AN ANALYSIS OF THE ROLE OF POLYMORPHISMS WITHIN THE TNF LOCUS AND THE DEVELOPMENT AND PROGRESSION OF COLORECTAL ADENOCARCINOMA

by

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B.Sc.

A THESIS SUBMITTED FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

to

THE UNIVERSITY OF GLASGOW

from RESEARCH CONDUCTED IN THE UNIVERSITY DEPARTMENT OF SURGERY, GLASGOW ROYAL INFIRMARY

MARCH 1995

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ABSTRACT

Colorectal cancer represents the second most common form of malignancy in the Western World and with over 20,000 deaths in the UK each year this disease represents a major health problem. The genetic component of this malignant disease has received much attention over the past few decades and it is now becoming clear that development of colorectal cancer is a muti-stage process and that a number of genes are involved at various stages of the process.

Central to any malignant disease is the question of why the immune system allows a tumour to grow given that under the correct conditions the host immune system is capable of mounting an immune response. Central to the modulation of the immune response is a family of cytokines termed the tumour necrosis factors (TNFα, Lymphotoxin-α (LTα) and LTβ). Low levels of TNFα have been shown in colorectal tumours and it has been suggested that the constant exposure of tumour cells to low levels of TNFα may result in the cells becoming desensitised to the cytotoxic activity TNFα. Why such low levels of TNFα production should occur are however unclear.

Recently a number of polymorphisms have been identified within the region of the TNF genes and a number of studies have demonstrated a link between specific alleles of these polymorphisms and a number of autoimmune and non-autoimmune diseases and a possible genetic predisposition to either over- or under-production of TNFα.

The genotypes at six of the TNF polymorphism (TNFa, TNFc, TNFd, TNFe, -308 TNFα, LTα *Nco*I) loci of 100 individuals with clinically defined colorectal cancer were compared to those of 115 normal non-malignant control individuals. The data presented in this thesis clearly identifies that alleles of the TNF polymorphisms are

markers of not only the presence of colorectal cancer but also the aggressiveness of the disease. In particular alleles of the TNFa, TNFd and TNFe microsatellites seem to be associated with the presence (TNFa3 and a11; TNFd3; TNFe3) or absence (TNFa1, a5, a9 and a13; TNFe2) of colorectal cancer whilst an extended genotype involving the LTα *Nco*I RFLP and the -308 TNFα RFLP may also be involved. Similar data is observed in a small study of 23 gastric adenocarcinoma patients.

An examination of the relationship between these polymorphisms and Dukes' staging also suggests that these markers may be involved in the aggressiveness of this malignant disease with alleles of both the RFLPs and the microsatellites showing associations with different Dukes' stages, whilst an association is also shown with the presence or absence of secondary disease.

It is still unclear whether these polymorphisms are functional or whether they represent markers for a near-by gene which is involved in the process of colorectal tumourigenesis. The possible reasons for these associations are discussed and future strategies for determining the role of these polymorphic markers in the carcinogenesis of colorectal cancer are suggested.

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DECLARATION

I declare that the work presented within this thesis has been carried out solely by myself, except where indicated below. The sample preparation from the colorectal patients and the polymorphism analysis for both the normal control individuals and the colorectal cancer patients was carried out by myself.

The work of Hui- Hui Oh, who was an undergraduate student within the department of Surgery during the academic year 1993-1994, contributed significantly to the preliminary study of the polymorphisms in Gastric cancer, although this work was performed under my supervision.

The normal control DNA was a kind gift from I Galbraith of the Tissue Typing Unit, Glasgow Royal Infirmary.

DEDICATION

To Olive

ACKNOWLEDGMENTS

I am extremely grateful to Dr. Grant Gallagher, who both as a supervisor and friend has helped me through the three and a half years of my study for the Degree of PhD. His encouragement, support and enthusiasm for all of the work which his group undertakes has provided me with a stimulating environment in which to develop as an independent researcher, and my commitment to continued collaboration and friendship with Grant will continue for many years to come.

I am also grateful to the Head of the Department, namely Professor T.G. Cooke, who not only funded my three years of study, but also gave me much encouragment during my time within his unit.

I am also indebted to Professor C.S. McArdle, Professor of Surgery within Glasgow Royal Infirmary, who supplied me with the majority of patient material and data for this study.

I would like to take this opportunity to thank the clinical and nursing staff attached to the University Department of Surgery for there support. In particular I would like to thank Heather, Liz and Patricia, the units research nurses, who assisted with the collection of blood and follow up data from the cancer patients.

I must also thank the staff of the Tissue Typing Laboratory, within the Royal Infirmary, and in particular Ian Galbraith and Nancy Henderson who supplied me with the DNA samples for the normal control individuals.

I am also grateful for the support of all of the secretarial and academic staff within the University Department of Surgery, Glasgow Royal Infirmary. I especially wish to acknowledge the support of Mrs. W. Hughes who as the Professors secretary has

assisted me greatly during my time within the department. The assistance of Mr. R. Wright who as the laboratory manager helped me during those unexpected and often frought periods when materials failed to arrive, is also acknowledged.

I would like to thank the remainder of the Post Docs., laboratory staff and students - whose list of names would seem endless - who assisted me in numerous ways and who have made my time within the department a pleasure. I wish them all sucess in the future.

I would last but not least like to thank my family and my wife Olive for their support and encouragment.

Introduction

1.1 Colorectal Cancer

Recent data now supports the idea that colorectal cancer is a multi-stage disease, which primarily afflicts the elderly. A number of rare familial syndromes have recently been identified in which colorectal cancer presents atypically. Through the study of these familial syndromes, the genetic processes that cause colorectal adenocarcinoma have begun to be unravelled. The influence that the environment has on the development of colorectal cancer is also unclear; scientists have been unable to identify any major high risk factors to date. Therefore the story remains incomplete and new studies are required to examine the fine details of the interactions between the genetic and environmental factors.

1.1.1 Epidemiology

Colorectal cancer represents the second most common form of malignancy in the western world, second only to lung cancer, and represents a major health problem. Annually this disease accounts for over 20,000 deaths in the United Kingdom and over 70,000 deaths in the United States of America. Despite an improvement in the five-year survival rate over the last 50 years, the incidence of this disease has risen and thus the mortality rate has changed little in the same period of time.

The general population risk, in the UK, of developing colorectal cancer is calculated at 1 in 50 but a recent analysis of the Scottish incidence data demonstrate that the actual life time risk is 1 in 23 for men and 1 in 33 for women, with a mortality rate of 1 in 50 for men and 1 in 65 for women (Dunlop, MG., 1992). Almost 20% of all incidence of colorectal cancer can be attributed to a genetic predisposition and thus, the life-time risk for members of the families of these patients increases dramatically. The life time risk for an individual with one relative affected is estimated at 1 in 17, with one first-degree and one second-degree relative affected

this rises to 1 in 12, with one relative under 45 years old affected the risk is 1 in 10, increasing to 1 in 6 with two first degree relatives affected and finally reaching 1 in 2 in individuals with a dominant family history (Houlston, R. S., *et. al.*, 1990).

The age-specific incidence of colorectal cancer rises progressively from the second to the seventh decade (Figure 1.1), whilst men have more incidence of cancer of the rectum than women the sexes are represented almost equally in respect to incidence of colorectal cancer (McArdle, C. S., *et. al.*, 1990).

1.1.2 Aetiology

A number of recent studies have suggested that both genetic and environmental factors have an influence in the development of colorectal cancer. The role of genetic factors in the predisposition to colorectal cancer will be discussed later in this chapter, however it is worth noting that the interaction between environmental and genetic factors is unclear, but the influence of these factors is not mutually exclusive.

To date, no major risk factors have been identified which correlate with the development of colorectal cancer. Factors such as diet are becoming the major focus of attention as the general public are becoming aware of the influence of diet on the development of a number of common cancers. In countries where diseases associated with dietary factors are common, such as coronary heart disease, which is associated with a low fibre and a high fat diet, it has been shown that there is a concordant increase in the incidence of colorectal cancer (Trichopoulou, A., *et. al.*, 1992).

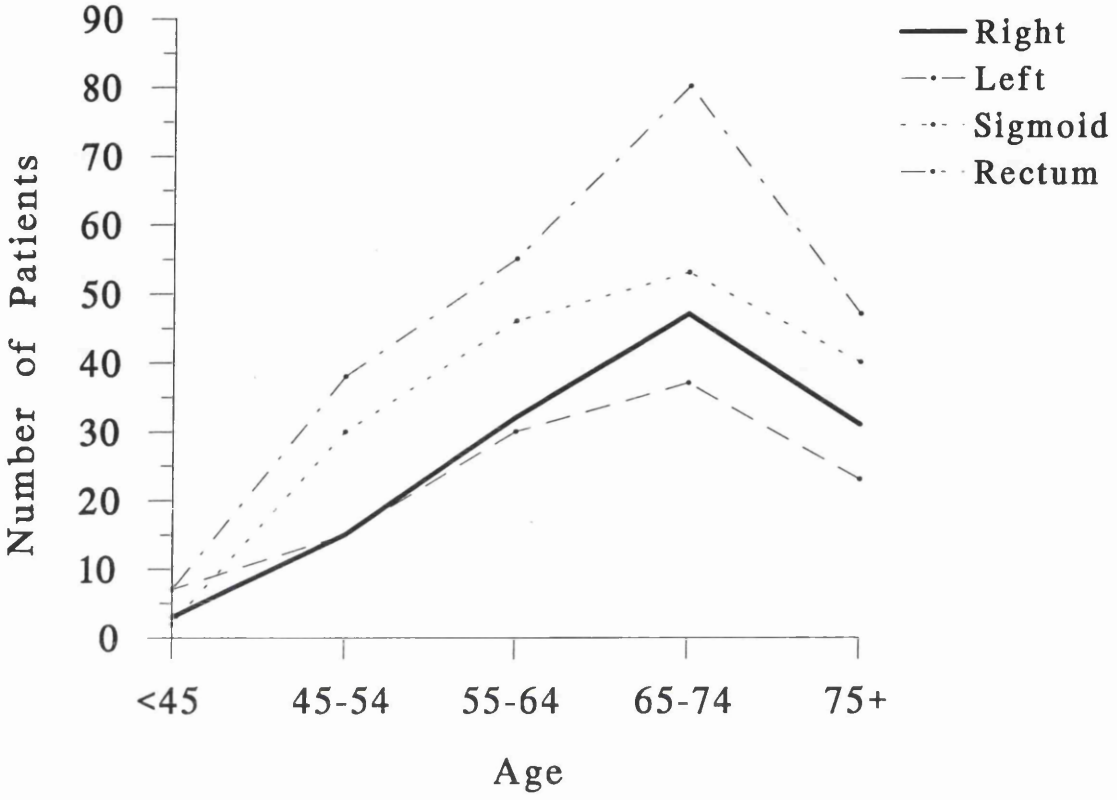

Figure 1.1. Concordant increase in the incidence of colorectal cancer, at various sites, from the second to the seventh decade. (Adapted from McArdle, C.S. *et. al.*, 1990).

The hypothesis that fibre intake is associated with the incidence of colorectal cancer has been based on observations from studies in the third world; however, two major studies by Drasar and Irvine (1973) and Armstrong and Doll (1975) have found no association between dietary fibre and the geographical incidence of colorectal cancer. Burkitt, in 1971, first proposed that increased stool bulk produced by high fibre intake reduces the time taken for the bolus to transit through the large bowel and thus reducing the potential exposure time of colonic mucosa to carcinogens. To date, however, there is no clear evidence to suggest that high fibre content of the stool shortens large bowel transit time.

Increased intake of aryl-hydrocarbon hydroxylases has also been suggested as an alternative explanation to the fibre hypothesis. These compounds, such as those found in cabbage, have been shown to inactivate noxious chemicals in the gut, and in animal studies these same compounds have been found to prevent experimentally induced tumours in rats (Wattenberg, L. W., & Loub, W. D., 1978).

The opposing theory to the fibre hypothesis is that decreased animal fat intake may lessen the risk of developing colorectal cancer. A study, in 1975, by Wynder of the incidence of colorectal cancer in 21 countries showed a clear relationship between high animal-fat diets and the incidence of this disease. Wyder suggested that people with a high animal fat intake showed elevated levels of bile acids and neutral steroids in their faeces. Reddy and Wynder (1977) subsequently showed that colorectal cancer patients do have higher levels of bile acids and neutral steroids in their faeces than normal controls. The bacterium *Clostridium paraputrificum* present in faecal material is known to dehydrogenate steroids to produce carcinogens (Hill, M. J., *et. al.*, 1975).

A number of other studies have attempted to examine more precisely the agents which may cause the development of colorectal cancer.

1). **Fecapentaenes,** are a family of potent mutagenic compounds detectable in human faeces. Levels of these compounds in the stool have been correlated with the incidence of colorectal cancer in high and low risk populations in South Africa (Ehrich, M., *et. al.*, 1979), whilst Bruce (1987) has recently demonstrated a positive association with fecapentaene levels and the incidence of colon polyps.

2). **3-Ketosteroids,** are a breakdown product of cholesterol and have been shown to induce mutagenesis *in vitro* and *in vivo* (Susuki, K., *et. al.*, 1986; Smith, LL., 1981).

3). **Pyrolysis products,** such as benzo[a]pyrene, are produced by frying meats at high temperatures and have been shown to be carcinogenic in experimental animals (Weisburger, J.H., *et. al.*, 1986).

4). **Bile acid,** levels are directly related to the intake of fat and are thought to induce the proliferation of cells lining the gut lumen (Suzuki, K., and Bruce, WR., 1986).

5). **Dietary calcium,** has been shown in experimental animals, to be able to modulate the damage caused by free bile acids by forming insoluble bile salt complexes (Bird, RP., *et. al.*, 1985). Garland *et. al.*, (1985) have shown, in a 19-year prospective study of men with colorectal cancer, that men tend to have a lower intake of calcium than do health control individuals.

6). **Alkaline pH** is known to allow the accumulation of abnormal levels of free bile acids and other potential mutagens (Van Dokkum, W., *et. al.*, 1983). Studies in both South Africans and Americans have also revealed a

correlation between high faecal pH and the incidence of colorectal cancer (Garland, C., *et. al.*, 1985;Walker, ARP., and Walker, AJ., 1986).

The primary prevention of colorectal cancer is an attractive proposition and with the belief that diet forms such a major factor in the development of this malignant disease, a variety of dietary controlled intervention therapies have been postulated and tried in the clinic. A number of case control and population studies have shown that increased intake of fibre in the diet is of benefit, whilst studies in Nebraska, Hawaii, and of Seventh-Day Adventists have confirmed that increased dietary intake of fat and cholesterol can be associated with increased risk of colorectal cancer.

With the advent of numerous biomarkers of colorectal cancer, such as colonic-crypt-cell proliferation index, faecal occult blood and the markers proposed in this study it is tempting to suggest that identification of those in the general population at elevated risk of developing colorectal cancer may benefit from a controlled "anti-cancer" diet.

1.1.3 Clinical risk factors

A number of risk factors have now been determined for colorectal cancer and these can be split into two broad groups.

1.1.3.1 Genetic risk factors

Although there are a number of hereditable syndromes associated with high risk of colorectal cancer the two most important of these are familial adenomatous polyposis (FAP) and hereditary non-polyposis colorectal cancer (HNPCC).

Although FAP is an extremely rare condition (accounting for only 1% of colorectal cancer patients), without clinical treatment all affected individuals develop colorectal cancer (Lipkin, M., *et. al.*, 1980). FAP affected individuals develop pan-colonic adenomatous polyps which are not present at birth. By the age of adolescence more than 1,000 individual polyps may be seen throughout the entire length of the colon.

The gene responsible for the development of FAP, and also the related Gardner's syndrome (Gardner's syndrome consists of polyposis coli, osteomas and desmoid tumours), was initially localised to human chromosome 5q (Herrera, L., *et. al.*, 1986), and subsequent linkage analysis by Vogelstein and colleagues (1988) confirmed this localisation. Initial analysis of this region on chromosome 5q showed four genes within this region (FER, MCC, TB2 and APC). Analysis of the sequence of the FER gene has now shown that it produces a 94-kDa polypeptide that exhibits protein tyrosine kinase activity (Hao, Q.L., *et. al.*, 1989; Feller, S.M. and Wong, T.W., 1992).

The "mutated in colorectal carcinoma" (MCC), TB2 and the adenomatous polyposis coli (APC) genes all map within a 200 kb fragment of 5q21. The MCC gene encodes a 829 amino acid polypeptide with a 19-residue stretch showing homology to the G protein-coupled m3 muscarinic acetylcholine receptor (Groden, J., *et. al.*, 1991). This region also exhibits putative sequences which specify G-protein specificity. MCC is therefore thought to be involved in the Ca^{++} / phosphoinositide cell signalling pathway, possibly as a negative receptor. Initial data on the MCC gene showed the existence of an number of point mutations within this gene resulting in non-conservative amino acid changes (Kinzler, K.W. 1991). Analysis of 90 FAP kindreds however failed to reveal any germline mutations.

The APC gene is predicted to encode for a 2843 amino acid polypeptide of which 25% of the N-terminus shows sequence homology to myosins, intermediate filaments, and plakoglobin (the cytoplasmic component of gap junctions) and 75% of the C-terminus shows short region homology with the yeast RA12 gene which is thought to play a role in *ras* regulation (Peifer, M., & Weischaus, E., 1990; Fukui, Y., *et. al.*, 1989). Loss of heterozygosity (LOH) studies have demonstrated a loss of 5q in 20% - 50% of patients with sporadic colorectal cancer (Solomon, E., *et. al.*, 1987; Vogelstein, B., *et. al.*, 1988; Miki, Y., *et. al.*, 1991). Studies of FAP patients have failed to demonstrate allelic loss of the APC gene (Okamoto, M., *et. al.*, 1988; Vogelstein, B., *et. al.*, 1988) suggesting that a single gene mutation may be enough to allow polyp development, whilst loss of the normal allele is required for carcinoma formation.

Two other forms of inherited colorectal cancer have recently been identified. These syndromes (termed Lynch type I and Lynch type II) are collectively referred to as hereditary nonpolyposis colorectal cancer (HNPCC; Fitzgibbons, R.J., *et. al.*, 1987). HNPCC accounts for between 4% and 13% of colorectal cancer cases (FAP accounts for approximately 1%). It is impossible to clinically distinguish the difference between HNPCC patients and cases of sporadic colorectal cancer as neither show the pan-colonic polyposis seen in FAP. HNPCC families are generally defined as those in which a minimum of three relatives in two generations have been diagnosed with colorectal cancer and with at least one of these relatives having been first diagnosed at less than 50 years of age (Vasen, H.F.A., *et. al.*, 1991). It has also been demonstrated that as well as malignancy of the colon, HNPCC patients can have malignancies of the endometrium, stomach, biliopancreatic system and urinary tract (Lynch, HT, *et. al.* 1991).

Genetic changes in the tumour cells of patients with HNPCC are characterised by instability of short di- and tri-nucleotide repeats, and the accumulation of a large

number of other mutations throughout the cells genome (Aaltonen, L.A., *et. al.*, 1993; Thibodeau, S.N., *et. al.*, 1993; Ionov, Y., *et. al.*, 1993). Initial linkage analysis mapped the gene predisposing to HNPCC to human chromosome 2 (Peltomaki, P., *et. al.*, 1993). Analysis of the tumours from HNPCC patients demonstrated that there was no loss of heterozygosity in 14 tumours examined and only 1 of 46 tumours from individuals with sporadic colorectal cancer showed loss of heterozygosity. The gene (hMSH2) on chromosome 2p16-15, responsible for the predisposition to HNPCC, has now been characterised and shows a high degree of homology to the MSH2 gene in yeast. In yeast, mutations in either the MLH1, MSH2 or PMS1 genes (which are involved in DNA mismatch repair) results in a 100- to 700-fold increase in genome instability (Strand, M., *et. al.*, 1993).

More recently a number of other human mismatch repair homologues have been identified. Theses include a gene located on chromosome 3p21.3-23 which shows a high degree of homology with the yeast gene MLH1, and has thus been termed hMLH1. Chromosome 3-linked HNPCC shows the characteristic microsatellite instability of chromosome 2-linked HNPCC (Lindblom, A., *et. al.*, 1993). Mutations in the hMLH1 gene have now been identified and in particular the H6 tumour cell line, which has no mismatch repair activity, has no wild-type hMLH1(Papadopoulos, N., *et. al.*, 1994).

1.1.3.2 Pre-existing disease

A number of diseases are now shown to be associated with increased risk of developing colorectal cancer. In particular, in patients with an inflammatory bowel disease there is a well recognised 30-fold increase in their risk of developing colorectal cancer.

Specific inflammatory bowel diseases, such as ulcerative colitis (UC), show an increased risk of developing colorectal malignant disease concordant with the level of bowel involvement, age of onset, disease severity and duration of disease (Mir-Modjlessi, S.H., *et. al.*, 1986). Although only about 1% of patients diagnosed with colorectal cancer have a history of inflammatory bowel disease, Ekbom *et. al.* (1990) found that the incidence of colorectal cancer in patients with UC was 5.7 times greater than the expected general population risk.

Crohn's disease is also known to confer an increase in risk of developing colorectal cancer (Hamilton, S.R., 1989). Although Crohn's disease is not associated with colorectal cancer as frequently as ulcerative colitis, atypical presentations of bowel adenocarcinomas are often seen at a younger than normal age (Kirsner, J.B. & Shorter, R.G., 1987). The bowel tumours in these patients may arise at the site of the inflammatory bowel disease or may occur at sites of previous bowel surgery (Weedon, D.D., *et. al.*, 1973).

People who have previously presented with malignant disease of the large bowel are also at an elevated risk of developing a second colorectal tumour. The possibility of developing a second primary tumour either coincident with the initial tumour or subsequently is increased at least three-fold (Morson, BC., 1976). It is interesting that clustering of adenocarcinomas of the breast, ovary, colon and rectum in the same patients has also been identified and are similar to those patterns observed in familial adenocarcinoma syndromes (Burbank, F., 1971).

Previous surgical interventions such as cholecystectomy and ureterosigmoidostomy have been reported to have an increased risk of developing large bowel cancers, though the mechanism by which this may occur is unclear.

It is now well recognised that there is a progression from adenoma to carcinoma involved in the development of cancers of the large bowel (See later). Large villous adenomas are 8 to 10 times more likely to contain malignant foci than small tubular adenomas (Muto, T., *et. al.*, 1975) and as expected the larger the number of adenomas a patient has the greater the risk of subsequently developing colorectal cancer (McDermott, F.T., *et. al.*, 1981).

1.1.4 The progression to malignancy

The hypothesis that there is a progressive shift from a non-malignant disease, normally a neoplastic polyp or adenoma, to the development of a malignant adenocarcinoma of the colon and rectum was first suggested by Morson (Morson, B.C., 1974), and the idea of an "adenoma-carcinoma sequence" is now widely accepted. It is proposed that normal intestinal epithelial cells pass through three histologically identifiable, precancerous stages between normal epithelium and histological colorectal cancer (Figure 1.2).

A histological examination of 1961 colorectal tumours by Muto and colleagues (Muto, T., Bussey, H.J.R., Morson, B.C., 1975) showed that over 14% of the tumours had some degree of non-malignant adenomatous tissue present. In the same study the authors report that 60% of the tumours confined to within the submucosa had an adenomatous component, whilst this was reduced to 20% in those infiltrating into the bowel wall, and only 7% in tumours infiltrating into the extramural fat. Although this study does not, in itself, provide overwhelming evidence for the adenoma-carcinoma sequence it does suggest that a number of adenocarcinomas do develop from a premalignant stage. Data from autopsy studies in the USA, where there is a high incidence of colorectal cancer, have shown that at over the age of fifty-five, 58% of males and 47% of females have one or more adenoma present at the time of death.

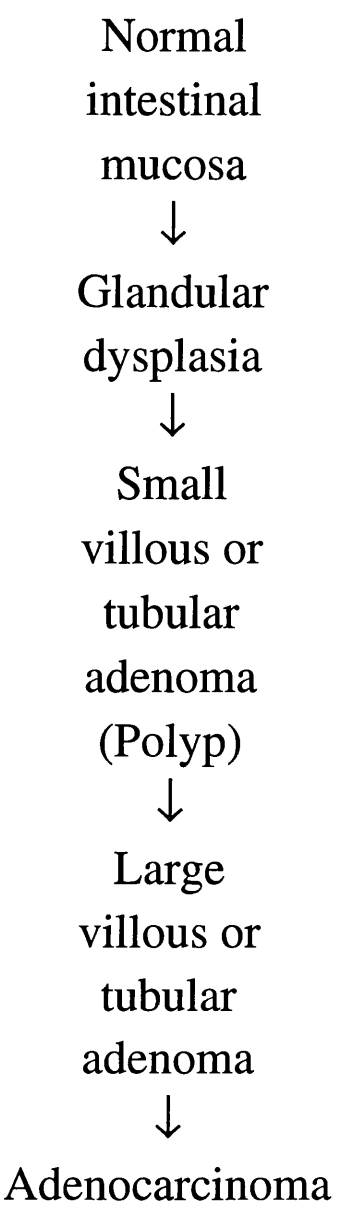

Figure 1.2. Diagramatic representation of the histological stages of the adeno-carcinoma sequence of colorectal cancer. Arrows represent the flow through increasing malignant potential. (Adapted from, Introduction to the cellular & molecular biology of cancer: second edition. Ed. Franks, LM., and Teich, NM., Oxford University Press, 1991).

It is clear, however, that the presence of an adenoma is not necessarily a predisposing factor to the development of colorectal cancer. The histological type, size of the adenoma, degree of dysplasia and number of polyps all contribute to the malignant potential associated with adenomas. Between 60%-70% of adenomas are tubular, 10% are villous and 20%-30% are tubulovillous upon histological examination. Muto *et. al.* (1977) report that the occurrence of malignancy in tubular adenomas is 5%, this figure rises to 22% for the intermediary tubulovillous adenomas and rises to a high of 40% in villous adenomas. Malignant change is also more common in larger villous adenomas. Muto *et. al.* (1977) as well as reporting the association with histological type of adenoma, also reported an association with size of adenoma. The authors reported that of adenomas less than 1 cm in diameter, over 1% showed any sign of malignancy, whilst over 45% of those over 2 cm in diameter had a malignant component. It is interesting that a recent study by De Benedetti showed a higher level of APC mutations in tubulovillous and villous adenomas than in tubular adenomas (De Benedetti, L., *et. al.*, 1994).

Although both histology and size of an adenoma correlate with malignant potential, the degree of epithelial dysplasia seen within adenomas has also been shown to correlate with the malignant potential. Studies have also correlated the number of polyps present within the bowel with the development of malignant disease. The risk of developing a subsequent adenoma a number of years after the initial detection of the first adenoma rises with the number of adenomas initially detected, whilst the risk of developing malignant disease rises concurrently (Bond, J.H., 1993).

1.1.5 Screening for disease

It is clear that there is an necessity to try and develop techniques by which the premalignant stages of the adenoma to carcinoma process of colorectal tumourigenesis can be detected and it is now widely accepted by clinicians that

detecting premalignant changes in the colon and rectum can facilitate the improvement of the mortality rate of colorectal cancer.

There are a number of methods available for the clinical screening of patients at risk from developing colorectal cancer. The classic physical examinations such as 'digital rectal examination', rigid sigmoidoscopy or flexible sigmoidoscopy result in a level of discomfort for the patients and also vary in their level of sensitivity.

Digital rectal examination has been part of the normal physical examination in a number of countries for sometime, however the sensitivity of this technique is low.

Rigid sigmoidoscopy remains the least popular examination with patients, and even though it is an inexpensive procedure its usefulness is severely limited to the short length of the bowel which can be covered. However a study by Hertz *et. al.* (1960) showed that of 26,000 patients over the age of 45 screened, 58 had asymptomatic cancer which resulted in a cure rate of 90%.

Flexible sigmoidoscopy remains the standard test for the examination of the bowel for the presence of asymptomatic or pre- malignant disease. The longest of the scopes, of about 45cm, will allow the easy detection of almost 65% of neoplastic polyps and small cancers. Atkin *et. al.* (1993) suggest that once-only sigmoidoscopy between the age of 55-60 years could prevent approximately 19% of colorectal cancers in this age group in the UK.

Recently there has been a search for more sensitive techniques for the detection of the premalignant stages in the development of colorectal cancer. One such technique which may facilitate this is the process of detection of "aberrant mucosal crypt cell production rate" (CCPR; Rooney, P.S., *et. al.,* 1993). This process involves the examination of non-neoplastic rectal biopsy material taken during

endoscopy. Biopsy material is incubated, *in vitro,* in vincristine to induce metaphase arrest. The resultant samples are then examined to determine the number of crypts in metaphase arrest and these figures compared with biopsy samples from either adenoma negative controls or from a group of vegetarian control volunteers. Rooney *et. al.* showed a clear association with high numbers of crypt cells in metaphase in patients with adenomas compared to both the normal and vegetarian controls.

At the opposite end of the scale is the non-invasive technique of faecal occult blood (FOB) testing or "Haemoccult". This process involves the sampling of faecal material to detect small quantities of blood that could be predictive of neoplastic or malignant changes in the bowel, and is based upon three systems.

1) Guaiac impregnated paper slides which detects the presence of haemoglobin in the faecal material by the colourimetric determination of its peroxidase activity (Mandel, J.S., *et. al.* 1989).

2) The HemoQuant test (SmithKline Diagnostics) which detects faecal haemoglobin content by the conversion of heme to fluorescent porphyrins (Ahlquist, D.A., *et. al.* 1984).

3) The immunodetection of faecal haemoglobin using an antibody based system (Saito, H., *et. al.* 1985).

The standard Haemoccult test fails to detect occult blood when it should in 1 in every 5 test performed (a false negative rate of 20%). This failure rate can be improved by increasing the number of tests a patient is given but this increase in sensitivity produces a highly unacceptable high false positive rate. There is still no conclusive evidence to support the use of this technique as a routine method of screening for colorectal cancer.

1.1.6 Biomarkers of disease development and progression

Of all the identifiable biomarkers of malignant disease several of these antigens have been suggested as useful markers in colorectal cancer. In 1969 it was first shown that CEA was elevated in the plasma of 35 out of 36 patients with colorectal cancer and that the plasma titre fell after "successful" surgery whilst levels equivalent to normal were found in patients bearing non-colorectal tumours or in patients with benign disease (Thompson, D.P.M., *et. al.* 1969). Carcinoembrionic antigen (CEA is a 200 kD glycoprotein expressed on the cell-surface) was originally identified in the foetal liver, intestine and pancreas during the first six months of development, however, it is now clear that CEA can be found, in adults, in malignant diseases of the liver, colon and pancreas but cannot be found in normal or benign tissues.

The largest study of this antigen to date has been a cumulative study of 35,000 samples from 10,000 patients and controls (Hansen, H.J., *et. al.* 1974). In this study, of 1425 non-smoking healthy individuals, 98.7% had CEA levels below the value of 5.0 ng/ml, however of 857 normals with a history of smoking, CEA was elevated in 33%. In a further 576 patients who had no clinical evidence of cancer, 23 were subsequently demonstrated to have colorectal cancer by barium enema and CEA levels of greater than 2.5 ng/ml were found in 18 of the 23. The authors also suggesting a correlation with CEA and stage of disease. CEA levels of greater than 5 ng/ml was found in 4% of Dukes' A disease patients, 25% of Dukes' B disease patients, 45% of Dukes' C disease patients and 65% of patients with clinically detectable metastatic disease at time of initial surgery (Hansen, H.J., *et. al.* 1974). It is clear that CEA lacks the specificity required to be used successfully in screening or diagnosis of colorectal cancer since it lacks both disease and stage specificity; elevated levels of CEA have also been found to have varying degrees of usefulness in breast cancer and in one study CEA was seen to be elevated in 60% of women

with metastatic breast cancer (Schwartz, M.K., 1987). The lack of specificity is confirmed by Sandler *et. al.* (1984) who suggest a false-negative rate of 24%.

The mucin-type glycoproteins have been suggested to have limited use in the screening for colorectal cancer. These antigens include CA 19-9, CA 195 and TAG 72. CA 19-9 is derived from a colon tumour cell line, but is not elevated in the serum of many patients with colorectal cancer. In one study CA 19-9 was found to be elevated in only 42% of colorectal cancer patients compared to a 72% rise in CEA (Koprowski, H., *et. al.* 1981). Levels of the CA 195 antigen (an antigen similar to CA 19-9) have been shown to be elevated in 72% of patients with active colorectal cancer compared with 57% with raised CEA and 61% with raised CA 19-9. The final antigen in this family, termed TAG 72, is a high molecular weight glycoprotein and is reported to be elevated in 54% of patients with advanced colon cancer and 55% of patients with advanced gastric cancer.

The clinical usefulness of these antigens in the analysis of disease development and progression is questionable. A number of studies have used CEA levels to direct surgery for local recurrence. In a retrospective study of 22 patients with elevated CEA, 86% of the patients were observed to have recurrent disease at laparotomy, however only 27% of these patients had a resectable tumour (Martin, E.W., *et. al.*, 1977). In all the studies which have examined the usefulness of CEA in directing surgery to recurrent disease, tumour was found in between 78%-100% of patients, however like the study of Martin *et. al.*, the resectability rate in these patients is low suggesting that CEA may be a marker for late disease, at least in recurrent tumours. The specificity of the mucin-type glycoproteins is also questionable. Studies have shown that both CA 19-9 and CA 195 are elevated in individuals with benign disease.

1.1.7 Molecular events in disease development

The molecular changes involved in the development of colorectal cancer are complex, and involve the alteration of the normal balance between oncogenes and tumour suppressor genes. Although its is becoming clear that these genetic changes are fundamental to the development of tumours, its is still accepted that these changes cannot be considered in isolation of environmental factors. It is now widely accepted that most tumours arise due to a combination of both environmental and genetic factors.

A number of genes have been now been identified that play a role in the development of both sporadic and familial colorectal cancer. Figure 1.3 shows a diagramatic representation of the mutational events that occur during the development of colorectal cancer.

1.1.7.1 Normal mucosa to hyperproliferative epithelium

The earliest event in the development of any tumour is a change which allows normal cells to escape from the cell-cycle to become hyperproliferative. The normal cell cycle is now well characterised, however the reason why premalignant cells escape the stringent control of the cell cycle is unclear.

Genetic instability is a common factor to both solid and leukaemic tumours. The recent identification of alterations in a number mismatch repair genes in both sporadic and hereditary non-polyposis colorectal cancer (HNPCC), suggests that early changes in genomic stability may be primary in the development of cancer. In an article in Nature, which was published concordantly with the reports describing the initial identification of mismatch repair errors in HNPCC, Vogelstein suggested

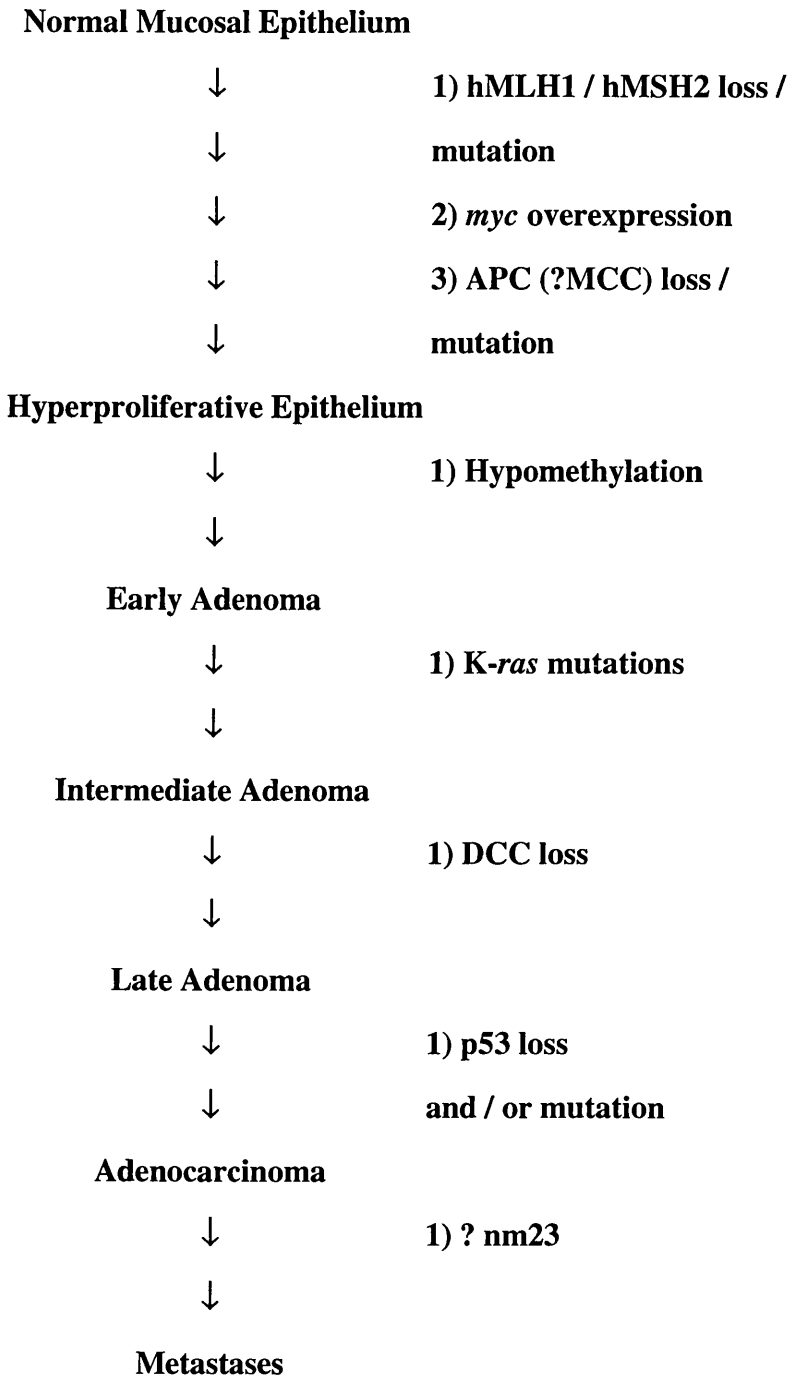

Figure 1.3. Model of mutational events in the development and progression of colorectal carcinogenesis. (Adapted from Fearon and Vogelstein, 1990).

that alterations in the function of mismatch repair genes could result in the genetic changes commonly associated with colorectal tumourigenesis; such as those seen in APC. (For a fuller description of the HNPCC associated genes please see section 1.1.3.1.)

A number of other genes have been shown to be altered at this early stage of tumour development. The *myc* gene, located on chromosome 8q24, is the cellular homolog of the transformation gene of avian myeloblastosis virus. This gene produces two polypeptides of 62 kD and 64 kD in size, and although the natural function of these two polypeptides is unknown, structure analysis has implicated them either in the regulation of transcription or as accessory factors for DNA polymerase. Increased levels of both *myc* mRNA and the p62 and p64 peptides have been identified in colorectal cancer (Finley, G.G., *et. al.*, 1989). It has now been shown that up to 50% of colorectal tumours with elevated *myc* expression have an associated deletion within the region of APC, whilst those tumours with no APC deletions show no overexpression of *myc* (Erisman , M.D., 1989). The reason for this interaction between *myc* and APC is unknown. The result of expression of *myc* by cells is now known to be dependent on the coexistence of growth factors within the cell's microenvironment. If growth factors are present within the cells microenvironment then the cell is prevented from entering the Go phase of the cell cycle and continues to proliferate, however if growth factors are absent, then the cell selects a path towards programmed cell death.

The adenomatous polyposis coli (APC) gene has been widely studied in colorectal cancer due to its recent identification as the cause of FAP. Recent studies have shown that alterations in APC are an early event in the development of the majority of colorectal cancers, with recent LOH studies demonstrating a loss of 5q in between 20% and 50% of patients with sporadic colorectal cancer (Solomon, E.,*et. al.*, 1987;

Vogelstein, B., *et. al.*, 1988; Miki, Y., *et. al.*, 1991). For a more detailed description of FAP and APC please see section 1.1.3.1 of this thesis.

The MCC gene is also located on chromosome 5q21. This gene was initially thought to be the gene responsible for FAP (Kinzler, K.W., *et. al.*, 1991), however it is now accepted that there are no genetic mutations in MCC associated with FAP. Studies of MCC in sporadic colorectal cancer have shown that the gene is mutated in at least 15% of patients (Nishisho, L., *et. al.*, 1991), whilst LOH studies have suggested that allelic loss occurs in over 40% of patients (Ashton-Rickardt, P.G., *et. al.*, 1991; Miki, Y., *et. al.*, 1991). The role this loss plays in colorectal tumourigenesis is unclear as all three of these studies were performed prior to the identification of APC within such a close proximity, and it is possible that deletions within this region cover both APC and MCC.

1.1.7.2 DNA hypomethylation

Methylation of genomic DNA is a normal event in both prokaryotes and eukaryotes where it appears to be involved in the control of gene expression and chromosome condensation. The methylation status of DNA from non-malignant polyps and colorectal adenocarcinoma specimens has been shown to differ from that observed in normal colonic mucosa. A decreased or hypo-methylation status has been observed in both colonic polyps and adenocarcinomas suggesting that this reduction in DNA methylation is an early event which precedes the development of malignancy (Goelz, S.E., *et. al.*, 1985). It is possible that loss of methylation of specific genes such as c-erbB-2 and *myc* may confer growth advantage to preneoplastic cells (Holzmann, K., *et. al.*, 1992; Sharrad, R.M., *et. al.* 1992).

1.1.7.3 The *ras* family of oncogenes

Numerous studies have examined the role of *ras* expression in colorectal cancer. The *ras* family consists of three genes (H-*ras*, K-*ras* and N-*ras*) which encode a series of 21 kD guanine nucleotide-binding proteins which are expressed on the cytoplasmic side of the cell membrane ($p21^{ras}$). The most common alterations of *ras* in colorectal cancer involve the K-*ras* gene, whilst H-*ras* and N-*ras* mutations are rare (Bos, J.L., 1989). *Vogelstein et. al.* (1988) reported that *ras* mutations occur in only 9% of adenomas of less than 1 cm in size, compared to 58% in larger adenomas and 41% in primary adenocarcinomas. Mutations in *ras* have been reported to be similar in both sporadic adenomas and tumours and in adenomas and tumours of patients with FAP (Sasaki, M., 1990) suggesting that *ras* mutations may indicate a common pathway of development of both sporadic and familial colorectal cancer.

Recently attempts have been made to associate *ras* mutations with stage of disease. In 1991, Oudejans *et. al.*, reported an increase in mutations of both K-*ras* and N-*ras* in patients with either liver or lung metastases (50% and 57% respectively) whilst only 37% of patients with "non-metastatic" Dukes' B tumours showed mutations in *ras*. Recently Moerkerk *et. al.* (1994) have reported a difference in the type of mutation in codon 12 of K-*ras* and the association with metastatic propensity of Dukes' B and C colorectal tumours.

Although gene transfer studies have failed to show that mutations in *ras* alone are capable of inducing a complete transformed phenotype (Sager, R., *et. al.*, 1983) there is some evidence to suggest that *ras* can result in partial transformation and an increase in proliferation of primary epithelial cells (Burns, J.S., *et. al.*, 1993).

1.1.7.4 LOH on chromosome 18

The putative tumour suppressor gene termed DCC ("deleted in colorectal cancer") has now been localised to chromosome 18q. Allelic loss of DCC has been shown to be a rare event in small early adenomas, but is observed in over 50% of late stage adenomas (Vogelstein, B., *et. al.*, 1988) and over 70% of colorectal adenocarcinomas (Vogelstein, B., *et. al.*, 1989) and is thus thought to be a late event in colorectal tumourigenesis (Fearon, E.R., *et. al.*, 1990). The DCC gene encodes a transmembrane protein which shows sequence homology with members of the Ig supergene family, and in particular the neural cell adhesion molecule (N-CAM; Edelman, G., 1988). Studies using antisense DCC mRNA, transfected rat fibroblasts have shown that if expression of DCC is inhibited then cells can acquire a tumourigenic phenotype, but also grow *in vitro* as suspension rather than as a monolayer (Narayanan, R., *et. al.*, 1992). This suggested that DCC is involved in cell-matrix interactions rather than cell-cell adhesion. The loss of DCC may be important in promoting a more invasive phenotype in an already transformed cell.

1.1.7.5 p53 and the malignant cell cycle

The gene for p53 maps to human chromosome 17p13.1. The gene encodes a 53 kD nuclear protein which shows almost pan-expression and shows a high level of conservation across species (Jenkins, J.R., *et. al.* 1988). p53 was originally thought to be the product of a dominant oncogene due to its ability to transform a number of cell lines (Jenkins, J.R., *et. al.*, 1984). Recent evidence has suggested that the initial transformation studies were flawed in that they used mutant p53 clones rather than the wild-type. It is thought that, in some cases, mutant p53 is capable of binding to wild type p53 and thus inactivating the wild-type molecule. A number of recent studies have now shown that p53 can revert transformed human cancer cell lines,

including colon and bladder, to an untransformed phenotype (Baker, S.J., *et. al.*, 1990; Chen, P.L., *et. al.*, 1990).

The function of p53 is not entirely clear. Emerging evidence suggests that p53 may be be important in the control of the cell-cycle of stressed cells (Lane, DP., 1992) and that p53 may function to control the passage from G_1 to S-phase in cells with damaged DNA (Hartwell, L., 1992; Lane, DP., 1992). Expression of p53 in cells with damaged DNA may allow the cell time to repair their DNA by causing cell-cycle arrest, if this repair subsequently fails then the cell can be pushed into apoptosis (Lane, D.P., 1992). A number of factors can result in the disruption of the normal function of wild-type p53. Figure 1.4 shows a diagramatic representation of the ways in which such inactivation may occur. The most common form of mutation in human tumours, including colorectal tumours, is a missense mutation in one allele of p53 followed by a deletion of the second allele (Baker, S.J., *et. al.* 1990).

A number of studies have attempted to correlate p53 overexpression with disease prognosis in colorectal cancer. Starzynska *et. al.*, (1992) report a significant association with p53 overexpression and clinical outcome with a progressive increase in levels of p53 associated with Dukes' staging and an association between high levels of p53 and early reccurence of disease. Remvikos *et. al.* (1992) also report an association between p53 and clinical outcome but failed to show any association with Dukes' staging.

