Aberrant DNA Methylation as a Diagnostic and Predictive Marker of Ovarian Cancer

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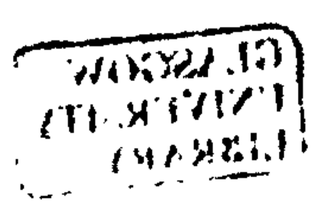

To Fraser, with love

Abstract

Aberrant methylation of CpG islands (CGIs) is associated with transcriptional silencing of key tumour suppressor genes in cancer and is a frequent epigenetic event in epithelial ovarian cancer (EOC). It has been shown to be involved in many aspects of tumour progression including chemoresistance. Methylation of CGIs can be detected in tumour DNA released into plasma, which means it has potential clinical use both as an early diagnostic and prognostic/predictive marker in EOC. The methylation status of 24 CGIs in a retrospective group of 142 EOCs and 16 non-tumour adjacent tissues were analysed using methylation-specific PCR (MSP) and Combined Bisulphite Restriction Analysis (COBRA) methods. CGI methylation of at least one of these loci was a frequent event in both early (78%) and late stage (60%) disease. A group of loci were identified as being methylated in 64% of early stage tumours (CGIs linked to the *OPCML*, *RASSF1A* and *HIC1* genes). The *HIC1* CGI was frequently methylated in matched non-tumour adjacent tissues, but not in normal ovarian surface epithelium, potentially representing an early epigenetic event in the carcinogenic process present even before apparent morphological change.

Differential methylation hybridisation (DMH) of a 12K CGI microarray using ovarian cell lines identified methylation of a CGI located at the *LMX1A* gene. This CGI was shown by MSP to be a potential early epigenetic marker methylated in 75% of early stage ovarian tumours. 87.5% of the early stage tumours examined were methylated in at least one of four loci (*LMX1A*, *OPCML*, *RASSF1A* or *HIC1*). The clinical application of this group of methylated CGIs was examined in matched plasma from chemonaive patients with EOC for similar methylation changes. Methylation of *LMX1A* was detected in 43.3% of all plasma samples and in 48.2% of those patients with methylated *LMX1A* in their tumour. When methylation was detected in plasma, it was always detectable in the corresponding tumour. Therefore, detection of *LMX1A* methylation in plasma has a sensitivity of 48.2% and a specificity of 100%.

In late stage chemonaive ovarian tumours, methylation in tumour of CGIs associated with *BRCA1*, *GSTP1* or *MGMT* correlated with an improved response to chemotherapy ($p=0.013$). In addition, a non-random pattern of methylation was observed which demonstrated that there is an underlying biological mechanism leading to co-methylation of specific genes, but the cause of this remains unidentified.

In addition to identifying methylation of the *LMX1A* CGI as a potentially early epigenetic event, DMH of ovarian cell lines also identified both the *LMX1A* CGI and a CGI at the *NR2E1* gene, within a group of ranked sequences, whose methylation status optimally discriminate between cisplatin sensitive and resistant cell lines. CGI methylation of these genes was associated with a transcriptionally repressed state. Methylation of these CGIs was observed in 61.8% and 12.6% of chemonaive ovarian tumours respectively, but not in normal ovarian surface epithelium. Comparison of matched pairs of chemonaive ovarian tumours and post-chemotherapy residual samples showed that methylation of *NR2E1* and *LMX1A* increased in 33% and 25% respectively following chemotherapy, which is in keeping with selection of methylation of these genes during platinum based chemotherapy and a potential role for these genes in platinum resistance mechanisms.

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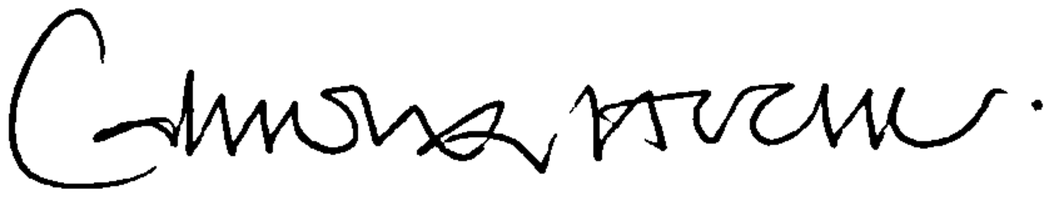
Declaration

I, Catriona Hardie, declare that I am the sole author of this thesis. All of the references have been consulted by myself in the preparation of this manuscript and all of the work described herein was performed by myself, except where otherwise stated. This work has not been previously accepted for a higher degree.



