	1	0
T95M absent	111	62
P-value = 1.000 (not significant)		
M128K present	1	0
M128K absent	111	62
P-value = 1.000 (not significant)		
R142H present	4	1
R142H absent	108	61
P-value = 0.656 (not significant)		
OR = 2.259, CI 0.247-20.671		
R151C present	26	14
R151C absent	86	48
P-value = 1.000 (not significant)		
OR = 1.037, CI 0.495-2.171		
I155T present	4	2
I155T absent	108	60
P-value = 1.000 (not significant)		
OR = 1.111, CI 0.198-6.245		
R160W present	31	11
R160W absent	81	51
P-value = 0.195 (not significant)		
OR = 1.774, CI 0.820-3.840		
R163Q present	12	5
R163Q absent	100	57
P-value = 0.610 (not significant)		
OR = 1.368, CI 0.459-4.080		
A171G present	2	0
A171G absent	110	62
P-value = 0.539 (not significant)		
D294H present	7	5
D294H absent	105	57
P-value = 0.757 (not significant)		
OR = 0.760, CI 0.231-2.503		

TABLE 33

Correlation between *MC1R* Variants and Red Hair in all DNA Samples Available

	Red Hair	Other than Red Hair
L44I present	1	0
L44I absent	23	150
P-value = 0.138 (not significant)		
V60L present	8	45
V60L absent	16	105
P-value = 0.812 (not significant)		
OR = 1.167, CI 0.466-2.921		
D84E present	2	6
D84E absent	22	144
P-value = 0.601 (not significant)		
OR = 2.182, CI 0.414-11.498		
V92M present	2	25
V92M absent	22	125
P-value = 0.378 (not significant)		
OR = 0.455, CI 0.100-2.057		
R142H present	3	2
R142H absent	21	148
P-value = 0.019 * significant (not significant after Bonferroni correction)		
OR = 10.571, CI 1.668-67.009		
R151C present	7	33
R151C absent	17	117
P-value = 0.440 (not significant)		
OR = 1.460, CI 0.558-3.818		
R160W present	11	31
R160W absent	13	119
P-value = 0.018 * significant (not significant after Bonferroni correction)		
OR = 3.248, CI 1.327-7.949		
R163Q present	1	16
R163Q absent	23	134
P-value = 0.474 (not significant)		
OR = 0.364, CI 0.046-2.880		

Bonferroni adjusted significance level for multiple tests = 0.006