1.1.7.6 nm23

Reduced expression of the nm23 gene has been identified in metastatic breast cancer and has been associated with a poor prognosis (Barnes, R., *et. al.*, 1991). The gene encoding nm23 has now been mapped to human chromosome 17p21 and encodes a nucleoside diphosphate (NDP) kinase (Gilles, A-M., *et. al.*, 1991) which is thought

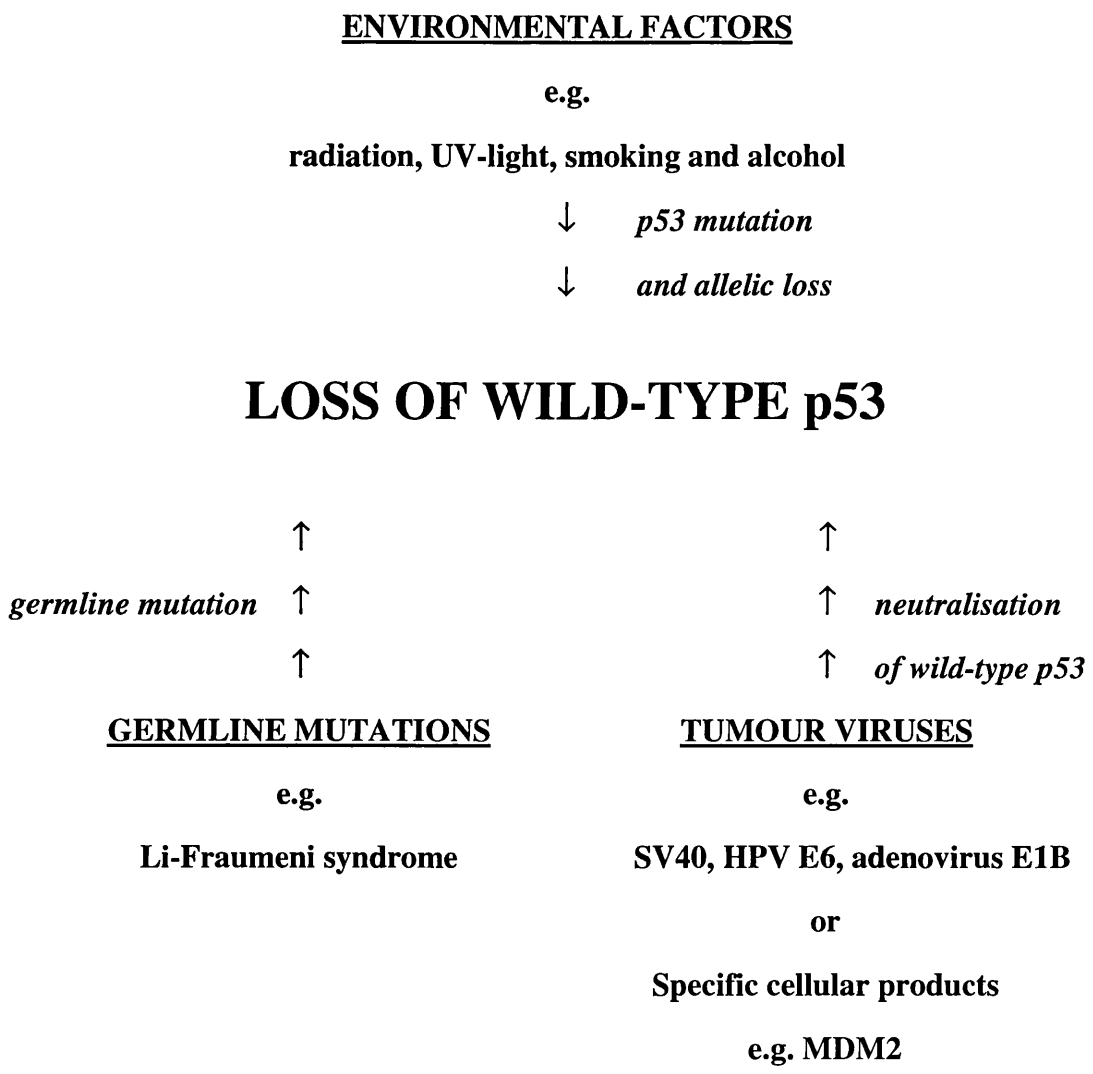

Figure 1.4. A number of factors can result in the inactivation of the normal function of p53 in malignant and premalignant cells.

to play an important role in the formation of $p21^{ras.GTP}$ complexes (Ruggieri, R., and McCormick, F., 1991).

Reduced expression has also been observed in colorectal cancer. Cohn *et. al.* (1991) have reported an association with allelic loss of nm23-H1 and distant metastases in colorectal cancer. In this study 73% of patients with deletions in nm23 had clinical evidence of matastases after 25 months of follow-up, compared to only 20% who had no evidence of nm23 deletions. The true role of nm23 in metastasis is still unclear, however studies have shown that transfection of nm23 results in the loss of metastatic phenotype in a number of animal models (Leone, A., *et. al.*, 1991).

1.2 The host / tumour interaction

In parallel to the expansion of the tumour cell population, solid tumours develop a stromal component, which varies in proportion with the malignant cells depending on the tumour type. This stromal component consists of new blood vessels, a fibrotic reaction and an inflammatory cell infiltrate (Dvorak, H.F., 1986). Interactions between the stromal component and the malignant cells form an important part of the modulation of the growth of solid tumours.

It has always been difficult to define the role which the host immune system plays in the prevention, control and eradication of malignant cells. Numerous studies, including the transplantation of chemical induced syngeneic rodent tumours (Foley, E.J., 1953; Prehn, R.T., & Main, J.M., 1957), have demonstrated the ability of the host immune system to recognize and reject malignant cells. Burnet's theory of immunological surveillance (Burnet, F.M., 1970) proposed that a major function of the immune system was the detection and elimination of cells which became disparate from the host due to some form of somatic mutation (e.g. tumour cells). Rygaard and Povlsen (1976), however, demonstrated that athymic nude mice did not develop spontaneous tumours at a higher rate than immunologically intact mice, and Prehn (1976) demonstrated that in some cases host T-cells could be seen to potentiate the growth of immunogenic transplanted syngeneic tumours. These studies provided a conflicting view of the role of the host immune system in the host / tumour interaction in the 1970s.

With the advent of advanced techniques in molecular and cellular immunology a number of the initial questions regarding the host / tumour interaction have been answered. Burnet's theory of immune surveillence now seems at least in part to be correct. It is now clear that immune system evolved to provide a mechanism for the control of viral infections and that T-cells are central to this response (Zinkernagel,

R.M., & Doherty, P.C., 1979). T-cells, however, still have an important role in the control of malignant disease. Epstein-Barr virus-induced (EBV) B-cell lymphomas are seen with increased frequency in patients that have undergone specific T-cell depletion (Moss D.J., *et. al.* 1977; Thorley-Lawson, D.A., *et. al.* 1977; Thorley-Lawson D.A., *et. al.* 1980) whilst UV-induced squamous cell cancers occur with high frequency in renal transplant patients (Hardie, I.R., *et. al.* 1980) suggesting that in a number of cases T-cells can control the development of a malignant phenotype.

1.2.1 T-cells

It is clear that tumour cells can express potentially immunogenic antigens on their surface in that it is possible to direct an immune response against tumours by the *in vitro* enrichment of lymphocytes (see below). The failure of T-cells to detect these determinants is probably due to an ineffective antigen presentation mechanism either by the tumour cells themselves or by professional antigen presenting cells (APC) which results in T-cell anergy by failing to provide the required costimulatory signals (Mueller,D.L., *et. al.*, 1989). Others have suggested that rapidly proliferating tumours result in the induction of a suppressor T-cell phenotype (Fujimoto, S., *et. al.*, 1976; Gorelik, E., 1983), however the idea of a suppressor T-cell subset has lost favour over the last few years. A final hypothesis as to the inability of T-cells to respond to tumours *in vivo,* is the idea that the effector T-cells cannot clonally expand at a sufficient rate to control tumour growth (De Boer, R.J., *et. al.*, 1985).

Cytotoxic T-cells were originally perceived to recognise integral membrane proteins. It is now clear, from the analysis of the mechanism of recognition by CD8+ve cytotoxic T-cells (Tc) of virally infected cells, that Class-I restricted Tc recognise intracellular proteins that are synthesised and degraded in the cytoplasm before translocation to the endoplasmic reticulum to be inserted into the cleft of the Class-I MHC molecule and finally transported through the Golgi resulting in the expression

on the cells surface in association with the MHC (Bjorkman, P.J., *et. al.*, 1987a,b; Nuchtern, J.G., *et. al.*, 1989).

Studies by Rosenberg have shown that it is possible to to augment the function of T-cells by treatment of lymphocytes with high doses of interleukin-2 (IL-2) *in vitro.* This results in the development of a population of cells exhibiting non-specific lymphokine activated killer function (LAK cells) which can be reinfused into the patient with or without concurrent infusion of IL-2. This form of adoptive immunotherapy does however carry a significant toxicity.

Studies in humans and experimental animals have shown that T-cell clones isolated from tumour infiltrating lymphocyte (TIL) preparations can be expanded in tissue culture to produce a large number of T-cells exhibiting a unique specificity against the initial tumour (Chapdelaine, J.M., *et. al.*, 1979; Belldegrun, A., *et. al.*, 1989). The treatment of patients with malignant melanoma, by such a method, can result in a response to the therapy in over 40% of patients (Rosenberg, S.A., *et. al.*, 1988)

1.2.2 Macrophages

Although T-cells constitute a sizable fraction of the total white cell infiltrate into tumours, numerous other cell types are present. Undoubtedly the most important cell, next to T-cells, is the tumour-associated macrophage (TAM).

Derived from bone marrow promonocytes, and passing through a blood-borne monocyte phase, macrophages (Mϕ) represent a large part of the tissue bound mononuclear phagocytic population. This phagocytic population is found throughout the bodys' connective tissue and surrounding the basement membrane of small blood vessels, but is particularily concentrated in areas such as the lung

(alveolar Mϕ), the liver (Kuppfer cells) and the lining of the spleen sinuosoids and lymph node medullary sinuses.

It is thought that TAMs are recruited from blood-borne monocytes by the production of a number of compounds, including monocyte chemotactic factor (MCP; Martinet, N., 1992). This recruitment requires the production of a concentration gradient from the secreting malignant cell to the blood vessels (Keller, H.V., 1977). A number of studies have now identified the existance of compounds such as MCP within the vicinity of tumours and this model of recruitment is now well accepted (Martinet, N., 1992).

A number of the functions of macrophages, such as the clearance of obsolete red blood cells (RBC), are continuous processes. However, their participation, in the defence against tumours requires the recruitment and subsequent specific activation of the cell (for review see Fidler, I.J., & Poste, G., 1982).

TAMs exhibit a wide range of functions, including the sectretion of:

1) **cytotoxic factors**, e.g. tumour necrosis factor (TNF;see later)
2) **angiogenic factors**, e.g. fibroblast growth factor (FGF; Polverni, P.J *et. al.*, 1984)
3) **lytic enzymes**, e.g. plasminogen activator (PA; Mussoni, L., *et. al.*, 1988)
4) **immunomodulatory factors**, e.g. prostaglandin E_2 (PGE_2; Varesio, L., *et. al.*, 1979)
5) **acute phase-response proteins**, e.g. interleukin 6 (IL-6; Erroi, A., *et. al.*, 1989).

The role these Mϕ-derived factors play in the host / tumour interaction remains unclear in that a number of the factors produced can have pro- and anti-tumourigenic

activities. The swing from the protumourigenic to antitumourigenic activity of Mϕ will depend on the interactions of the individual compounds produced by the cell, which in themselves will be controlled by the level of activation of the Mϕ.

Immunohistichemical analysis of a number of human tumours including breast (Gottlinger, H.G., *et. al.*, 1985), colon (Allen, C., & Hogg. N., 1985) and skin (Broker, E.B., *et. al.*, 1988) have demonstrated the presence of Mϕ in the stromal compartment of these tumours. Allen and Hogg (1987) have demonstrated that Dukes' C colonic cancers have a larger Mϕ infiltrate than than less advanced tumours.

The presence of TAM in solid tumours makes them an attractive target for therapeutic agents. The results of a number of rodent studies are hopeful in that they demonstrate that activated Mϕ express an anti-tumour phenotype, however this response is only noticible with a high macrophage to tumour cell ratio (Allavena, P., *et. al.*, 1987). It would be futile to attempt to describe the fine details of the interaction between host tumour cells and TAMs. Relevent to this thesis is the central role of the Mϕ in the production of TNF and this will be discussed in more detail in a subsequent section of this thesis.

1.2.3 The major histocompatibility complex

The major histocompatibility complex (MHC) is located on the short arm of chromosome 6, within the band 6p21.3 (Lamm, L.U., & Olaisen, B., 1985), and consists of three linked gene clusters. These gene clusters are termed the MHC class I (class I), MHC class II (class II) and MHC class III (classIII) regions and contain a series of highly polymorphic genes which encode for a series of cell surface and secreted peptides.

The class I region containes at least 17 highly related genes including the classical transplantation antigens HLA-A, HLA-B and HLA-C (Strachan, T., 1987). These antigens are expressed as 43 kD peptides non-covalently linked to the 11 kD molecule β_2-microglobulin. The class I genes are now known to be central to the recognition of viral or foreign antigens by $CD8^+$ cytotoxic T-cells (CTLs).

The class II region, commonly termed HLA-D, is subdivided into four subregions containing the genes for the α and β domains for HLA-DP, HLA-DZ / DO, HLA-DQ and HLA-DR (Trowsdale, J., 1987). The products of the class II genes are found mainly on cells of the immune system (specifically on B-cells, Mϕ and dendritic cells) and are involved in antigen presentation to $CD4^+$ T-cells and B-cells.

The class III region of the MHC contains a number of seemingly unrelated genes (Dunham, I., *et. al.*, 1987; Browning, J.L., *et. al.*, 1993) including the second and fourth components of the complement system (C2 and C4), the steroid 21-hydroxylase (21-OHase), the heatshock protein 70kD (HSP-70) family and the tumour necrosis factor genes (TNF-α, LT-α and LT-β; see section 1.3).

1.2.3.1 The MHC and malignancy

The majority of studies examining the role of the MHC in malignancy have been performed on experimental animal models with the studies on human material being confined to *in vitro* experiments or to immunohistochemical analysis of biopsy material. Studies of animal models have shown that a number of tumour systems show a significant down regulation of specifically MHC class I (or H-2 in the mouse) expression on the tumour cells, others show an up regulation of specific molecules of the MHC whilst other tumours show the development of novel class I molecules due to recombination or mutation (for review please see Doherty, P.C., *et. al.*, 1984).

In the majority of tumours down regulation of class I molecules is associated with a concordant loss of β_2-microglobulin expression, however in colon tumours the down regulation may be due to a loss of β_2-microglobulin expression (Garrido, F., & Ruiz-Cabello, F., 1991). Loss of class I expression is seen in approximately 14% of human primary colorectal adenocarcinomas (Ruiz-Cabello, F., *et. al.*, 1991), compared to 10% of human melanomas (Lopez Nevot, M.A., *et. al.*, 1987), and 20% of carcinomas of the lrynx (Esteban, F., *et. al.*, 1989).

A number of studies have attempted to correlate loss of class I expression with a high metastatic propensity. Altered expression of class I on the surface of circulating "metastatic" cells may allow them to escape from detection by the host immune system. In 1987 Durrant, *et. al.*, demonstrated a large variation in the expression of class I and class II MHC on the surface of colorectal cancer cells, but failed to demonstrate any association with MHC expression and clinical outcome or pathological staging. Goepel, *et. al.*, (1991) report a partial or complete loss of monomorphic determinants in 60% of primary colorectal tumours, whilst two respective lymphnode metastases showed a reduce level of expression relative to their primary tumour and four showed increased expression. The authors also examined the specific loss of HLA-A2 and report a partial or complete loss of this polymorphic determinant in all of the tumours examined with a similar loss in the associated metastases.

Epithelial cells rarely express class II MHC molecules, however over 50% of colorectal tumours express HLA-DR (Durrant, L.G., *et. al.*, 1987). The pattern of staining was consistantly associated with the most poorly differentiated tumours, however in the majority of tumours the level of staining was poor (Durrant, L.G., *et. al.*, 1987, Rognum, T.O., *et. al.*, 1983). The loss of class II has also been reported on the metastases from colorectal tumours although the figures vary from 50% to 100% (Durrant, L.G., *et. al.*, 1987, and Thompson, J.J., *et. al.*, 1982, respectively).

It has been suggested that expression of HLA-DR on the surface of primary tumours is capable of increasing the tumours immunogenicity (Fossati, G., *et. al.*, 1984). However a study of metastatic melanoma cells demonstrated that cells expressing high levels of HLA-DR antigens have the potential to inhibit the immune response of autologous T-cells (Taramelli, D., *et. al.*, 1984). This is in conflict with the view of Durrant *et. al.* (1987) who suggest that in colorectal cancer the metastatic cells are absent of Class II on their surface. It may be that in the melanoma model the excessively high levels of class II MHC result in the induction of tolerance to the target antigen.

γIFN has the ability to upregulate the expression of HLA-DR antigens (and HLA-A, B and C) on a number of cell types, including tumour cells (Durrant, L.G.,1987). Durrant *et. al.* (1987) demonstrate that 2/4 secondary tumours express the HLA-DR antigen, however they also demonstrate that young dividing cells, when grown *in vitro,* show consistent negative staining for HLA-DR. On treatment of these cells with γIFN upregulation of the MHC molecule can be observed. This would be consistent with the idea that metastases are produced from MHC negative or MHC down regulated cells which can easily evade the immune system.

The true function of the MHC in colorectal tumourigenesis is unclear, with a number of reports showing conflicting degrees of expression. It is of course widely accepted that tumours represent a heterogeneous population and it may be this fact that results in the discrepancies seen between studies. However it seems safe to say that the MHC plays an extremely important part in the host / tumour interaction. Any factor or factors that can affect the expression of the MHC will have major implications on the intricate interactions which occur between host and the tumour. It is clear that loss of expression of HLA antigens is of benefit to tumours in that it allows them to evade the immune system. However studies in experimental animals have also

demonstrated that an increase in class-I expression is associated with a decrease in natural killer (NK) cell sensitivity (Stern, P., *et. al.*, 1980).

There are very few studies which have examined whether specific MHC antigens are involved in the predisposition to malignancy. A number of MHC haplotypes have been associated with lung cancer including Aw19 and B5 which seem to confer resistance to the progression of bronchogenic carcinoma (Rogentine, G.N., *et. al.*, 1977), whilst more recently HLA-DR7 has been suggested to confer resistance to lung cancer (Romano, P.J., *et. al.*, 1991). Other tumours such as nasopharyngeal cancer, which has been associated with Bw40 (Simons, M.J., *et. al.*, 1975,) and eosophageal cancer, which has been associated with B40 (Simons, J.M., *et. al.*, 1977), all show associations with class I antigens although only in specific ethnic groups. Recently, however, an association has been demonstrated between specific HLA-DR and DQ antigens and the susceptibility to papillomavirus-type specific cervical carcinoma (Apple, R.J., *et. al.*, 1994). In this study the authors report an association with with DRB*1501 and DRQB1*0602 with HPV16-type specific cervical cancer whilst DR13 exerts a strong protective effect. This result suggets that perhaps specific MHC haplotypes can influence the way in which a tumour is seen by the immune system with certain MHC haplotypes allowing for good tumour-specific antigen recognition and others resulting in poor antigen presentation.

1.3 Tumour Necrosis Factor and Lymphotoxin

In the late 19th Century Dr WB Coley (1891) attempted to treat a number of patients who presented with sarcomas, to the Memorial Sloan-Kettering Cancer Center, with a preparation of killed Gram-negative bacteria, which he termed Coley's toxin. On treatment with this toxin these patients tended to show a remarkable (although generally short lived) regression of their tumours. It was almost a century later before a group from the same centre became the first to describe a factor distinct from bacterial endotoxin which could induce haemorraghic necrosis of transplanted murine tumours, and thus proposed the existance of tumour necrosis factor or TNF (Carswell, E.A., *et. al.*, 1975).

Today the tumour necrosis factors (TNFs) are a closely related family of compounds which are individually refered to as tumour necrosis factor alpha (TNFα), lymphotoxin alpha (LTα; previously TNFβ) and lymphotoxin beta (LTβ). These three compounds are members of the rapidly growing group of compounds, commonly termed cytokines.

Although the initial studies examined the role of TNFα in the host response to malignant disease, this compound has now been demonstrated to be important in the host response to numerous other, non-malignant, diseases. Recent advances in recombinant technology have allowed a greater understanding of the function of this compound and within the last few years major advances have been seen in the use of this compound as a therapeutic agent.

1.3.1 Biological function.

Tumour Necrosis Factor was originally described in the middle of the 1970's. Carswell *et. al.* (1975) originally described a soluble factor which could be identified

in the sera of animals treated with reticuloendothelial stimulators, bacterial endotoxins or lipopolysaccharide (LPS). Mannel *et. al.* (1981) subsequently described the macrophage as being the principal source of TNFα and that this same molecule was an important effector molecule in the non-specific tumouricidal activity of macrophages. TNFα appeared to be a preferential mediator of cytotoxicity for tumour and transformed cells and received its name due to its ability to cause haemorrhagic necrosis of subcutaneous tumours in mice. Thence came the expectation that TNFα could provide a major key to therapy of malignant disease.

The cloning of the two original forms of TNF (TNF-α and LT-α (Lymphotoxin)) in 1984 by Gray *et. al.* and Pennica *et. al.* and the expression of these genes in *E. coli.* allowed further work to be performed using this recombinant source of the protein. Sugerman *et. al.* (1985) showed that TNFα was capable of stimulating proliferation of normal cells, Dayler *et. al.* (1985) showed that TNFα could stimulate collagenase production whilst Bertolini *et. al.* (1986) showed that TNFα could cause bone resorption. A review by Aiyer and Aggarwal (1987) describes a number of other activites of TNFα on normal cells including effects on the metabolism of fibroblasts, neutrophils, hepatocytes and leucocytes. Tracey *et. al.* (1986) first suggested that TNFα had the ability to induce similar effects to those resulting from endotoxin-induced shock and Beutler *et. al.* (1985), claim that TNFα is the primary mediator in endotoxin-induced shock. However in the experiments of Beutler *et. al.* (1985), the authors fail to show a complete block of the shock following administration of anti-TNFα antibodies, suggesting that other mediators are also important.

Numerous studies have examined the ability of TNFα to induce tumour necrosis *in vivo* or tumour cell lysis *in vitro*. Carswell *et. al.* (1975) were the first to show that serum from mice challenged with *Bacillus calmette-guerin,* and subsequently injected with endotoxin, contained a compound which could cause haemorrhagic killing of a number of murine tumours *in vivo* including the s-180 sarcoma in CD-1

Swiss mice, the BP8 leukaemia in C3H mice and the chemically induced Meth A sarcoma in BALB/c mice. This same group showed that the effect of the serum was greatest if the tumours were allowed to develop for at least five days prior to the treatment with the serum, thus suggesting that a vascularised tumour was essential to the action for the endotoxin treated serum to be effective.

Administration of concentrations of TNF-α several-folds higher than the antitumour dose results in a severe weight loss in experimental animals. This weight loss, or cachexia, is accompanied by an inhibition of lipoprotein lipase (LPL; an essential protein which is involved in the clearence of triglyceride from the blood) secretion by adipose tissue (Cerami, A., *et. al.*, 1985). Cessation of TNFα administration, however, results in a rapid recovery, and a gain in weight (Lee, *et. al.*, 1987).

TNF is now known to have a large spectrum of effects on a large number of cell types. TNF causes the adhesion of neutrophils to endothelial cells (Gamble, J.R., *et. al.*, 1985) and may be the primary cause of endotoxin induced neutropenia. TNF has also been shown to be growth inhibitory in that it causes vascular endothelial cell remodelling in which the cells flatten, become overlapping, rearrange their actin filaments and lose stainable fibronectin (Schweigerer, L., *et. al.*, 1987). In addition to causing adhesion of neutrophils to endothelial cells, TNF also acts on neutrophils to enhance their production of superoxide (Shalaby, M.R., *et. al.*, 1985), induce the release of lysozymes and hydrogen peroxide and also induces neutrophil degranulation (Kiobanoff, S.J., *et. al.*, 1986).

TNF-α is now known to be synthesised by a number of cell types, including cells of the haematopoietic and non-haematopoietic lineages (Beutler, B., 1990; Spriggs, D.R., *et. al.* 1988; Jevnikar,A.M., *et. al.* 1991) although it is now recognised that macrophages are one of the major sources of TNFα (Mannel, D.N., *et. al.*, 1981). Although macrophages do express receptors for TNFα, little is known about the

function of TNFα on the macrophage although it has been shown to be chemotactic to these cells (Ming, W.J., *et. al.*, 1987). TNFα is known to induce the production of GM-CSF by a number of cell lines and may cause the activation of macrophages indirectly via this pathway (Munker, R., *et. al.*, 1986).

TNFα also exerts an effect on lymphocytes. TNFα acts to induce the upregulation of TNFR on primary cultured T-cells (Scheurich, P., *et. al.*, 1987) and has also been shown to induce the expression of the HLA-DR antigen and the high affinity IL-2 receptor thus resulting in an enhanced proliferative response of T-cells, particularily to IL-2. Conversely TNF has been shown to inhibit pokeweed mitogen induced proliferation and differentiation of B-cells (Kashiwa, H., *et. al.*, 1987).

Although this is by no means a comprehensive description of the intricate interactions that TNF and LT are involved in, it can be clearly seen that TNFα, as well as having direct cytotoxic effects, also acts as an important immune modulator and is one of the primary initiators and modulators of the immune response.

TNFα exists in solution as a homotrimer of 17 kD subunits each synthesised from a 26kd transmembrane propeptide that itself appears to exhibit biological activity when membrane bound (Kreigler,M., *et. al.* 1988), whilst LT-α (originally TNF-β) is a 25 kD soluble protein, synthesised primarily by lymphocytic cells (Paul,N.L., and Ruddle, N.H., 1988). Although antigenically distinct, it displays approximately 30% sequence homology with TNF-α and also close homology with the newly cloned LT-β (Browning, J.L., *et. al.* 1993). The function of both lymphotoxins still remains unclear.

Within the last year a new member of the TNF gene family has been identified and with its discovery a change in the nomenclature of the three TNF proteins has arisen. This new molecule, termed Lymphotoxin-β (LT-β; Browning, J.L., *et. al.* 1993), has

recently been cloned. LT-β is a 33 kD glycosylated transmembrane protein. LT-α (originally TNF-β) is present on the surface of activated T-cells, B-Cells and lymphokine activated killer cells (LAK cells) heterodimerised to the LT-β protein. No function has yet been ascribed to this new molecule although human peripheral blood mononuclear cells (PBLs) stimulated with either anti-CD3 antibody or interleukin-2 (IL-2) express mRNA for both lymphotoxins (Browning, J.L., *et. al.* 1993). It is interesting that this heterodimeric molecule is incapable of binding to either of the two known TNFα receptors (p55 and p75; see below). Crowe *et. al.* (1994) have recently identified a receptor specific for LTβ, however little is know about its function. Browning *et. al.* (1993) hypothesise that the surface-bound LT-α β dimer may be important in cell-cell contact-dependent programmed cell death, but this still remains unproven, whilst Crowe *et. al.* (1994) suggest that, since LTα knock-out mice display a disfuction in lymphoid organ development, yet $TNFR_{55}$ or $TNFR_{75}$ knock-out mice develop a normal lymphoid organ system, the LTα-LTβ complex and the LTβR may play an important role in immune development.

Two separate receptors have been identified for TNF-α, which also show a high degree of specificity for LT-α but a low degree of specificity for LT-β. These receptors, termed p55 and p75 (or sometimes p60 and p80) differ in both their molecular weight and also in the mechanism by which they mediate the biological effects of TNF-α (Brockhaus, M., *et. al.*, 1990). The p55 receptor is expressed on a wide range of cell types and expression of p55 seems to be sufficient to result in the cytotoxic action of TNF-α on tumour cells *in vitro* (Balkwill, F.R., *et. al.*, 1993), independent of the expression of the p75 receptor (Higuchi, M., & Aggarwal, B.B., 1994). The p75 receptor shows a more specific pattern of expression and is found primarily on lymphoid and myeloid cells (Balkwill, F.R., *et. al.*, 1993). Whether or not the p75 receptor plays a function in the cytotoxicity of TNF-α is debatable, with a number of studies suggesting that in some cases this receptor is capable of

inducing cytotoxicity upon crosslinking with specific antisera (Medvedev, A.E., *et. al.*, 1994).

1.3.2 TNF and human malignant disease

A number of studies have examined the role of TNFα in human solid tumours with the majority of these studies having looked at breast and ovarian cancer, although a number of studies have examined other tumour types including colorectal cancer.

Both immunohistochemical and *in situ* hybridisation techniques have demonstrated the presence of TNF-α protein and TNF-α mRNA within colorectal tumour biopsy samples (Beissert, S., *et. al.*, 1989). The source of this material tends to be associated with tumour associated macrophages (TAM). Semiquantitative data from Beissert *et. al.* (1989) suggest that, whilst levels of TNF-α mRNA were detectable in all of the samples examined, less than 1% of the TAMs were synthesising immunoreactive TNF-α. By using a Northern blot technique Naylor *et. al.* (1992) were able to support the idea that TNF-α is produced within colorectal tumours. This study, however, failed to positively identify the nature of the TNF-α secretory cells. The authors suggested that these cells could either be macrophage derived or a subset of T-cells.

Studies of TNFα mRNA expression in breast cancer have demonstrated that over 90% of breast tumours have detectable mRNA localised to sporadic cells within the stroma (Vitolo, D., *et. al.*, 1992). A variety of breast epithelial tumour cell lines have been shown to secrete TNF-α *in vitro* (Spriggs, D.R., *et. al.*, 1988). These cells often show resistance to the cytotoxic activity of TNF-α, and it has been suggested that low levels of TNF-α secretion can result in the desensitisation of tumour cells to TNF (Spriggs *et. al.*, 1987). Indeed it has been demonstrated that TNF-α induces the internalisation of the p55 receptor and the extracellular release of the p75 receptor

(Higuchi, M., & Aggarwal, B.B., 1994). It is entirely possible that this internalisation results in the loss of the sensitivity to TNFα-mediated cytotoxicity, whilst the p75 receptor acts to bind and neutralise free TNFα.

There are a number of ongoing clinical trials involving the use of TNFα. These trials, like those of other cytokines, have been severely restricted due to the level of toxicity of high dose TNFα. The response of patients to recombinant TNF-α (rTNF α) as a single therapeutic agent have been mixed. Studies in metastatic breast cancer involving the administration of rTNFα as a single therapeutic agent have had no success (Budd, G.T., *et. al.* 1991), however the regional administration of rTNFα to metastatic lessions in the liver of patients has shown a partial response in 14% of patients with primary colorectal cancer (Mavligit, G.M., *et. al.*, 1992).

A number of studies have attempted to assess the feasibility of administering rTNFα in conjunction with a number of other therapeutic agents. A phase 1B trial involving the administration of rTNFα in conjunction with γ-IFN has shown no significant increase in the biological response of patients with widely disseminated cancer (Schiller, J.H., *et. al.*, 1992). Recent studies involving isolated limb perfussion to treat advanced melanoma have given some hope to the use of TNFα in the therapy of malignant disease. Studies involving the infusion of rTNFα and Melphalan into melanoma patients presensitised with γ-IFN results in an increase in complete response from 50% to almost 90% (Lienard, D., *et. al.*, 1994), whilst studies involving patients with irresectable soft tissue sarcomas demonstrates a limb salvage rate of almost 90% (Eggermont,A.M.M., *et. al.*, 1994).

Although these studies have not been performed on large numbers of patients, the results are promising. Perhaps the use of mutated forms of TNF-α such as those which only bind to the p55 receptor (Vaan Ostade, X., *et. al.*, 1993) may facilitate

reduction of the toxicity associated with TNF therapy and may allow more widespread use of rTNF in the therapy of malignant disease.

1.3.3 The TNF genes

The tumour necrosis factor genes (TNF-α, LT-α and LT-β) are located within the region p21.1 to p21.3, of human chromosome 6. By using the technique of Southern blotting Spies *et. al.* (1986) proposed that the two original TNF (TNFα and LTα) genes map to two possible regions either between HLA-DR and HLA-A or slightly centromeric to HLA-DP. These results were confirmed by chromosome *in situ* hybridisation. In 1987 Dunham *et. al.*, using pulsed-field gel electophoresis, mapped the human TNF-α and LT-α genes to their now accepted position approximately 250 kb centromeric to HLA-B (Figure 1.5). The most recent member of the TNF gene family (LTβ) has also been located to within the class III region of the MHC, approximately 2 kb centromeric to TNF-α (Figure 1.5; Browning, J.F., *et. al.*,1993).

1.3.3.1 The TNF-α gene

TNF-α is coded for by a 3634 bp gene consisting of 4 exons which results in a 157 amino acid mature protein with a 76 amino acid signaling sequence (Nedwin, G.E., *et. al.* 1985). The 5' untranslated region of TNF-α is 180 bp long and begins at nucleotide 615 of the published sequence. Intron one is 607 bp in length and disect the genomic sequence at codon 62. The second intron is 187 bp in length and disects the sequence within the second codon of the mature protein sequence. The third intron is of 301 bp in length and occurs within codon 18 of the mature protein sequence. There is a TATA box located at position 590, an AGC putative site of anitiation of transcription located at position 615, an AATAAA site located at position 3368 and a putative site of polyadenylation (poly(A)) at position 3382

(Nedwin, G.E., *et. al.* 1985). There is also an AU-rich region which is involved in determining mRNA stability is located at position 3050 (Shaw, G., *et. al.*, 1986).

1.3.3.2 The LT-α gene

The sequence of both the TNF-α and LT-α mature proteins show approximately 28% homology (Pennica, D., *et. al.* 1984) with a maximal homology of 56% at the nucleotide level in the coding region of exon 4 of both genes. The introns also show a very low degree of homology at only 35% (Nedwin, G.E., *et. al.* 1985).

Like TNF-α, the LT-α gene is constructed of 4 exons and 3 introns and is coded for by a 3037 bp stretch of DNA. The first intron of LT-α is 287 bp in length and disects the 5' untranslated region 9 bp before the beginning of the coding region. The second intron is 86 bp in length and disects the signal sequence one amino acid residue before the begining of the coding sequence of the mature protein. The third intron is 247 bp long and disects the coding sequence at amino acid 35 of the mature protein. The site of initiation of transcription is located at position 818 and the poly(A) site is at position 2854. The TATA box is located at position 790 and the AATAAA region located at position 2735. Again, like TNF-α, there is also an AU-rich region at the 3' end of the gene (at position 2587) which is involved in determining mRNA stability (Shaw, G., *et. al.*, 1986).

1.3.3.3 The LT-β gene

Like the other members of the TNF gene family LT-β has four exons and three introns and is coded for by a 2200 bp fragment of DNA (Browning, J.F., 1994). The promotor region of this gene contains a putative TATA box at position 109 and a CAAT element at position 266. The 5' untranslated region of LT-β is only 10 bp long and begins at nulcleotide 293. Intron one is 96 bp in length and disects the

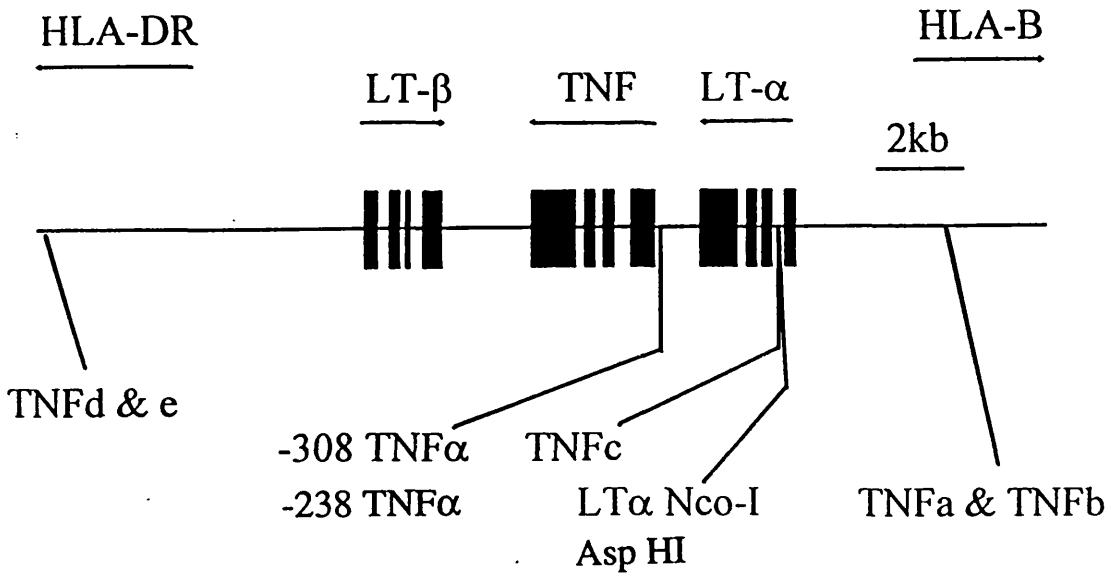

Figure 1.5. Diagramatic representation of the TNF locus showing the position of the TNFα, LTα and LTβ genes and there intron/exon arrangement and the relative position of the TNF polymorphisms.

genomic sequence between codon 54 and 55. The second intron is 83 bp in length and disects the sequence within codon 70. The third intron is of 395 bp in length and occurs within codon 94 of the protein sequence. The AU-rich motif that is common to the 3'-untranslated regions of both the TNF and LT-α genes is not present in the LT-β suggesting that the LT-β mRNA displays a differing degree of stability than TNF or LT-α. The site of initiation of transcription is located at position and the poly(A) site is at position 2127.

1.3.4 Polymorphisms within the TNF locus

The number of polymorphic regions identified to date within the human genome has not been formally calculated but there are at least 10 within the TNF locus. These polymorphic regions are divided into two distinct types comprising of restriction fragement length polymorphisms (LT-α *Eco*RI RFLP, LT-α *Nco*I RFLP, -308 TNF-α RFLP, -238 TNFα RFLP and *Asp*HI LTα RFLP; Partanen, J. and Koskimies, S., 1988; Messer, G., *et. al.*, 1991; Wilson, A.G., *et. al.* 1992; D' Alfonso, S. & Richiardi, P.M., 1994; Ferencik, S., *et. al.*, 1992) and five microsatellite polymorphism (TNFa, TNFb, TNFc, TNFd and TNFe; Jongeneel, C.V., *et. al.*, 1991 and Udalova, I.A., *et. al.* 1993). These regions exhibit various degrees of polymorphism and span the entire length of the TNF gene cluster (Figure 1.5).

1.3.4.1 LT-α *Eco*RI RFLP

With the strong association between TNFα and autoimmune disease there has always been a search for polymorphisms within the region of the TNF genes which could be associated with the secretion pattern of TNFα seen in various autoimmune diseases. The *Eco*RI RFLP described by Partanen and Koskimies (1988) is a biallelic polymorphism with the polymorphic restriction enzyme site locate in the 3' untranslated region of exon 4 of the LT-α gene. This RFLP produces two alleles

defined by Southern hybridisation of a common 2.4kb and a rare 2.5kb which was present in 4 of the 67 (6%) unrelated individuals in the original study. This RFLP shows no association with the common autoimmune-associated HLA antigens B8, DR3 or B27 but does show a strong association with B40 (Partanen, J. and Koskimies, S., 1988). Since the 2.5kb allele of the *Eco*RI RFLP is so rare little work has been performed regarding the function or associations of this polymorphism in disease.

1.3.4.2 LT-α *Nco*I RFLP

The LT-α *Nco*I RFLP was initially defined by Southern hybridisation with a TNF-α probe to give two alleles of 5.5kb (termed the B*1 allele) and 10.5kb (termed the B*2 allele) and for a number of years it was thought that the polymorphic *Nco*I site was within the TNF-α gene (Fugger, L., *et. al.*, 1989a). Messer *et. al.* (1991), however, mapped a 56.8kb region of a number of overlapping genomic DNA clones and by direct sequencing located the polymorphic restriction site to within the first intron of the LT-α gene, at position 252. This polymorphism results in a guanine to adenine substitution to create the *Nco*I recognition site. Messer *et. al.* (1991) also showed an associated amino acid substitution at position 26 of the LT-α gene with an asparagine associated with B*1 allele and threonine with the B*2 allele.

A number of studies have examined the role of this RFLP in various autoimmune and non-autoimmune diseases, and like the *Eco*RI RFLP this polymorphism seems to show a high degree of association with an extended MHC. The B*2 allele shows strong association with HLA-DR4 and -B15, whilst the B*1 allele shows an association with HLA-A1, -B8, -DR3, in normal control individuals (for review see Verjans, G.M.G.M., *et. al.*, 1992).

Unlike the *Eco*RI RFLP, functional analysis of the various LT-α RFLP genotypes have demonstrated an association with specific genotypes and the production of TNF α *in vitro*. The most recent study by Pociot *et. al.* (1993a) shows a distinct pattern of TNF-α secretion, but interestingly not LT-α secretion, associated with various LT-α RFLP genotypes. The authors report a significantly higher production of TNF-α by lipopolysaccharide (LPS)-stimulated monocytes from individuals homozygous for the B*2 allele than those levels seen in B*1 homozygous individuals, whilst individuals heterozygous at this locus showed an intermediate level of TNF-α under the same stimulatory conditions. Although the LT-α RFLP is located within the gene for LT-α and is also associated with a variant amino acid within the LT-α protein, Pociot *et. al.* (1993a) report no difference in the level of LT-α secretion by phytohemagglutinin (PHA)-stimulated peripheral blood mononuclear cells (PBMCs). These results are in contrast to those reported by Messer *et. al.* (1991), who reported an increased level of LT-α secretion associated with the TNFB*1 allele, in PHA stimulated T-cells. In the study by Messer *et. al.* (1991a) the authors report no association between TNF-α secretion in PHA-stimulated PBMCs from individuals homozygous for either of the two LT-α alleles, whilst individuals homozygous for the B*1 allele showed a significant increase in LT-α production over those individuals homozygous for the B*2 allele.

The reason for the differences seen between these two studies is unclear. It is now accepted that these alleles form part of an extended MHC haplotype which includes alleles of the other TNF associated polymorphisms. Although neither of the other RFLPs have been associated with distinct patterns of TNF-α or LT-α secretion, alleles of the microsatellite polymorphisms, particularily the TNFa microsatellite, have been reported to show association with TNF-α secretion. It is probable that alterations in the control of TNF-α and LT-α secretion cannot be associated with one polymorphism alone. The genetic control of production seems to be affected by numerous factors such as MHC and TNF microsatellite genotypes as well as the

TNF RFLP genotypes. Classical mechanisms known to alter the levels of TNF-α or LT-α *in vitro* and *in vivo* will also play an important role in the degree of response seen upon stimulation of various cell types and will contribute to the differences reported in the two studies described above.

1.3.4.3 -308 TNF-α RFLP

The -308 TNF-α RFLP is one of two RFLPs that have been identified, to date, within the gene for TNF-α. Like all RFLPs the -308 RFLP is a biallelic polymorphism describing two alleles, a common allele termed TNF1 and a rare allele termed TNF2 (Wilson, A.G., *et. al.*, 1992). This polymorphism results in the substitution of a guanine by an adenosine in the promotor region at position -308, relative to the site of initiation, in the rare TNF2 allele.

Like the LT-α RFLP this polymorphism shows a strong association with alleles of the MHC. Wilson *et. al.* (1993) have shown a strong association with the uncommon, TNF2 allele, and the extended HLA haplotype HLA-A1, -B8, -DR3. This extended haplotype has now been expanded to include the alleles of the LT-α RFLP as well as alleles of the TNFa, TNFb and TNFc microsatellites (see bellow). This haplotype now extends to DQB1*0201, DQA1*0501, DRB1*0301, TNF2, LT-α B*1, B8, A1/2, C4A *null* and has been observed in control individuals, in patients with insulin-dependent diabetes mellitus (IDDM; Pociot, F., *et. al.*, 1993b) and in patients with systemic lupus eryuthematosis (SLE; Wilson, A.G., *et. al.*, 1994a).

Constructs involving the two TNF-α allelic promotors upstream of a CAT reporter system result in a six to seven fold increase in transcription in both PHA stimulated and unstimulated Raji B-cell line transfected with the TNF2 bearing promotor (Wilson, A.G., *et. al.*, 1994b). Studies by a group in The Netherlands on similar CAT constructs have failed to show a similar effect. In this study a CAT construct

containing the region -619 to +108 of the two allelic promotor regions of TNF-α, failed to show any difference upon transfection into the Jurkat T-cell line (Brinkman, B.M.N., *et. al.*, 1994). The differences between the results of the two groups remains unexplained. It is unclear in the paper by Wilson *et. al.* what region of the TNF-α promotor was used in the study. Its is possible that these regions differ between the two studies and that differences in the number of transcriptional factor binding sites such as NFκ-B binding sites, have been missed. However this hypothesis remains unproven.

Studies of CAT constructs of the two polymorphisms cannot be directly related to differences in production of TNF-α *in vivo* and the study of the *in vivo* effects has yet to be performed.

1.3.4.4 The *Asp*HI RFLP

A further RFLP has been reported within the same intron of the LTα gene as the LT α *Nco*I RFLP (Ferencik, S., *et. al.*, 1992). Like all of the RFLPs this polymorphism results due to the substitution of a single base resulting in the formation or destruction of a restriction endonuclease recognition sequence. Like the *Eco*RI RFLP there is very little further data available regarding this polymorphism. It has been reported that there is no linkage between the two RFLP loci in this intron or with alleles of the MHC (Ferencik, S., *et. al.*, 1992).

1.3.4.5 -238 TNFα RFLP

A recent report by D' Alfonso and Richiardi (1994) identifies a further RFLP within the promotor region of the TNFα gene. It has been shown that changes in nucleotide sequence can result in changes in the superhelical structure of genomic DNA and that these changes can influence the electrophoretic mobility (Calladine, C.R., *et. al.*,

1988). Using this knowledge D' Alfonso and collegues were able to detect a polymorphism distinct from the -308 TNFα RFLP at position -238 of the TNFα gene. This region of the promotor forms part of the highly conserved Y box which has been shown to be essential for MHC class II promotor function (Benoist, C. & Mathis, D., 1990).

Again this polymorphism seems to form part of an extended MHC haplotype and the authors report a number of associations including the rare TNFA-A allele with HLA-DR3. To date no further studies have examined the relationship between this polymorphism and disease although it seems likely that these studies will be forthcoming since this polymorphism seems to occur in a very important part of the TNFα promotor.

1.3.4.6 TNF microsatellite polymorphisms

Although the two RFLPs within the TNF locus can provide invaluable evidence to the function of TNF in various diseases, their usefulness is limited due to their small degree of polymorphism. Recently five regions of dinucleotide repeat microsatellite polymorphism have been found distributed within the TNF locus (Nedospasov, S.A., *et. al.*, 1991; Jongeneel, C.V., *et. al.*, 1991, Udalova, I.A., *et. al.*, 1993). These loci show varying degrees of polymorphism.

The TNFa and TNFb polymorphisms consist of AC/GT and TC/GA repeat units respectively. These two loci are found within a 200 bp region located 3.5 kb upstream of the LT-α promotor and show 13 and 7 alleles respectively (Nedospasov, S.A., *et. al.*, 1991; Jongeneel, C.V., *et. al.*, 1991). The TNFc polymorphism is biallelic and consists of TC/GA repeat units and is found within the first intron of the LT-α gene (Nedospasov, S.A., *et. al.*, 1991). The two most recently described misrosatellite polymorphisms have been termed TNFd and TNFe and are located 8-

10 kb downstream of the TNF-α gene. These polymorphisms both consist of TC/GA repeat units and have 7 and 3 alleles respectively (Udalova, I.A., *et. al.*, 1993).