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Abbreviations

A	adenosine
aa	amino acid
Ab	antibody
APAF-1	Apoptotic Peptidase Activating Factor-1
APC	Adenomatous Polyposis Coli
APS	adenosine 5'phosphosulphate
ATP	adenosine triphosphate
5-azaC	5-azacytidine
bp	base pair
BRAF	v-RAF murine sarcoma viral oncogene homolog B1
BRCA1	Breast Cancer Associated 1
BSA	bovine serum albumin
C	cytosine
°C	degrees celsius
CASP8	Caspase 8
cDNA	coding DNA
CGI	CpG island
CIMP	CpG island methylator phenotype
cm^2	centimetres squared
COBRA	Combined Bisulphite Restriction Analysis
CR	complete response
CT	computed tomography
DAC	5-aza-2'-deoxycytidine
DAPK	Death-Associated Protein Kinase
DcR1	Decoy Receptor 1
DEPC	diethyl pyrocarbonate
dH_2O	distilled water
DMH	Differential Methylation Hybridisation
DMSO	dimethyl sulphoxide
DNMT	DNA Methyltransferase
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
DTT	dithiothreitol
EBV	Epstein-Barr virus

E.coli	*Escherichia coli*
EDTA	ethylene-diamino-tetraacetic acid
EOC	epithelial ovarian cancer
FANCF	Fanconi Anaemia, Complementation Group F
FAS	TNF Receptor Superfamily, member 6
FBS	fetal bovine serum
FDA	Food and Drug Administration
FIGO	International Federation of Gynaecology and Obstetrics
g	gram
G	guanine
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GSTP1	Glutathione S-Transferase π
HAT	Histone Acetyl Transferase
HDAC	Histone Deacetylase
H&E	Haematoxylin and Eosin
HIC1	Hypermethylated In Cancer 1
hMLH1	Human MutL Homologue 1
hMSH2	Human MutS Homologue 2
HMT	Histone Methyltransferase
HOXA5	Homeobox A5
HP	high performance
HP1	Heterochromatin Protein 1
ICF	Immunodeficiency, Centromeric region instability, Facial anomalies
Ig	immunoglobulin
IgLON	immunoglobulin LSAMP, OPCML/OBCAM and neurotrimin family
iOSE	immortalised ovarian surface epithelium
IP	intraperitoneal
IPTG	isopropyl-β-D-thiogalactoside
IUPAC	International Union of Pure and Applied Chemistry
IV	intravenous
IVM	*in vitro* methylated DNA
k	kilo
l	litre
LB	Luria-Bertani
LMX1A	LIM homeobox transcription factor 1, alpha
LOH	loss of heterozygosity

LPA	linear polyacrylamide
LSAMP	limbic system-associated membrane protein
m	milli
M	molar
MALT	Mucosa Associated Lymphoid Tissue
MBD	Methyl Binding Domain
MCJ	Methylation-Controlled J
MDR	multidrug resistance
MGMT	O-6-Methylguanine-DNA Methyltransferase
MINT25	Methylated In Tumour 25
MMR	mismatch repair
mRNA	messenger ribonucleic acid
MSP	Methylation-specific PCR
MZ	monozygotic
n	nano
NEGR1	neuronal growth factor 1
NFκB	nuclear factor Kappa B
nOSE	normal ovarian surface epithelium
NR2E1	Nuclear Receptor subfamily 2, group E, member 1
OPCML	Opioid binding Protein/Cell adhesion Molecule-Like
OS	overall survival
p	pico
p value	probability value
PAM	Prediction Analysis for Microarrays
PBMC	peripheral blood mononuclear cell
PBS	phosphate buffered saline
PCA	Principal Components Analysis
PCNA	proliferating cell nuclear antigen
PCR	Polymerase Chain Reaction
PD	progressive disease
PE	PBS/EDTA
PFS	progression free survival
PMN	peripheral blood mononuclear cell
PPi	pyrophosphate
PR	partial response
PTEN	Phosphatase and Tensin homologue 1

PS	performance status
qRT-PCR	Quantitative Reverse Transcriptase PCR
R	purine (adenosine or guanine)
RASSF1A	Ras Association domain Family 1
RCT	randomised-controlled trial
RLGS	Restriction Landmark Genomic Scanning
RNA	ribonucleic acid
rpm	revolutions per minute
RT	Reverse Transcription
RTase	reverse transcriptase
RT-PCR	Reverse Transcriptase PCR
SAM	S-adenosylmethionine
SD	stable disease
SDS	sodium dodecyl sulphate
SFRP	Secreted Frizzled-Related Protein
SNP	single nucleotide polymorphism
SOCS-3	Suppressor of Cytokine Signalling 3
SSC	sodium chloride-sodium citrate
SWOG	Southwest Oncology Group
T	thymine
TBE	tris-borate-EDTA
TBS	tris buffered saline
TE	tris-HCl EDTA
TMS1	Target of methylation-induced silencing
Tris	2-amino-2-(hydroxymethyl)propane-1,3-diol
TSG	tumour suppressor gene
μ	micro
U	unmethylated
US	ultrasound
UV	ultraviolet
WHO	World Health Organisation
xg	centrifugal force
X-Gal	5-bromo-4-chloro-3-indolyl-β-D-galactoside
Y	pyrimidine (thymine or cytosine)