A recent study by Pociot *et. al.* (1993a) has shown an association between the TNFa2 allele and high secretion of TNF-α by LPS stimulated monocytes (~3.4 ng/ml) whilst TNFa6 was associated with a significantly lower level of secretion of TNF-α (~2.4 ng/ml), compared to a base line taken as 3 ng/ml. TNFc2 positive monocytes also displayed a significantly high level of TNF-α secretion, although the exact value for this was not presented. Like the LT-α RFLP this study failed to show any association with TNFa or TNFc alleles and the secretion of LT-α by PHA stimulated PBMCs (Pociot, F., *et. al.*, 1993a). The only report of an association with alleles of the other microsatellite loci describes an association with the TNFd3 allele and an elevated level of TNF-α secretion in endotoxin stimulated monocytes from patients homozygous for the TNFd3 allele as compaired to patients heterozygous for the TNFd3 allele (Turner, D.M., *et. al.*, 1994).

Like the three RFLPs within the TNF locus, the alleles of the five microsatellites seem to form part of an extended MHC haplotype. Four such haplotypes have been identified which correlate with TNF-α secretory capacity of LPS stimulatyed monocytes (Pociot, F., *et. al.*, 1993a). These haplotypes are, in order of increasing TNF-α secretion, as follows

DRw6, TNFB*1, TNFa4, B40, A2

DQw8, DR4, TNFB*1, TNFa6, B44, A2

DQw2, DR3, TNFB*1, TNFa2, B8, A1

DQw8, DR4, TNFB*2, TNFa2, B115, A2.

Although the authors report a difference in the mean level of TNF-α secretion between these four groups the differences are not significant.

Analysis of 101 cell lines from the Human Leukocyte Antigen Workshop reference panel for alleles of the five microsatellite polymorphisms and the LT-α ,Nco-I RFLP have identified a number of common extended MHC haplotypes (for a complete description of these haplotypes please see the ref. Udalova, I.A., *et. al.*, 1993). The authors report a number of unusual differences between the cell lines. Whilst a number of the cell lines were shown to be identical for their extended haplotype, a number of lines extended haplotypes were created by recombination, which in one case occured between the TNFb microsatellite locus and the HLA-B locus. Other cases were identified where cell lines shared the same HLA-serological haplotype but showed differing TNF alleles. These cell lines have been used as control for HLA typing due to the fact that the majority of them are homozygous for the serologically-defined HLA alleles. On analysis of the TNF alleles of several of these cell lines Udalova *et. al.* (1993) report that some of these lines show heterozygosity for alleles of the TNF locus. Those cell lines which showed heterozygosity for one of the TNF alleles, also showed heterozygosity for one of the HLA alleles. It is now clear that alleles within the TNF locus show a high degree of association with alleles of the HLA system. The ability to subdivide extended HLA haplotypes by incorporating haplotypes of the TNF polymorphisms may allow a more detailed analysis of the association of the MHC and in particular TNF with human diseases.

1.3.5 TNF polymorphisms and human disease

TNFα is now widely accepted to be one of the major mediators of autoimmune disease, with alterations in the level of TNFα being implicated in the pathogenisis of diseases such as RA, SLE and IDDM. Within the last few years a number of groups

have attempted to determine whether this alteration in TNFα secretion is genetically controlled, and in particular whether this loss of normal control can be associated to one or more polymorphism within the TNF locus.

In 1989, Fugger *et. al.*, (1989a) performed a screen of the TNF locus in an attempt to ascertain the degree of polymorphism within the TNF locus. Of 20 restriction enzymes the authors examined only Nco-I displayed a polymorphic pattern when hybridised in a Southern blot with a TNF-α cDNA probe. In this original study the authors wrongly assumed that the Nco-I polymorphism that they had detected was within the TNF-α gene, however they were the first group to document an association with the decrease in the presence of a particular allele of this RFLP, in this case the TNFB*2 allele, and and the presence of the autoimmune disease primary biliary cirrhosis (pBS). Studies by the same group showed a further association with alleles of the Nco-I RFLP and three of four other autoimmune disease (Fugger, L., *et. al.*, 1989b). A high degree of association was seen between the TNFB*1allele and pauciarticular juvenile rheumatoid arthritis (P-JRA), primary Sjogren's syndrome (pSS) and SLE, whilst a decrease in the frequency of the TNFB*2 allele was observed in patients with P-JRA, pSS, SLE and RA, however the decrease was only significant in the SLE group. The authors also note a strong association between HLA-A8 and the TNFB*1 allele and suggest that any decrease in the observed frequency of the TNFB*2 allele could be a secondary effect of this strong linkage disequilibrium.

A number of groups have also examined the role of the LT-α Nco-I polymorphism in insulin-dependent diabetes mellitus (IDDM). IDDM is characterised by a mononuclear cell infiltration of the pancreatic islets resulting in the autoimmune destruction of the pancreatic β-cells (Kolb-Bachofen, V and Klob, H., 1989). TNF-α has been widely implicated in the pathogenesis of this disease, and the disease is known to be associated with the HLA antigens DR3 and DR4, with individuals

displaying both of these antigens having the highest relative risk (Svejgaard, A., *et. al.*, 1986). Initial studies by Pociot *et. al.* (1989) demonstrated that the TNFB*2 allele is found more commonly associated with the HLA-DR3 haplotype in IDDM patients than in normal control individuals, however this study fails to show an independent association with the TNFB*2 allele and this disease. Further studies in normal individuals have demonstrated that the IL-1β and TNF-α response of monocytes from these individuals can be associated with the LT-α RFLP. LPS and/or gamma interferon and/or TNF-α stimulated monocytes from individuals homozygous for the TNFB*2 allele demonstrated a higher level of TNF-α and IL-1β than heterozygous individuals, however this pattern of response was also demonstrated in patients with recent-onset and long-standing IDDM (Molvig, L., *et. al.*, 1990). Two further studies involving groups headed by J Nerup of the Steno Memorial Hospital in Gentofte, Denmark, and involving both Pociot and Molvig, have continued to examine the role of the LT-α Nco-I RFLP in IDDM (Pociot, F., *et. al.*, 1991; Honen, J., *et. al.*, 1992). The first of these studies reinforced the idea of an involvement of the TNFB*2 allele in IDDM, however the second study, which examined IDDM in a Finnish population, failed to show any association between the TNFB*2 allele and DR4/DQ8-associated IDDM. There are now a number of other studies demonstrating linkage between this RFLP and disease. Messer *et. al.*(1994) have recently demonstrated an association between both the LTα *Nco*I RFLP and the -308 TNFα RFLP in dermatitis herpetiformis, whilst Tomita *et. al.* (1993) have demonstrates an association with the TNFB*1 allele and SLE, however Goldstein and Sengar (1993) report no independent correlation in this disease. Interestingly, and possibly some what surprisingly, an association has been described between this locus and the C4 'null' allele and recurrent spontaneous abortion in Finnish couples (Laitinen, T., *et. al.*, 1992), however the reason for this association remains unclear.

Attempts to show linkage of disease to polymorphic markers at the TNF locus had, until 1991, been restricted to the use of the RFLP technique. In 1991 Nedospasov *et.*

al. reported the first recorded series of microsatellite polymorphisms within the region of the TNF genes (see section 1.3.4.4). Pociot *et. al.* (1993a) were the first group to use these microsatellites, in conjunction with HLA haplotypes, and the LT-α Nco-I RFLP, to examine, in detail, the the role of the MHC, and in particular the TNF locus, in IDDM. In a group of 26 DR3/DR4 heterozygous patients with IDDM, the authors found a significantly higher frequency of the TNFa2 and a lower frequency of the TNFa6 allele than healthy HLA-DR matched controls. Twelve of this same group of patients also demonstrated an extended haplotype , DSQw8, DR4 (Dw4), C4A3, TNFB*2, TNFa2 and B15, identical to the DR4 haplotype which the authors report correlates with an elevated TNF-α response in LPS stimulated monocytes.

Until recently all of the polymorphic loci within the TNF genes have been found either distant from the coding sequence of the TNF proteins or within the gene for LT-α, however a polymorphic site within the promotor region of the TNF-α gene has recently been identified (Wilson, A.G., *et. al.*, 1992; see section 1.3.4.3). The rare allele of this polymorphism does show an association with the common autoimmune disease HLA haplotype A1, B8, DR3 (Wilson, A.G., *et. al.*, 1993). This HLA haplotype is known to be common in diseases such as IDDM, SLE, Graves' disease and celiac disease however, unlike the LT-α RFLP, this polymorphism shows no association with IDDM (Pociot, F., *et. al.*, 1993b). An abstract submitted to the British Society for Rheumatology in 1994, by Wilson *et. al.* examines the relationship between this polymorphism and systemic scleroderma (SSc). In this study of 19 patients no association between the alleles of this polymorphism and the presence of the disease was noted, however some association was observed between the rare allele and the presence of anti-PM-Scl antibodies. Two further studies of the -308 TNF-α RFLP have examined the relationship between alleles of this polymorphism and autoimmune disease, and in both cases no independent association between alleles of this locus and either SLE (Wilson, A.G.,

et. al., 1994) or ankylosing spondylitis could be demonstrated (Verjans, G.M, *et. al.*, 1994).

Although there is considerable evidence to suggest that TNFα plays an important role in tumourigenesis only one study to date has examined the role of polymorphisms within the TNF locus and human malignancy. A number of associations between various HLA alleles and lung cancer have been reported including Aw19, B5 and DR7 conferring resistance to this disease and A29 conferring susceptibility (Rogentine, G.N., *et. al.*, 1977; Terasaki, P.I., *et. al.*,1977; Romano,P.J., *et. al.* 1991). A recent study of the LT-α polymorphism in a group of 135 lung cancer patients has identified an association between TNFB*2 homozygosity and resistance to lung cancer and also with an improved prognosis of patients with the disease (Shimura, T., *et. al.*, 1994).

It is clear from these studies that polymorphisms at the TNF locus could be of great importance in the attempt to understand the role of TNFα and the MHC in human disease. However, the mechanism behind the effects seen of these polymorphisms is still not understood and will require further study. Studies performed in the mouse perhaps give the best possible chance to allow a better understanding of the function of the TNF polymorphisms. There are a number of studies that have demonstrated a relationship between murine TNF locus polymorphisms and disease. The New Zealand White (NZW) mouse strain probably represents the best murine model for studying the association between TNF polymorphisms and disease. This mouse when crossed with the New Zealand Black (NZB) mouse results in an F1 generation which are susceptible to a lupus nephritis. An RFLP in the mouse TNFα gene has been related to the H-2 susceptibility of this disease and studies have shown that this polymorphism correlates with reduced TNFα production by NZW mice peritoneal macrophages and it has also been shown that treatment with rTNFα can delay

disease onset (Jacob, C.O. and McDevitt, H.O., 1988). The contribution of the NZW parent to the disease has been mapped to a single dominant allele within or very close to the mouse MHC (Kotzin, B.L. and Palmer, E. 1987). Although the RFLP described by Jacob and McDevitt does not define a unique allele corresponding to susceptibility to this disease a recent study by Jacob and Hwang (1992) identifies a unique TNFα microsatellite allele in the NZW mouse and an associated unique HSP70 allele and the authors suggest that these polymorphisms could be important in defining inter-individual differences which result in susceptibility to disease, whilst in an earlier paper Jongeneel *et. al.* (1990) also describe a particular TNFα microsatellite allele unique to the NZW mouse strain. An association has also been described between a two TNFα polymorphisms in the mouse and resistance to toxoplasmic encephalitis (Freund, Y.R., *et. al.*, 1992), whilst Vincek *et. al.* (1993) also show a correlation with a TNFα polymorphisms and the reduction of cutaneous immunity on exposure of mice to UV-B.

It is far easier in the mouse to determine whether the relationships identified between TNF polymorphisms and disease are truly linked to the function of the TNF genes or whether they are just markers of a disease association with the MHC, or other close by genes, in that it is easy to treat mice with either recombinant TNFα or with antibodies against TNFα. It will be by extrapolating from the mouse studies that we will be able to gain the most insight into the function of TNF polymorphisms. For example in Jacob and McDevitt's (1988) lupus nephritis model it is clear that treatment with TNFα can significantly delay the onset of the disease and that therefore the TNF polymorphism must be at least a marker of impaired TNFα production.

1.4 Aims of this project

The role of TNF in the tumourigenesis of colorectal cancer is still unclear, however it has been shown that TNF is produced in very small quantities within colorectal tumours. A number of studies have shown that polymorphisms within the TNF locus can alter the secretion of TNFα by stimulated monocytes and a number of further studies have shown significant correlations between these polymorphisms and a number of disease in which TNF has been implicated as a major factor in the disease. It was therefore of interest to examine the role of these polymorphisms in colorectal carcinogenesis. Outlined below is a list of the six major aims of this work.

1. To develop a protocol for examining the association between polymorphisms within the TNF locus and malignant disease.

2. To correlate genotypes and alleles of the TNF polymorphisms with the presence of colorectal adenocarcinoma.

3. To establish if there is any correlation with genotypes and alleles of the TNF polymorphisms and the commonly used Dukes' system for the pathological grading of colorectal tumours.

4. To establish if any correlations could be detected between the genotypes and alleles of these polymorphisms and the progression of colorectal cancer patients to either local recurrence or metastatic disease subsequent to a curative resection of the primary tumour.

5. To determine if extended genotypes exist between the alleles of the polymorphisms at the TNF locus and to examine the role of such extended genotypes in colorectal cancer.

6. To examine briefly, in a small pilot study, if similar correlations could be made between these polymorphisms in gastric adenocarcinoma.

Subjects

2.1 Colorectal adenocarcinoma patients

The follow-up clinic of Professor C. S. McArdle was used as a source for all of the patients represented in this thesis. Patients attending this clinic had previously either received surgery for the resection of a primary colorectal tumour within Glasgow Royal Infirmary under the care of C. S. M. or had been referred to the Unit for treatment of hepatic metastases from primary colorectal tumours. Patients undergoing surgery for colorectal cancer under the care of C. S. M. are requested to attend a follow-up clinic every 3 months for the first year, followed subsequently by yearly appointments, or until further intervention is required. All patients undergo colonoscopy and computed tomographic scanning (CT scanning) at routine intervals. In total 100 consecutive patients were entered into this study. Figure 2.1 shows a complete list of the clinical data obtained for all of the patients in this study.

2.1.1 Epidemiology

The most recent statistics for Scotland shows that colon and rectum cancer accounted for 2,957 cases of cancer in 1991 representing 13% of all cancers (Directorate of information services, Scottish health statistics, 1993). The male-to-female ratio reported in this same study for colon was 1:1.18 and for rectum was 1.24:1 giving an overall ratio for colorectal cancer of 1:1.06. The male-to-female ratio in the study group presented here is 1.13:1 with 53 males and 47 females. Chi squared analysis of the male to female ratio from our population and the 1991 data for Scotland shows no significant deviation from the expected frequency (χ^2= 0.58, ρ<0.45).

Code #	D.O.B	D.O.Death	Last Seen	Stage	D.O.Op	Clinical	Type of Op.	Pathology
10001	17/07/45	25/06/94	25/06/94	B	21/08/91	Mets + Local	AP Resection	Mod well Diff Adenocarc
10002	01/07/41		27/04/94	C	25/01/92	Mets + Local Recur	Anterior Resection	Mod Well Diff Adenocarc
10003	12/10/60		28/04/94	C	07/02/92	Clear	Right Hemicolectomy	Transmural Infil Adenocarc
10004	17/10/18	09/02/94	09/02/94	B	30/06/92	Clear	Anterior Resection	Mod Well Diff Adenocarc
10005	09/03/29		30/06/94	B	21/04/92	Clear	Right Hemicolectomy	Mod Well Diff Adenocarc
10006	08/05/40	14/02/93	14/02/93	C	13/10/92	Widespread Mets.	Sigmoid Colectomy	Mod Well Diff Adenocarc
10007			04/07/94			Liver Mets.		
10008	18/11/20		30/06/94	B	06/10/92	Clear	Right Hemicolectomy	Mod Diff Adenocarc
10009	18/09/26		28/04/94	B	29/09/92	Clear	Left Hemicolectomy	Mod Well Diff Adenocarc
10010	09/08/36		19/07/94			Liver Mets.	Initial surgery at Inverclyde	
10011	13/11/26	29/06/94	29/06/94	C	29/09/92	Local Recur	AP Resection (Anterior 4/91)	Mod Diff Adenocarc
10012	06/07/19		31/03/94	C	13/08/91	Clear	Left Hemicolectomy	Well Diff Adenocarc
10013	09/11/90		25/11/93	B	09/11/90	Clear	Extended Right Hemicolectomy	Mod Diff Adenocarc
10014	10/03/37		14/06/94	B	16/07/91	Mets	Right Hemicolectomy	
10015	12/11/16		28/10/93	C	04/08/92	Mets	Sigmoid Colectomy	Mod Diff Adenocarc
10016	12/03/39		30/04/94	B		Clear	Sigmoid Colectomy	Mod Diff Adenocarc
10017	04/01/25		26/05/94	C	02/06/92	Clear	Anterior Resection	Mod Well Diff Adenocarc
10018	30/12/31		31/03/94	C	22/01/91	Clear	Hemicolectomy	
10019	06/03/31				19/11/92	Liver Mets.	Initial Surgery in Gateshead	
10020	02/01/14		26/05/94	B	24/11/92	Local Recur	Hemicolectomy & Part. Gast.	Mod Diff Invasive Adenocarc
10022	05/02/17				21/04/93	Clear		
10023	08/08/29		30/06/94	A	22/12/92	Clear	Sigmoid Colectomy	Mod Diff Adenocarc
10025	19/01/20		30/06/94	B	02/02/90	Clear	Anterior Resection	Mod Well Diff Adenocarc
10026	11/11/35		14/06/94	B	04/04/89	Mets.		Mod Well Diff Adenocarc
10027	29/05/34		16/12/93	A	13/12/80	Clear	Anterior Resection	Mod Diff Adenocarc
10028	16/09/34		30/06/94	B	19/01/90	Clear	Sigmoid Colectomy	Well Diff Adenocarc
10029	09/07/21	21/04/94	24/01/94	C	27/07/92	Clear	Right Hemicolectomy	Poorly Diff Adenocarc
10030	04/07/29		31/03/94	B	25/08/92	Clear	Sigmoid Colectomy	Invasive Mod Diff Adenocarc
10031	24/04/28	25/10/93	25/10/93	B	25/08/92	Clear	AP Resection & Hysterectomy	Mod Diff Adenocarc
10032	09/12/23		26/05/94	B	03/12/91	Clear	Sigmoid Colectomy	MOd Diff Adenocarc
10033	27/11/07		28/04/94	B	26/08/92	Clear	Right Hemicolectomy	Mod Diff Adenocarc
10034	24/08/22		31/03/94	C	13/08/91	Clear	Hartmann's Proceedure	Mod Diff Adenocarc
10035	27/06/21		31/03/94	B	24/03/92	Clear	Right Hemicolectomy	Poorly Diff

Code #	D.O.B	D.O.Death	Last Seen	Stage	D.O.Op	Clinical	Type of Op.	Pathology
10036	01/10/32		30/06/94	B	06/02/91	Clear	Hartmann's Proceedure	Mod Well Diff Adenocarc
10037	14/08/12			B	10/12/91	Clear	Anterior Resection	
10038	28/12/44		26/05/94	C	19/05/92	Clear	Right Hemicolectomy	Poorly Diff Adenocarc
10039	09/08/34		14/06/94	C	09/05/92	Mets	Hepatectomy (11/91 Hemi.)	
10040	10/08/56			C	22/12/92	Mets	Right hemicolectomy	Mod Well Diff Adenocarc
10041	01/11/14	29/12/93	29/12/93	D	19/01/93	Mets.	Right Hemicolectomy	Mod Diff Adenocarc
10042	11/11/29		14/10/93	A	13/05/92		AP Resection	Mod Diff Adenocarc
10043	03/11/29		07/02/94	B	09/08/88	Clear	Total Colectomy	Mod Diff Adenocarc
10044	02/09/31		30/06/94	B		Clear	AP Resection	Mod Well Diff Adenocarc
10045	07/04/29	17/04/94	17/04/94	C	17/05/91	Mets	Sigmoid Colectomy	Mod Well Diff Adenocarc
10046	12/01/30		28/04/94	B	07/05/91	Clear	Anterior Resection	Mod Diff Adenocarc
10047	18/10/19		30/06/94	B	26/02/91	Clear	Right Hemicolectomy	Mod Well Diff Adenocarc
10048	03/02/38		26/05/94	B	27/05/90	Clear	Sigmoid Colectomy	Mod Well Diff Adenocarc
10049	26/03/33		07/02/94	C	09/09/88	Clear	Sigmoid Colectomy	Well Diff Adenocarc
10050	31/03/20			A	05/02/93		Right Hemicolectomy	Mod Diff Adenocarc
10051	09/08/55			C	31/01/92	Liver Mets	Sigmoid Colectomy	Mod Diff Adenocarc
10052	04/12/20		26/05/94	B	31/07/90	Local Recur	Left Hemicolectomy	
10053	21/05/20		30/06/94	A	28/12/88	Clear	Transverse Colectomy	Mod Well Diff Adenocarc
10054	27/06/27		26/05/94	C	09/02/88	Clear	Perineal Resection	Invasive Well Diff Adenocarc
10055	07/12/22		31/05/94	A	29/11/88	Local + Mets	Right Hemicolectomy (2/92 Rec)	
10056	12/03/39		31/03/94	B	05/10/91	Clear	Extended Right Hemicolectomy	Mod Diff Adenocarc
10057	23/07/39		26/05/94	B	15/01/91	Clear	Left Hemicolectomy	Well Diff Adenocarc
10058	15/11/21	20/07/93	20/07/93	A	08/05/90	Clear	Sigmoid Colectomy	Mod Well Diff Adenocarc
10059	15/04/33		26/05/94	B	02/06/92	Clear	Anterior Resection	Well Diff Adenocarc
10060	12/03/26	20/10/93	20/10/93	C	17/02/93	Mets	Right Hemicolectomy	Mod Well Diff Adenocarc
10061	17/09/16		30/06/94	B	17/09/91	Clear	Right Hemicolectomy	Mod Diff Adenocarc
10066	19/02/22		30/06/94	Bx2	12/05/94	Clear	Right Hemicolectomy	Mod Diff Mucinous Adenocarc
10068	12/08/25		30/06/94	C	03/09/91	Clear	Anterior Resection	Mod Diff Mucoid Adenocarc
10069	03/01/06		31/03/94	B	16/10/90	Clear	Extended Right Hemicolectomy	Ulcerating Mod Well Diff Adeno
10070	03/08/33		24/02/94	C	18/01/89	Clear	Right Hemicolectomy	Mod Diff Adenocarc
10071	25/09/22		30/06/94	B	01/05/90	Mets	Partial colectomy	Well Diff Adenocarc
10072	02/12/16		28/10/93	B	16/03/93	Clear		Mod Diff Adenocarc
10073	12/06/05		14/12/93	B	13/12/90	Mets	Right Hemicolectomy	Mod Diff Adenocarc

Code #	D.O.B	D.O.Death	Last Seen	Stage	D.O.Op	Clinical	Type of Op.	Pathology
10074	21/03/26		28/04/94	C	22/02/93	Clear		Mod Well Diff Mucin Sec Adenoc
10075	26/03/29		28/04/94	C	04/05/93	Clear	Anterior Resection	Poorly Diff Adenocarc
10076	30/05/15		09/11/93	B	03/10/89	Clear		Mod Well Diff Adenocarc
10077	05/08/22		13/07/94	B	29/06/93	Local	-Date of Rectal Biopsy	Poorly Diff Adenocarc
10078	20/06/28		26/05/94	B	15/10/93	Clear	Sigmoid Colectomy	Adenocarc
10079	07/07/39		19/04/94	D	20/10/93	Mets.	Hemicolectomy	Mod Diff Adenocarc
10080	31/07/40		28/04/94	C	30/07/93	Clear		Mod Diff Adenocarc
10081	07/11/31		26/05/94	B	22/06/93	Clear	Right Hemicolectomy	Poorly Diff Adenocarc
10082	10/08/26		26/05/94	B	03/08/93	Clear	Hemicolectomy	Mod Diff Adenocarc
10083	18/05/26		30/06/94	B	15/06/93	Clear	Right hemicolectomy	Mod Well Diff Adenocarc
10084	08/05/14		28/10/93	B	10/10/89	Clear	Sigmoid Colectomy	Mod Diff Adenocarc
10085	13/03/10		07/02/94	B	23/04/87	Clear		Mod Well Diff Adenocarc
10086	29/12/33		28/10/93	B	28/02/85	Clear	AP Resection	Mod Well Diff Adenocarc
10087	14/08/27		26/05/94	C	09/11/93		AP Resection	Mod Diff Adenocarc
10088	18/04/15		07/07/94	B	09/09/85	Clear	Colectomy	Mod Diff Adenocarc
10089	25/04/13		21/09/93	B	23/10/93	Clear	Right Hemicolectomy	Poorly Diff Adenocarc
10090	03/11/22		26/05/94	C	28/01/92	Mets	Anterior Resection	Mod Well Diff Adenocarc
10091	05/08/29		26/08/93	B	07/05/93	Clear		Well Diff Infiltrat Adenocarc
10092	06/05/33	27/11/93	27/11/93			Mets	Initial Surgery Hirmyres	
10093	22/12/32		26/05/94	B	16/11/93		Right Hemicolectomy	Mod Diff Mucoid Adenocarc
10094	10/10/24		28/04/94	B	27/04/93	Clear	Sigmoid Colectomy	Mod Well Diff Adenocarc
10095	27/03/43	19/06/94	19/06/94			Mets		Invasive Adenocarc
10096	15/06/22		28/07/94	B	15/06/93	Clear	Right Hemicolectomy	Mod Diff Adenocarc
10097	28/08/20		25/11/94	B	02/12/86	Clear	Right hemicolectomy	Mod Diff Adenocarc
10098	28/11/23		31/03/94	B	09/03/93	Clear	Anterior Resection	Mod Diff Adenocarc
10099	03/08/0440		25/11/94		28/03/89	Clear	Sigmoid Colectomy	
10100	15/03/26		31/03/94	B	25/07/93	Clear	Hartmann's Proc.	Mod Diff Adenocarc
10101	22/05/41		31/03/94	C	04/09/93	Clear	Sigmoid Colectomy	Well Diff Adenocarc
10102	19/09/44		18/03/94	D	17/07/92	Mets.	Hartmann's Proc.	Mod Well Diff Adenocarc
10103	26/03/29		28/04/94	C	04/05/93		Anterior Resection	Poorly Diff Adenocarc
10104	06/05/43	12/10/93	12/10/93	D		Mets		
10105	22/01/27	20/11/93	20/11/93	D		Mets.	Initial Surgery at Victoria	
10106	27/08/36		08/07/94	D		Mets.	Initial Surgery at Inverness	

Figure 2.1. Complete list of the clinical data obtained for all of the patients in this study

It is widely accepted that cancer is a disease of the elderly and colorectal cancer is no exception. The mean age of patients in this study group was 66 with a maximum age of 88 and a minimum age of 34.

Family history was obtained, where possible, from all of the patients entering into this study. No patient reported any form of family history of colorectal adenocarcinoma that they could recall and no further examination of the families of these patients was undertaken due to the restraints of time on both the clinical and scientific staff within the department. These results are unusual given the frequency of hereditary colorectal cancer. From a population of 100 individuals statistically you would expect at least five individuals to have HNPCC and at least one individual to have FAP, however with out further analysis these data remain unavailable.

2.1.2 Pathology

Pathology reports were obtained for all of the patients which had undergone surgery within Glasgow Royal Infirmary. Full pathological data was obtained for 81 of the 100 (81%) patients entered into this study and is detailed in Figure 2.1. Of 81 primary tumours where pathological reports were available, these were graded as follows:

8 well-differentiated adenocarcinomas,

1 invasive well-differentiated adenocarcinoma,

25 moderately well-differentiated adenocarcinomas,

1 moderately well -differentiated mucin-secreting adenocarcinoma,

1 ulcerating moderately well-differentiated adenocarcinoma,

31 moderately-differentiated adenocarcinomas,

2 moderately-differentiated invasive adenocarcinomas,

1 moderately-differentiated mucoid adenocarcinoma,

8 poorly-differentiated adenocarcinomas,

1 transmural invasive adenocarcinoma,

1 invasive adenocarcinoma,

1 adenocarcinoma.

Although these descriptions are clinically useful, scientifically they are meaningless since they give no indication of the true invasiveness of the tumours. The original and most widely-used system for the grading of rectal tumours is that described by Dukes (Dukes, C.E., 1932). This staging system proposed a method of describing the level of penetration of the tumour mass into the bowel wall with stage A indicating penetration into, but not through, the bowel wall, stage B indicating penetration through the bowel wall and stage C indicating involvement of lymph nodes regardless of the degree of bowel wall penetration. A number of refinements have been made to this classification system to take into account the finer levels of penetration of the bowel wall and the degree of nodal metastases. The Dukes' staging system is now commonly used in the classification of all colorectal adenocarcinomas.

The original Dukes' staging system did not contain any classification for haematologically disseminated metastatic disease. In 1949, Dukes characterised a fourth stage in which he described the disease as being beyond the limits of surgical resection. In 1967 Turnbull *et. al.* formally defined this fourth stage as stage D, in which distant metastasis had occurred independent of the degree of penetration of the tumour through the bowel wall or lymph node involvement.

Figure 2.2 shows a comparison of the more descriptive American Joint Committee on Cancer and the Union Internationale Contre le Cancer staging classification system for colorectal cancer, with Dukes' staging. This system takes into account a

Primary Tumour (T)

TX	Not Assessable
T0	No tumour in resected sample
Tis	Carcinoma in situ
T1	Invades submucosa
T2	Invades muscularis propria
T3-T4	Serosa present:
	T3 Invades through muscularis propria into subserosa
	T4 Invades through serosa into peritoneal cavity or into contiguous organs
	Serosa absent:
	T3 Invades through muscularis propria
	T4 Invades other organs (vagina, prostate, ureter, kidney)

Regional Lymph Nodes (N)

NX	No assessable nodes
N0	No regional node metastases
N1	1-3 nodes positive
N2	4 or more nodes positive
N3	central nodes positive

Distant Metastases (M)

MX	Not assessable
M0	No distant metastases
M1	Metastases present

Corrrelation with TNM and Dukes' Staging

Dukes' A	T1N0M0
	T2N0M0
Dukes' B	T3N0M0
	T4N0M0
Dukes' C	T(any)N1M0
	T(any)N2M0
	T(any)N3M0
Dukes' D	T(any)N(any)M1

Table 2.2. Comparison of the original Dukes' staging system for colorectal malignancy with the AJC/UICC classification system.

number of important clinical factors such as degree of invasion, degree of lymph node metastases and degree of distant metastases.

Dukes' staging was available for 92 of the 100 patients represented in this study. These included 7 (8%) stage A tumours, 50 (54%) stage B tumours, 28 (30%) stage C tumours and 7 (8%) stage D tumours. In a study of 70 patients from the west of Scotland by Finlay and McArdle (1986) the authors reported a distribution of 10% stage A tumours, 43% stage B and 47 % stage C. If we remove the stage D patients from this study and perform a Chi square analysis on the relative distribution of the three stages between the two studies we find that there is no significant difference between the Dukes' stage distribution between the two studies (χ^2=3.99, ρ=0.0678).

2.1.3 Survival analysis

The date of initial operation, date of "last seen" within the Department of surgery and date of death were recorded where possible for all patients within this study. Figure 2.3 shows a Kaplan-Mier survival analysis for 68 patients for whom date of initial operation and date of last appointment in clinic or date of death was available. The three-year survival rate for these patients is over 75%. The high survival rate seen within this population precludes a formal analysis of the difference in survival between subgroups of these patients determined by sex, Dukes' staging, site of disease and pathology of primary tumour. However it is widely accepted that there is some difference in mortality rate between males and females with women having a more favourable prognosis than men (Chapuis, P.H., *et. al.*, 1985).

The degree of invasiveness as defined by Dukes' staging is known to have a significant effect on the survival of colorectal cancer patients. There is an expected five-year survival in the west of Scotland of 100% for stage A, 67% for stage B, 39% for stage C and 6% for individuals with overt liver metastases (Finlay, I.G, and

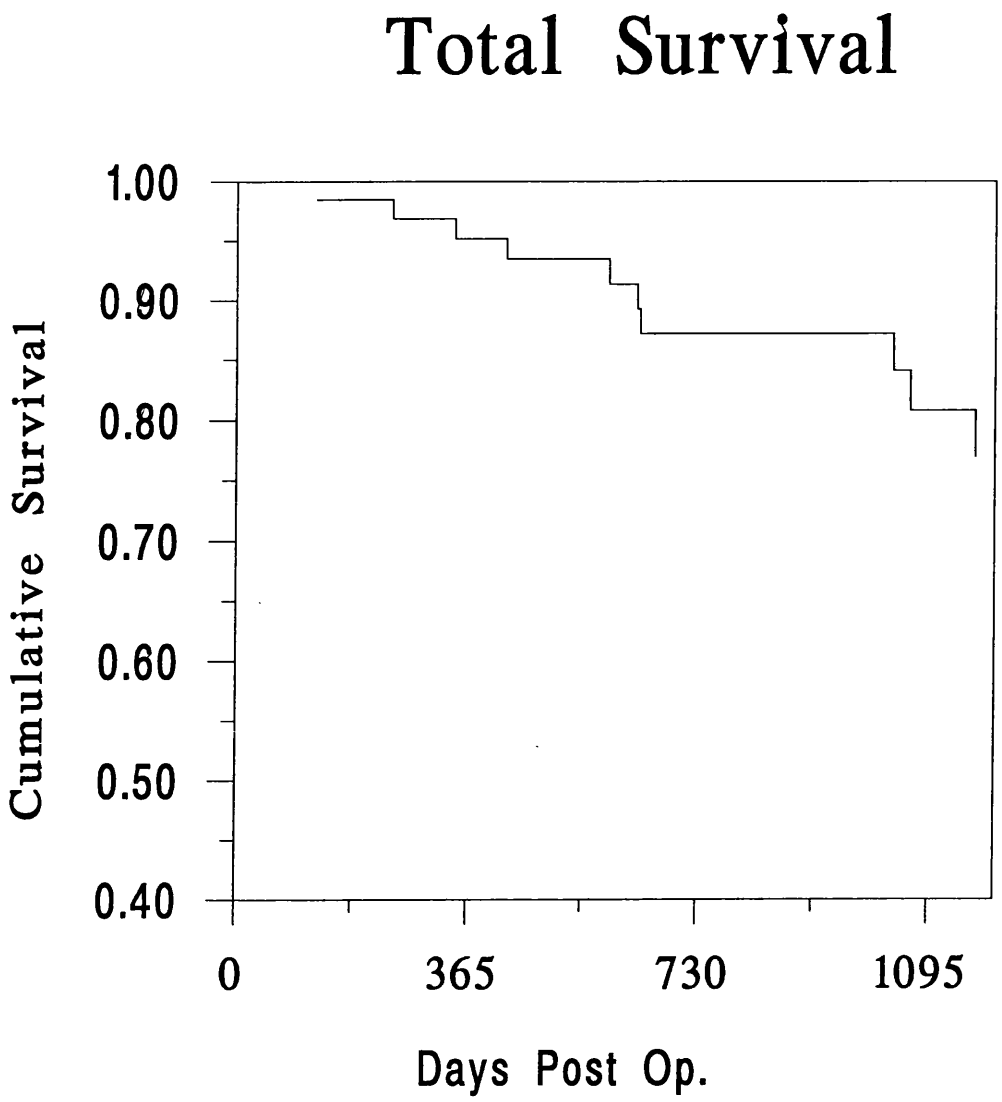

Figure 2.1. Kaplan-Meir survival curve for 68 patients for whom complete follow up data was available.

McArdle, C.S., 1986). Eisenberg *et. al.* (1982) report five-year survival figures of 82% for stage A, 73% for stage B and 40% for stage C whilst Willet *et. al.* (1984) report 97% for T1N0M0 tumours (~Stage A), 90% for T2N0M0 tumours (~Stage A), 78% for T3N0M0 tumours (~Stage B), 63% for T4N0M0 tumours (~Stage B), 74% for T2N1M0 tumours (~Stage C), 48% for T3N1M0 tumours (~Stage C) and 38% for T4N1M0 tumours (~Stage C).

Although these figures are interesting in terms of survival for these patients they have no bearing on the results of this study and are therefore reported only for completeness.

2.1.4 Metastatic disease

It was important to ascertain whether any association between polymorphisms at the TNF locus and the presence of colorectal cancer also showed an association with the development of metastatic disease within this study population. Development of metastatic disease was determined following standard clinical procedures including the use of CT scanning. 24 of the 100 patients within this study either had metastatic disease at the time of initial surgery or subsequently developed metastatic disease with or without local recurrent disease, whilst 4 patients developed local recurrent disease (Figure 2.1).

2.2 Control individuals

115 samples of peripheral blood genomic DNA from control individuals were obtained as a generous gift from the department of Tissue Typing within Glasgow Royal Infirmary.

This DNA represents a random sample obtained from individuals entering a number of studies within the Tissue Typing department and including a number of volunteers for bone marrow donation. Very little data is available regarding these individuals, however no individuals at the time of donation of blood were reported to have any form of malignant disease. Data regarding the sex of the donors were available for 69 of the 115 controls. 36 of the 69 control individuals are male and 33 are female, giving a male to female ratio of 1.09:1 compared to a ratio of 1.13:1 for the colorectal cancer patients. Chi square analysis of the sex distribution of the two populations shows no difference between the two groups (χ^2=0.01, ρ=0.92).

Materials and Methods.

All reagents used within this study were obtained from Sigma unless otherwise stated.

3.1 Isolation of genomic DNA.

Since a good source of full-length genomic DNA was central to the success of this study it was decided from the outset that the traditional 'salting out' method for the isolation of genomic DNA would be used, rather than turning to (and having to rely upon) commercially available kits which at the time seemed only to be able to offer a low success rate at a high cost.

In theory the Polymerase Chain Reaction (PCR) is capable of amplifying one fragment of intact genomic DNA. In practice, however, it is clear that a number of factors do influence the ability of Taq DNA polymerase to amplify genomic DNA. The key factor in the reaction is of course the target DNA, and although many other factors can influence the reliability of the reaction, none of these will enable successful amplification with a poor preparation of the target DNA.

3.1.1 Collection of peripheral venous blood

7 ml of whole peripheral venous blood from consenting patients was collected into either pre-chilled blood tubes or into a "Vacutainer" containing 54µl of 0.34M tri-potassium ethylene diamino tetra-acetic acid (K_3 EDTA) and placed on ice prior to processing. Whole blood samples were stored for no longer than 3 hours prior to the isolation of peripheral blood mononuclear cells (PBMCs).

3.1.2 Isolation of PBLs from peripheral venous blood

K_3 EDTA-treated peripheral venous blood was removed from the collection tubes and placed into 50ml centrifuge tubes (Greiner). Three volumes of a red blood cell (RBC) lysis solution (8.3% w/v ammonium chloride, 0.037% w/v EDTA, 1% w/v potassium hydrogen carbonate) was placed into each of the 50ml tubes and the contents mixed gently. Tubes were then incubated at room temperature for 15 minutes. Whole cells remaining were collected by centrifugation at 1,400 rpm for 10 mins at room temperature. The supernatant was discarded and the process repeated a further two times. At this stage if whole RBCs were still present in the white cell pellet then the process of incubation in the RBC lysis solution was repeated one further time. However, if the white cell pellet was seen to be free from RBC contamination then the resultant cells were resuspended in a 500 µl volume of sterile phosphate buffered saline (PBS: 0.8% w/v sodium chloride, 0.02% w/v potassium chloride, 0.144% w/v disodium hydrogen phosphate, 0.02% w/v potassium dihydrogen phosphate pH 7.4) and transferred to 1.5 ml microcentrifuge tubes. The resultant purified PBL suspension was then stored at a temperature of -20 °C until required for DNA purification.

3.1.3 Isolation of full-length genomic DNA from whole cells.

Human PBLs were isolated as above and allowed to thaw on ice if frozen. The PBLs were lysed as follows.

To each 500µl sample of PBL suspension, 500µl of a 0.6M solution of sodium acetate was added (final concentration of 0.2M) and the solutions mixed gently. 150 µl of a 10% w/v aqueous solution of sodium dodecyl sulphate (SDS; final concentration of 1% w/v), 150µl of a 1 mg/ml solution of Proteinase K (final concentration of 0.1mg/ml; Stratagene) and 200µl of sterile water were subsequently

added to the PBL sodium acetate mixture and the solutions were again mixed gently. Digestion was performed at 37°C for a period of 48 hours.

The resultant viscous solution was split between two 1.5 ml microcentrifuge tubes and the cellular protein removed by a phenol:chloroform extraction. An equal volume of Tris EDTA (10mM Tris-Cl, 0.5mM EDTA, pH 8.0) saturated phenol:chloroform (1:1 v/v) was added and the solutions mixed. The aqueous and solvent layers were separated by centrifugation in a microcentrifuge at 13,500 rpm for 10 mins at room temperature. The upper aqueous layer (containing the genomic DNA) was then aspirated by pipetting and placed into a new microcentrifuge tube and a fresh equal volume of Tris EDTA saturated phenol:chloroform was added. The solutions were again mixed and the aqueous and solvent layers again separated by centrifugation and the upper aqueous layer was aspirated and placed into a further new microcentrifuge tube and this time an equal volume of chloroform was added. The solutions were mixed and the aqueous and solvent layers were separated again by centrifugation and the aqueous layer aspirated and placed into a further new microcentrifuge tube. Genomic DNA was precipitated by adding a 10th volume of 5M ammonium acetate and 1 ml of ice cold 100% pure ethanol. The precipitated genomic DNA was removed from the mixture by spooling onto a sealed glass pasture pipette. The DNA, still attached to the pipette, was washed in 70% pure ethanol, then sterile water and left to resuspend in a 500µl volume of sterile, UV treated, water overnight at 37°C.

Once the DNA had resuspended a 10µl sample was removed, diluted in 990µl of sterile water, and used to calculate the concentration of the stock DNA by measuring absorbance at 260nm. One $O.D._{260}$ unit was taken to represent 50µg per ml of undiluted genomic DNA. The genomic DNA was aliquoted, coded and then stored at 4°C.

3.2 Confirmation of integrity of genomic DNA

In order that the integrity of the isolated genomic DNA be controlled I have adopted a scheme by which all DNA samples were analysed by horizontal submarine gel electrophoresis on a 1% w/v LE SeaKem agarose gel (FlowGen).

3.3 Genotype determination at the -308 TNFα RFLP Locus

3.3.1 PCR primers

Oligonucleotide PCR primers were synthesised according to published sequences (Wilson, A.G., *et. al.*, 1992) on an Applied Biosystems automated synthesiser.

-308TNFα 5' 5'- AGGCAATAGGTTTTGAGGGCCAT -3'

-308TNFα 3' 3'- TCCTCCCTGCTCCGATTCCG -3'

Primers were obtained as an ammonia elution and were subsequently deprotected by incubation at 56°C over night. The primers were purified by precipitation, by adding a 10^{th} of a volume of 5M ammonium acetate (ammonium acetate was used due to its ability to volatilize at 37°C during the subsequent rotary evaporation) and 2 volumes of ice-cold 100% pure ethanol and incubating at -70°C for 1 hour. The precipitated oligonucleotide was pelleted by centrifugation at 14,000 rpm and 4°C for 30 mins straight from the -70°C freezer. The resultant oligonucleotide pellet was washed in 70% pure ethanol, centrifuged for a further 15 mins and the ethanol evaporated off by centrifugation in a rotary evaporator. The lyophilised oligonucleotide pellet was resuspended in a 500µl volume of sterile, UV treated water and the concentration calculated by measuring the optical density at 260nm (one $O.D._{260}$ unit is taken to represent 33µg per ml of single stranded oligonucleotide DNA) and the molarity of

each of the primer solutions calculated and noted for future reference. (Note- no further dilution of the stock solutions were made at this point). The resultant stock primer solutions were stored at -20°C until required. Before use primers were diluted to give a 10μM working solution to be used at a 1:10 dilution to give a final concentration of 1μM per reaction.

3.3.2 PCR amplification

250ng of genomic DNA was added to 45μl of a PCR master mix containing 1μM of each primer, 1 unit of Taq DNA polymerase (Applied Biotechnology), 2mM dATP, dTTP, dCTP, dGTP (GibcoBRL), 1.5mM magnesium chloride (Applied Biotechnology) and a 1x final concentration of reaction buffer (Applied Biotechnology). The samples were overlayed with 50 μl of light mineral oil to eliminate evaporation during cycling and centrifuged briefly to separate the two layers.

The samples were subjected to temperature cycling on a Biometra Uno Block thermocycler as follows

Step 1	Melting temp	94°C for 180 seconds	
	Annealing temp	60°C for 60 seconds	
	Extension temp	72°C for 60 seconds	
Step 2	Melting temp	94°C for 60 seconds	
	Annealing temp.	60°C for 60 seconds	} x35
	Extension temp.	72°C for 60 seconds	
Step 3	Final extension	72°C for 300 seconds	

After amplification the PCR products were stored at 4°C until required for analysis.

3.3.3 Nco-I restriction endonuclease digestion of PCR products.

The basis of the -308TNFα RFLP is that the PCR amplification produces a 117bp fragment which incorporates a polymorphic Nco-I restriction endonuclease site within the 3' end of the PCR product. In order to assign genotypes, the resultant PCR products were therefore subjected to Nco-I restriction endonucleases digestion.

To a 10μl aliquot of PCR product 1.5 μl of appropriate 10 x buffer (GibcoBRL), 5 units of Nco-I restriction endonuclease (GibcoBRL) and sterile deionised water to a final volume of 20μl was added on ice. All reagents were mixed by vortexing and centrifuged briefly and then incubated at 37°C for 1 hour. The resultant products were analysed on a 9% polyacrylamide gel as described below.

3.3.4 Polyacrylamide gel electrophoresis (PAGE) of PCR products

10μl of restriction endonuclease digested PCR products and 10μl of the undigested PCR products were electrophoresed in parallel on 9% non-denaturing polyacrylamide gel on a 20cm x 20cm Protean II gel electrophoresis system, (Biorad) in TAE buffer, with ϕX174 digested with Hae III (GibcoBRL) as a molecular weight marker. Electrophoresis was performed at a constant current of 30 mA per gel until the bromophenol blue in the gel loading buffer was 2cm above the bottom of the gels. The gel assembly was dismantled and the gels submerged in 1x TAE containing 0.4 μg/ml w/v ethidium bromide and incubated for 30 minutes before being rinsed in 1x TAE. The resultant banding pattern was visualised and photographed under translumination with an ultraviolet (UV) light source.

Genotypes were assigned as follows;

1) **homozygosity** for the **T1 allele** as a band of **97bp** in size, plus a band of **20bp**;

2) **heterozygosity** for **T1** and **T2 alleles** as a band of **107bp** size, plus bands of **97bp** and **20bp** in size;

3) **homozygosity** for **T2 allele** as band of **107bp** in size only, (Figure 3.1).

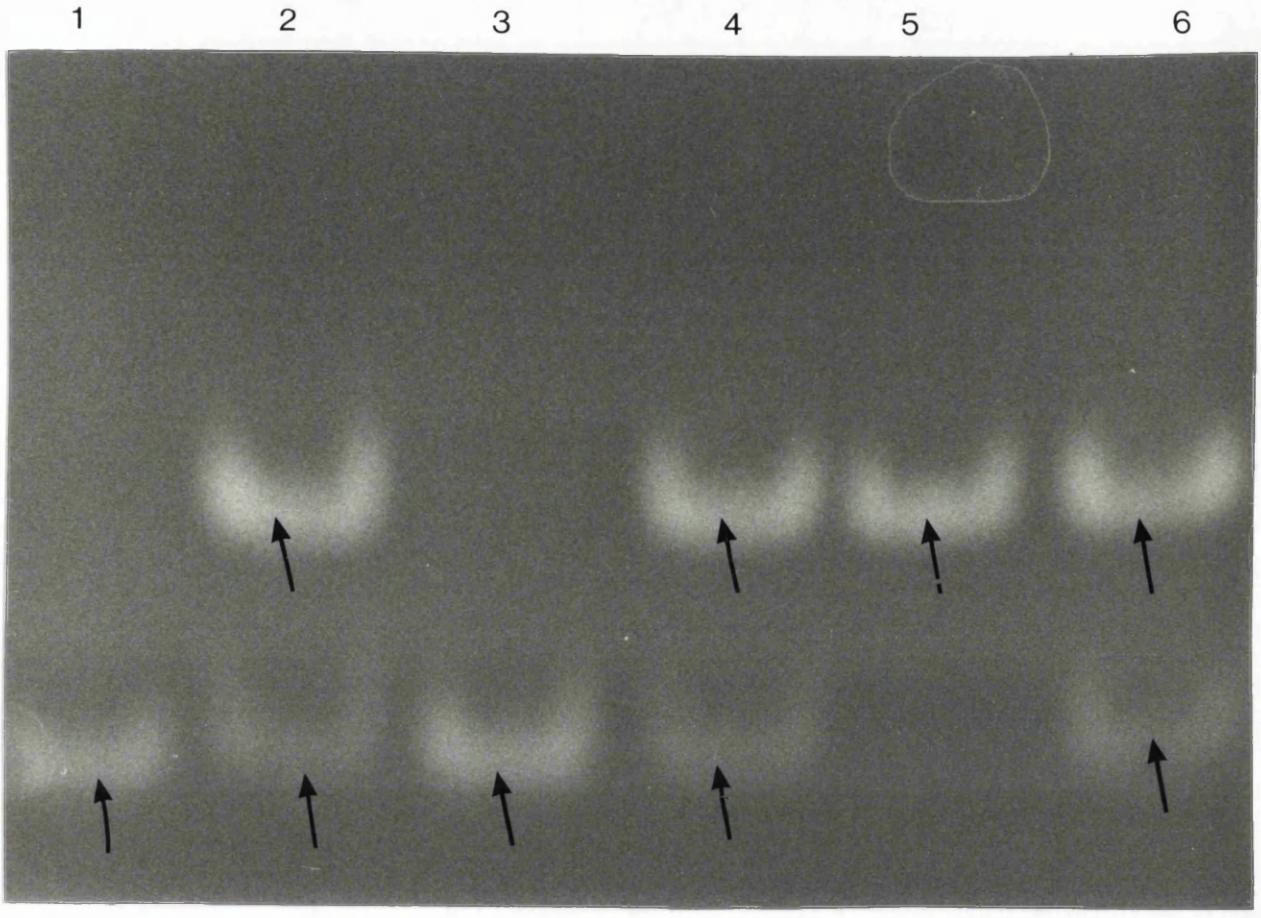

Figure 3.1. Polyacrylamide gel showing the three possible -308 TNFα RFLP genotypes. Sequence specific polymerase chain rection products are digested with *Nco*I restriction endonuclease give give two possible products per individual. Lanes 1 and 3 -homozygous TNF2 allele, Lane 2, 4 and 6 - hetezozygous for TNF1 and TNF2 alleles, Lane 5 homozygous for TNF1 allele. Note- although not shown on this photograph ϕX174 digested with *HaeIII* was routinely used as a size marker.

3.4 Genotype determination at the LTα Nco-I RFLP Locus

3.4.1 PCR primers

Oligonucleotide PCR primers were synthesised according to the published sequence (Messer,G., *et. al.*, 1991) on an Applied Biosystems automated synthesiser.

LT-α Nco-I 5' 5'- CCGTGCTTCGTGCTTTGGACTA -3'

LT-α Nco-I 3' 3'- AGAGCTGGTGGGGACATGTCTG -3'

The primers were deprotected and the resultant stock primer solution stored at -20°C until required as previously described (see section 3.3.1).

3.4.2 PCR Amplification

250ng of genomic DNA was added to 45µl of a PCR master mix containing 1µM of each primer, 1 unit of Taq DNA polymerase (Applied Biotechnology), 2mM dATP, dTTP, dCTP, dGTP (GibcoBRL), 1.5mM magnesium chloride (Applied Biotechnology) and a 1x final concentration of PCR reaction buffer (Applied Biotechnology). The samples are overlayed with 50 µl of light mineral oil to eliminate evaporation during cycling and centrifuged briefly to separate the two layers.

The samples were subjected to 40 cycles on a Biometra Uno Block thermocycler as follows

Step 1 Initial Melt 95°C for 6 mins

Step 2	Melting temp	95°C for 60 seconds	
	Annealing temp.	64°C for 60 seconds	} x40
	Extension temp.	72°C for 60 seconds	
Step 3	Final extension	72°C for 5 mins	

After amplification the PCR products were stored at 4°C until required for analysis.

3.4.3. Nco-I restriction endonuclease digestion of PCR products.

The basis of the LTα Nco-I RFLP is that the PCR amplification produces a 750bp fragment which straddles a polymorphic Nco-I restriction endonuclease site. In order to assign genotypes the resultant PCR products were therefore subjected to Nco-I restriction endonucleases digest.

To a 10µl aliquot of PCR product 1.5 µl of appropriate 10 x buffer (GibcoBRL), 5 units of Nco-I restriction endonuclease (GibcoBRL) and sterile deionised water to a final volume of 20µl were added on ice. All reagents were mixed by vortexing and centrifuged briefly and then incubated at 37°C for 1 hour. The resultant products were analysed on a 2% LE SeaKem agarose gel (Flowgen) as described below.

3.4.4 Agarose gel electrophoresis of PCR products

10µl of restriction endonuclease digest PCR products were electrophoresed in parallel on a 2% agarose gel in a TAE buffer containing 0.4 µg/ml of Ethidium Bromide, with ϕX174 digested with Hae III (GibcoBRL) as a molecular weight marker. Gels were electrophoresed at a constant current of 50 mA until all bands were clearly resolved.

Genotypes were assigned as follows;

1) **homozygosity** for **TNFB*1 allele** as bands of **500bp** and **250bp** in size only;

2) **heterozygosity** for **TNFB*1** and **TNFB*2 alleles** as a band of **750bp** size, plus bands of **500bp** and **250bp** in size;

3) **homozygosity** for the **TNFB*2 allele** as a single band of **750bp** in size (Figure 3.2).

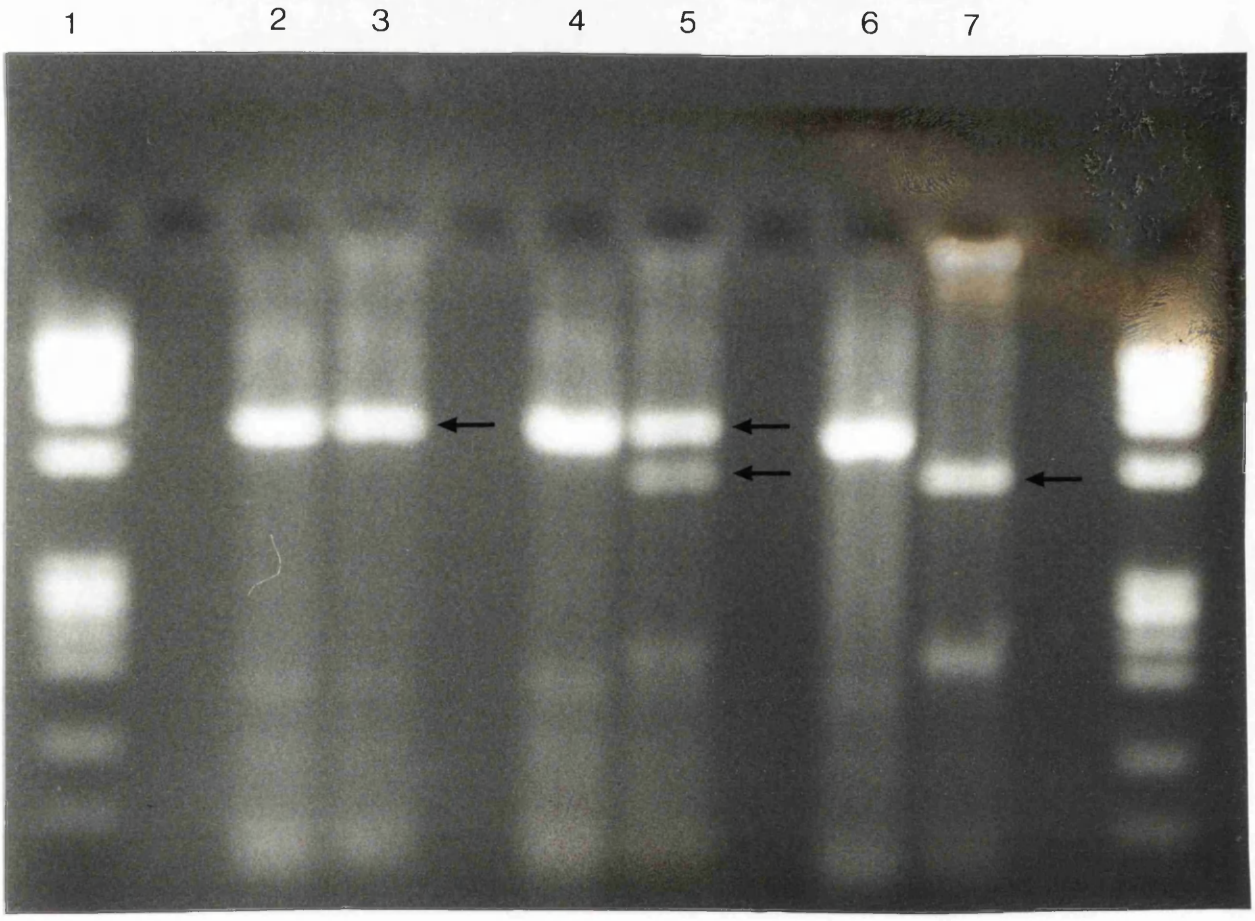

Figure 3.2. Agarose gel showing the three possible LTα *Nco*I RFLP genotypes. Sequence specific polymerase chain rection products are digested with *Nco*I restriction endonuclease give give two possible products per individual. Lane 1 - ϕX174 *Hae*III digested ladder, Lane 2 - undigested PCR product from TNFB*2 homozygous individual, Lane 3 - *Nco*I digested PCR product from Lane 2, Lane 4 - undigested PCR product from heterozygous individual, Lane 5 - *Nco*I digested PCR product from Lane 4, Lane 6 - undigested PCR product from TNFB*1 homozygous individual, Lane 7 - *Nco*I digested PCR product from Lane 6.

3.5 Genotype determination at the four TNF microsatellite polymorphism loci

3.5.1 PCR primers

Oligonucleotide PCR primers were synthesised according to published sequences (Udalova, I.A., *et. al.*, 1993) on an Applied Biosystems automated synthesiser.

TNFa 5' 5'- GCCTCTAGATTTCATCCAGCCACAG-3'

TNFa 3' 5'- CCTCTCTCCCCTGCAACACACA-3'

TNFc 5' 5'- GGGAGGTCTGTCTTCCGCCG-3'

TNFc 3' 5'- CGTTCAGGTGGTGTCATGGG-3'

TNFd 5' 5'- AGATCCTTCCCTGTGAGTTCTGCT-3'

TNFd 3' 5'- CATAGTGGGACTCTGTCTCCAAAG-3'

TNFe 5' 5'- GTGCCTGGTTCTGGAGCCTCTC-3'

TNFe 3' 5'- TGAGACAGAGGATAGAGACAG-3'

The primers were deprotected and the resultant stock primer solution were stored at -20°C, as previously described (see section 3.3.1), until required.

3.5.2 PCR amplification

100ng of genomic DNA were added to 18µl of a PCR master mix containing 1µM of each primer, 1 unit of Taq DNA polymerase (Applied Biotechnology), 2mM dATP, dTTP, dCTP, dGTP (GibcoBRL), 1.5mM magnesium chloride (Applied Biotechnology), 0.4µCi of α^{32}P dCTP (Amersham) and a 1x final concentration of PCR reaction buffer (Applied Biotechnology). The samples are overlayed with 50 µl

of light mineral oil to eliminate evaporation during cycling and centrifuged briefly to separate the two layers.

The samples were subjected to 40 cycles on a 96 well Biometra Uno Block thermocycler as follows

Step 1	Initial Melt	94°C for 5 mins	
Step 2	Melting temp.	94°C for 25 seconds	
	Annealing temp.	60°C for 60 seconds	} x40
	Extension temp.	74°C for 60 seconds	
Step 3	Final extension	74°C for 10 mins.	

After amplification the PCR products were stored, under appropriate conditions for the storage of radioactive material 4°C, until required for analysis.

3.5.3 Polyacrylamide gel electrophoresis of microsatellite PCR products.

3.5.3.1 Production of $\gamma^{32}P$ labelled 10bp ladder

1µl of 10bp ladder (GibcoBRL) was end-labelled with $\gamma^{32}P$ ATP (Amersham) by incubating with 2µl of 10x T4 polynucleotide kinase buffer (0.5M Tris.Cl pH 7.6, 0.1M $MgCl_2$,50mM dithiothreitol, 1mM spermidine HCl, 1mM EDTA pH 8.0), 5µl of $\gamma^{32}P$ ATP (~50µCi; Amersham), 11µl sterile deionised H_2O and 1µl T4 polynucleotide kinase (1 Unit/µl). The reaction mixture was centrifuge briefly before being incubated at 37°C for 30 mins.

3.5.3.2 Non-denaturing PAGE

3µl aliquots of the radiolabelled PCR products were analysed on a 15% non-denaturing polyacrylamide gel in TAE buffer on a Base Ace vertical sequencing gel rig (Stratagene) with 2 lanes of the γ ATP labelled 10bp ladder as size references (plus M31A (American Tissue Culture Collection) in the case of TNFa,b and c). Gels were run overnight at 22 mA until the bromophenol blue had run off the bottom and the xylene cyanol was within 5 cm of the bottom of the gel. The resultant gels were removed from the glass plates and dried onto Whatman 3MM filter paper on a vacuum gel dryer (Biorad).

The dry gels were incubated with X-ray sensitive photographic film overnight to produce an autoradiographic image of the gel and the electrophoretic mobility for each of the samples was measured in reference to the 10bp ladder and compared with that of the cosmid M31A which has a known TNF genotype of a8, b2 and c1 or with reference to each other in the case of TNFd and TNFe (Figures 3.3, 3.4, 3.5, 3.6). Although it is common to see shadow bands running at lower molecular weights than the actual alleles the TNFa microsatellites produced shadow bands of a higher molecular weight. This phenominon was consistant throughout the whole of this study and alleles were taken to be the most dense bands on the gels (see figure 3.3)

In order to eliminate operator bias a number of gels were analysed by two seperate individuals, the author of this thesis and Dr Grant Gallagher. In some cases duplicate samples were also run on separate gels and amplified on seperate occasions in order to confirm allele calls.

3.5.3.3 TNF allele sizes

TNFa	Size bp
1	93
2	95
3	97
4	99
5	101
6	103
7	105
8	107
9	109
10	111
11	113
12	115
13	117

TNFc	Size bp
1	96
2	98

TNFd	Size bp
1	105
2	107
3	109
4	111
5	113
6	115
7	117

TNFe	Size bp
1	108
2	110
3	112

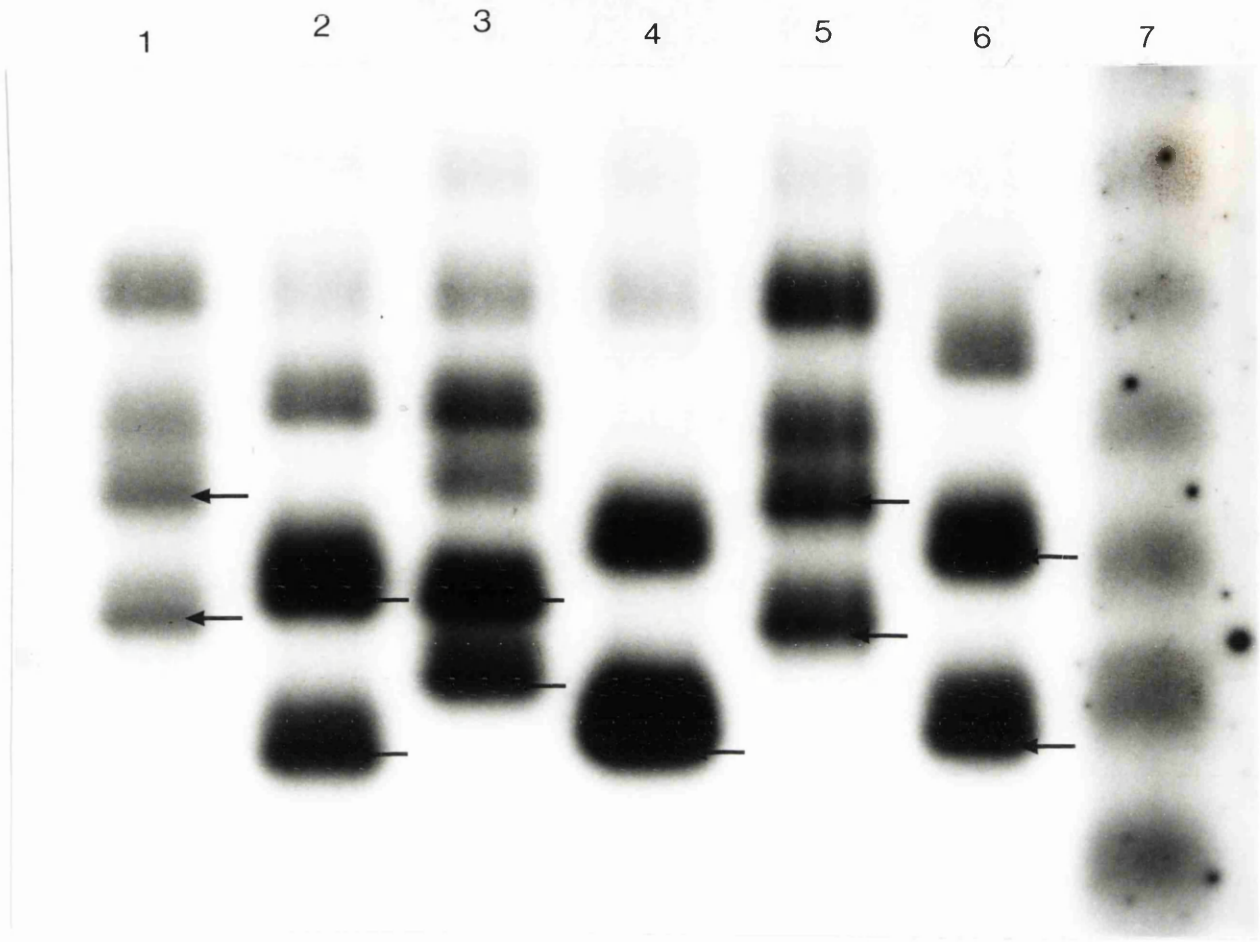

Figure 3.3. Section from a representative polyacrylamide gel showing a range of the possible TNFa microsatellite alleles. Sequence specific α ^{32}P dCTP labelled polymerase chain reaction products are separated on a 15% non-denaturing polyacrylamide gel. Lane 1 - 10103 with genotype TNFa7,a11, Lane 2 - 10104 with genotype TNFa3,a8, Lane 3 - 10105 with genotype TNFa5,a8, Lane 4 - 10106 with genotype TNFa3,a3, Lane 5 - 10069 with genotype TNFa6,a11, Lane 6 - 10070 with genotype TNFa3,a10, Lane 7 - 10bp ^{32}P γATP labelled 10bp ladder (GibcoBRL). Bands representing the alleles are marked with arrowheads.

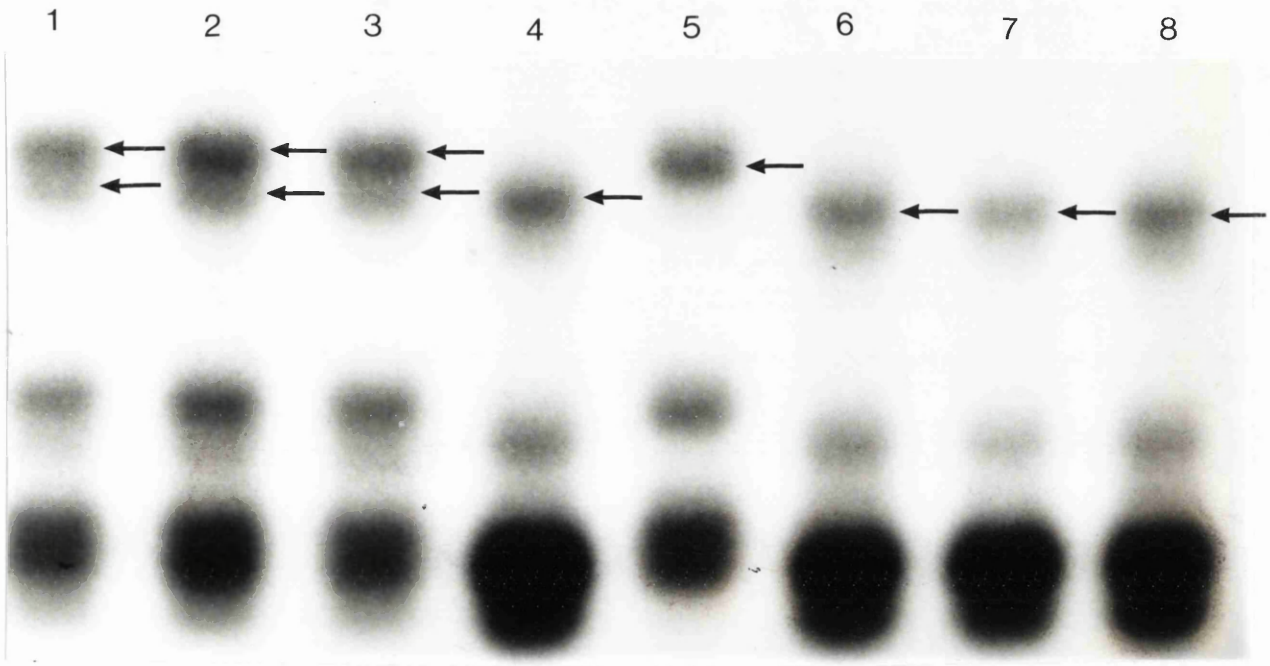

Figure 3.4. Section from a representative polyacrylamide gel showing the two possible TNFc microsatellite alleles. Sequence specific $\alpha^{32}P$ dCTP labelled polymerase chain reaction products are separated on a 15% non-denaturing polyacrylamide gel. Lane 1 - 10089 with genotype TNFc1,c2, Lane 2 - 10090 with genotype TNFc1,c2, Lane 3 - 10091 with genotype TNFc1,c2, Lane 4 - 10092 with genotype TNFc1,c1, Lane 5 - 10093 with genotype TNFc2,c2, Lane 6 - 10094 with genotype TNFc1,c1, Lane 7 - 10095 with genotype TNFc1,c1, Lane 8 - 10096 with genotype TNFc1,c1. Bands representing the alleles are marked with arrowheads.

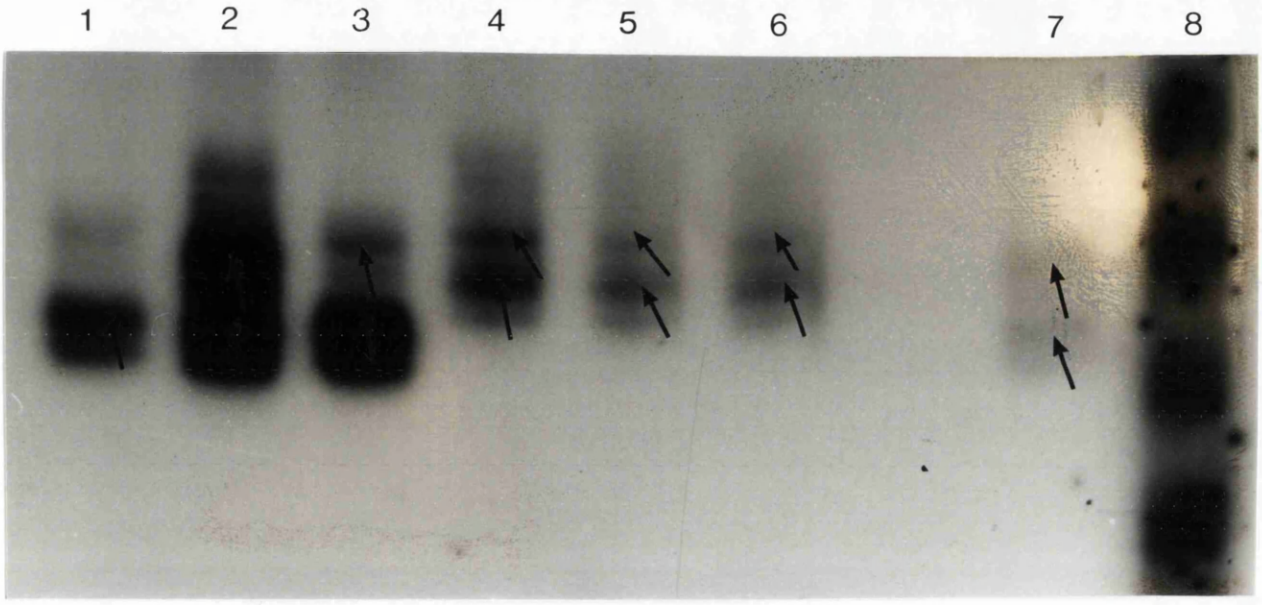

Figure 3.5. Section from a representative polyacrylamide gel showing a range of the possible TNFd microsatellite alleles. Sequence specific α ^{32}P dCTP labelled polymerase chain reaction products are separated on a 15% non-denaturing polyacrylamide gel. Lane 1 - 20068 with genotype TNFd1,d1, Lane 2 - 20069 with genotype TNFd1,d5, Lane 3 - 20070 with genotype TNFd2,d6, Lane 4 - 20071 with genotype TNFd4,d7, Lane 5 - 20072 with genotype TNFd4,d7, Lane 6 - 20073 with genotype TNFd4,d7, Lane 7 - 20075 with genotype TNFd2,d6, Lane 8 - 10bp γATP labelled 10bp ladder (GibcoBRL). Bands representing the alleles are marked with arrowheads.

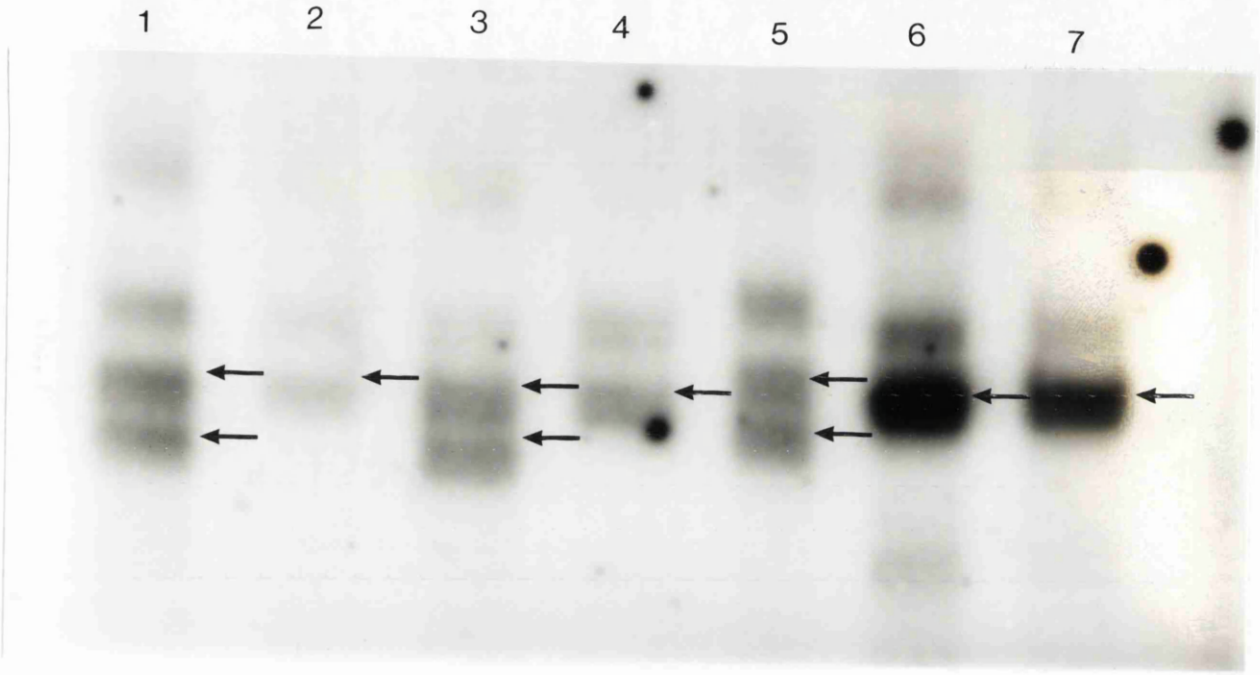

Figure 3.6. Section from a representative polyacrylamide gel showing a range of the possible TNFe microsatellite alleles.

Sequence specific α^{32}P dCTP labelled polymerase chain reaction products are separated on a 15% non-denaturing polyacrylamide gel. Lane 1 - 20011 with genotype TNFe1,e2, Lane 2 - 20012 with genotype TNFe2,e2, Lane 3 - 20013 with genotype TNFe1,e2, Lane 4 - 20014 with genotype TNFe2,e2, Lane 5 - 20015 with genotype TNFe1,e3, Lane 6 - 20016 with genotype TNFe2,2, Lane 7 - with genotype TNFe2,e2. Bands representing the alleles are marked with arrowheads.

3.6 Statistical Analysis

All data were compiled in tabulated form on Excel 4 for Windows on a Packard Bell PC. Statistical analysis was performed in all cases by Chi-square test on the Minitab for Windows (Minitab version 10.1) computer program running on a standard PC. Reference was made to the book Practical Statistics for Medical Research by Douglas G. Altman. Further reference was made to a number of statisticians including the group of Dr. Gordon Murray in the University of Glasgow.

Alleleic frequencies were calculated following the protocol of Nedospasov *et. al.* (1991) using the following equation

$$\text{Allelic frequency of allele X} = \frac{\text{No of occurences of X in the given population}}{\text{(No.of individuals in Population) X 2}}$$

It is assumed that there are no "null" alleles at any of these loci and therefore where only one band was observed on the gel this was taken to indicate homozygosity for that particular allele. This was taken into consideration during the calculations by counting this as two occurences of the particular allele.

Statistical significance was taken at the 95% level with $\rho<0.05$ being taken as significant. Comparisons of the frequency of individual allelels of the various polymorphisms are reported in various sections of this thesis. For each of these instances two ρ values are stated. The first ρ is derived directly from the Chi-square analysis, whilst the second (ρc) takes into account the Bonferroni correction required for multiple analysis. Although considerable emphasis is placed on the uncorrected ρ values in the discussion of the results of this thesis it must be remembered that it is the corrected (ρc) values that indicate true statistical significance in this study.

Results

4.1 Associations between alleles of polymorphisms at the TNF locus and the presence of colorectal cancer

There is compelling evidence to suggest that TNF may have a role in the development and progression of colorectal cancer (see section 1.3.2 of this thesis). Since a number of alleles of the polymorphisms located within the TNF locus have been associated with either over- or under-production of TNF (see section 1.3.4 of this thesis) it was of interest to determine if there is any association between alleles of these polymorphisms and the presence of colorectal cancer. 100 individuals with histologically proven colorectal cancer were obtained following the criteria as described in section 2.1 of this thesis and were compared to a panel of 115 control individuals who had no form of malignant disease at the time of collection of blood. Table 4.1 shows a complete list of the genotypes at all of the six polymorphisms examined in both the colorectal cancer population and the normal control group.

4.1.1 -308 TNFα RFLP analysis

The -308 TNF-α RFLP is the only polymorphism examined in this thesis which is located within the gene encoding TNF-α. It is characterised by a single base substitution at position -308 within the promotor region of TNF-α. It is identified in genomic DNA by performing a sequence specific polymerase chain type reaction to amplify a 107bp stretch of DNA which incorporates a polymorphic Nco-I restriction endonuclease site. Restriction digestion results in the identification of a bi-allelic polymorphism resulting in the ability to assign one of three possible genotypes to any individual. These genotypes are termed homozygous for the TNF1 allele (T1/T1), homozygous for the TNF2 allele (T2/T2) or heterozygous for both alleles (T1/T2).

Code No.	TNFa	TNFc	TNFd	TNFe	LT-alpha Nco-I	308 TNF-alpha
10001	3,3	1,2	6,7	2,3	B*1/B*2	T1/T1
10002	10,11	1	7,7	3,3	B*1/B*2	T1/T1
10003	2,2	1,2	7,7		B*2/B*2	T1/T2
10004	2,2	1,2	4,6	2,3	B*2/B*2	T1/T2
10005	2,2	1,2	6,6	2,3	B*2/B*2	T1/T2
10006	2,2	1	6,6	3,3	B*2/B*2	T2/T2
10007	4,10	1			B*1/B*2	T1/T2
10008	6,10	1,2	3,3		B*1/B*2	T1/T2
10009	3,10	1,2	3,4	3,3	B*2/B*2	T1/T2
10010	1,6	1,2	5,5		B*2/B*2	T1/T2
10011	3,5	1	1,2	3,3	B*1/B*2	T2/T2
10012		2	3,4			T1/T2
10013		1	2,3			T1/T1
10014			5,5			
10015		1,2	3,3	3,3		T1/T1
10016			1,3			
10017		1,2	3,3	1,3		T1/T1
10018		1	2,2			T1/T1
10019	7,12	1				
10020		1,2	1,2	2,2	B*2/B*2	T1/T1
10022	7,11	1			B*1/B*2	T1/T1
10023	3,3	1,2	3,3		B*2/B*2	T1/T1
10025	9,10	1	3,3		B*2/B*2	T1/T1
10026	7,7	1,2	4,4	1,3	B*1/B*2	T1/T1
10027	2,4	2			B*2/B*2	T1/T1
10028	2,4	1,2	5,5		B*2/B*2	
10029	10,12	1	5,5		B*2/B*2	
10030	7,10	1	4,5			
10031	7,7	1	5,5		B*1/B*1	T1/T1
10032	3,11	1,2		1,3	B*1/B*2	T1/T1
10033	3,11	1	4,4		B*1/B*2	
10034	2,9	2			B*2/B*2	
10035	3,7	1,2	2,3		B*1/B*2	T1/T2
10036	6,11	1	2,2		B*1/B*2	
10037	3,9	2	2,2		B*2/B*2	
10038		1,2	1,2		B*2/B*2	T1/T1
10039	3,4	1			B*2/B*2	T2/T2
10040	7,11	1	2,2			T1/T1
10041	8,11	1	2,2		B*1/B*2	T1/T1
10042	3,3	2	3,3		B*2/B*2	T1/T1
10043	7,11	1	4,4		B*2/B*2	T1/T1
10044	7,7	1,2				T1/T1
10045	4,4		2,4			
10046	8,11	1		3,3	B*2/B*2	T1/T1
10047	3,11	1,2	3,6	3,3		T1/T1
10048	11,12	1			B*2/B*2	
10049	10,10	1	3,4		B*2/B*2	T1/T1
10050	3,11	1,2	3,4	1,2	B*2/B*2	T1/T1
10051		1	3,4			T1/T2
10052		1,2	1,4	1,2	B*1/B*2	T1/T2
10053		1	2,4			T1/T2
10054	3,3	1,2	2,4			T1/T2

Code No.	TNFa	TNFc	TNFd	TNFe	LT-alpha Nco-I	308 TNF-alpha
10055	3,3	1	2,2			T1/T2
10056		1,2	3,3	3,3	B*2/B*2	T1/T1
10057	3,3	1	2,4			T1/T2
10058		1	4,4			T1/T2
10059		1,2	5,5	3,3	B*2/B*2	T1/T1
10060	3,6	1,2			B*1/B*2	T1/T1
10061	3,9	1,2	4,4			
10066	3,3	2			B*2/B*2	T1/T1
10068	3,10	1,2	5,6		B*2/B*2	T1/T1
10069	6,11	1	5,5	1,3	B*1/B*2	T1/T1
10070	3,10	1,2	6,7	3,3	B*2/B*2	T1/T2
10071	6,10	1,2		1,3	B*1/B*2	
10072	7,10	1		1,3	B*2/B*2	
10073	7,10	1		3,3	B*1/B*2	
10074	2,7	1,2	5,5	3,3	B*1/B*2	
10075	7,11	1		3,3	B*1/B*2	
10076	2,10	2	5,6	3,3	B*2/B*2	T1/T1
10077	3,10	2		2,3	B*1/B*2	T1/T1
10078	5,11	1,2	4,5	2,3	B*1/B*2	T1/T1
10079	3,7	1	4,5	3,3	B*1/B*1	T1/T2
10080	3,11	1		3,3	B*1/B*2	T1/T2
10081	5,5	1	1,2	3,3	B*1/B*1	T1/T2
10082	3,11	2	3,3	3,3	B*2/B*2	T1/T1
10083	4,7	1,2	1,5	3,3	B*1/B*2	T1/T1
10084	4,8	1	3,4	2,3	B*1/B*2	T1/T2
10085	8,11	1		3,3	B*1/B*2	T1/T1
10086	8,11	1,2	4,4	3,3	B*1/B*2	T1/T1
10087	7,12	1	3,5	2,2	B*1/B*2	T1/T1
10088	7,12	1	3,5	3,3	B*1/B*2	T1/T1
10089	3,11	1,2	1,3	3,3	B*1/B*2	T1/T2
10090	3,11	1,2		1,3	B*1/B*2	T1/T2
10091	3,7	1,2	3,3	1,3	B*1/B*2	T1/T1
10092	10,10	1	4,4	3,3	B*2/B*2	T1/T1
10093	5,10	2	2,4	2,2	B*2/B*2	T1/T1
10094	6,6	1	1,2	3,3	B*1/B*1	T1/T1
10095	10,10	1	2,4	2,3	B*2/B*2	T1/T1
10096	6,10	1	3,3	2,3		T1/T1
10097	6,11		3,4	1,3		
10098	6,10	1,2	3,4	2,2		
10099	3,11	1,2	3,3			T1/T1
10100	3,3	1,2	4,4	3,3		T1/T1
10101	4,8	1	1,3	3,3		
10102	6,8	1,2	3,5	1,3		T1/T1
10103	7,11	1	3,3	3,3		T1/T1
10104	3,8	1,2	2,4	2,3		T1/T1
10105	5,8	1,2		2,3		T1/T1
10106	3,3	1,2	1,4	1,3		T1/T2

Code No.	TNFa	TNFc	TNFd	TNFe	LT-alpha Nco-I	308 TNF-alpha
20001	5,10		4,5	3,3	B*1/B*2	
20002	6,9	1,1	3,5		B*1/B*2	T1/T1
20003	2,10	2,2	4,6		B*2/B*2	T1/T1
20004	7,12	1,1	4,4	3,3	B*2/B*2	T1/T1
20005	5,10	1,2	5,5	2,2	B*2/B*2	T1/T1
20006	7,11	1,1			B*2/B*2	T1/T1
20007	2,4	1,2	3,5	3,3	B*1/B*2	T1/T2
20008	4,10	1,1	3,3		B*1/B*2	T1/T2
20009	7,10	1,2	3,3	2,2	B*2/B*2	T1/T1
20010	9,10	1,1	3,3		B*2/B*2	T1/T1
20011	2,6	1,1	5,5	1,2	B*1/B*2	T1/T1
20012	4,9	1,2	4,5	2,2	B*1/B*2	T1/T2
20013	2,6		4,4	1,2	B*1/B*2	T1/T1
20014	2,6	1,1	4,4	2,2	B*1/B*1	T1/T2
20015	2,7	1,2	2,2	1,3	B*2/B*2	T1/T1
20016	7,7	1,1	1,3	2,2	B*2/B*2	T1/T1
20017	6,6		2,3	2,2	B*1/B*1	T1/T1
20018	6,10	1,1	4,4		B*1/B*2	T1/T1
20019	2,11			1,2	B*2/B*2	T1/T1
20020	2,11	1,1	3,3	2,2	B*1/B*2	T1/T2
20021					B*1/B*2	T1/T2
20022			7,7		B*1/B*2	T1/T2
20023			6,6		B*2/B*2	T1/T1
20024					B*2/B*2	T1/T1
20025			2,2		B*2/B*2	
20026						
20027	8,8	1,1			B*1/B*2	
20028	4,9	1,2			B*2/B*2	
20029	5,10	1,2			B*2/B*2	
20030	9,10				B*2/B*2	
20031	5,10	1,2			B*1/B*2	
20032	5,10	1,2			B*1/B*2	
20033	2,6	1,1			B*1/B*2	
20034	2,5	1,1			B*2/B*2	
20035	2,2	2,2			B*2/B*2	
20036	2,8		1,3		B*2/B*2	
20037	1,1	1,1			B*1/B*2	
20038	1,5	1,2			B*1/B*2	
20039	1,4	1,1			B*1/B*2	
20040	4,10				B*2/B*2	
20041	1,8	1,2			B*2/B*2	
20042	6,6	1,1			B*1/B*2	
20043	5,5	1,1			B*1/B*2	
20044	4,4	1,1	1,3		B*1/B*2	
20045	2,9				B*1/B*2	
20046	2,7	1,1				
20047	6,12	1,1	4,6	2,2	B*2/B*2	
20048	8,8	1,1	5,5	1,2	B*1/B*2	T1/T1
20049	6,9	1,2	5,5	3,3	B*2/B*2	T1/T1
20050	7,9	1,1	4,6		B*1/B*1	T1/T2
20051	6,7	1,1	3,4		B*1/B*1	T2/T2
20052	4,10	1,2	4,5	2,2	B*2/B*2	T1/T1

Code No.	TNFa	TNFc	TNFd	TNFe	LT-alpha Nco-I	308 TNF-alpha
20053	4,4	2,2	5,5	1,2	B*2/B*2	T1/T1
20054		1,1	4,4	2,2	B*2/B*2	T1/T1
20055	12,12	1,2	4,4	2,2	B*2/B*2	T1/T1
20056	12,12	1,1	2,4	2,2	B*2/B*2	T1/T1
20057	6,12	1,1	1,4	2,3	B*1/B*2	T1/T2
20058	4,6	1,2	3,5	1,3	B*1/B*2	T1/T2
20059		1,2	2,3	3,3	B*2/B*2	T1/T1
20060		2,2	2,3		B*1/B*2	T1/T1
20061	6,6		3,3	2,2		T1/T1
20062	4,4	1,2	1,3		B*1/B*1	T1/T2
20063		1,1	4,4		B*2/B*2	T1/T1
20064	6,6	2,2	1,3			T1/T1
20065	4,4	1,1				T1/T1
20066			3,3	3,3	B*1/B*2	T1/T1
20067	13,13	1,1	2,2	2,3	B*2/B*2	T1/T1
20068	6,11	1,2	1,1	2,3		T1/T2
20069		1,2	1,5	3,3	B*1/B*2	T2/T2
20070	6,13	1,2	2,6		B*1/B*2	T1/T2
20071	11,11	1,2	4,7	3,3		T1/T1
20072	12,13	2,2	4,7		B*1/B*1	T1/T2
20073	8,10	1,1	4,7	1,1	B*2/B*2	T1/T1
20074	7,7	1,2				T1/T2
20075	4,11		2,7	3,3	B*1/B*2	T1/T2
20076	11,11				B*2/B*2	T1/T1
20077	11,13		4,4	3,3	B*1/B*1	T1/T2
20078	10,10				B*2/B*2	T1/T1
20079	10,10		4,4	3,3		T1/T1
20080	5,5		4,4	2,3	B*1/B*2	T1/T1
20081	10,10		4,6		B*2/B*2	T1/T1
20082	8,8				B*1/B*2	T1/T2
20083	10,10			1,2	B*2/B*2	T1/T1
20084	9,10		2,4	2,2	B*2/B*2	T1/T1
20085	9,10		2,4	2,2	B*2/B*2	T1/T1
20086	9,9		1,4			T1/T2
20087			3,5		B*2/B*2	T1/T1
20088			1,1	2,2	B*1/B*2	T1/T2
20089	8,9		1,4		B*1/B*1	T2/T2
20090	3,8		2,4	3,3		T1/T2
20091	2,6		2,4			T1/T2
20092	5,5		3,6	3,3	B*1/B*2	T1/T1
20093	5,5		4,6	3,3	B*1/B*2	T1/T1
20094	1,8		4,4	3,3	B*1/B*2	T1/T1
20095	3,3		4,6	3,3	B*1/B*1	T1/T2
20096	2,4		6,6	3,3	B*1/B*1	T1/T1
20097			5,5			T1/T2
20098	8,9		4,4	3,3		T1/T1
20099	8,8		5,5		B*2/B*2	T1/T1
20100	2,3	1,2	3,3	1,2	B*1/B*1	T1/T2
20101	1,9	2,2	4,5	2,2		T1/T1
20102	7,7	1,1	2,4		B*2/B*2	T1/T1
20103	7,7	1,1		2,2	B*2/B*2	T1/T1
20104	3,8	1,2		2,2	B*1/B*2	T1/T1

Code No.	TNFa	TNFc	TNFd	TNFe	LT-alpha Nco-I	308 TNF-alpha
20105	3,3	1,2	5,5		B*1/B*2	T1/T1
20106	6,6	1,2	2,7		B*2/B*2	T1/T1
20107		1,2	1,5		B*1/B*1	T2/T2
20108	1,5	1,2	2,2			T1/T1
20109	1,1	1,2	1,5		B*1/B*2	T1/T2
20110	1,9	1,1	1,2		B*1/B*2	T1/T2
20111	3,3	1,1	2,2			T1/T2
20112	3,5	1,1	6,6		B*1/B*1	T1/T1
20113	4,4	1,2	3,7		B*1/B*2	T1/T1
20114		1,1			B*1/B*2	T1/T2
20115	2,2	2,2	5,5		B*2/B*2	T1/T1

Table 4.1 Complete genotypes at the six TNF polymorphisms in a) the colorectal cancer patients, and b) the normal control individuals. Colorectal cancer patients are coded 10001 to 10106 whilst the normal individuals are coded 20001 to 20115.

The -308 TNF α RFLP genotype for each individual was determined by the method described in section 3.3 of this thesis. Genotypes were determined for 86 (75%) of the normal control individuals and 79 (79%) of the colorectal cancer patients.

Genotype and allelic frequencies were calculated for the two populations and plotted as separate histograms. Figure 4.1 shows a histogram of the frequencies of the three possible genotypes of the -308 TNF-α RFLP of the normal and colorectal cancer populations. Examination of the relative frequencies between the two groups, by chi-square analysis, demonstrates that there is no significant difference between the genotype frequencies of the two groups (χ^2=0.630, ρ=0.3649, df=2). Figure 4.2 shows a histogram of the frequency of the TNF1 and TNF2 alleles in the two populations. As expected from the results of the genotype analysis, chi-square analysis shows that there is also no statistically significant difference between the alleleic frequencies of the two populations, (χ^2=0.607, ρ=0.3780, df=1).

4.1.2 LT-α Nco-I RFLP analysis

The LT-α Nco-I RFLP (also known as the TNF-β Nco-I RFLP) is the most widely studied of the polymorphisms associated with TNF. Like the -308 TNF-α RFLP it is associated with a polymorphic Nco-I site which is located within the first intron of the gene for LT-α. Identification of genotypes of individuals was performed in a similar manner to that used for the analysis of the -308 TNF-α RFLP, and involves the sequence specific amplification of a 750bp stretch of genomic DNA which again incorporates a polymorphic Nco-I site. The restriction digestion of the resultant PCR product allows the identification of a bi-allelic polymorphism which results in the ability to assign one of three possible genotype as either homozygous for the TNFB*1 allele (B*1/B*1), homozygous for the TNFB*2 allele (B*2/B*2) or heterozygous for both alleles (B*1/B*2).

Genotypes were determined according to the method described in section 3.4 of this thesis. LT-α Nco-I RFLP genotypes were determined for 98 (85%) of the normal individuals and 80 (80%) of the colorectal cancer patients.

As for the -308 TNF-α RFLP, both genotype and allelotype frequencies were calculated for the two populations and were plotted as seperate histograms. Figure 4.3 shows a histogram of the genotype frequency of the LT-α RFLP between the two populations. Chi-square analysis of the frequencies of the two populations shows no significant difference between the genotypes of the two groups (χ^2=2.506, ρ=0.1428, df=2). Figure 4.4 shows a histogram of the allelic frequencies found in the normal and colorectal cancer populations. As expected from the analysis of the genotype distribution, no significant difference could be demonstrated between the frequency of the individual alleles of the two populations (χ^2=1.754, ρ =0.1253, df=1).

4.1.3 TNF microsatellite polymorphism analysis

RFLPs, by nature, are bi-allelic, thus any attempt to identify linkage analysis with these loci is restricted due to the small number of alleles possible at each locus. With the recent advent of microsatellite technology, which identifies regions of high degree of polymorphism, the problems associated with the RFLPs can potentially be resolved. There are in total five sites of microsatellite polymorphism within the TNF locus which exhibit varying degrees of polymorphism. Of these five microsatellites alleles of TNFa, TNFc and TNFd loci have been associated with alterations in the secretion of TNF-α by stimulated monocytes (see section 1.3 of this thesis). To determine the genotypes at each of the microsatellite loci for any individual genomic DNA is subjected to a polymerase chain reaction, sequence specific for each of the microsatellite loci. The resultant PCR products are variable in length and vary by 2bp between alleles.

4.1.3.1 TNFa microsatellite

The TNFa microsatellite is the most polymorphic of the microsatellites found within the TNF locus. It is located 3.5kb upstream of the LT-α gene and has 13 possible alleles. TNFa microsatellite genotypes were obtained for 98 (85%) of the control population and 81 (81%) of the colorectal cancer population. Figure 4.5 shows the alleleic frequencies in the control and colorectal cancer populations. A 2 x 13 chi-square analysis of the allelic frequencies between the two groups demonstrates that there is a significant association between alleles of the TNFa microsatellite and the presence of colorectal cancer (χ^2=37.962, ρ=0.0001, df=12).

On closer analysis it can be seen that a number of the alleles show either over- or under-representation in the colorectal cancer population. Table 4.2 contains a complete analysis of the relative frequencies of each of the 13 TNFa alleles in both the normal and colorectal cancer populations. It can be seen from this table that the alleles TNFa3 and TNFa11 show a higher frequency in the colorectal cancer population (TNFa3 - χ^2=4.44, ρ=0.035, df=1, ρc=0.455); TNFa11 - χ^2=8.03, ρ=0.005, df=1, ρc=0,065) than compared to the control group. Whilst these two alleles show an over expression in the cancer population the TNFa1, TNFa5, TNFa9 and TNFa13 alleles all show under expression in the cancer population (TNFa1 - χ^2=6.83, ρ=0.009, df=1, ρc=0.117; TNFa5 - χ^2=4.63, ρ= 0.031, df=1, ρc=0.403; TNFa9 - χ^2=5.34, ρ=0.02, df=1, ρc=0.26; TNFa13 - χ^2=4.19, ρ=0.04, df=1, ρc=0.52) when compared to the control population.

Of the 91 theoretically possible genotypes at the TNFa locus 49 were identified in the normal population whilst only 35 were seen in the colorectal cancer population. Eight (10%) of the colorectal cancer patients were homozygous for the TNFa3 allele whilst a further seven were TNFa3/a11 (8%). Of the normal individuals, only three (3%) were homozygous for the TNFa3 allele whilst there were no individuals in the

normal population with the genotype TNFa3/a11. Of the remaining colorectal cancer patients who were positive for allele TNFa3, three were positive for TNFa10, one was positive for TNFa8 , one was positive for TNFa5 , one was positive for TNFa6 and one was positive for TNFa9. Of the remaining TNFa11 positive colorectal cancer patients three were also TNFa8 positive, three were TNFa7 positive, two were TNFa6 positive, one was TNFa5 positive, one was TNFa10 positive and one was TNFa12 positive.

Of the four TNFa alleles which are under-represented in the colorectal cancer the most common genotypes associated with these alleles were TNFa5/a5 and TNFa5/a10. Four (4%) normal individuals were genotype TNFa5/a5 and five (5%) were TNFa5/a10, whilst only one colorectal individual was TNFa5/a5 (1%) and of the 81 colorectal cancer patients typed for TNFa none was found to be TNFa5/a10. The other three alleles represented within the colorectal cancer population were found in association with a number of other alleles.

Two alleles of the TNF locus have been shown to be associated with altered secretion of TNF-α. TNFa2 has been shown to correlate with increased secretion of TNF-α in LPS stimulated monocytes, whilst TNFa6 has been shown to correlate with reduced secretion. It is interesting to note that four (5%) of the colorectal cancer patients are homozygous for TNFa2, whilst this genotype is found in only two (2%) of the normal individuals. Five (5%) of the normal individuals are homozygous for TNFa6, whilst only one (1%) colorectal cancer patient is homozygous for TNFa6.

4.1.3.2 TNFc Microsatellite

The TNFc polymorphism is located within the first intron of the LT-α gene, and is the least polymorphic of the microsatellites within the TNF locus, with only two

identifiable alleles. The TNFc genotype was determined using a sequence specific polymerase chain type reaction, as described in section 1.3.4.4, for 71 (62%) of the normal individuals and 96 (96%) of the colorectal cancer population.

Figure 4.6 shows a graph of the allelic frequencies of the two TNFc alleles within the colorectal cancer and normal populations. The allele TNFc2 has been reported to correlate with overexpression of TNF-α in stimulated monocytes, however, it can be seen that there is no difference in allelic frequencies between the two groups (χ^2=0.057, ρ=1.624, df=1). Analysis of the genotype at the TNFc locus also fails to show any difference between the two groups (data not shown; χ^2=0.774, ρ= 0.3395, df=2).

4.1.3.3 TNFd microsatellite

The TNFd microsatellite polymorphism is co-localized with the TNFe polymorphism, 8-10 kb down stream of the TNF-α gene, and has seven possible alleles. Again the specific genotypes of the TNFd polymorphism were determined using a sequence specific polymerase chain reaction, and were obtained for 83 (72%) of the normal control individuals and 78 (78%) of the colorectal cancer individuals.

Figure 4.7 shows the allelic frequency of each of the seven TNFd alleles in the two populations. 2 x 7 chi-square analysis of the allelic usage between the two groups shows that there is no overall difference between the two populations (χ^2=5.719, ρ =0.1171, df=6). Table 4.3 shows the actual frequencies of each of the TNFd alleles in the two populations. The TNFd3 allele has been shown to correlate with overexpression of TNF-α in stimulated monocytes. Analysis of the frequency of this allele in the two populations demonstrates a significant over representation of this allele in the colorectal cancer population (χ^2=4.583, ρ=0.019, df=1, ρc=0.133).

Of the 28 possible genotypes at the TNFd locus seen within the normal individuals 22 were observed in the normal population and 19 were seen in the colorectal cancer patients. The majority of genotypes found in both populations are homozygous genotypes. The genotype TNFd2/d2 is found in 5 (6%) normal individuals and 6 (8%) colorectal individuals, the genotype TNFd4/d4 is found in 12 (14%) normal individuals and 8 (10%) colorectal individuals, the genotype TNFd5/d5 is found in 9 (11%) normal individuals and 8 (10%) colorectal individuals. Although these homozygous alleles are common in the two populations there is no significant difference between the two groups. A difference can be observed in the number of individuals homozygous for the TNFd3 allele between the two populations with 6 (7%) of the normal individuals having the genotype TNFd3/d3, whilst 12 (15%) of the colorectal individuals display this genotype. Chi-square analysis, however, fails to prove this difference is statistically significant (χ^2=2.693, ρ=0.063, df=1, ρ c=1.764).

4.1.3.4 TNFe microsatellite

As described above the TNFe microsatellite locus is co-localized with the TNFd microsatellite 8-10 kb down stream of the TNF-α gene, and has three possible alleles. As for the other microsatellite polymorphisms, the specific genotypes at the TNFd locus were obtained for 51 (44%) of the normal individuals and 55 (55%) of the colorectal cancer patients, by sequence specific PCR.

The allelic frequency for each of the three alleles of both the normal individuals and the colorectal cancer patients can be seen in figure 4.8. 2x3 Chi-square analysis shows an extremely high difference between the alleles observed in the normal individuals and the colorectal cancer patients (χ^2=24.413, ρ<0.0000, df=2).

Table 4.4 shows a list of the allelic frequencies at each of the three alleles of the TNFe locus in both the normal and colorectal cancer patients. Chi-square analysis confirms that TNFe2 is significantly under-represented in the colorectal cancer population (χ^2=23.485, ρ<0.0000, df=1, ρc<0.0000), whilst the TNFe3 allele shows a significant over-representation in the cancer group (χ^2=19.357, ρ<0.0000, df=1, ρc<0.0000).

All six possible genotypes at the TNFe locus have been identified within this study group, however the frequency of a number of the genotypes does differ significantly between the two groups. There is a significant difference between the frequency of the TNFe1/e2 genotype (frequency- 0.14 and 0.04, normal and colorectal respectively; χ^2=3.467, ρ=0.038, df=1, ρc=0.228), the TNFe1/e3 genotype (frequency- 0.04 and 0.2, normal and colorectal respectively; χ^2=6.358, ρ=0.007, df=1, 0.042) and also the TNFe2/e2 genotype (frequency- 0.37 and 0.07, normal and colorectal respectively; χ^2=14.001, ρ=0.0001, df=1, ρc=0.0006) in the normal and colorectal cancer populations.

4.1.3.5 Family analysis

It is common in this sort of study to include a number of families in order to confirm mendelian inheritance of the alleles and also to confirm integrity of allele calls. Large extended families were not available at the initial time of starting this study however three small partial families were found to be included within the control individuals. Allele calling was performed on these individuals before family pedigrees were disclosed and although small errors can be seen in families one and two (underlined alleles) these pedigrees provide reassurance that allele calling was consistent. The pedigrees were as follows.

Family One

Father -

20064 (a6,6,c2,2,d1,3,e?,LTα?,-308T1,T2)

Sons -

20057 (a6,12,c1,1,d1,4,e2,3,LTαB*1,B*2,-308T1,T2)

20058 (a4,6,c1,2,d3,5,e1,3,LTαB*1,B*2,-308T1,T2)

Family Two

Three siblings -

20068 (a6,11,c1,2,d1,1,e2,3,LTα?,-308T1,T2)

20069 (a?,c1,2,d1,5,e3,3,LTαB*1,B*2,-308T1,T2)

20070 (a6,13,c1,2,d2,6,e?,LTαB*1,B*2,-308T1,T2)

Family Three

Three siblings -

20083 (a10,10,c?,d?,e1,1,LTαB*2,B*2,-308T1,T1)

20084 (a9,10,c?,d2,4,e2,2,LTαB*2,B*2,-308T1,T1)

20085 (a9,10,c?,d2,4,e2,2,LTαB*2,B*2,-308T1,T1)

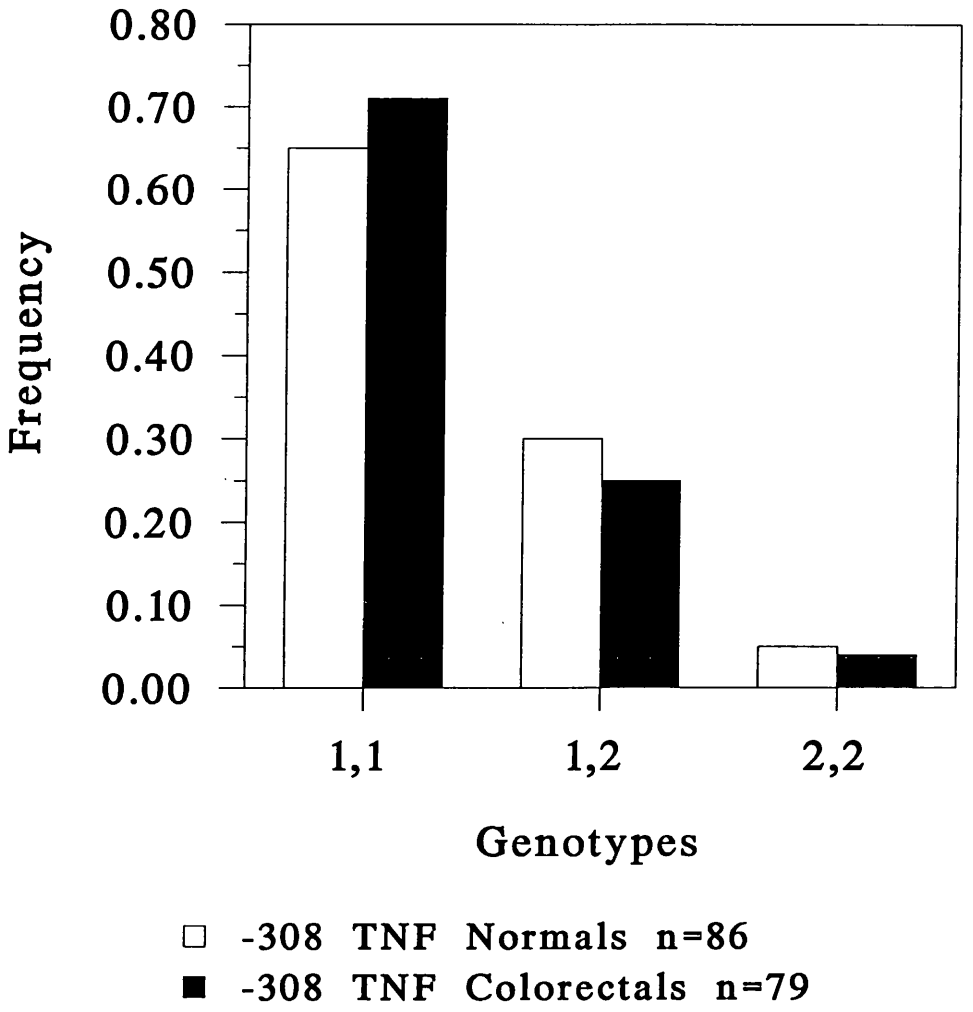

Figure 4.1. Histogram showing the distribution of the three possible -308 TNFα RFLP genotypes in the normal and colorectal cancer populations. Chi-square analysis shows no significant association between genotypes at this locus and the presence or absence of colorectal cancer.

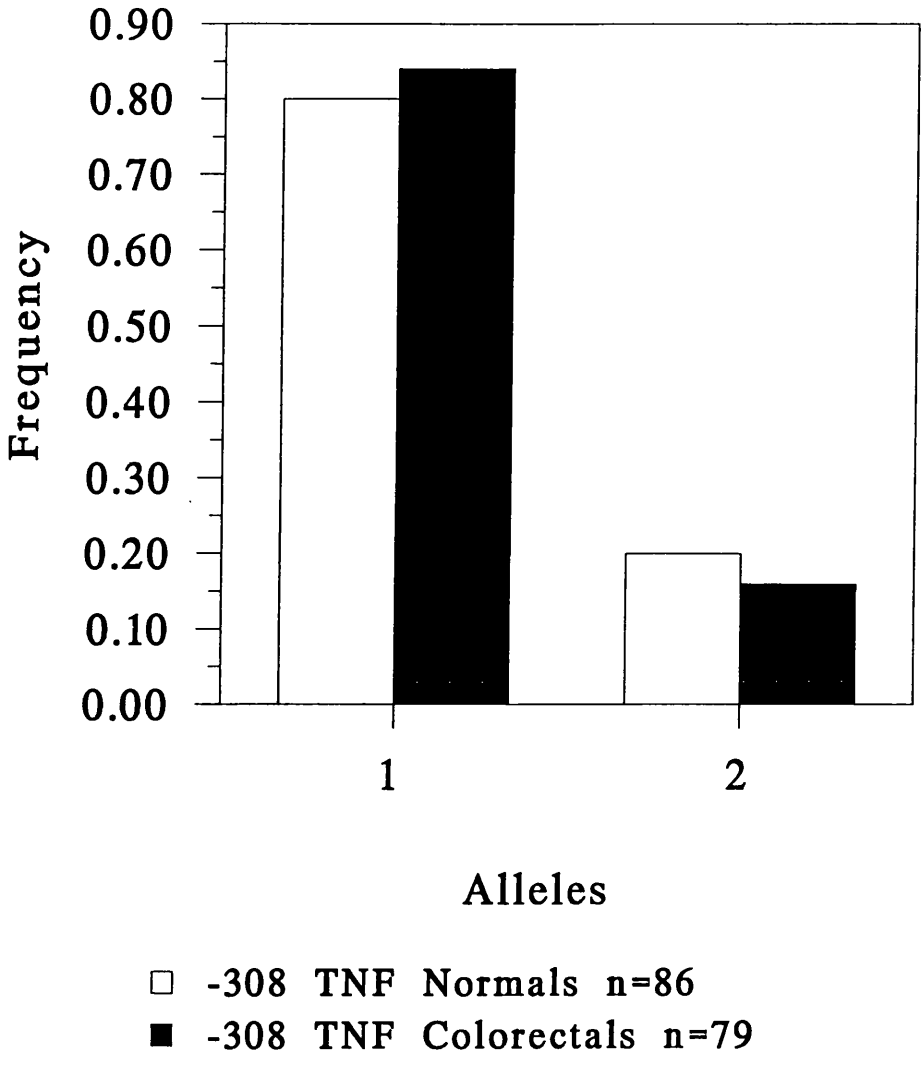

Figure 4.2. Histogram showing the distribution of the two possible -308 TNFα RFLP alleles in the normal and colorectal cancer populations. As for the -308 TNFα genotypes, Chi-square analysis shows no significant association between alleles at this locus and the presence or absence of colorectal cancer.

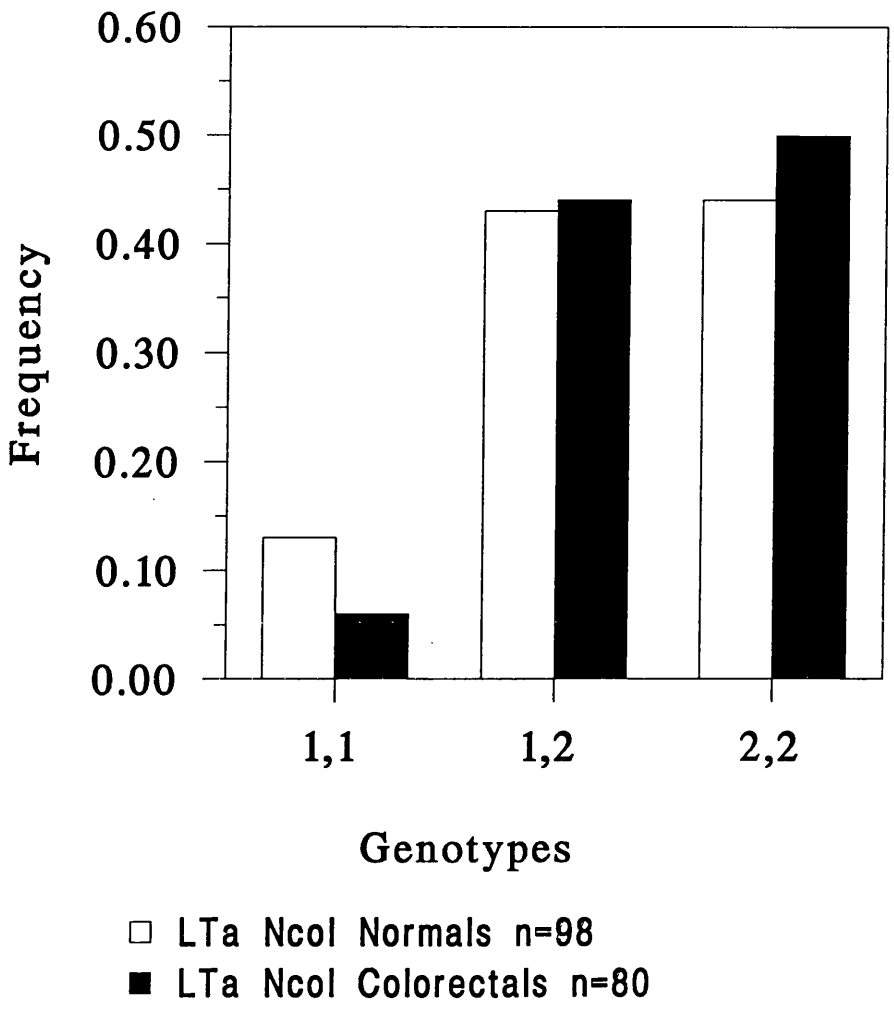

Figure 4.3. Histogram showing the distribution of the three possible LTα *Nco*I RFLP genotypes in the normal and colorectal cancer populations. Chi-square analysis shows no significant association between genotypes at this locus and the presence or absence of colorectal cancer.

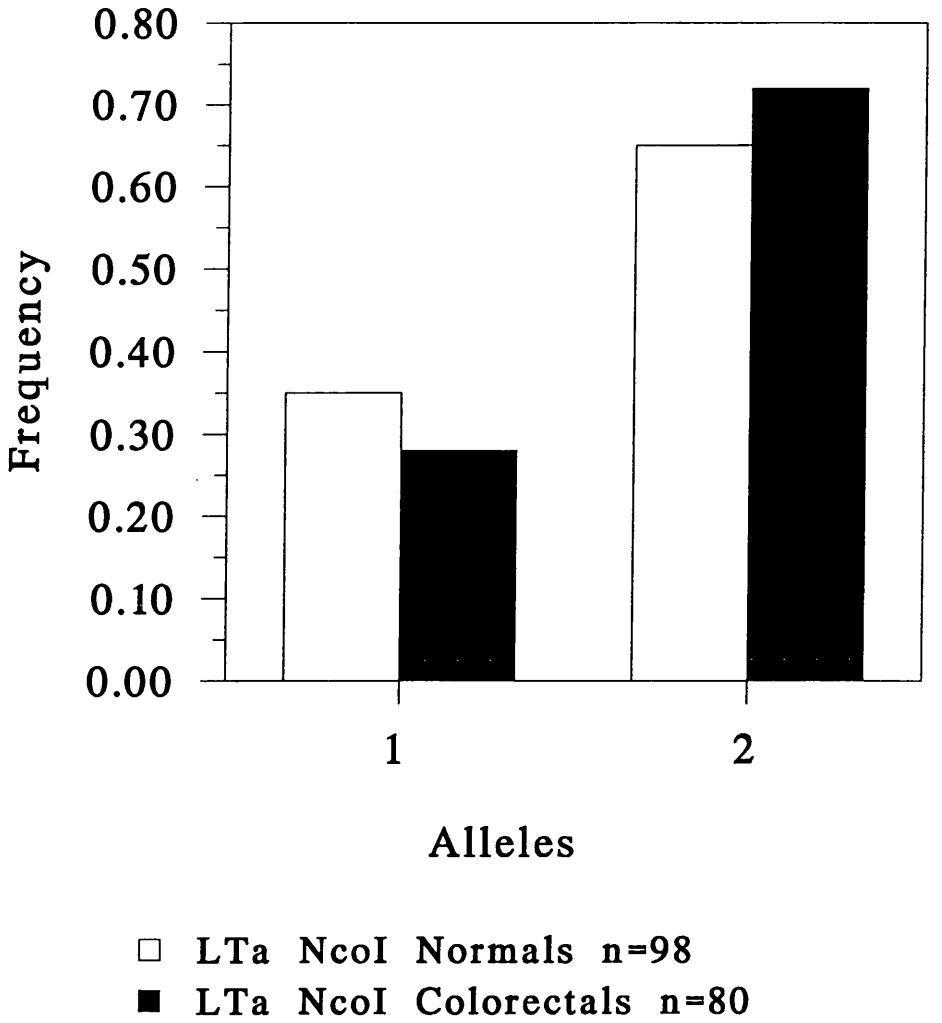

Figure 4.4. Histogram showing the distribution of the two possible LTα *Nco*I RFLP alleles in the normal and colorectal cancer populations. Again Chi-square analysis shows no significant association between alleles at this locus and the presence or absence of colorectal cancer.

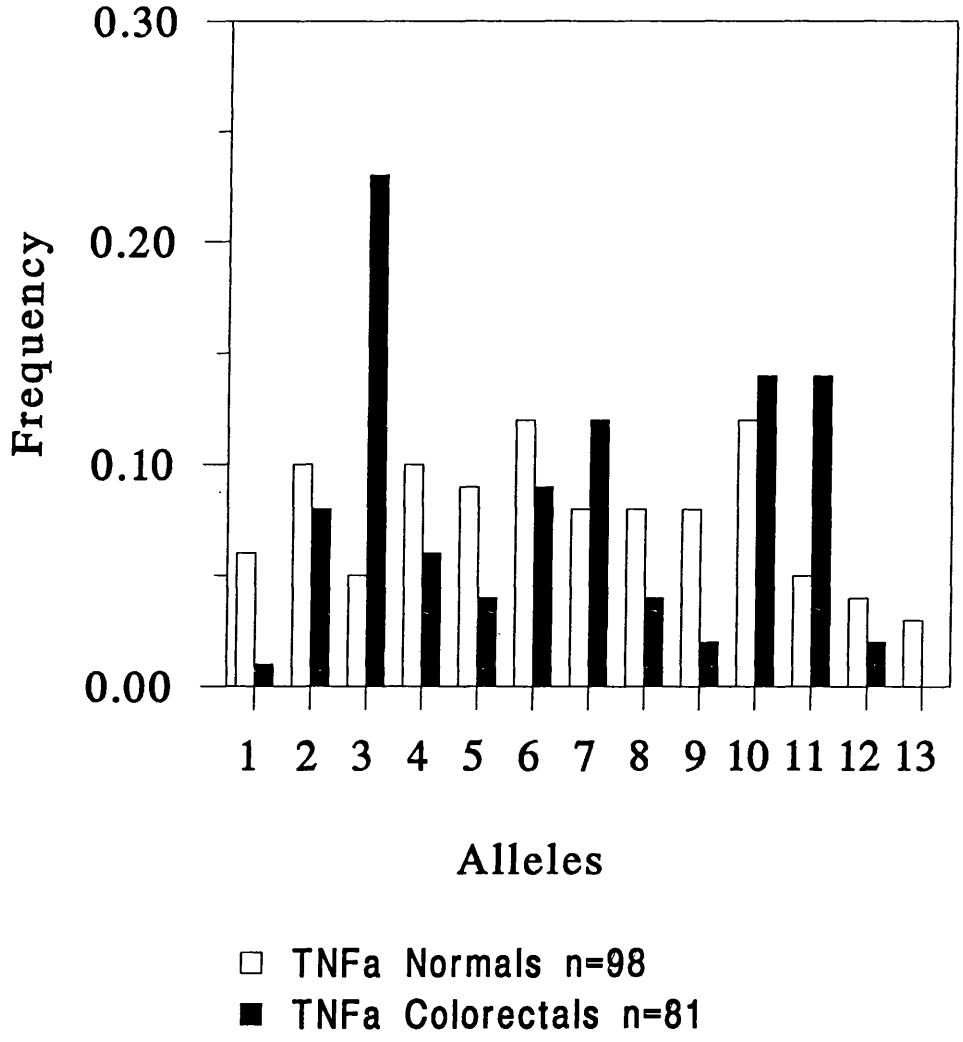

Figure 4.5. Histogram showing the distribution of the 13 TNFa microsatellite alleles in the normal and colorectal cancer populations. Chi-square analysis shows a significant difference between the distribution of the TNFa alleles at this locus and the presence or absence of colorectal cancer.

TNFa	Normals	Colorectals	Chi-square	P-value
1	0.06	0.01	6.83	0.009
2	0.1	0.08	0.5	ns
3	0.05	0.23	4.44	0.0006
4	0.1	0.06	1.88	ns
5	0.09	0.04	4.63	0.031
6	0.12	0.09	1.21	ns
7	0.08	0.12	2.32	ns
8	0.08	0.04	2.1	ns
9	0.08	0.02	5.34	0.02
10	0.12	0.14	0.34	ns
11	0.05	0.14	8.03	0.005
12	0.04	0.02	0.68	ns
13	0.03	0	4.19	0.04

Table 4.2. Breakdown of the allelic frequencies and associated Chi-square values of the 13 TNFa alleles in the normal and colorectal cancer populations. A significant linkage, by Chi-square, can be seen with 6 of the 13 TNFa alleles and either the presence or absence of colorectal cancer. TNFa3 and TNFa11 are over represented in the cancer population, whilst TNFa1, TNFa5, TNFa9 and TNFa13 are all under represented in the cancer group.

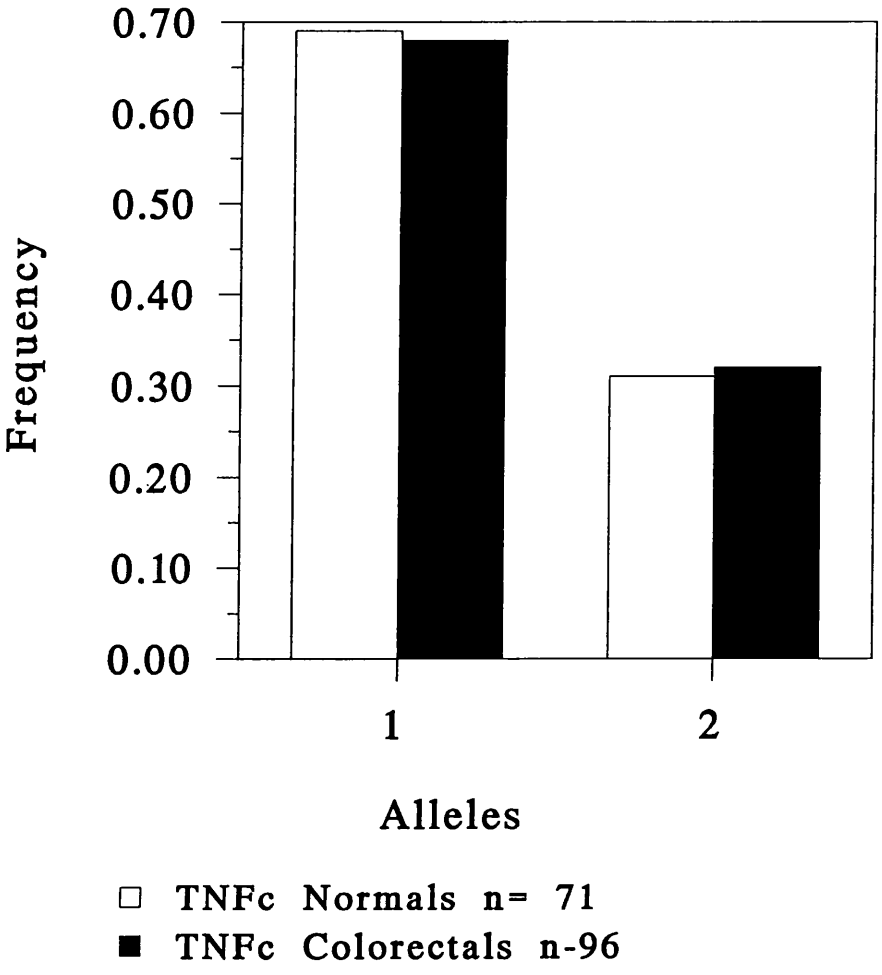

Figure 4.6. Histogram showing the distribution of the 2 TNFc microsatellite alleles in the normal and colorectal cancer populations. Chi-square analysis shows no significant difference between the distribution of the TNFc alleles and the presence or absence of colorectal cancer.

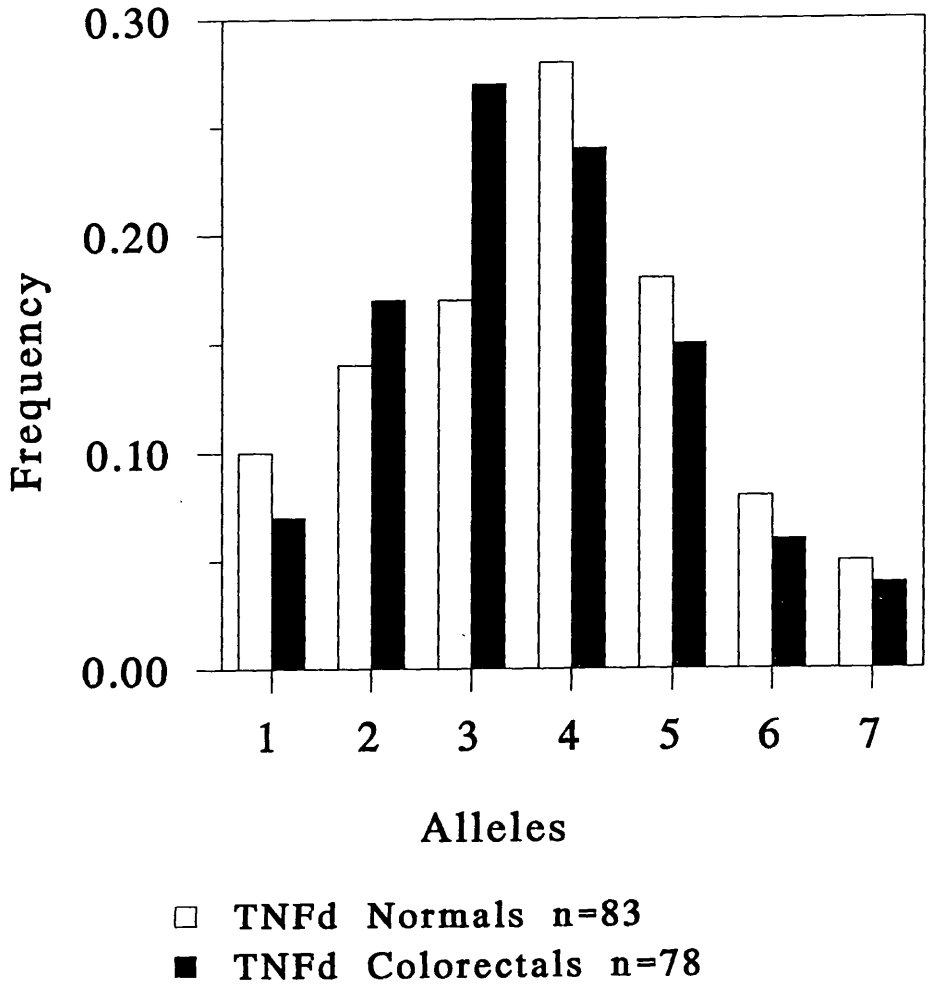

Figure 4.7. Histogram showing the distribution of the 7 TNFd microsatellite alleles in the normal and colorectal cancer populations. Chi-square analysis shows no significant difference between the distribution of the TNFd alleles and the presence or absence of colorectal cancer.

TNFd	Normals	Colorectals	Chi-square	P-value
1	0.1	0.07	1.05	ns
2	0.14	0.17	0.04	ns
3	0.17	0.27	4.58	0.019
4	0.28	0.24	0.94	ns
5	0.17	0.15	0.28	ns
6	0.08	0.06	0.5	ns
7	0.05	0.04	0.19	ns

Table 4.3. Breakdown of the allelic frequencies and associated Chi-square values of the 7 TNFd alleles in the normal and colorectal cancer populations. A significant linkage, by Chi-square, can be seen with TNFd3 and the presence colorectal cancer.

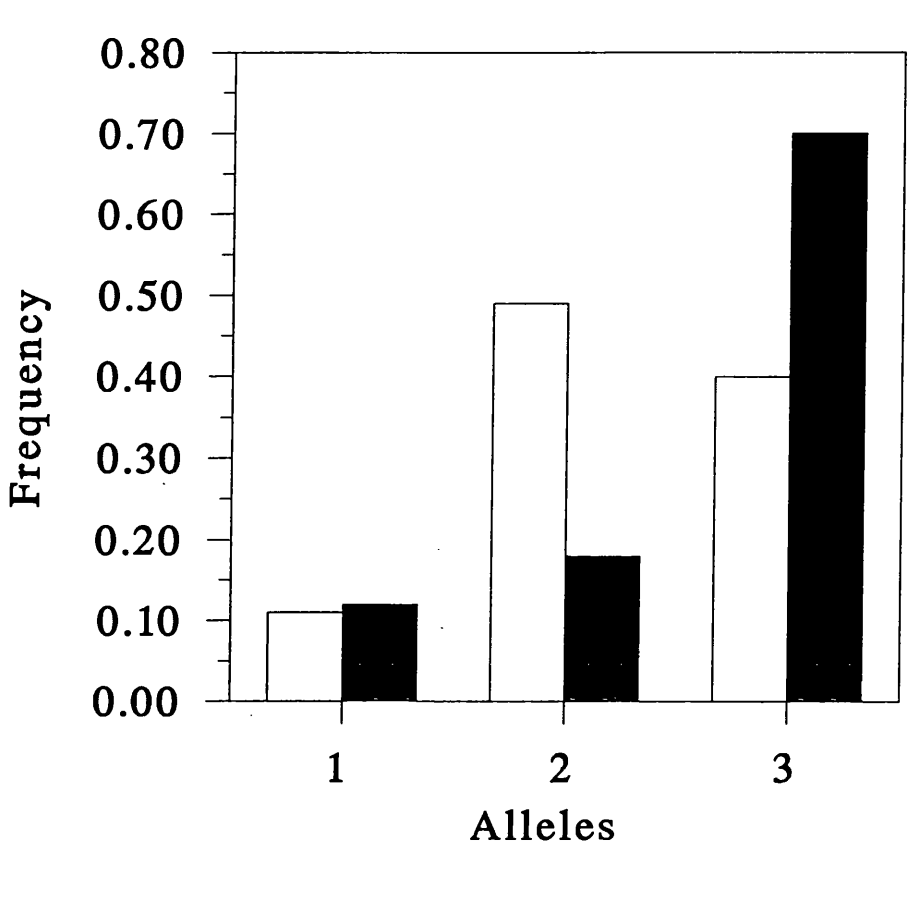

Figure 4.8. Histogram showing the distribution of the 3 TNFe microsatellite alleles in the normal and colorectal cancer populations. Chi-square analysis shows no significant difference between the distribution of the TNFe alleles and the presence or absence of colorectal cancer.

Normals	Colorectals	Chi-square	P-value
0.11	0.12	0.08	ns
0.49	0.18	23.48	<0.000
0.4	0.7	19.36	<0.000

Table 4.4. Breakdown of the allelic frequencies and associated Chi-square values of the 3 TNFe alleles in the normal and colorectal cancer populations. A significant linkage, by Chi-square, can be seen with TNFe2 and the absence of colorectal cancer, whilst TNFe3 correlates with the presence of colorectal cancer.

4.2 Association between alleles at the TNF locus and Dukes' staging in colorectal adenocarcinoma

Dukes' staging was initially described in 1932 as a method of pathologically grading rectal tumours. By 1967 this system had been adapted to include colonic tumours. Dukes' staging generally correlates with the degree of aggresiveness of a colorectal tumour and has been shown to correlate well with prognosis, and to date this system still remains the most widely used system for the pathological grading and clinical assesment of colorectal cancer. Dukes' staging was available for 92 (92%) of the colorectal cancer patients in this study (see section 2.1.2). Having established that a number of alleles of the polymorphisms within the TNF locus correlate with the presence of colorectal cancer it was of interest to determine if either the same alleles or a group of other alleles could be correlated with the pathological grading of the colorectal tumours.

4.2.1 -308 TNFα RFLP and Dukes' stage

Although no significant differences in the alelles of the -308 TNFα RFLP were identified between the normal and colorectal cancer individuals, it was still of interest to determine if any differences could be observed between the different Dukes' stages in the colorectal cancer patients.

Figure 4.9 shows a histogram of the frequency of the three possible genotypes at the -308 TNFα RFLP locus in colorectal cancer patients group. A 3x4 Chi-square analysis of the genotype frequencies within each of the four Dukes' stage groups shows that there is no overall difference between the four groups (χ^2=9.019, ρ=0.056, df=6). Closer analysis does however show that the genotype TNF2/2 is only found in those patients that are Dukes' C (χ^2=7.747, ρ=0.003, df=1, ρc=0.009).

Figure 4.10 shows the allelic frequencies of the two -308 TNFα alleles in the four Dukes' stage groups. Again, as for the genotype frequencies, there is no significant difference suggesting a linkage between -308 TNFα RFLP alleles and Dukes' stage (χ^2=5.153, ρ=0.069, df=3).

4.2.2 LTα Nco-I RFLP and Dukes' stage

The LTα RFLP genotype of 64 patients with histologically proven colorectal cancer and for whom Dukes' staging was available, were examined to determine if there is linkage between the LTα genotype and Dukes' stage.

Figure 4.11 shows the histogram of the genotype frequencies of each of the three possible genotypes at the LTα RFLP locus in the four Dukes' stage groups. A 3x4 chi-square analysis of the genotype frequencies produces a borderline value for chi-square (χ^2=8.898, ρ=0.058, df=6), suggesting that, although no statistically significant at 95%, there may be some association between the LTα genotype and Dukes' staging. Closer analysis of the frequencies demonstrates that all of the individuals with Dukes' A tumours were the genotype TNFB*2/B*2 (χ^2=4.545, ρ=0.019, df=1, ρc=0.057).

Figure 4.12 Shows a histogram of the allelic usage between the four Dukes' stage groups. A 2x4 chi-square analysis of this data fails to show any significant linkage between alleles of this locus and Dukes' staging (χ^2=4.551, ρ= 0.087, df=3), although as expected there is a significant association with the TNFB*2 alelle and Dukes' A (χ^2=3.392, ρ=0.039, df=1).

4.2.3 TNF microsatellites and Dukes' stage

Alleles of the TNF microsatellites are the only alleles that show any linkage with the presence of colorectal cancer and since Dukes' staging is the most widely used marker for disease severity and it was of interest to determine if alleles of the TNF microsatellites show any association with Dukes' stage.

4.2.3.1 TNFa microsatellite

The TNFa genotype of 78 colorectal cancer patients for whom Dukes' staging was available were examined to determine if linkage occurs between the TNFa locus and Dukes' staging.

Figure 4.13 shows the allelic frequency of the 13 possible alleles at the TNFa locus in the four Dukes' stage groups. A 13x4 chi-square analysis of the frequency of the 13 alleles in the four Dukes' groups was not possible due to the small number of Dukes' A patients (computer analysis reports that the chi-square analysis is invalid due to a significant number of cells having expected values of less than 1.0). Closer analysis of the individual alleleic frequencies shows two major differences between the four groups. The allele TNFa3 shows a high degree of association with Dukes' stage A (χ^2=12.650, ρ=0.0002, df=1, ρc=0.0026). An association between TNFa8 and Dukes' stage D is also noted (χ^2=12.128, ρ=0.0003, df=1, ρc=0.0039).

4.2.3.2 TNFc microsatellite

The TNFc genotype of 90 colorectal cancer patients for whom Dukes' staging was available were examined to determine if any linkage could be seen between alleles at the TNFc locus and Dukes' staging.

Figure 4.14 shows the allelic frequency of the two alleles of the TNFc locus in the four Dukes' stage groups. A 2x4 chi-square analysis of the allelic frequencies between the four groups fails to predict any linkage between alleles at this locus and Dukes' staging (χ^2=1.090, ρ=0.24, df=3). Although there is a slight increase in the frequency of the TNFc2 allele in the Dukes' A group than compared to the other Dukes' stage groups this difference is not significant (χ^2=0.545, ρ=0.41, df=1).

4.2.3.3 TNFd microsatellite

The TNFd genotype of 76 colorectal cancer patients for whom details of Dukes' stage had been obtained were examined in order to ascertain whether there is any linkage between alleles at the TNFd locus and Dukes' stage.

Figure 4.15 shows the allelic frequency of the seven alleles of the TNFd locus in the four Dukes' stage groups. Analysis using a 4x7 chi-square test demonstrates a significant linkage between alleles at the TNFd locus and Dukes' staging (χ^2=24.981, ρ=0.027, df=18).

In terms of TNFα production, the most important TNFd allele is the TNFd3 allele. Analysis of the frequency of this allele in the four Dukes' stage groups shows a over-representation of this allele in the Dukes' A patients when compared with Dukes' D patients individually (χ^2=4.338, ρ=0.022, df=1), however this is not significant when compared to the other Dukes' stage groups combined (χ^2=1.75, ρ=0.13, df=1). An under-representation of the TNFd3 allele is observed in the Dukes' D patients, however this is not significant by chi-square (χ^2=2.771, ρ=0.06, df=1, ρc=0.42).

The TNFd5 allele is absent in the Dukes' A patients, and this difference is statistically significant when compared to the frequency of this allele in the other three Dukes' stage groups (χ^2=3.469, ρ=0.038, df=1). However since there are only

a small number of Dukes' A patients in this study the exact meaning of this relationship is still unclear.

4.2.3.4 TNFe microsatellite

TNFe microsatellite genotypes were obtained for 55 colorectal cancer patients for whom Dukes' staging was available. Statistical analysis of these data was performed to determine if any linkage was present between alleles at the TNFe locus and Dukes' staging.

Figure 4.16 shows the allelic frequency of the three alleles of the TNFe locus in each of the three Dukes' stages. 3x4 chi-square analysis shows a borderline figure for suggesting that there may be some linkage between the TNFe locus and Dukes' staging (χ^2=9.412, ρ=0.05, df=6).

The TNFe3 allele is not represented in the Dukes' stage A patients, and 2x2 chi-square analysis confirms that this difference is significant (χ^2=4.753, ρ=0.017, df=1, ρc=0.051) however since there is only one individual represented within the Dukes' A patients this result is probably invalid.

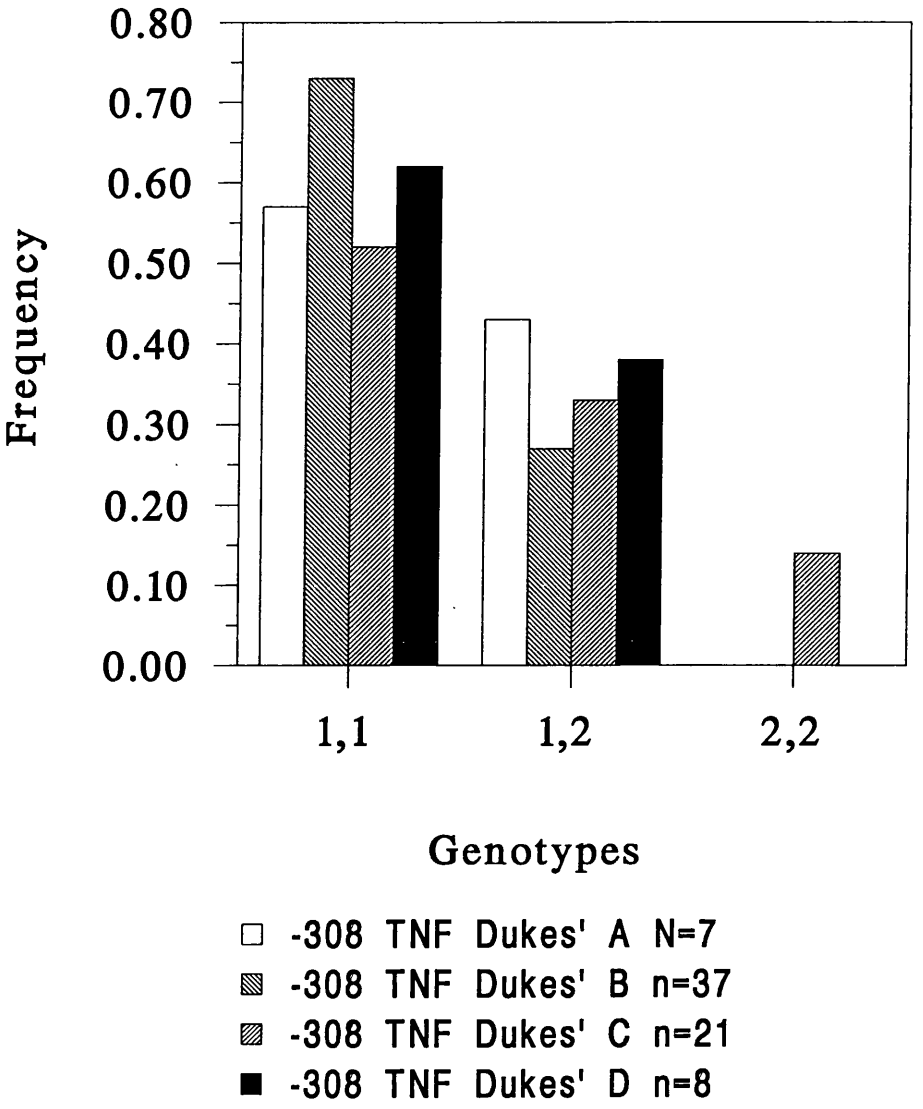

Figure 4.9. Histogram showing the distribution of the three possible -308 TNFα RFLP genotypes in the four Dukes' stage groups. Chi-square analysis shows a significant association between the TNF2/2 genotype and Dukes' stage C, however no further differences are noted.

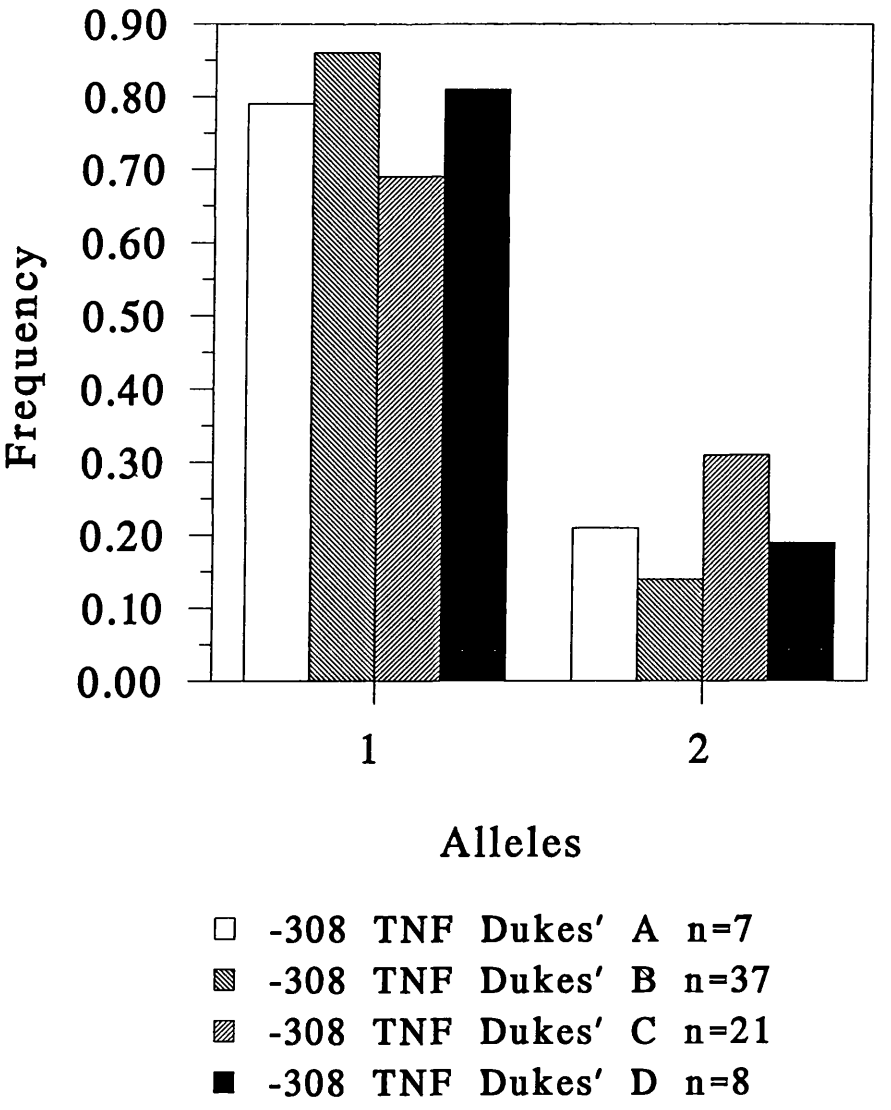

Figure 4.10. Histogram showing the distribution of the two possible -308 TNFα RFLP alleles in the four Dukes' stage groups. Chi-square analysis shows no significant association between alleles at this locus and Dukes' staging.

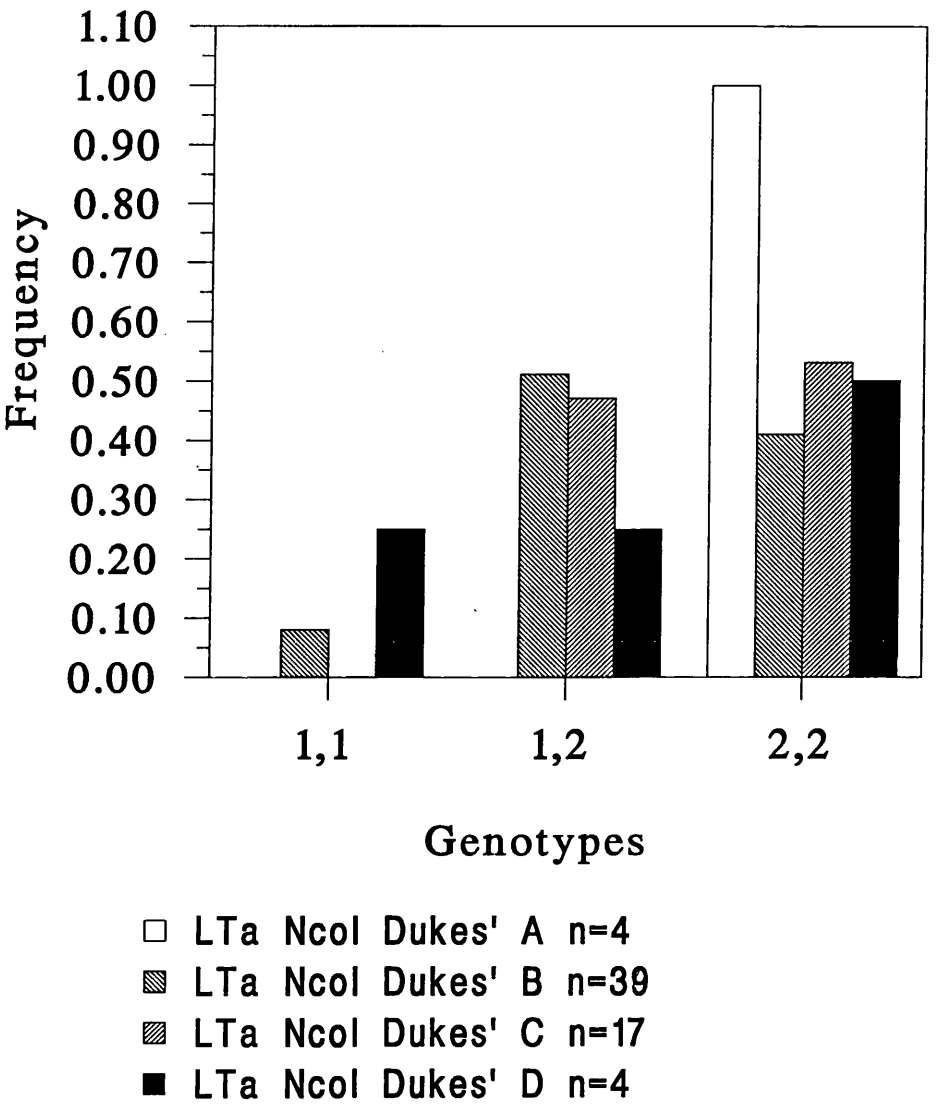

Figure 4.11. Histogram showing the distribution of the three possible LTα *Nco*I RFLP genotypes in the four Dukes' stage groups. Chi-square analysis shows a borderline association between the LTα *Nco*I genotype and Dukes' stage. Closer analysis demonstrates a significant association between the TNFB*2/B*2 genotype and Dukes' stage A, howver since the number of individuals with stage A is so small the true relevence of this result is unclear.

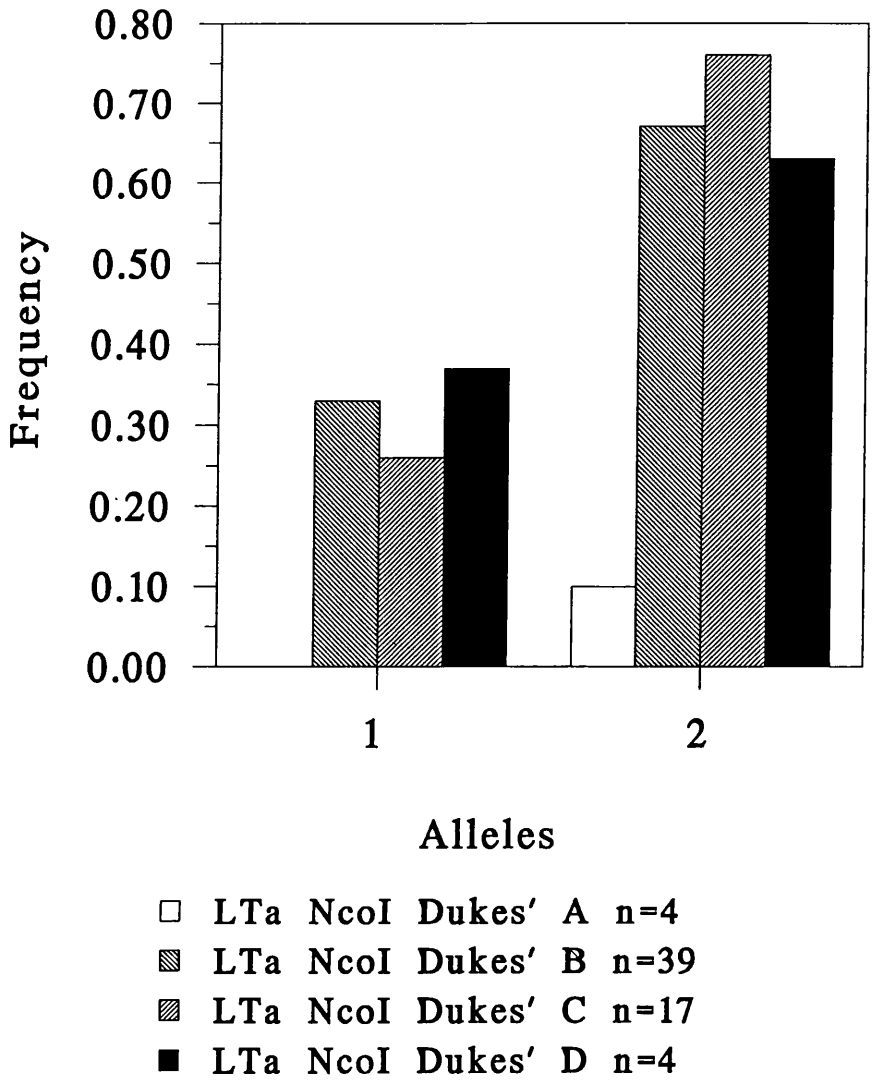

Figure 4.12. Histogram showing the distribution of the two possible LTα *Nco*I RFLP alleles in the four Dukes' stage groups. Chi-square analysis shows an association between the TNFB*2 allele and Dukes' stage A, however no further differences are noted.

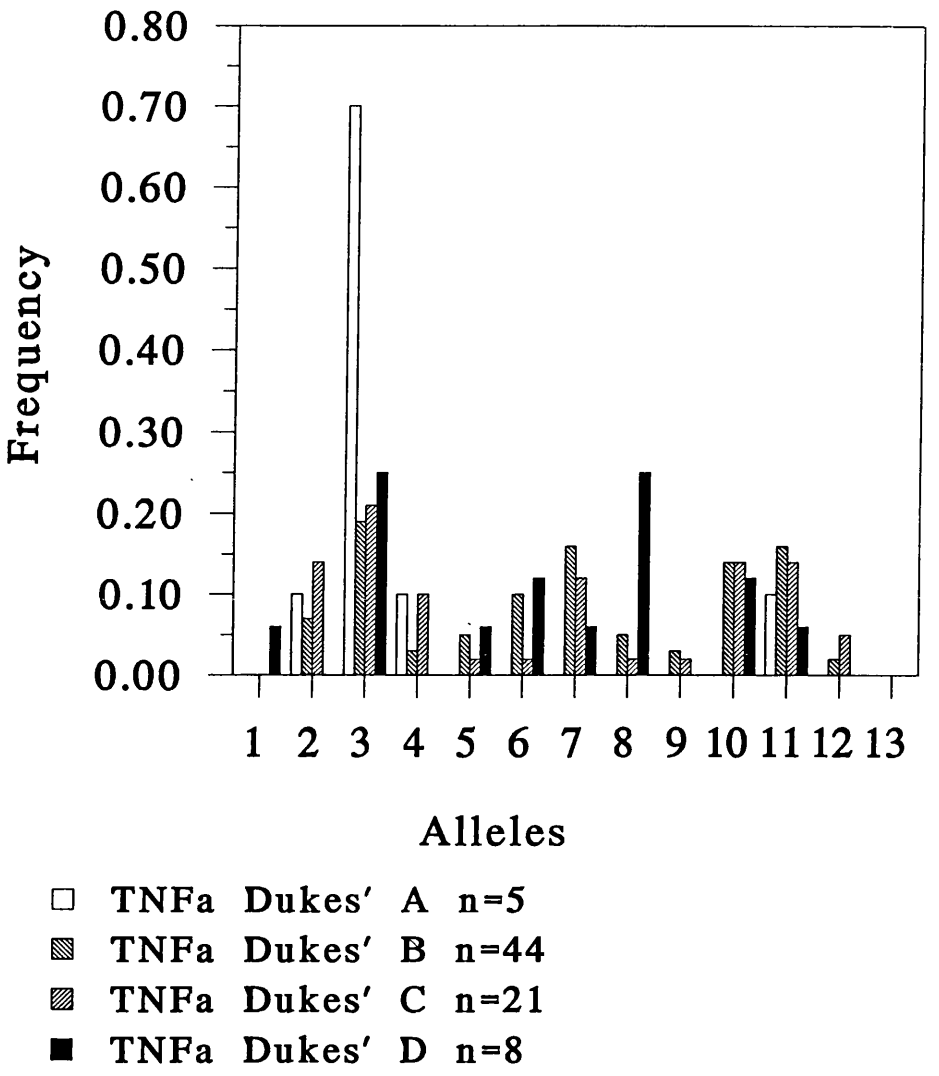

Figure 4.13. Histogram showing the distribution of the 13 TNFa microsatellite alleles in the four Dukes' stage groups. Chi-square analysis shows a significant association between the TNFa3 allele and Dukes' stage A and also a significant association between TNFa8 and Dukes' stage D.

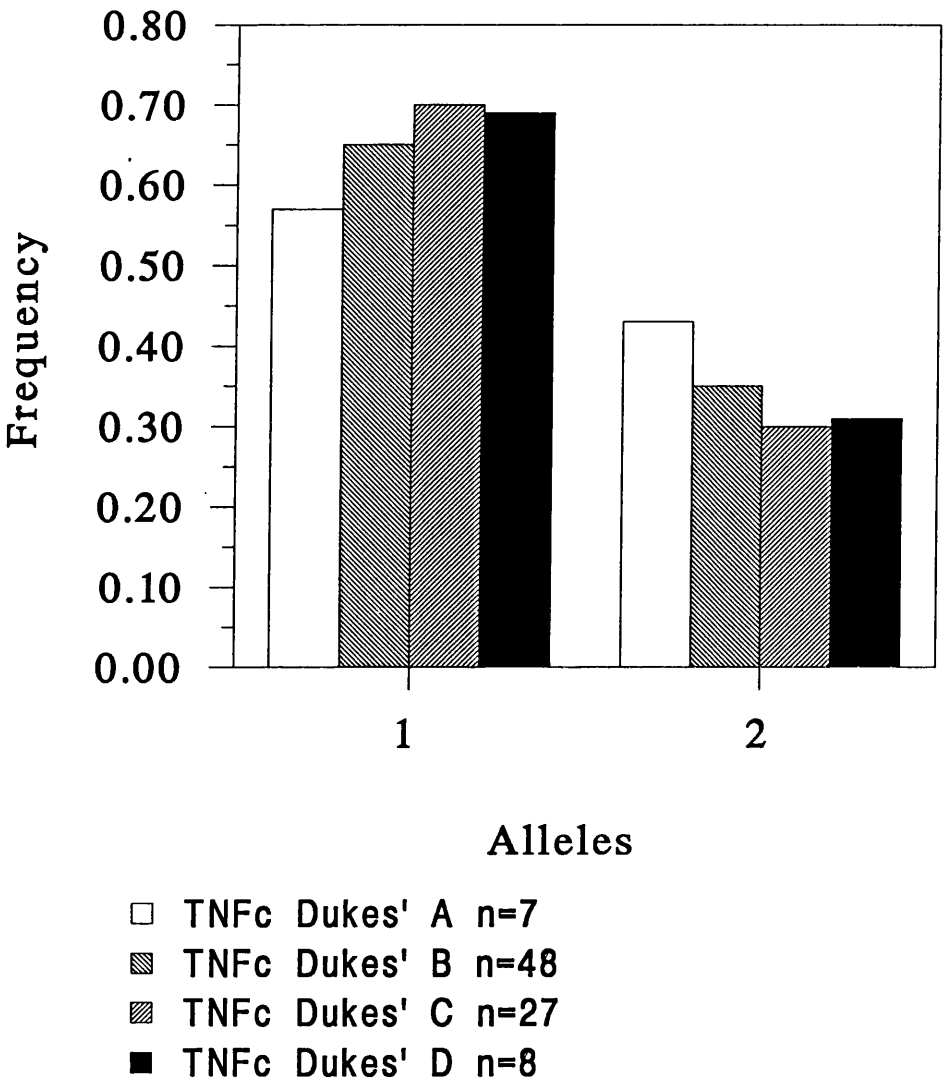

Figure 4.14. Histogram showing the distribution of the 2 TNFc microsatellite alleles in the four Dukes' stage groups. Chi-square analysis fails to shows any association between the TNFc alleles and Dukes' stage.

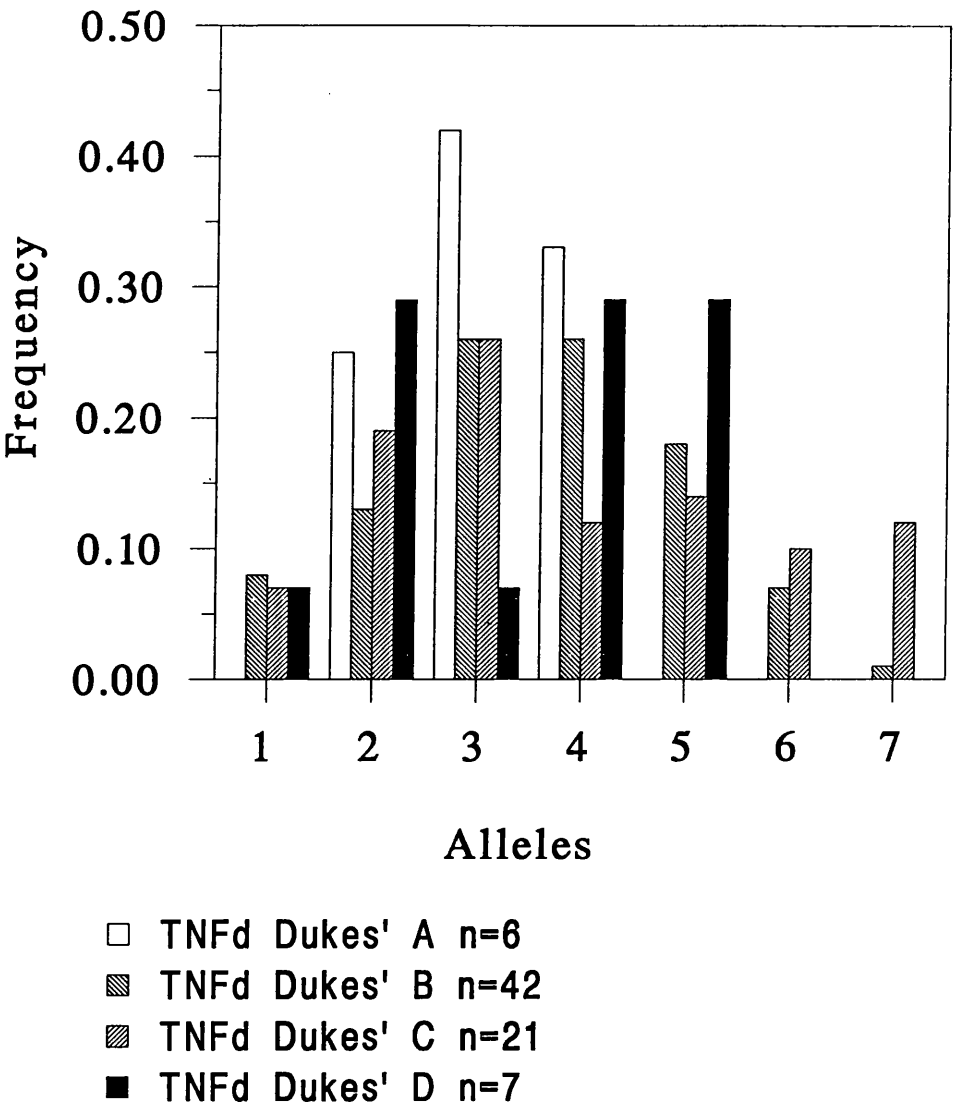

Figure 4.15. Histogram showing the distribution of the 7 TNFd microsatellite alleles in the four Dukes' stage groups. Chi-square analysis shows any association between the TNFd3 allele and Dukes' stage A when compared to Dukes' stage d, but not with stage B or C. The TNFa5 allele shows a significant association with Dukes' stage A when compared to the other three stages, however since the numbers of Stage A tumours was small the exact meaning of this result is unclear.

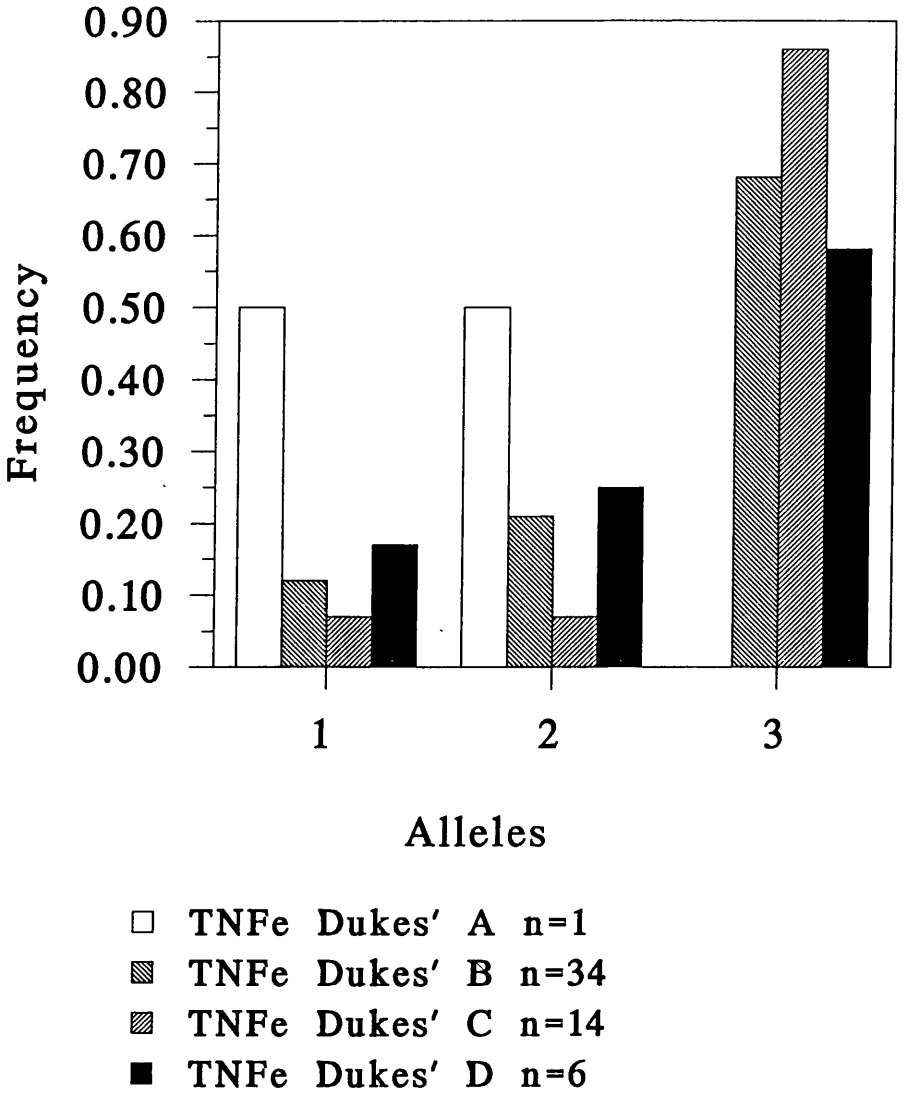

Figure 4.16. Histogram showing the distribution of the 3 TNFe microsatellite alleles in the four Dukes' stage groups. Chi-square analysis shows any association between the TNFe3 allele and Dukes' stage B, C and D when compared to Stage A, however since the numbers of Stage A tumours is small the exact meaning of this result is unclear.

4.3 Association between alleles at the TNF locus and disease progression in colorectal adenocarcinoma

Figures from a 1984 study suggest that out of 100 patients 51 will be cured by surgical intervention. Of the other 49, 44 will die due to the presence of metastatic disease or local recurrence and 5 will die with unresectable primary disease (August *et. al.*, 1984).

Follow up data was available for almost all of the patients represented within this study and on the 1st of July 1994 the number of individuals that had survived for greater than two years from the date of their initial operation and the number of individuals that had developed recurrent disease, in the form of either metastatic disease or local recurrence, were calculated. These sub groups of patients were analysed for their TNF polymorphism genotypes in an attempt to determine whether alleles of any of the polymorphisms could be correlated with the progression of colorectal cancer.

4.3.1 -308 TNFα RFLP and disease progression

Thirty colorectal cancer patients for whom -308 TNFα RFLP genotype was available were found to have survived disease free for greater than two years from the time of their initial operation. The genotypes of these disease free patients were compared with the genotypes of twenty three patients who had developed either metastatic disease or local recurrence within two years from the date of their initial operation.

Figure 4.17 shows the genotype frequencies of each of the two groups. Chi-square analysis shows that there is no linkage between alleles at this locus and the progression of colorectal cancer (χ^2=4.162, ρ=0.06, df=2). Figure 4.18 shows the allelic frequence of the same group of patients and chi-square analysis of this data

confirms that there is no link between the -308 TNFα RFLP locus and the progression of colorectal cancer to either local recurrence or the development of metastatic disease (χ^2=2.06, ρ=0.1, df=1).

4.3.2 LTα Nco-I RFLP and disease progression

The LTα Nco-I RFLP genotype was available for 23 colorectal cancer patients who had survived for greater than two years disease free and 19 patients with either metastatic or recurrent disease. The genotypes of these two groups were compared in an attempt to determine whether the LTα Nco-I RFLP locus is linked with disease progression.

Figure 4.19 shows a histogram of the genotype frequencies of the two colorectal cancer groups. Chi-square analysis of the genotype frequencies of the two groups shows a significant association between the genotype of the LTα Nco-I RFLP and the progression of colorectal cancer (χ^2=5.324, ρ=0.035, df=2). The TNFB*1/B*2 genotype is over represented in the metastatic/recurrent population than in the normal population (χ^2=3.359, ρ=0.04, df=1, ρc=0.12). Whilst the TNFB*2/B*2 genotype is under represented in the metastatic/recurrent population (χ^2=4.709, ρ=0.018, df=1, ρc=0.054).

Figure 4.20 shows a histogram of the allelic frequencies of the LTα RFLP alelles between the two populations. Again chi-square analysis demonstrates a significant association between alleles of this RFLP and the progression of colorectal cancer (χ^2=4.073, ρ=0.026, df=1). As expected the TNFB*1 allele is over represented in the metastatic/recurrent population, whilst the TNFB*2 allele is under represented in this same group.

4.3.3 TNF microsatellite polymorphisms and disease progression

Again, due to the fact that the two RFLPs within the TNF locus show a low degree of polymorphism, it was of interest to examine if the more polymorphic microsatellite loci showed any association with the progression of colorectal cancer.

4.3.3.1 TNFa microsatellite

The TNFa microsatellite genotype was obtained for 28 colorectal cancer patients with no evidence of recurrent disease 2 years after their initial surgery and for 23 patients with either local recurrent disease or distant metastases. The allelic frequency of the 13 TNFa alleles was compared between the two poulations to determine if alleles of this polymorphism could be correlated with disease progression.

Figure 4.21 shows the allelic frequency of the 13 alleles at the TNFa locus in both populations. Due to the small numbers involved in this particular grouping a 2x13 chi-square analysis is not possible. Analysis of individual alleles does, however show a number of differences. Allele TNFa3 shows an over representation in the recurrent/metastases patients as does TNFa4, TNFa9 and TNFa10 however neither of these differences reaches statistical significance (TNFa3 - χ^2=1.642, ρ=0.13, df=1, ρc=1.69; TNFa4 - χ^2=0.44, ρ=0.48, df=1, ρc=6.42; TNFa9 - χ^2=2.539, ρ=0.07, df=1, ρc=.91; TNFa10 - χ^2= 1.577, ρ=0.14, df=1, ρc=1.82).

Analysis of the allelic frequency of the TNFa2 and TNFa11 alleles shows that both are under represented in the recurrent/metastases patients. However, on chi-square analysis, only the difference of the TNFa11 alleleic frequency is statistically significant before correction(TNFa2 - χ^2=2.821, ρ=0.058, df=1, ρc=0.754; TNFa11 - χ^2=3.672, ρ=0.033, df=1, ρc=0.429). Other minor differences can be seen

between these two poulations however statistical analysis was thought not to be warranted due to the low frequency of the alleles concerned.

4.3.3.2 TNFc microsatellite

The TNFc microsatellite genotype was obtained for 34 of the patients that had survived 2 years disease free from the time of initial operation and 25 of the recurrent/metastases patients. As for the TNFa microsatellite the allelic frequency of the two TNFc alleles was compared between the two populations.

Figure 4.22 shows the allelic frequency of the two TNFc alleles between the two populations. Chi-square analysis of this data shows that there is no significant difference between the allelic frequencies in the two populations (χ^2=0.193, ρ=0.825, df=1).

4.3.3.3 TNFd microsatellite

The TNFd genotype of 28 colorectal cancer patients with no sign of recurrent or metastatic disease two years post initial surgical intervention and 19 patients with recurrent/metastatic disease were obtained. Again the allelic frequencies of the populations were compared.

Figure 4.23 shows the allelic frequencies of the seven TNFd alleles in the two colorectal cancer populations. A 2x7 chi-square analysis of the allelic frequencies between the two poulations shows no significant difference between the two populations (χ^2=7.89, ρ=0.075, df=6).

Closer analysis of the allelic frequencies between the two populations shows two major differences. The TNFd1 allele shows a significant over representation in the

recurrent/metastases population (χ^2=5.21, ρ=0.013, df=1, ρc=0.091) and the TNFd3 allele shows a significant under representation in this same population (χ^2=4.471, ρ=0.02, df=1, ρc=0.14).

4.3.3.4 TNFe microsatellite

The TNFe genotype of 13 colorectal cancer patients clear from any recurrent disease 2 years post operative and 20 patients with recurrent/metastatic disease disease were obtained. As for the other TNF polymorphisms, the allelic frequencies at this locus were compared between the two populations.

Figure 4.24 shows the allelic frequency of the three TNFe alleles between the two populations. A 2x3 chi-square analysis shows no association between the TNFe alleles and the progression of colorectal cancer. No other association can be seen at this locus.

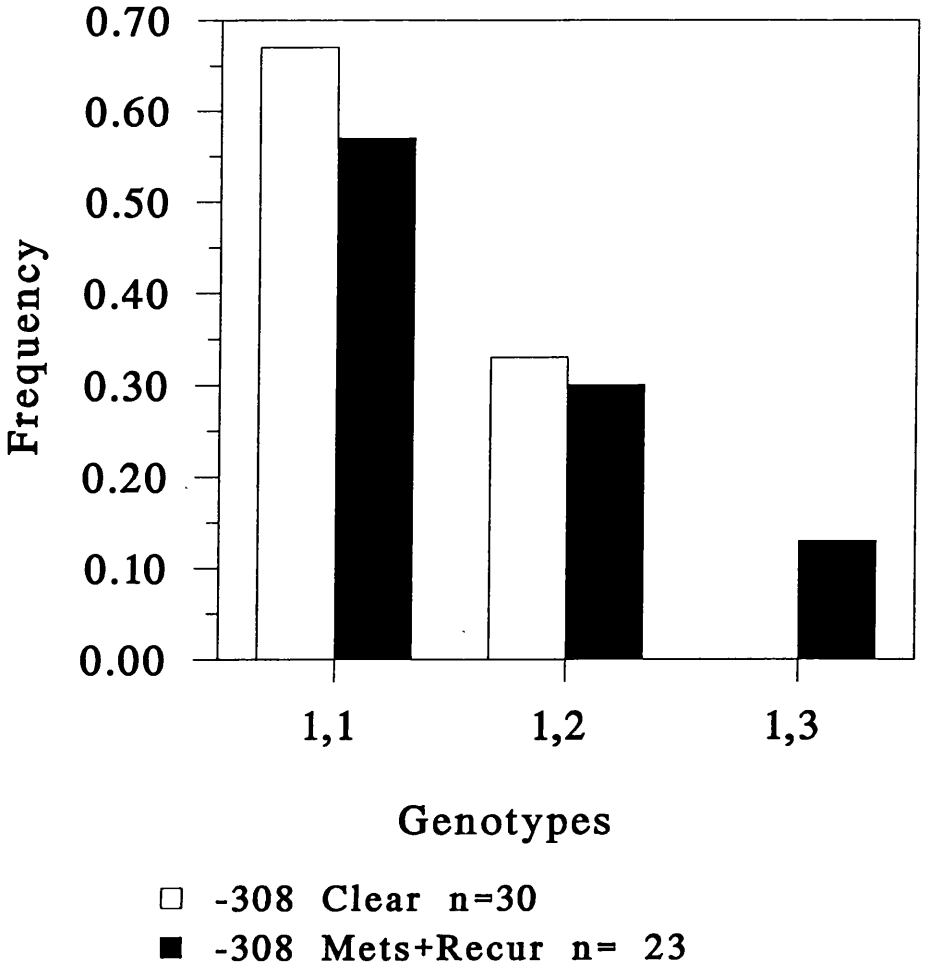

Figure 4.17. Histogram showing the distribution of the three possible -308 TNFα RFLP genotypes in the 2 years clear and metastatic/local recurrence individuals. Chi-square analysis shows that there is no association between the -308 TNFα genotype and disease progression.

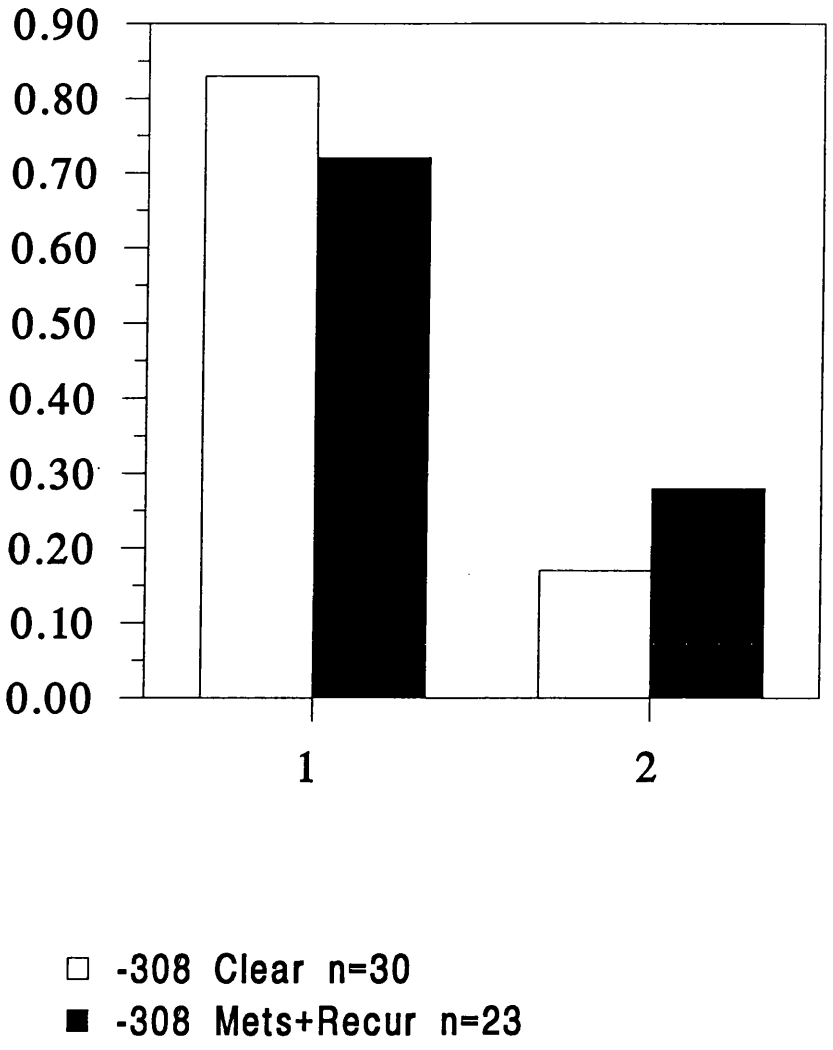

Figure 4.18. Histogram showing the distribution of the two possible -308 TNFα RFLP alleles in the 2 years clear and metastatic/local recurrence individuals. Chi-square analysis shows that there is no association between the distribution of the -308 TNFα alleles and disease progression.

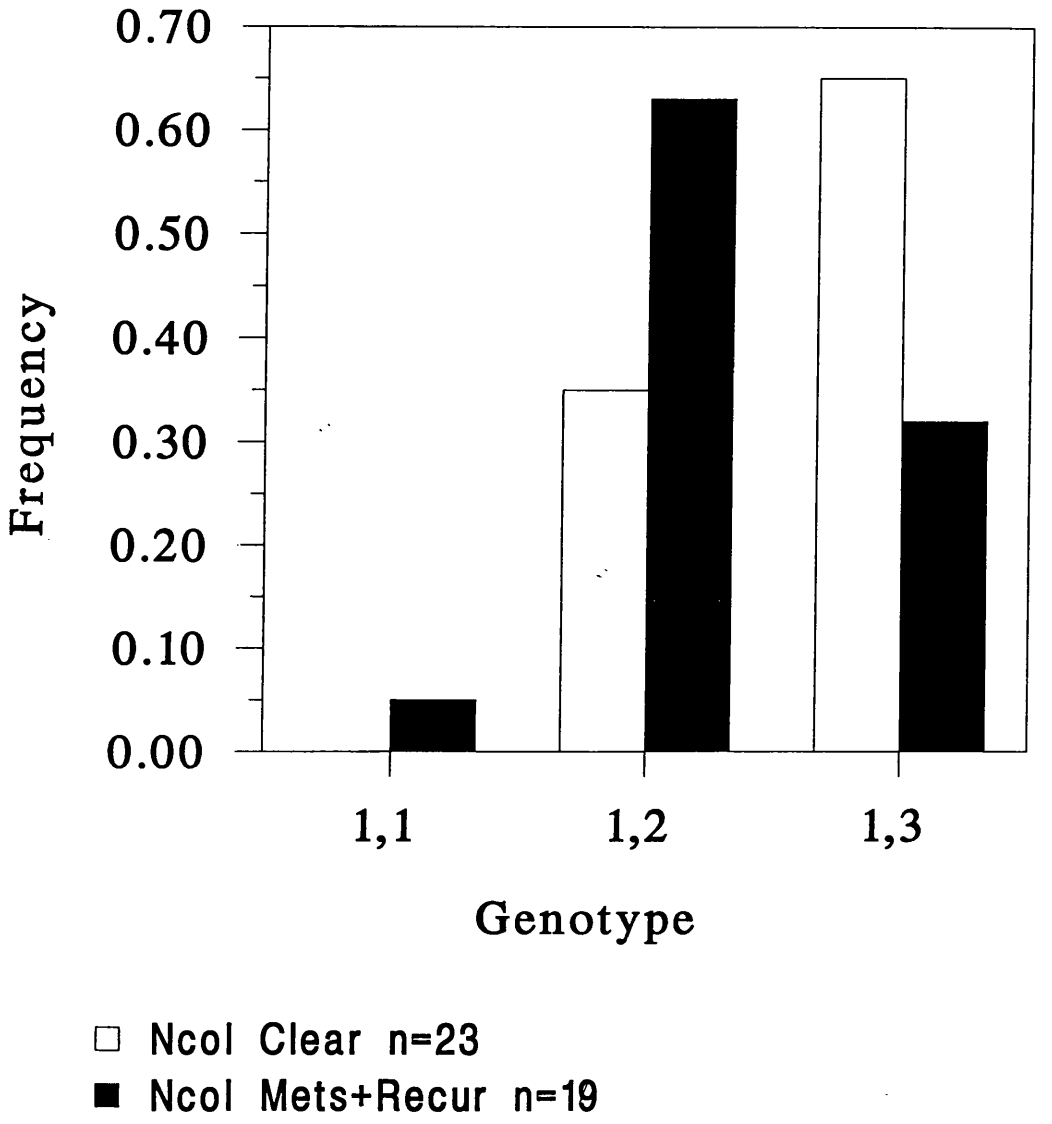

Figure 4.19. Histogram showing the distribution of the three possible LTα *Nco*I RFLP genotypes in the 2 years clear and metastatic/local recurrence individuals. Chi-square analysis shows a significant association with the TNFB*1/*2 genotype and the presence of metastatic disease, and a reciprocal association with the TNFB*2/B*2 genotype and two years disease free survival.

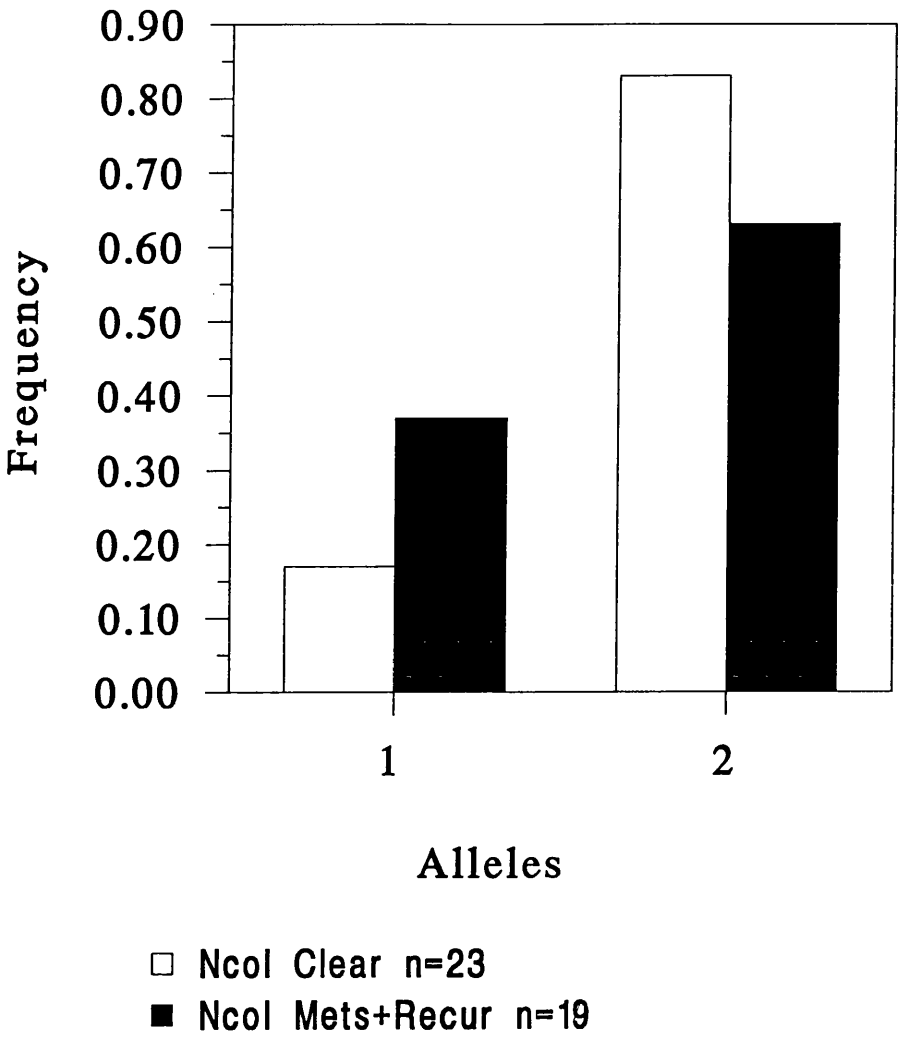

Figure 4.20. Histogram showing the distribution of the two possible LTα *Nco*I RFLP alleles in the 2 years clear and metastatic/local recurrence individuals. Chi-square analysis shows a significant association with the TNFB*1 allele and the presence of metastatic disease, and a reciprocal association with the TNFB*2 allele and two years disease free survival.

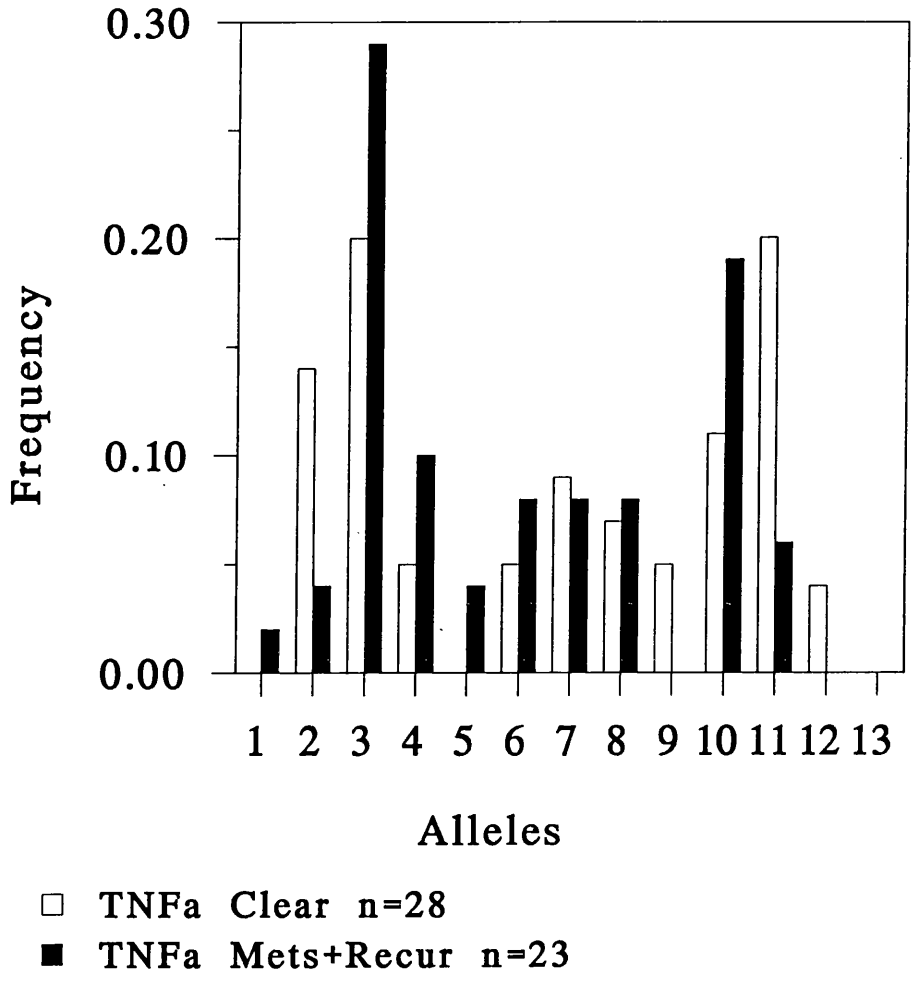

Figure 4.21. Histogram showing the distribution of the 13 possible TNFa microsatellite alleles in the 2 years clear and metastatic/local recurrence individuals. Although a number of small differences can be observed between the distribution of the TNFa alleles in these two groups, Chi-square analysis shows only a significant association with the TNFa11 allele and two years disease free survival.

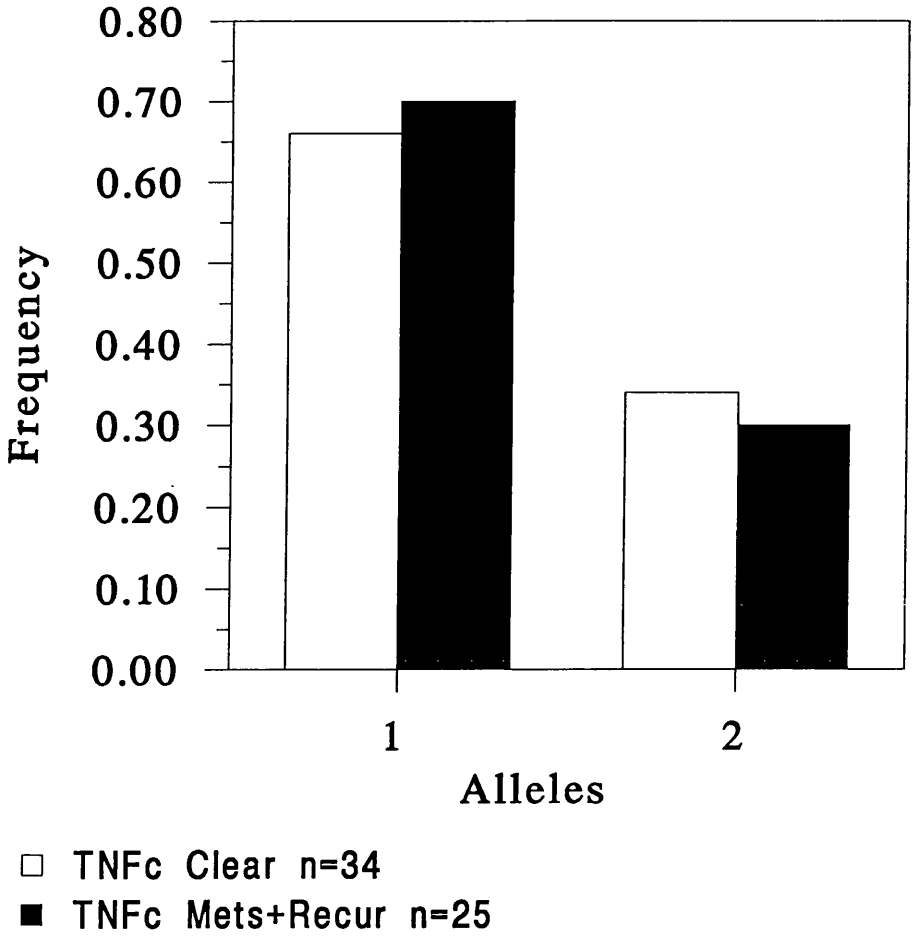

Figure 4.22. Histogram showing the distribution of the 2 possible TNFc microsatellite alleles in the 2 years clear and metastatic/local recurrence individuals. Chi-square analysis shows no significant association with the TNFc alleles and either two years disease free survival or metastatic/local recurrence.

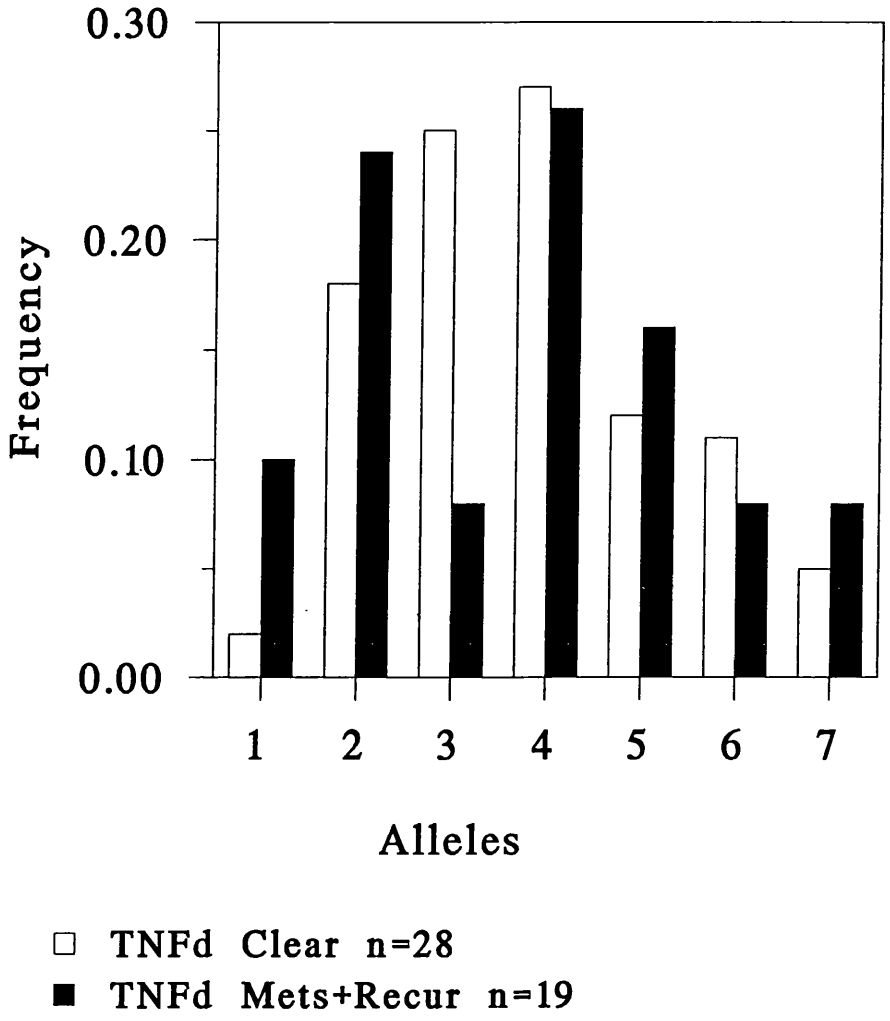

Figure 4.23. Histogram showing the distribution of the 7 possible TNFd microsatellite alleles in the 2 years clear and metastatic/local recurrence individuals. Chi-square analysis shows asignificant association with the TNFd1 allele and metastatic/local recurrence whilst the TNFd3 allele is associated with two years disease free survival.

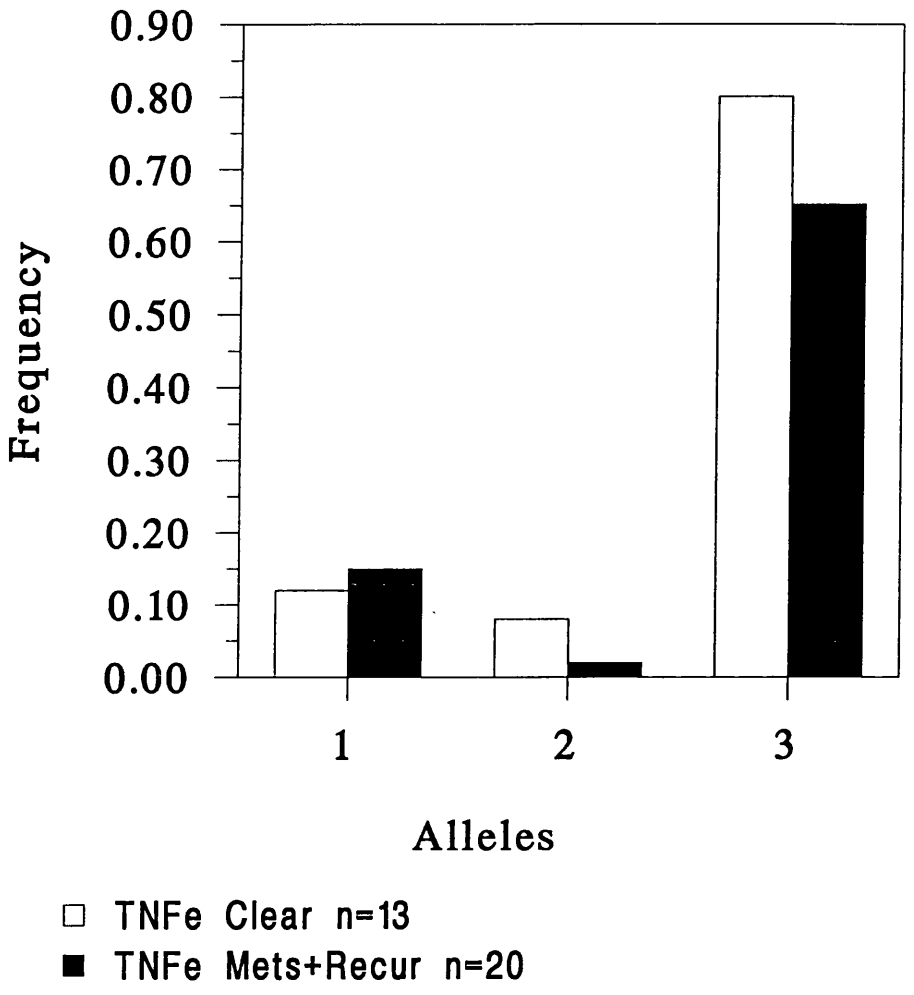

Figure 4.24. Histogram showing the distribution of the 3 possible TNFe microsatellite alleles in the 2 years clear and metastatic/local recurrence individuals. Chi-square analysis shows no significant association with the TNFe alleles and either metastatic/local recurrence or two years disease free survival.

4.4 Extended genotypes of the five TNF polymorphisms

It is now clear that the region of the MHC is highly polymorphic. However, it is widely accepted that alleles of the HLA show some degree of linkage in that they form extended haplotypes spanning from the HLA-A locus, across the class III region to the DP locus. A number of studies have now suggested that these extended haplotypes can also include alleles from the polymorphisms, including the TNF polymorphisms, found within the class III region of the MHC.

4.4.1 Extended genotypes involving the two RFLPs

As described in section 1.3.4.3 an extended genotype has been suggested between the LTα Nco-I RFLP locus and the -308 TNFα RFLP locus. This association involves linkage between the TNFB*1 allele and the TNF2 allele. It was of interest to determine if this association can be seen within the control population described within this study, and to see if there was any difference in this linkage between the normal and colorectal groups.

For all of the control individuals who were typed at both the LTα RFLP and the -308 TNFα RFLP the possible genotypes over these two loci were calculated. 76 such individuals can be identified, resulting in a total of 304 possible genotypes since it is impossible to determine the individual pairing of alleles. Of these 304 possible genotype the TNFB*2/TNF1 genotype accounts for 58% of the genotypes, the TNFB*1/TNF1 accounts for 23%, the TNFB*1/TNF2 genotype accounts for 14 % and the TNFB*2/TNF2 accounts for the final 5%.

Figure 4.25 shows a comparison of these extended genotype frequencies between the normal control individuals and the whole colorectal cancer population. Both the TNFB*1/TNF1 and the B*2/T1 extended genotypes are observed with equal

frequency between the two populations. However, the TNFB*1/TNF2 extended genotype shows a significant under representation in the colorectal cancer population (χ^2=8.347, ρ=0.002, df=1, ρc=0.008), and the TNFB*2/TNF2 extended genotype shows a significant over representation in the colorectal cancer population (χ^2=9.678, ρ=0.001, df=1, ρc=0.004).

The relationship between this extended genotype and the pathological staging of the colorectal cancer patients was also examined. Figure 4.26 shows a graph of the genotype frequences in each of the four Dukes' stages. A 4x4 chi-square analysis of the frequencies demonstrates a significant difference between the four groups (χ^2=51.848, ρ<0.0000, df=9). Closer analysis shows that the TNFB*1/TNF1 genotype is more frequent in the Dukes' B group than in the Dukes' C group (χ^2=4.022, ρ=0.003, df=1, ρc=0.012), whilst the TNFB*2/TNF2 genotype is more common in the Dukes' C genotype than in the Dukes' B genotype (χ^2=13.527, ρ=0.0001, df=1, ρc=0.0004).

Analysis of this extended genotype in the 2 years clear verses the metastatic/recurrent patients also shows an association between this extended genotype and disease progression (Figure 4.27; χ^2=14.56, ρ=0.001, df=3). Significant differences in the genotype frequencies can be seen at three of the four genotypes, with an under representation of the TNFB*2/TNF1 genotype in the metastatic/recurrent patients (χ^2=13.52, ρ=0.0001, df=1, ρc=0.0004) and an over representation of both the TNFB*1/TNF2 (χ^2=3.91, ρ=0.03, df=1, ρc=0.12) and the TNFB*2/TNF2 genotypes (χ^2=4.072, ρ=0.03, df=1, ρc=0.12).

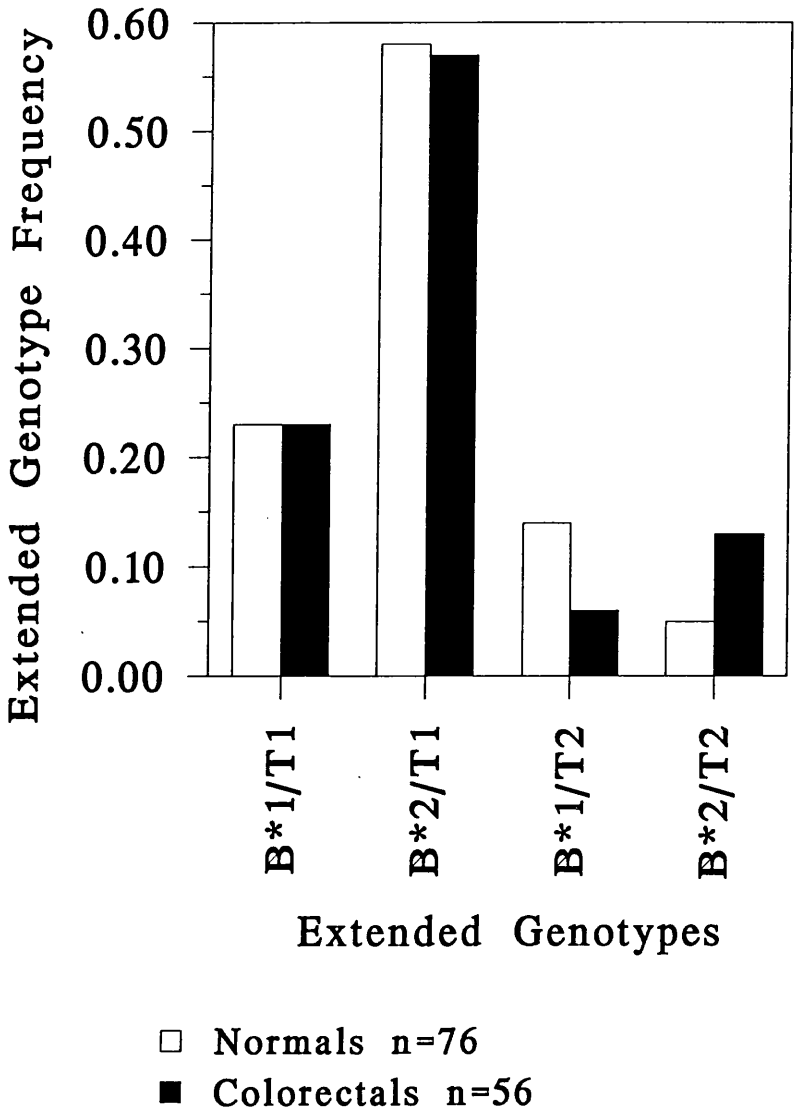

Figure 4.25. Histogram showing the distribution of the 4 possible extended RFLP genotypes in the normal and colorectal cancer populations. Chi-square analysis shows a significant under representation of the TNFB*1/TNF2 genotype in the colorectal cancer individuals and an over representation of the TNFB*2/TNF2 genotype in this same group.

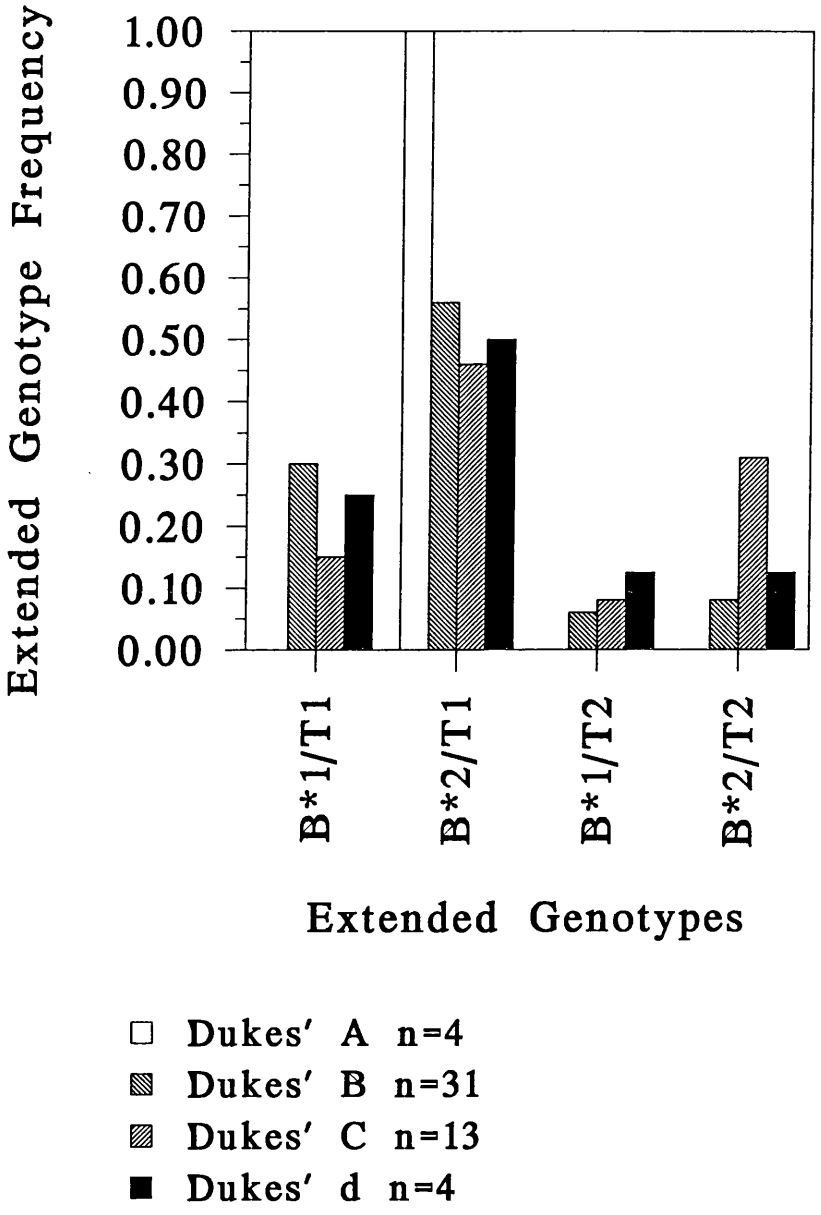

Figure 4.26. Histogram showing the distribution of the 4 possible extended RFLP genotypes in the four Dukes' stage groups. Chi-square analysis shows that the TNFB*1/TNF1 genotype is more common in the Dukes' stage B group than in the stage C group whilst the TNFB*2/TNF2 genotype is more common in the stage C group than in the stage B group.

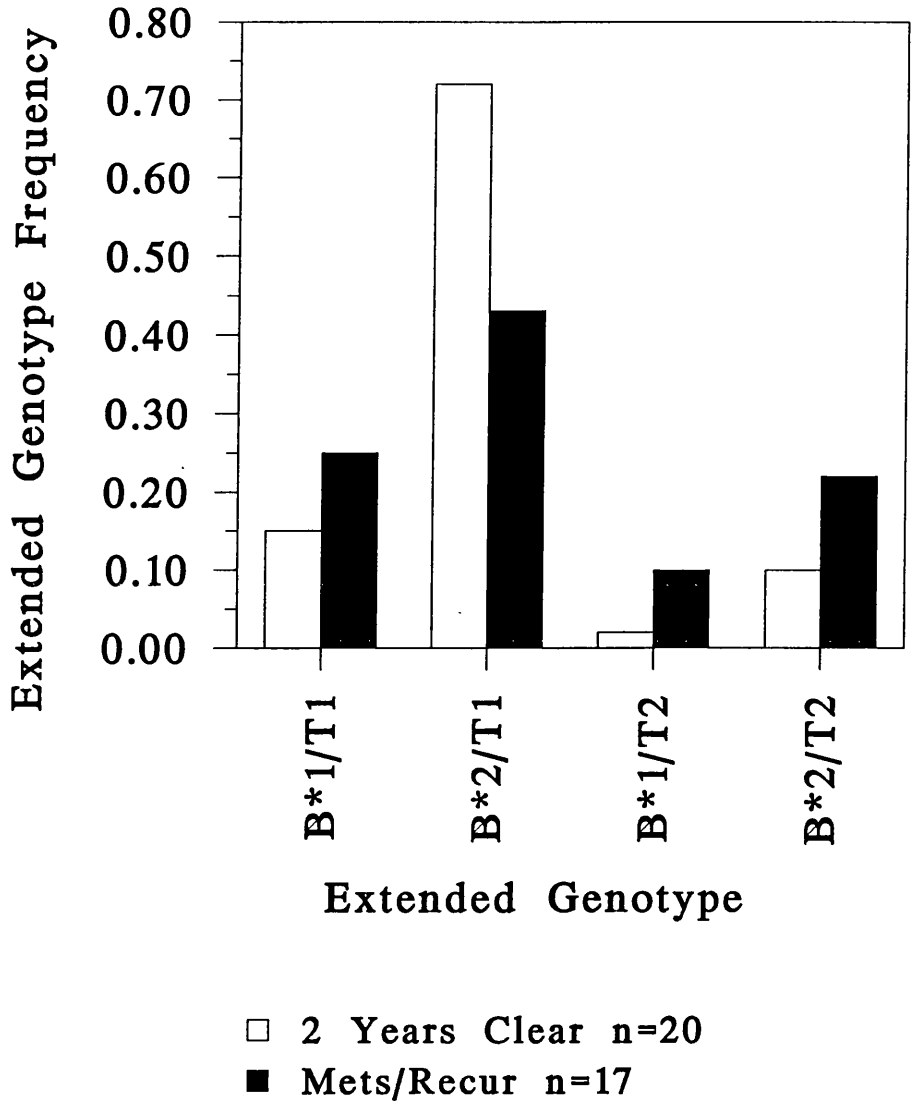

Figure 4.27. Histogram showing the distribution of the 4 possible extended RFLP genotypes in the two years disease free clear and the metastatic/local recurrence individuals. Chi-square analysis shows that the TNFB*2/TNF1 genotype is more common in the two years disease free individuals and that the TNFB*1*2/TNF2 and the TNFB*2/TNF2 genotypes are more common in the metastatic/local recurrence individuals.

Gastric Cancer

The following chapter has been included to demonstrate the similarities seen at both the TNFa microsatellite polymorphism and the LTα Nco-I RFLP in an other human adenocarcinoma. The work was performed primarily by an undergraduate student working within the department of surgery, at Glasgow Royal Infirmary, as part of her final year course in biochemistry at the University of Glasgow. Although the work was performed on a very limited sample of patients, it helps to extend the investigation into the role of these polymorphisms in human cancer.

5.1 Gastric cancer

If gastric cancer is defined as cancer of either the stomach or oesophagus, then this disease accounted for 1697 cases of cancer in Scotland in 1991 (Directorate of Information Services, Scottish Health Statistics, 1993), with over 97% of gastric cancers being adenocarcinomas of the stomach. Gastric cancer is the fifth most common malignancy in the western world and has a male to female ratio of 2:1. Like colorectal cancer, gastric cancer has seen a steady decline in its frequency of the past 50 years (Sandler, R.S. and Holland, K.L., 1987), however there has been little change in the 5-year survival rate which has remained between 7% and 11%. Diet is still thought to be the major risk factor for this malignant disease, however other studies have implicated exposure to compounds such as coal dust or asbestos (Mewhouse, M., 1981).

Since the study group involved is so small, readers are refered to the chapter in Surgical Oncology (Ed. C.S. McArdle) by D. Cunningham entitled "The management of gastric cancer", for a more detailed review of this disease.

5.2 Gastric cancer and TNF

There is very little evidence in the literature to indicate a role for TNFα in the development of Gastric adenocarcinoma. Monocytes from gastric cancer patients recieving BCG therapy in combination with 5-flurouracil, adriamycin and mitomycin C, have been shown to secrete elevated levels of TNFα (Zembala, M., *et. al.*, 1993). Apart from this study there seems to be no further evidence to suggest a role for TNF α *in vivo*. However, since there is little data to support the hypothesis for a role of TNFα in gastric cancer it therefore seemed likely that this tumour typre would be a good control for the analysis of the TNF polymorphisms in colorectal cancer.

5.3 TNFa polymorphisms

The TNFa genotypes of 23 patients with primary gastric adenocarcinoma were determined using the methods as outlined in chapter 2 of this thesis. Table 5.1 shows the individual genotypes for each of the 25 patients. Figure 5.1 shows a graph of the TNFa allelic frequencies in the normal control individuals and the 23 gastric adenocarcinoma patients. Chi-square analysis of the frequencies of the two groups is not possible due to the small number of gastric patients however a number of differences in the frequency of the individual alleles can be seen. Table 5.2 shows the individual alleleic frequencies of the normal and gastric patients. Chi-square analysis shows that the only significant difference is that of the frequency of the TNFa3 allele (χ^2=10.772, ρ=0.0006, df=1, ρc=0.0078).

It was of interest to determine if the allelic frequencies at the TNFa microsatellite polymorphism were equivalent between the colorectal and gastric populations. Figure 5.2 shows a graph of the allelic frequency of the TNFa polymorphism in these two populations. Again chi-square analysis of the two populations is not possible due to the small number of gastric patients, however the two populations look

similar. Table 5.3 shows the individual frequencies at of the 13 alleles in both groups. The only difference between the two populations is in the frequency of the TNFa5 allele which is over represented in the gastric cancer patients (χ^2=4.565, ρ=0.019, df=1, ρc=0.247).

5.4 LTα *Nco*I RFLP

The LTα Nco-I RFLP genotype of 23 individuals with histologically proven gastric adenocarcinoma were obtained by using the method as described in section 3.4 of this thesis. Figure 5.3 shows a graph of the frequencies of the three possible genotypes at the LTα Nco-I RFLP locus in 98 normal control individuals and 23 gastric adenocarcinoma patients. Chi-square analysis demonstrates a significant link between genotypes at the LTα Nco-I RFLP and the presence of gastric cancer (χ^2=8.192, ρ=0.008, df=2). Closer analysis shows that there is a decrease in the frequency of the B*1/B*1 genotype (χ^2=3.418, ρ=0.039, df=1, ρc=0.117) and a significant increase in the frequency of the B*1/B*2 genotype (χ^2=7.191, ρ=0.004, df=1, ρc=0.012) in the gastric cancer patients. The observed difference the B*2/B*2 genotype frequencies fails to reach significance (χ^2=2.447, ρ=0.075, df=1, ρc=0.225).

Figure 5.4 shows a graph of the individual allelic frequencies of the two LTα Nco-I RFLP alleles in the normal and gastric individuals. Chi-square fails to show any difference between the alleleic frequencies of the two populations (χ^2=0.084, ρ=1, df=1).

Having identified a difference in the frequency of the LTα Nco-I RFLP genotypes in the gastric patients it was of interest to compare the genotype and allelic frequencies at this loci with those for the colorectal cancer patients since no association with the

presence of colorectal cancer can be asscribed to either genotypes or alleles at this locus.

Figure 5.5 shows the graph of the genotype frequencies of the 23 gastric patients in comparison with those of the colorectal cancer patients. Chi-square analysis demonstrates a significant difference in genotype frequencies between the two groups (χ^2=6.944, ρ=0.016, df=2). Closer analysis of the frequencies of the individual genotypes demonstrates an over representation of the B*1/B*2 genotype in the gastric cancer patients (χ^2=6.502, ρ=0.006, df=1, ρc=0.018) and an under representation of the B*2/B*2 genotype in the gastric patients (χ^2=4.133, ρ=0.025, df=1, ρc=0.075).

Figure 5.6 shows a graph of the individual allelic frequencies between the gastric and colorectal cancer populations. Chi-square analysis of this data fails to show any significant difference between the two populations (χ^2=1.325, ρ=0.179, df=1).

Patient Code	TNFa	LT NcoI
40001	5,7	B*2,B*2
40002	7,3	B*1,B*2
40003	3,10	B*2,B*2
40004	6,8	B*1,B*2
40005	3,10	B*2,B*2
40006	3,10	B*2/B*2
40007	3,6	B*1,B*2
40008	6,7	B*1,B*2
40009	2,4	B*1,B*2
40010	5,10	B*1,B*2
40011	5,11	B*1,B*2
40012	3,10	B*1,B*2
40013	5,10	B*1,B*2
40014	3,11	B*1,B*2
40015	3,7	B*1,B*2
40016	5,8	B*1,B*2
40017	3,7	B*1,B*2
40018	2,4	B*1,B*2
40019	11,12	B*2,B*2
40020	7,12	B*1,B*2
40021	6,9	B*2,B*2
40022	5,6	B*1,B*2
40023	6,11	B*1,B*2

Table 5.1 Complete genotypes at the TNFa microsatellite polymorphisms and the LTα *Nco*I RFLP in the 25 gastric adenocarcinoma cancer patients.

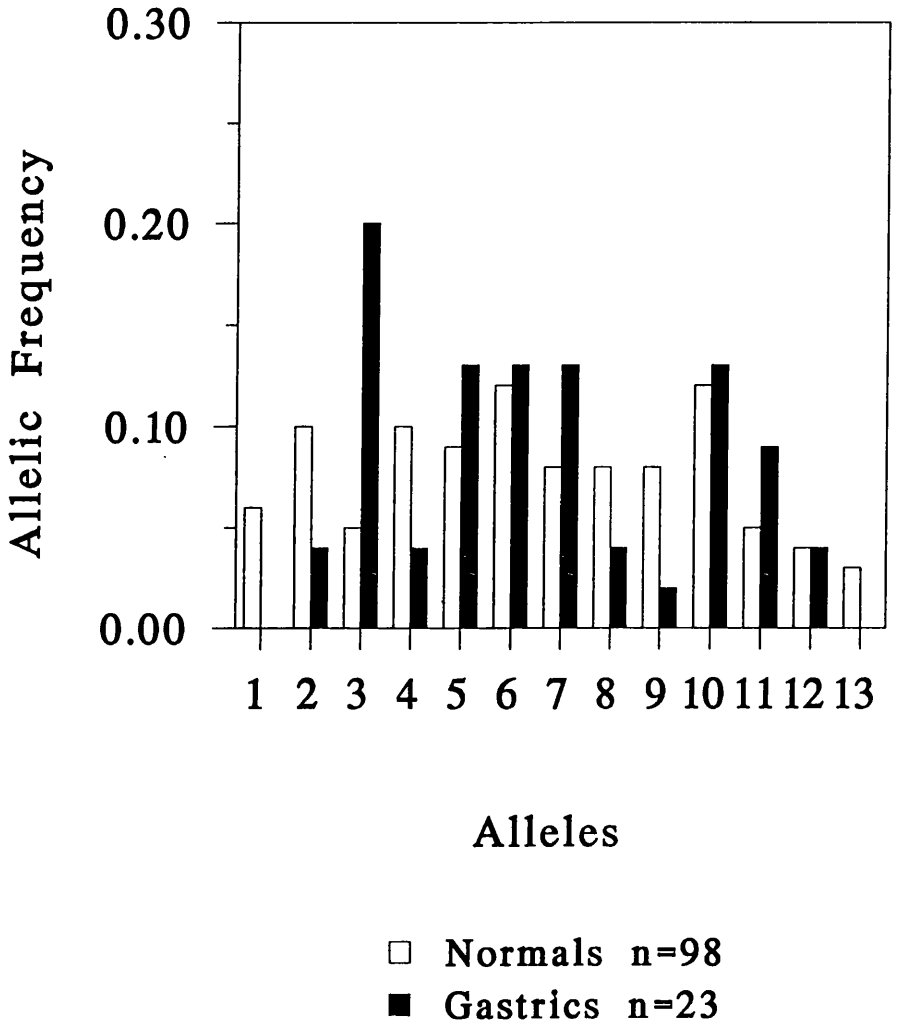

Figure 5.1. Histogram showing the distribution of the 13 TNFa microsatellite alleles in the normal and gastric cancer populations. Due to the small number of gastric cancer patients formal statistical analysis was not possible to compare these two groups

TNFa	Normals	Gastrics	Chi-square	P-value
1	0.06	0	2.705	ns
2	0.1	0.04	1.546	ns
3	0.05	0.2	10.77	0.0006
4	0.1	0.04	1.546	ns
5	0.09	0.13	0.827	ns
6	0.12	0.13	0.022	ns
7	0.08	0.13	1.366	ns
8	0.08	0.04	0.788	ns
9	0.08	0.02	2.046	ns
10	0.12	0.13	0.022	ns
11	0.05	0.09	0.883	ns
12	0.04	0.04	0.007	ns
13	0.03	0	1.198	ns

Table 5.2. Breakdown of the allelic frequencies and associated Chi-square values of the 13 TNFa alleles in the normal and gastric cancer populations. A significant linkage, by Chi-square, can be seen with the TNFa3 alleles and either the presence of gastric cancer. No other significant associations are observed.

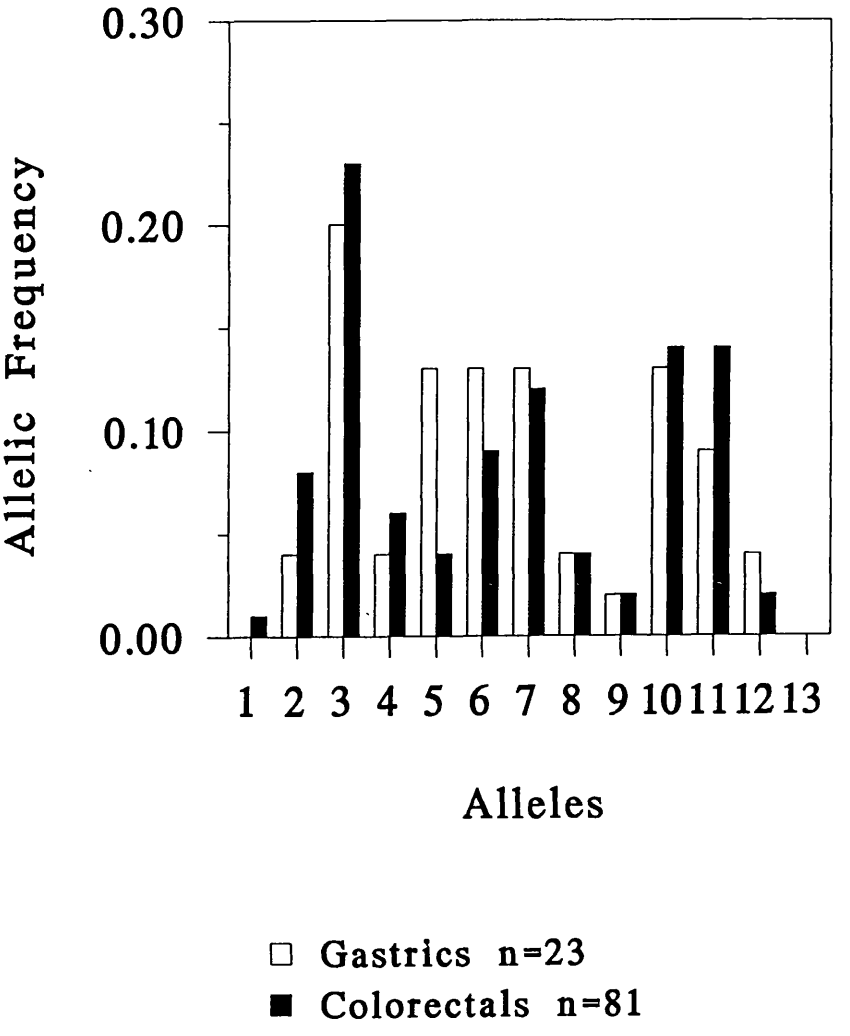

Figure 5.2. Histogram showing the distribution of the 13 TNFa microsatellite alleles in the colorectal and gastric cancer populations. Due to the small number of gastric cancer patients formal statistical analysis was not possible.

TNFa	Gastrics	Colorectals	Chi-square	P-value
1	0	0.01	0.285	ns
2	0.04	0.08	0.724	ns
3	0.2	0.23	2.267	ns
4	0.04	0.06	0.219	ns
5	0.13	0.04	4.565	0.019
6	0.13	0.09	0.799	ns
7	0.13	0.12	0.016	ns
8	0.04	0.04	0.016	ns
9	0.02	0.02	0.013	ns
10	0.13	0.14	0.04	ns
11	0.09	0.14	0.782	ns
12	0.04	0.02	0.451	ns
13	0	0	0	ns

Table 5.3. Breakdown of the allelic frequencies and associated Chi-square values of the 13 TNFa alleles in the colorectal and gastric cancer populations. The only significant difference between the two groups is the increased frequency of the TNFa5 allele in the gastric cancer individuals.

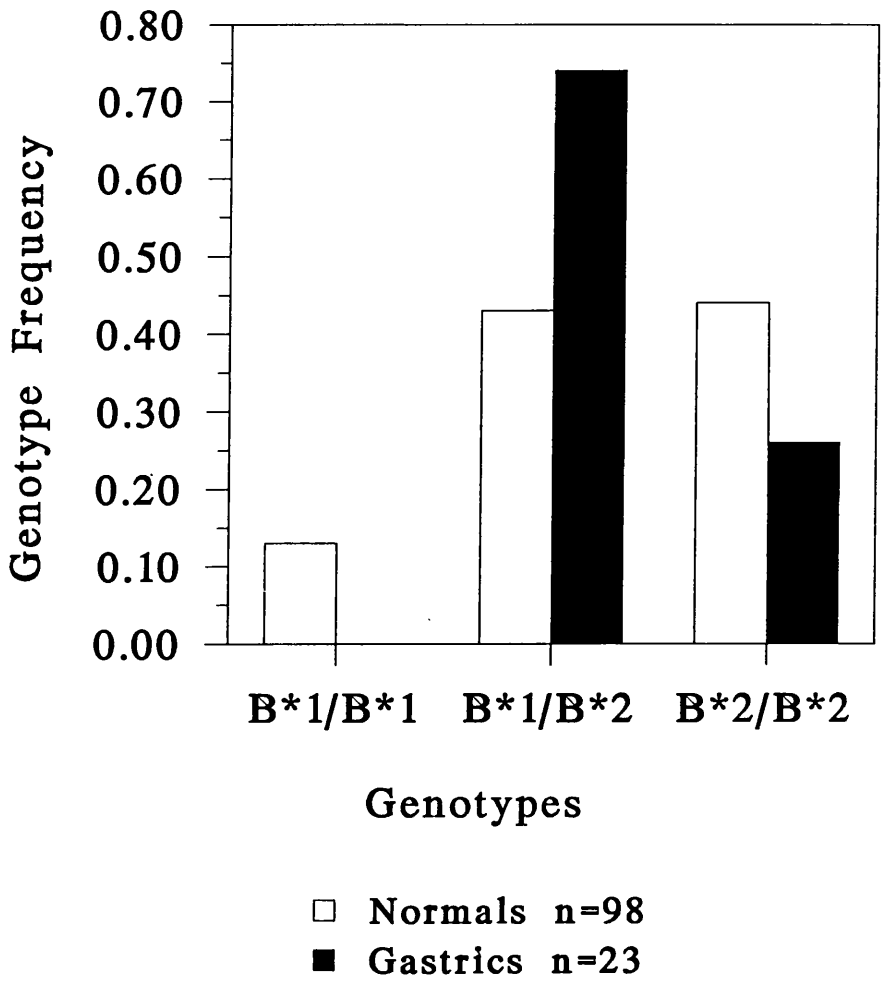

Figure 5.3. Histogram showing the distribution of the three possible LTα *Nco*I RFLP genotypes in the normal and gastric cancer populations. Chi-square analysis shows a significant association between the presence of gastric cancer and the B*1/B*2 genotype and a association with the absence of gastric cancer and the B*1/B*1 genotype.

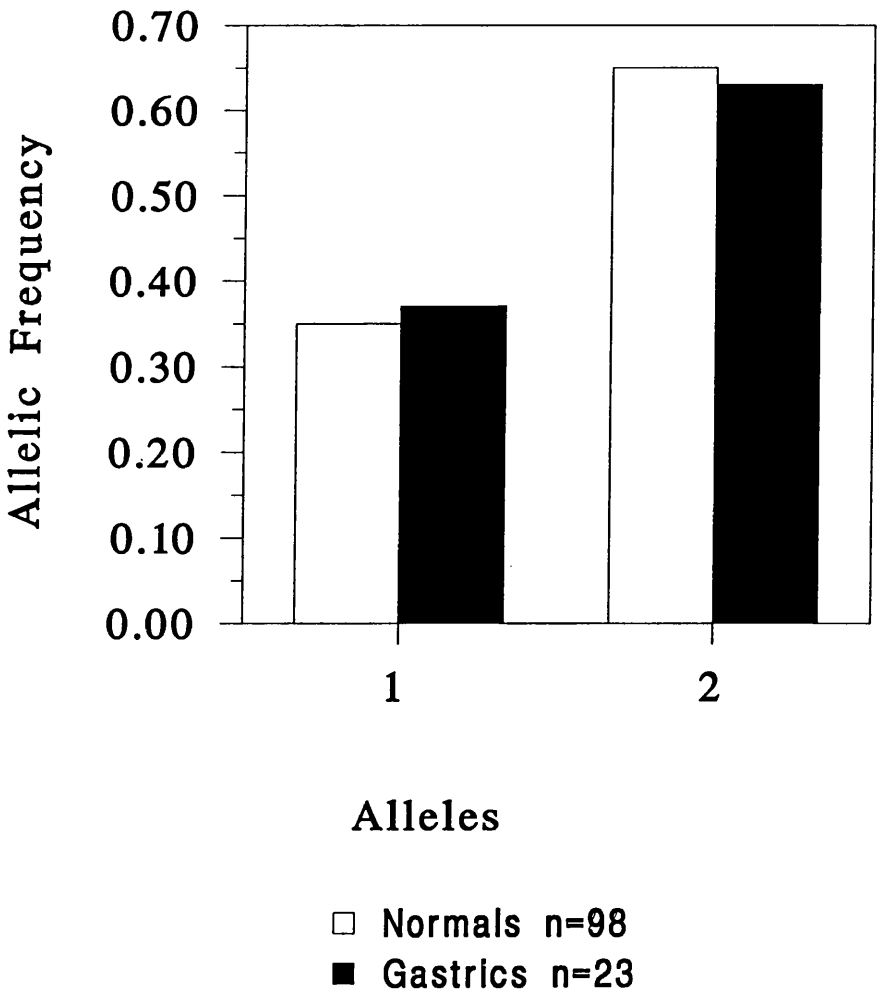

Figure 5.4. Histogram showing the distribution of the two possible LTα *Nco*I RFLP alleles in the normal and gastric cancer populations. Chi-square analysis shows no significant association between alleles at this locus and the presence or absence of gastric cancer.

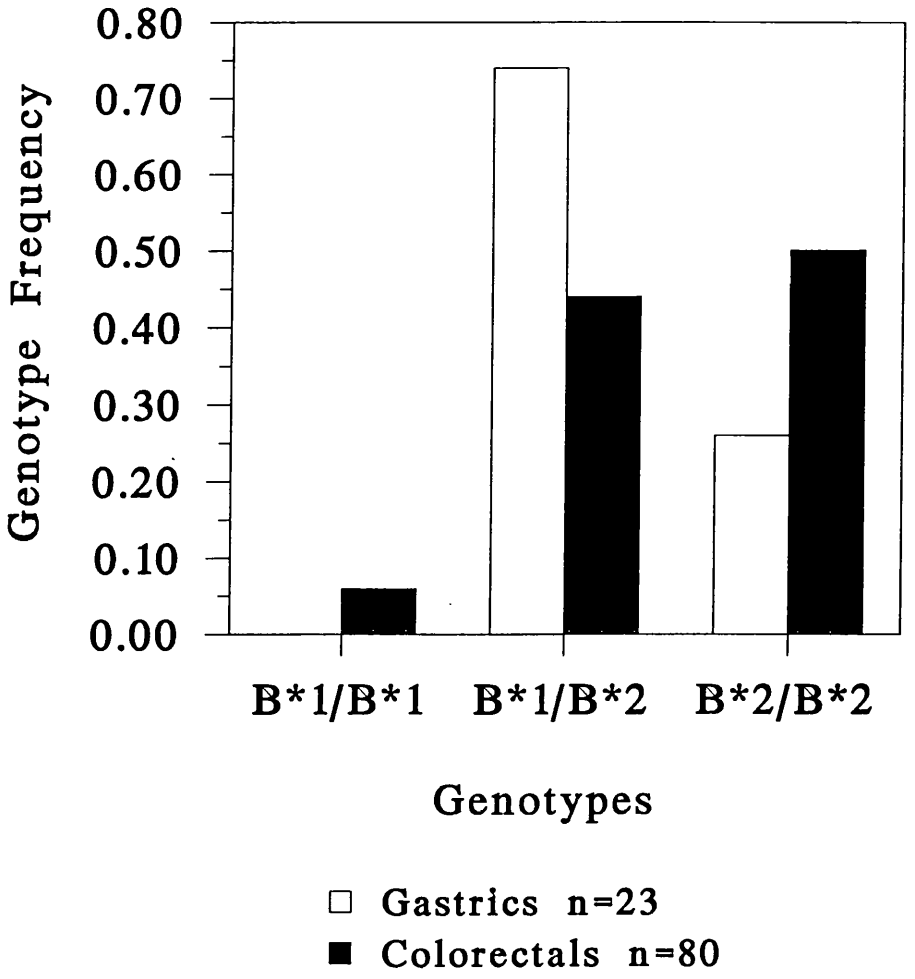

Figure 5.5. Histogram showing the distribution of the three possible LTα *Nco*I RFLP genotypes in the colorectal and gastric cancer populations. Chi-square analysis shows a significant difference between gastric and colorectal cancer with the frequency of the B*1/B*2 genotype being increase in the gastric cancer patients and frequency of the B*2/B*2 genotype being increased in the colorectal cancer patients.

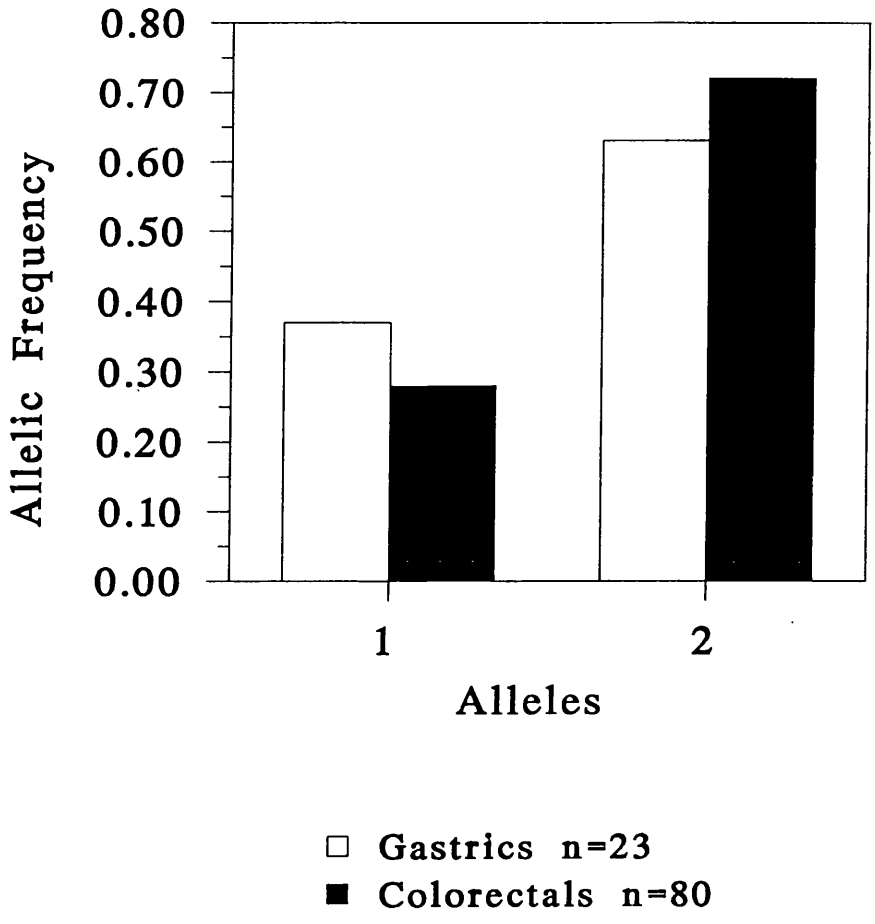

Figure 5.6. Histogram showing the distribution of the two possible LTα *Nco*I RFLP alleles in the colorectal and gastric cancer populations. Chi-square analysis shows no significant difference between alleles at this locus and gastric or colorectal cancer.

Discussion

Over the last few decades, the results of a number of major studies have provided an important insight into the mechanisms involved in the development and progression of colorectal adenocarcinomas. Despite such major improvements in the understanding of this common disease, the mechanism of treatment and the subsequent outcome has changed little in this same period.

The exact nature that genetic factors play in the tumourigenesis of colorectal neoplasms is still unclear. However, a common system of genetic changes, involving a number of recently identified oncogenes and tumour suppresser genes, has been suggested (Fearon, E.R. & Vogelstein, B., 1990). These genetic events, at least in part, support the idea of an adenoma-carcinoma sequence in which normal colonic mucosa progresses through a non-malignant adenoma stage before developing into a malignant adenocarcinoma (see section 1.1.7 and Figure 1.3 of this thesis).

Although the picture is becoming clearer, techniques which could facilitate the identification of individuals at an elevated risk of developing colorectal adenocarcinoma are still few and far between and those which do exist tend only to be used experimentally. Section 1 of this thesis describes a number of the biological and molecular markers that have been used in the past few years in an attempt to identify individuals in the general population who may be at risk of developing this malignant disease. Common to all of these markers is the problem associated with the stage at which they can detect the presence of abnormalities within the colorectal mucosa.

Markers such as the aberrant mucosal crypt cell production rate (Rooney, P.S., *et. al.*, 1993) require the detection of areas of change within the colon or rectum that can be detected and biopsied by colonoscopy. Other biochemical markers such as CEA (Thompson, D.P.M., *et. al.*, 1969) and the mucin-type glycoproteins, such as

CA19-9 (Koprowski, H., *et. al.*, 1981) can be found at elevated levels in individuals with malignant disease, but tend to lack sufficient sensitivity to be clinically useful and can often result in false positive and false negative results. Even the standard clinical forms of screening rely on the presence of noticeable changes within the colon or rectum and thus patients often tend to be presented for surgery too late to allow a sufficient cure rate.

The ability to identify individuals who may be at risk of developing colorectal cancer due to a genetic predisposition is therefore attractive. It is widely accepted that the host immune system is aware of the presence of a malignant lesion and that under the correct conditions is capable of mounting an immune response against the tumour (for example the work of Rosenberg and colleagues (1988)on the augmentation of T-cell cytotoxicity upon pre-treatment with IL-2 for the treatment of metastatic melanoma). Why the immune system therefore allows tumours to develop still remains a mystery.

Central to the modulation of the immune system is the family of compounds commonly termed tumour necrosis factors (TNFs). This family consists of three compounds termed TNFα, lymphotoxin-α (LTα) and LTβ. The most widely studied of these molecules is TNFα and many studies have shown TNFα to be involved in the pathogenesis of a number of autoimmune and non-autoimmune diseases; TNF is now regarded to play a pivotal role in the inflammatory response.

Levels of TNFα at both the mRNA and the protein level have been examined in a number of human solid tumours. Although a substantial amount of data is available on the presence of this molecule particularly in breast cancer (Spriggs, D.R., *et. al.*, 1988), there is very little data with regards to colorectal cancer. Those studies which have examined levels of TNFα mRNA and protein report a very low level of TNFα within the tumour with the majority of the material being associated with tumour

associated macrophages (Beissert, S., *et al,* 1983; Naylor, M.S., *et al,* 1992). It has been suggested that this low level of TNF may be responsible for the "desensitisation" of tumour cells to the cytotoxic activity of the tumour associated macrophage derived TNFα, however the reason why such a low level of secretion should occur is unclear.

Recently a number of polymorphisms have been described within the TNF locus and subsequent publications have reported alterations in the ability of cells to produce members of the TNF family associated with specific alleles of these polymorphisms (Pociot, F., *et. al.*, 1993a; Wilson, A.G., *et. al.*, 1994a; Turner, D.M., *et. al.*, 1994). The data reported in this thesis now suggest a role for these polymorphic markers in understanding the development and progression of colorectal adenocarcinoma, whilst a small preliminary study also suggests a similar role in gastric adenocarcinoma. Although the true function of these markers in the pathology which underlies these two malignant diseases still remains unclear, it is possible to make a number of comparisons with both other human disease which show associations with these markers and also with a number of studies which have examined the function of equivalent murine polymorphisms.

There are at least ten TNF associated polymorphisms located within a 12kb region of the human MHC class III region on chromosome 6. These polymorphisms can be split into two groups. The first group comprises five restriction fragment length polymorphisms, designated as the LT-α *Eco*RI RFLP, the LT-α Nco-I RFLP, the -308 TNF-α RFLP, the -238 TNFα RFLP and the *Asp*HI LTα RFLP (Partanen, J. and Koskimies, S., 1988; Messer, G., *et. al.*, 1991; Wilson, A.G., *et. al.* 1992; D' Alfonso, S. & Richiardi, P.M., 1994; Ferencik, S., *et. al.*, 1992), and the second comprises five di-nucleotide repeat microsatellite polymorphisms designated as the TNFa, TNFb, TNFc, TNFd and TNFe polymorphisms (Jongeneel, C.V., *et. al.*, 1991 and Udalova, I.A., *et. al.* 1993).

The data in this thesis shows a comparison of the allelic frequencies of the alleles of six of these TNF associated polymorphisms in a group of normal control individuals, a group of pathologically proven colorectal cancer patients and a small group of gastric cancer patients. Although the methodology used in this study has been adapted from a number of publications (see elsewhere in this thesis) it is standard practice in adapting and transferring such protocols to include a series of families to allow for a validation of the allele calling protocol. No large families were available at the beginning of this study however, three partial families have been subsequently identified within the control individuals. Although there are small anomalies at the TNFc locus for family one and at the TNFd locus for family two and although the genotypes are incomplete for a number of the individuals this data provides reassurance that the allele calling strategy used in this study produced consistently reproducible results in terms of accuracy of allele calling at all of the TNF polymorphism loci examined (see section 4.2.3.5).

Further reassurance of accuracy of allele calling is obtained from the examination of data from other groups. Four separate studies have reported population frequencies for the TNF microsatellite alleles (Nedospasov, S.A., *et. al.*, 1991; Jongeneel, C.V., *et. al.*, 1991; Udalova, I.A., *et. al.*, 1993, Crouau-Roy *et. al.*, 1993). Taken together these four studies demonstrate the wide variation in allelic frequencies at the TNFa and TNFc loci in a total of six different populations. Table 6.1 shows a direct comparison with the allelic frequencies of the normal individuals in this study and the six published populations. There are a number of other differences between the control population in this study and the six reported populations however without the raw data from these studies it is impossible to statistically compare the distribution of the alleles between the different studies. Crouau-Roy *et. al.* have, however, performed a statistic analysis of the distribution of the TNFa alleles in their four populations and report significant differences when these are compared. Although the allele frequencies from the normal population in this study do not directly match

		Crouau-Roy (1993)				Jongeneel (1991)	Nedospasov (1991)
TNFa	Normals	Basque	French	Greek	Danish	French	Russian
1	0.06	0.116	0.031	0	0.007	0.02	0
2	0.1	0.25	0.208	0.219	0.32	0.215	0.11
3	0.05	0.035	0.031	0.012	0.033	0.034	0
4	0.1	0.045	0.042	0.037	0.1	0.04	0.9
5	0.09	0.036	0.031	0.11	0.067	0.04	0.4
6	0.12	0.071	0.094	0.134	0.14	0.101	0.22
7	0.08	0.17	0.125	0.073	0.04	0.134	0.15
8	0.08	0	0.052	0	0	0.04	0
9	0.08	0.027	0	0.085	0.033	0	0.07
10	0.12	0.107	0.104	0.134	0.093	0.154	0.15
11	0.05	0.089	0.187	0.098	0.147	0.128	0.13
12	0.04	0	0.073	0.012	0.033	0.074	0.02
13	0.03	0.054	0.021	0.085	0.007	0.013	0.02
TNFc							
1	0.69	0.596	0.771	0.697	0.706	0.772	0.87
2	0.31	0.404	0.23	0.303	0.294	0.228	0.13

Table 6.1. Comparison of the allelic frequencies at the TNFa and TNFc loci of the normol control individuals with 6 published populations. A number of differences can be seen between the six populations and the allelic frequencies of the TNFa and TNFc alleles from this studyand also within the six published populations. [1]Crouau-Roy *et. al.*, 1993; [2]Jongeneel, C.V., *et. al.*, 1991; [3]Nedospasov, S.A., *et. al.*, 1991.

any of the allelic frequencies from either of the other studies there are sufficient similarities to provide sufficient reassurance that the allelic frequencies reported in this study are correct.

Having identified these differences it is tempting to speculate why these differences should occur. It is unfortunate that Crouau-Roy *et. al.* have not included MHC data in their study. The study by Jongeneel *et. al.* (1991) clearly demonstrates the existence of linkage between the TNF polymorphisms and both the MHC class I and class II regions. It is therefore almost certain that the differences reported between the six different populations, and indeed the normal population used in this study, is associated with the MHC background of the populations. Indeed the two independent studies of the French population (Jongeneel, C. V., *et. al.*, 1991 and Crouau-Roy, B., *et. al.*, 1993) show very similar frequencies of both the TNFa and TNFc alleles, with a few differences at the TNFb locus. Having established that the allele calling strategy from this study provides reproducible and comparable results it is now possible to further speculate on the exact meaning of the data presented within this thesis.

Of the ten TNF associated polymorphism loci, two restriction fragment length polymorphisms have gained much attention over the past few years. The LTα *Nco*I RFLP is located within the first intron of the LTα gene and results is a biallelic polymorphism resulting in three possible genotypes per individual. Associated with this polymorphism is an amino acid substitution at codon 26 of the mature LTα protein resulting in a asparagine to a threonine conversion (Messer, G., *et. al.*, 1991). Data from a number of groups has now suggested that the genotype at this locus correlates with a distinct pattern of TNFα, but not LTα, secretion by stimulated monocytes *in vitro*. In the most recent study (Pociot *et. al.*, 1993a), monocytes from individuals homozygous for the common B*2 allele were shown to produce a significantly higher amount of TNFα upon LPS-stimulation whilst individuals

homozygous for the rare B*1 allele showing low levels of secretion and heterozygous individuals secreting an intermediate level of TNFα. These results are in contrast with earlier results of Messer *et. al.* (1991) which show no association with TNFα secretion in PHA-stimulated PBMCs, but do show a distinct pattern of LTα secretion associated with the LTα *Nco*I RFLP genotype, whilst a study by Abraham *et. al.* (1993) also reports an association with the B*1 allele and high TNF α and LTα production. There are a number of reason why these discrepancies should occur and it is probable that the results obtained are dependent on a number of factors. These factors would include cell type, stimulus used, type of assay used to measure the cytokine and probably more importantly the MHC background of the cells.

More recently a polymorphism has been detected within the promotor region of the TNFα gene. This polymorphism is located at position -308 relative to the start codon of the TNFα gene. Like the LTα *Nco*I RFLP an number of studies have attempted to correlate alleles of this polymorphism with ability to produce TNFα. Studies involving a CAT construct reporter system have demonstrated that the rare TNF2 allele bearing promotor produces a 6-7 fold increase in reporter activity in the Raji B-cell line (Wilson, A.G., *et. al.*, 1994b) although in another study a similar CAT construct, but this time in the Jurkat T-cell line, failed to show any difference in reporter activity (Brinkman, B.M.N., *et. al.*, 1994).

Given the possible influence of these two polymorphisms on the ability of a cell to produce TNFα it was of great interest to examine whether the genetic component of colorectal carcinogenesis could include alleles of these two polymorphisms. However an examination of both the genotype and allelotype frequencies of these two polymorphisms in the panel of colorectal cancer and normal individuals failed to show any significant association with either specific individual alleles or with specific genotypes and the presence of histologically proven colorectal cancer.

The majority of publications which have described associations with alleles of these two RFLPs and either disease or the production of TNFα also showed that these two loci form part of a large extended haplotype which covers the MHC class I, class II and a number of the class III genes. Since there is no association between the presence of colorectal cancer and either alleles or genotypes of the -308 TNFα RFLP and the LTα *Nco*I RFLP individually this would suggest that the low level of TNFα seen within colorectal tumour biopsies (Beissert, S., *et. al.*, 1989) is not genetically predisposed by alleles of either of these two genotypes. However, when both of the polymorphisms are examined in the form of an extended genotype, there is an under representation of the TNFB*1/TNF2 genotype, which should predispose to the secretion of low levels of TNFα corresponding to the presence of the rare TNFB*1 allele but high levels of TNFα corresponding to the presence of the TNF2 allele and an over representation of the TNFB*2/TNF2, or high TNFα secretion, genotype. These results would therefore suggest that perhaps individuals with colorectal cancer are predisposed to secretion of high levels of TNFα and that therefore either TNFα is not involved in the initial process of tumourigenesis or that aberrant high expression of TNFα may be important in the initial process.

Pei *et. al.* (1991) have demonstrated the existence of extended MHC haplotypes involving polymorphisms of loci of the Class III region including the LTα *Nco*I RFLP. In this study, the authors clearly demonstrate an association between alleles of the LTα polymorphism, the HLA-B-associated transcript-3 gene (BAT3) and two RFLPs (termed R5A and M20A) located between the HLA-B locus and the TNF locus. The authors demonstrate that the extended haplotypes A1, B8, DR3 and A3, B7, DR2 both showed a characteristic haplotype profile at the four polymorphisms. However when the authors attempted to examine the association between these polymorphisms and individual HLA-B or -DR antigens they found that this was highly variable in that the alleles associated with B7 or DR2 and B8 or DR3 were not the same as those associated with the respective extended haplotypes. This

would suggest that these RFLPs were defined during the evolution of this part of the genome, prior to the selection of the common extended haplotypes. The authors suggest that since all of these polymorphisms are closer to HLA-B than they are to HLA-DR it is therefore no surprise that certain HLA-B antigens such as B14, B35, B44, Bw62, Bw57 and B27 demonstrate significant linkage disequilibrium with one or more of the class III polymorphisms. In general these associations were independent of HLA-DR. In fact most HLA-DR antigens in this study showed no association with any of the class III polymorphisms except for HLA-DR2 which showed a strong association with the BAT3 and the R5A polymorphisms and HLA-DR3 which shows a strong association with the BAT3, LTα, R5A and M20A polymorphisms. The ancestral MHC haplotype 8.1 (A1, B8, BfS, C4AQ0, C4B1, DR3) has been shown to include the TNFB*1 allele of the LTα *Nco*I RFLP (Dawkins, R.L., 1989; Abraham, L.J., 1991). A more recent study by Abraham *et. al.* (1993) demonstrated that the TNFα activity of supernatants from lymphoblastoid cell lines carrying the 8.1 haplotype was greater than in cell lines homozygous for a number of other ancestral haplotypes. The authors also show an association with the TNFB*1 allele and the increase in TNFα production which is in conflict with the view of Pociot *et. al.* (1993) who suggest that it is the TNFB*2 allele which is associated with high TNFα production. The authors do suggest, however, that since the LTα *Nco*I RFLP is within a non-coding region of the LTα gene that it is perhaps either another polymorphism within the TNF locus that is associated with the alterations in TNFα secretion or that it is the MHC ancestral haplotype which is directly responsible.

It has been demonstrated in RA patients that the HLA-DR antigens play an important role in the pathogenesis of this autoimmune disease. A study by Weyand *et. al.* (1992) demonstrated that individuals either homozygous or heterozygous for specific HLA-DR alleles display different extra-articular manifestations of the disease. They report that homozygosity for the common HLA-DRB1*0401 was common among

patients with major organ involvement, whilst combinations of either DRB1*0401 with B1*0404 or B1*0101 was typical in patients with nodular disease. A system similar to this may also involve the alleles of the TNF RFLPs. It may not just be the presence of the TNFB*2/TNF2 genotype that confers the susceptibility to the development of colorectal cancer but rather it may be the way in which the alleles of these two loci have come together due to inheritance with themselves and/or with other alleles of the TNF polymorphisms and the MHC, that may result in the development of the predisposition to the disease.

There are no data in the literature to suggest that the initial development of colorectal cancer is associated with a particular MHC haplotype although there are a number of studies that report altered expression of both class I and class II antigens (see section 1.2.3 of this thesis). A recent study by Shimura and co-workers described an association between TNFB*2 homozygosity and the protection from the development of lung cancer (Shimura, T., *et. al.*, 1994) and a number of MHC haplotypes have been associated with this disease. Of particular interest, Aw19 and B5 are associated with resistance to the progression of bronchogenic carcinoma (Rogentine, G.N., *et. al.*, 1977), whilst more recently HLA-DR7 has also been suggested to confer resistance to lung cancer (Romano, P.J., *et. al.*, 1991). It is probable that the effect on protection from the development of lung cancer is conferred by the MHC haplotype of the unaffected individuals rather than being independently associated with the LTα *Nco*I haplotype. Why there is an association between extended haplotype and the presence of colorectal cancer is unclear and perhaps a similar system exists in colorectal cancer as has been demonstrated in lung cancer by Shiumara *et. al.*(1994). It is possible that there is an as yet undetected association between the MHC and the susceptibility of colorectal cancer, and that rather than it being an independent association with the classical MHC antigens it is with an extended MHC haplotype encompassing the TNF polymorphisms. In order to fully establish whether there is an association between the genotypes of the two

RFLPs and the presence of colorectal cancer a study of the individuals extended MHC haplotype and that of their parents would be required. This would enable the determination of the inheritance pattern of the alleles of these genotypes and may provide a key to the genetic predisposition of non-familial colorectal cancer.

Although there is no data available regarding the MHC genotypes of the colorectal cancer individuals limited data is available for the control individuals. The small numbers involved do not allow for a formal analysis of association with TNF alleles and MHC however this data does at least provide reassurance that the control individuals represent a reasonable spread of the expected MHC genotypes in a South West of Scotland population.

Until recently polymorphic analysis of the TNF locus has been restricted to the use of RFLPs, however recently a panel of five microsatellites has been described within this locus (Jongeneel, C.V., *et. al.*, 1991; Nedospasov, S.A., *et. al.*, 1991; Udalova, I.A., 1993). Analysis of these more polymorphic microsatellite loci also identifies a number of associations with specific alleles and colorectal adenocarcinoma. The most polymorphic of the four microsatellites examined is the TNFa microsatellite which displays 13 possible alleles. An association with increased allelic frequency of the TNFa3 and TNFa11 alleles is observed in the colorectal cancer patients. Conversely TNFa1, TNFa5, TNFa9 and TNFa13 all show an association with the absence of, or protection from the development of, colorectal cancer. Like the RFLPs alleles of a number of the microsatellite polymorphisms have been associated with increased or decreased levels of secretion of TNFα. Of the two TNFa microsatellite alleles which have been associated with the increase or decrease in TNFα production (Pociot *et. al.*, 1993), namely TNFa2 (high production) and TNFa6 (low production), neither of these alleles shows any association with either the presence or absence of colorectal cancer in this population suggesting that the linkage between these markers and the presence of colorectal cancer may not

indicate a genetic predisposition to either over or under production of TNFα by colorectal cancer individuals.

The TNFc microsatellite has also been implicated in the control of the production of TNFα with TNFc2 positive monocytes displaying increased production upon stimulation (Pociot *et. al.*, 1993). However no difference is observed between the colorectal cancer individuals and the normal controls in terms of the allelotypes of the TNFc microsatellite again suggesting a lack of genetic susceptibility to aberrant TNFα production.

More recently the TNFd3 allele has been demonstrated to be important in the control of TNFα secretion in heart transplant biopsies (Turner *et. al.*, 1994). Analysis of the TNFd allelotypes of the normal individuals and the colorectal cancer patients demonstrates a difference between the frequency of the TNFd3 allele with the highest frequency being observed in the colorectal cancer patients. However like the association with LTα RFLP haplotye there is some question regarding the association with TNFd3 and high levels of TNFα. This finding has only been reported by one group in a small number of individuals who had undergone cardiac transplantation and it is possible that, since the individuals were receiving large amounts of immunosupressant therapy and were under significant immunological stress due to the organ transplant, the data could be influenced by the procedures which the patients were undergoing.

To date no associations has been demonstrated between alleles of the TNFe polymorphism and the production of TNFα, however analysis of the TNFe genotypes of the normal and colorectal cancer individuals demonstrates a significant difference between the allelotypes of the two populations. In particular TNFe3 is significantly over represented within the colorectal cancer population, whilst TNFe2 is significantly under represented within this same group. Like the TNFa3 allele,

these TNFd and TNFe associations could be markers of a linkage with a gene or a number of genes within this region that are associated with malignancy.

Why there should be this association with microsatellite alleles is even more unclear than for the RFLPs. The vast majority of studies that examine microsatellites use them as markers to detect linkage between regions of the genome and specific diseases. By performing linkage analysis of this sort it has been possible to identify numerous candidate genes important in a large number of human malignant and non-malignant diseases. It is therefore perhaps just luck that an association has been identified between alleles of the various TNF associated microsatellites and the presence of malignant disease. If these data do demonstrate a linkage between this region of the genome and colorectal cancer it would be easy to speculate which gene or genes within this region may be important in colorectal carcinogenesis since this region contains a number of important immunoregulatory genes. This region contains amongst other genes the TAP genes which are a family of genes involved in the transport of antigens derived from the cytoplasmic protein pool to a pre-golgi region where they are incorporated into the MHC class I molecule. Recently a down regulation of TAP1 has been demonstrated in colorectal carcinomas (Kaklamanis, L., *et. al.*, 1994). In order to identify which gene or genes are important would require a more detailed and larger study and would require more detailed statistical analysis. It must also be remembered that the TNFa microsatellite locus is 3.5kb away from the LTα gene and that to date there has been no search for a gene reported within this region. The sequence between the TNFa and b microsatellite loci and the LTα gene has recently been published and it is therefore now open for a formal analysis to be performed on this region to determine whether it contains an further gene (Iris, F.J.M., *et. al.*, 1993). Indeed loss of heterozygosity of chromosome 6 has been demonstrated in ovarian tumours (Foulkes, W.D., *et. al.*, 1993) suggesting that there may be a tumour suppressor gene on human chromosome 6. Data from the mouse also suggests that there could be a gene

involved in determining tumour susceptibility within the region of the MHC. A recent paper by Fijneman *et. al.* (1995) suggests that a susceptibility gene for alveolar lung tumours in the mouse is located between the HSP70.3 and the G7 genes within the H2 complex of the mouse although the exact gene has not yet been identified.

From this initial study it seems unlikely that the genetic polymorphisms which have been demonstrated to affect the control of TNFα production play any part in the initial development of colorectal adenocarcinoma. The specific association seen with various alleles does however suggest that there is some form of association with this region and a susceptibility to colorectal cancer.

It is also interesting to note the similar results observed when the TNFa genotypes are examined in a group of gastric adenocarcinoma patients. If the effect of specific alleles of the TNF polymorphisms are to be seen as markers for the development of adenocarcinomas in general then it would be expected that similar associations to those observed in colorectal adenocarcinoma would be observed in gastric adenocarcinomas. And indeed this is the case. In particular, again the TNFa3 allele shows a strong association with the presence of cancer. This would suggest that the TNFa3 microsatellite is a marker for a common event in the development of adenocarcinomas. Unlike the colorectal cancer group, however, the gastric cancer group also shows an independent association with genotypes of the LTα *Nco*I RFLP. The LTα *Nco*I RFLP association is with the heterozygous genotype and when the frequency of the individual alleles is examine the association is lost. This would suggest that the association between the LTα *Nco*I RFLP locus and gastric cancer is related to the combination of the two alleles and therefore possibly due to some form of inheritance pattern possibly associated with the MHC, rather than a function of these alleles on TNFα production.

In contrast to the idea that these microsatellites are just markers for genes associated with disease susceptibility there are a number of studies that have identified associations between specific alleles of microsatellites and gene function. If these polymorphisms are functional in a manner which results in an alteration of the secretion of TNFα, LTα and/or LTβ, then it is easy to see a number of ways in which they could be involved in tumourigenesis. In the mouse a polymorphism immediately upstream of the TATA box within in the 5' regulatory region of TNFα has been shown to correlate with resistance to the deleterious effects of UVB light on cutaneous immunity (Vincek, V., *et. al.*, 1993). In another study, Jongeneel and co-workers (1990) report the identification of an allele from a microsatellite within the TNFα promotor region of mice which is unique to the NZW strain of mice. These mice are defective in their ability to secrete TNF in response to LPS and when crossed with NZB mice are seen to develop a lupus-like glomerulonephritis which is curable on treatment with TNFα. This same microsatellite also shows an allele specific association with murine resistance to toxoplasmic encephalitis and has been shown to correlate with levels of TNFα mRNA in the brain of infected animals (Freund, Y.R., *et. al.*, 1992). If the effects of the TNF microsatellite polymorphisms observed in the mouse can be extrapolated to the human system then it is entirely proper to suggest that there is a relationship between the microsatellite polymorphisms and production of TNFs and the susceptibility to colorectal cancer.

In humans an allele-specific association has been demonstrated between a microsatellite located approximately 100 bp down stream of the H-*ras*-1 gene, and the presence of four common human cancers (breast, urinary bladder, acute leukaemia and colorectal; Krontiris, T.G. *et. al.*, 1993). The authors of this paper propose two possible reason for this association. The first proposal is that the rare alleles of this microsatellite are in linkage with a gene, possibly H-*ras* itself, which is involved in carcinogenesis. The second explanation they suggest is that the length of this microsatellite may alter the transcriptional control of genes within the vicinity

of this locus. In two previous papers it has been demonstrated that this region binds at least four members of the *rel*/NF-κB-family of transcriptional regulatory factors (Trepicchio, W.L., *et. al.*, 1992) and that an allele specific alteration in the transcriptional activity of this region is also present. A microsatellite within the intron which separates the diversity and joining segment of the human immunoglobulin heavy-chain has also been shown to bind a transcription regulation factor which, this time, seems to be a member of the mycHLH family of proteins (Trepicchio, W.L., *et. al.*, 1993).

TNFα is intimately involved in a wide number of immune functions and TNFα is now thought to be one of the central immunoregulators in autoimmune diseases such as RA where it can be found in excessive amounts in both the synovial fluid and membrane (Chu, C.Q., *et. al.*, 1991). (Section 1.3 of this thesis provides an introduction of the role of TNF in human malignancy.) As previously stated, a number of studies have suggested that although TNFα can be detected in colorectal tumour biopsies, this is at an insufficient level to induce an immune response (Beissert, S., *et. al.*, 1989; Naylor, M.S., *et. al.*, 1992) and that low levels of TNFα production may result in a desensitisation of the tumour cells to TNFα (Spriggs, D.R., *et. al.*, 1987). Given that the true level of TNFα production in the tumours of the colorectal cancer patients in this study has not yet been defined it is impossible to determine whether these polymorphisms are associated with either over or under production of TNFα and whether there is any pathological function of these genotypes. In order to determine if this hypothesis is correct, some form of quantitative estimation of the TNFα protein content of colorectal tumours would have to be performed and an analysis of the correlation between this and RFLP and microsatellite genotypes would need to be performed. A study such as this would require relatively small numbers in order to determine the correlation between the TNF RFLPs and the level of TNFα, however this would require significantly higher

numbers to fully determine whether the TNF microsatellites correlate with TNFα production.

Even if the TNF polymorphisms do not produce any change in the production of TNFα, LTα or LTβ then it is still possible that they may have some influence on the function of the MHC. Although, to date, there has been no description of susceptibility to colorectal adenocarcinoma being associated with specific MHC haplotypes, it may be possible that the inclusion of the TNF polymorphisms within such a study would enable a link to be identified.

Given the role of the MHC in antigen presentation it is possible to suggest that the presence of extended haplotype spanning the entire length of the MHC could play an important role in antigen presentation. It is clear that specific HLA haplotypes can function differently in different individuals. For example, not all individuals who are HLA-DR4 antigen positive will develop rheumatoid arthritis although 70-80% of RA patients express this antigen (Stastny, P., 1978).

The work of a number of groups on the role of TNF polymorphisms within autoimmune diseases points to a number of important MHC-dependent and MHC-independent associations with alleles of these polymorphisms and the presence of autoimmune disease (See section 1.3.5). Autoimmune diseases are characterised by the presence of a specific immunological reaction. The site of the autoimmune reaction is normally characterised by an over expression of MHC antigens, particularly class II antigens. The expression of these antigens on cells which would generally not express them at such high levels has led to the hypothesis that the aberrant expression of auto-antigens on the surface of cells due to the up regulation of Class II antigen expression significantly contributes to the initial and continual activation of auto-antigen-reactive T cells (Feldmann, M., 1989). These T-cells, in turn, can induce the production of inflammatory cytokines such as TNFα and IL-4

which are thought to be responsible for the pathogenesis of the autoimmune disease and also IFNγ, which can induce the expression of class II antigens on the surface of a number of cell types. It is widely accepted that IFNγ can synergise with TNFα to cause upregulation of MHC antigens on a number of other cell types.

The pathogenesis of an autoimmune reaction can be seen to be similar to the pathogenesis observed in tumours. It is possible to identify an ongoing inflammatory response characterised by an infiltration of cells of the immune system within a tumour and it is possible to identify similar mediators, such as TNFα and IFNγ, which are also observed at the site of an autoimmune reaction. Like the cells at the site of an autoimmune reaction, tumour cells express a number of different antigens on their surface, a number of which are presented in the context of the class I and class II molecules. Any genetic variation resulting in a difference in the ability of a persons cells to process and express these antigens within the MHC could result in the immune system failing to see these antigens and therefore failing to initiate an immune response against the target tumour cell. It has been clearly documented that there is altered MHC expression on colorectal tumour cells (see section 1.2.3 of this thesis) and therefore it is possible that any alteration in MHC expression influenced by TNF polymorphisms may facilitate in the evasion of malignant or pre-malignant cells in the host.

The data presented within this thesis clearly define an association between specific alleles of the TNF polymorphisms and the development and progression of colorectal adenocarcinoma and although it seems unlikely that the effects seen at the TNF polymorphisms in association to disease development are associated with the control of TNF production it was still of interest to determine whether these polymorphic markers could be associated with either disease severity or disease progression. Clinical data, in the form of pathological reports, was obtained for 81 of the 100 colorectal cancer patients and was used to determine Dukes' staging and

metastatic status at time of initial surgery. Dukes' staging was obtained for a further 11 patients from various other clinical records. The clinical data from the follow up clinic of Prof. C.S. McArdle was used to determine the time point at which patients either developed metastatic disease or local recurrence and also to identify individual patients who had survived over two years free from recurrent disease from the time of their initial surgery.

Dukes' staging still represents the most widely used form of clinical grading of colorectal malignancies. It provides a system for the classification of tumours into four broad groups dependant on the degree of bowel wall infiltration, lymphnode involvement and metastatic spread. Since Dukes' staging is essentially a measure of the aggressiveness of the tumour then it is possible that a patient's ability to produce TNFα could relate to the aggressiveness of their disease and therefore one would expect the less aggressive Dukes' A patients to be genetically predisposed to produce more TNFα than the more aggressive Dukes' stage B, C or d tumour bearing patients. Given this hypothesis it was considered important to ascertain if alleles of any of the TNF polymorphisms could be correlated with increased or decreased severity of the primary disease.

Analysis of the -308 TNFα RFLP shows a significant association between homozygosity for the TNF2 allele and the presence of a Dukes' C tumour however no further differences were observed. As previously stated the TNF2 allele has been reported to result in an increase in the activity of the promotor region of TNFα by up to seven fold, however this work is contradicted. Due to the small number of samples within the four Dukes' stage groups it is hard, though still tempting, to postulate that Dukes' C tumours could be secreting more TNFα than the other groups however a larger cohort of patients would be required to test this hypothesis. Analysis of the LTα *Nco*I RFLP genotypes of the same patients failed to show any significant difference between the two groups. Examination of the extended

genotypes of these same patients fails to demonstrate that all of the Dukes' A individuals are positive for the common TNF1 and TNFB*2 alleles, however due to the small number of Dukes' A patients found in this study it is impossible to suggest whether this finding has any real clinical significance. It is therefore impossible to concluded that predisposition to either over or under production of TNFα by either of the two RFLPs is involved in the aggressiveness of primary colorectal adenocarcinoma as determined by Dukes' staging and therefore the hypothesis, in terms of the TNF RFLPs, remains unproven.

Analysis of the microsatellite polymorphisms in the Dukes' staged colorectal cancer patients shows a clear association with a number of the TNF microsatellite alleles and the staging of colorectal adenocarcinoma. The TNFa3, which showed such a high frequency in the colorectal cancer patients than compared to the normal individuals, also shows an association with Dukes' staging. When compared to the other three stages combined, TNFa3 shows an over representation in the Dukes' stage A individuals. TNFa8 also shows an association with Dukes' staging in that its is found at a higher frequency in the Dukes' stage d, i.e. metastatic tumours. Again due to the low numbers of Dukes' staged patients in this study it is impossible to predict whether this TNFa3 or the TNFa8 association has any clinical relevance.

Analysis of the other three microsatellite loci fails to demonstrate any further significant differences between the Dukes' stage groups and alleles of these loci. Although a number of smaller differences are observed at these loci it is difficult to predict their significance due to the small number of individuals within each group.

Although there has been much improvement in terms of the treatment of primary colorectal adenocarcinoma, generally in terms of diagnosis, surgery and patients management the problems associated with secondary disease are still not fully resolved. Over 49% of individuals with colorectal cancer will die within two years

of initial diagnosis and of these almost 90% will die either from disseminated metastatic disease or from local recurrent disease. It can thus been clearly seen that this form of secondary disease still represents a major problem for clinicians. Why metastatic disease should occur in one patient and not in the other still remains a mystery. A number of genes have been suggested to be important in the metastatic process such as nm23 (Barnes, R., *et. al.*, 1991). However it still remains unclear if these markers will allow the separation of tumours which will metastasis from those which will not.

The data suggest a role for the TNF polymorphisms in the process of metastasis. If the polymorphisms are associated with functional changes in the ability to produce TNFα then it is possible that an over production of TNFα at either the site of the primary tumour or the secondary tumour could facilitate crossing of the endothelium by tumour cells.

Having demonstrated associations between alleles of the TNF polymorphisms and both the primary development, and possibly the aggressiveness as defined by Dukes' staging, of colorectal adenocarcinomas, it was logical to examine whether alleles of these polymorphisms could also be used to identify, retrospectively, those cancer patients who would develop secondary disease from primary colorectal cancer. Development of secondary disease was determined following extensive clinical follow up of all of the patients treated by Prof. C.S.M (GRI). Patients were also identified who had survived disease free two years from their initial surgery for primary disease and these were used as a control population.

Analysis of the two RFLPs demonstrates a difference between the two populations at both loci, however statistically significant differences are only observed at the LTα *Nco*I RFLP locus. Analysis of both genotypes and allelotypes of the -308 TNFα RFLP shows a slight difference between the frequency of the TNF2/2 genotype, with

this, homozygous genotype appearing more frequently in the metastatic disease group than compared to the non-metastatic group, however this difference is not statistically significant.

Analysis of the LTα *Nco*I RFLP locus demonstrates an association with the TNFB*1/B*2 genotype and the presence of secondary disease, whilst there is a decrease in the frequency of the TNFB*2/B*2 genotype in the same individuals. If the data suggesting that the TNFB*2/B*2 genotype is associated with increased ability to secrete TNFα is true then this result does not support the hypothesis that TNFα is important in metastasis. Analysis of the allelic usage in these patients demonstrates that, as expected from the genotype data, the TNFB*1 allele is over represented in the secondary disease population, and the TNFB*2 allele is under represented in this same group. There is some evidence to suggest that metastatic cells express less MHC antigen on their surface than non-metastatic cells and that this allows the cell to evade the host immune system, particularly NK cells (Goodrich, K.H., *et. al.*, 1993). TNFα is known to be able to induce MHC expression on the surface of a number of cell types, therefore a genetic predisposition to under produce TNFα may result in a decreased expression of MHC antigens on the surface of metastatic tumour cells there by facilitating their evasion of the host immune system.

The clinical relevance of these associations has yet to be ascertained and the true relevance of this finding can only be ascertained fully in a large scale, prospective, clinical trial carried out over a period of at least five years. This length of time would allow for the accumulation of a significant number of individuals and for these individuals to be followed up from time of initial diagnosis and treatment for a minimum period of two years. This data would then allow for an analysis of the potential clinical usefulness of this diagnostic marker in the determination of the

increased risk in of developing secondary disease in individuals with colorectal cancer.

It is therefore clear that polymorphisms within this region of the genome play an important part in the development of colorectal adenocarcinoma and progression of colorectal adenocarcinoma. The method by which this occurs at each stage still remains unclear but may be due to three possible mechanisms. The first mechanism is that there is a gene close to this region of the genome which is intimately involved in the process of carcinogenesis and that the phenomenon observed within this study is simple linkage disequilibrium. This first hypothesis would fit with the relationship between the TNFa3 allele and the presence of colorectal cancer, where it is hard to envisage such a central role for TNFα. The second mechanism is that these markers form part of a very large extended haplotype encompassing the whole of the MHC. These polymorphisms are not functional by themselves but instead when combined with a number of other polymorphisms are part of a large polymorphic region which is involved in altering the specificity of an individuals MHC repertoire. This hypothesis would again fit with the association with development of colorectal cancer in that it could be envisaged that altered expression of MHC antigens would allow a tumour cell to evade the immune system. This same hypothesis would also fit with the relationship to progression of colorectal cancer, where an ability to evade the immune response would give a tumour an advantage at both the primary and secondary sites. The third, and most attractive mechanism, is that polymorphisms within this region are involved in the transcriptional control of a gene or a number of genes, possibly TNF itself, and that the function of these transcriptional regulatory domains are affected due to differences in the size of the microsatellites, however it is still controversial as to whether the TNF RFLPs play a role in the control of TNFα, LTα or LTβ secretion and there is even less evidence to support a role for the microsatellite polymorphisms in the control of TNF gene activity.

It is clear that a great deal of further analysis is required to fully explain the association between this region of the genome and the development and progression of colorectal adenocarcinoma. Studies involving larger numbers of individuals will be the key to answering most of the unanswered questions posed by this study and thus it remains vital to continue to perform the basic allele association studies whilst in parallel endeavouring to ascertain whether these polymorphisms are functional or whether they just point to the presence of a tumour associated gene within the region of the MHC.

Future Studies

Given the controversy over the role of the TNF RFLPs and the microsatellites in the control of TNF production it is now vital that a study to determine the exact relationship that the polymorphisms have on *in vivo* TNF production is performed. It is after all the role of these markers *in vivo* that is important and not their role in the artificial tissue culture system that will influence the way that these markers are utilised. This study could take a number of forms, including the use of immunohistochemical or *in situ* hybridisation analysis or it could be performed on tumour derived cells including T-cells, macrophages and the tumour cells themselves.

It would also be important to examine the MHC background of the study individuals. Since it is clear that these polymorphisms are intimately associated with the MHC then it may prove fruitful to examine whether there is any susceptibility to colorectal cancer associated with the MHC. Studies of familial colorectal cancer may also provide some help in determining the role of these markers. Since it has been demonstrated that the mere presence of a particular allele, particularly of the RFLPs, may not be sufficient to infer the susceptibility then these alleles may be functioning in a recessive, dominant or codominant manner dependent on the particular marker that you look at.

Given the relative ease with which promotor function can be examined by using CAT reporter systems it may also be advisable to study the effects of the various microsatellites on the promotor activity of the various TNF genes. This would require an extensive study in which all of the permutations of TNFa, TNFb, TNFc and TNFd alleles would have to be examined to look for both dependent and independent associations.

There are of course five microsatellites within the TNF locus. This study does not report any analysis of the TNFb polymorphism. This was due to technical difficulties in amplifying the TNFb region. It is therefore imperative that the data be completed by performing an analysis of this locus and it may be advisable to use the technique of Nedospasov *et. al.* (1991) where the TNFa and b loci are co-amplified and the TNFb haplotye calculated from the size of the PCR product given that the corresponding TNFa allele size is already known.

The whole area of disease susceptibility is very complicated and unfortunately the role of the TNF polymorphisms in this larger picture is even less clear. It will only be with careful analysis and a larger study that any ground will be gain into trying to understanding the complex nature of this problem.

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