Chapter 1

Introduction

1 Introduction

1.1 Clinical diagnosis and first-line treatment of ovarian cancer

Ovarian cancer is a heterogeneous disease in its biological nature, aggressive characteristics and response to chemotherapeutic agents. Epithelial ovarian cancer (EOC) is considered to be the most aggressive neoplasm of the female genital tract, and remains the cause of more cancer-related deaths than a combination of both cervical and endometrial cancers (Ozols et al., 2004). EOC is the most common WHO (World Health Organisation) histological type and multiple subtypes of this exist, which are based on cell type and architecture of the tumour. At least 80% of EOCs arise from the coelomic epithelium of which 75% are serous cystadenocarcinomas. These are predominantly found in advanced ovarian malignancy. Less common types include mucinous, endometrioid, transitional cell (Brenner), carcinosarcoma, clear cell and unclassified carcinomas. Approximately 20% are germ-cell and sex cord-stromal cell tumours (Kaku et al., 2003).

Despite the many advances in diagnosis and treatment of ovarian cancer over the last four decades, approximately 7000 women develop this disease in the United Kingdom, with more than 4600 women dying annually (www.info.cancerresearchuk.org/cancerstats). The most important determinant of survival from ovarian cancer is tumour stage at diagnosis. Early stage ovarian cancer, which is limited to the ovary, can be treated successfully with surgery alone in 90% of patients, but due to its insidious onset, the majority (approximately 80%) present with incurative disseminated late stage disease (FIGO Stage III/IV) (International Federation of Gynaecology and Obstetrics) (Agarwal and Kaye, 2003). The five year survival rates for these advanced tumours dramatically decrease to 17-35% (Angioli et al., 2006). Ovarian tumours are known to spread via the lymphatic system and through seedling implantations on the peritoneum. Development of this disease is not associated with any specific clinical symptoms or signs. A diagnosis is therefore very difficult to make based on non-specific symptoms as these will often mimic upper gastrointestinal disease with abdominal fullness, dyspepsia and bloating (Cannistra, 2004).

Over the last four decades, surgical debulking has been the accepted initial management of patients with advanced ovarian cancer (Griffiths, 1975). One of the most important prognostic factors in the treatment of advanced ovarian malignancy is a crude measurement of the extent to which the tumour is optimally debulked. Patients with

residual tumour mass of greater than 2cm have a median survival of 12-16 months compared to 40-45 months if residual disease is less than 2cm (Mutch, 2002). However, the evidence for debulking surgery has previously mainly been based on small, retrospective studies (Allen et al., 1995; Hunter et al., 1992; Voest et al., 1989), but there is recent evidence to suggest that increased progression-free survival (PFS) may be associated with patients who are optimally debulked with less advanced disease (Crawford et al., 2005). Some authors have suggested that the underlying heterogeneous biological nature of ovarian tumours may dictate its potential surgical resectibility (Hogberg, 1995; Zanaboni et al., 1988). This has lead to some debate over whether or not neoadjuvant chemotherapy would improve survival (Inciura et al., 2006; Schwartz et al., 1999; Vergote et al., 2000). There are now randomised prospective studies examining this which are being undertaken by the European Organisation for Research and Treatment of Cancer 55971 (EORTC) and Chemotherapy or Up-Front Surgery studies. These studies are comparing neoadjuvant chemotherapy followed by surgery versus primary surgery followed by chemotherapy. Until conclusions are drawn from these studies and other future prospective trials, cytoreductive surgery will remain the recommendation for the first-line treatment of FIGO Stage III/IV disease.

The majority of patients present too late for curative removal of the tumour, hence, chemotherapeutic agents remain a key part of treatment for most women. Historically, women were treated with alkylating agents such as cyclophosphamide. However, adjuvant chemotherapy has advanced significantly over the last four decades with the introduction of platinum, and more recently, taxane-based chemotherapy (McGuire and Markman, 2003). Platinum analogues, such as cisplatin or carboplatin, are DNA damaging agents which mediate their effects by inducing intra- and interstrand crosslinks within DNA (Kartalou and Essigmann, 2001). In contrast, taxanes such as paclitaxel and docetaxel exert their cytotoxic effects by binding to and stabilising the tubulin polymer (Rowinsky and Donehower, 1995). For patients with early-stage tumours with an increased risk of relapse, i.e. those with Stage Ia/b high grade, Stage Ic and Stage II disease, the use of platinum-based chemotherapy can result in a 5 year disease-free survival of approximately 80% compared to approximately 65% in those patients who do not receive platinum adjuvant therapy (Young, 2003; Young et al., 2003). In advanced ovarian malignancy, two randomised-controlled trials (RCTs) in the 1990s established paclitaxel combined with a platinum agent as standard first-line chemotherapy (McGuire et al., 1996; Piccart et al., 2000), showing a particular increase in overall survival when compared to treatment with cyclophosphamide. Meta-analysis results showed that carboplatin has a lower toxicity profile than cisplatin in patients (Aabo et al., 1998), and this is now routinely used

following two larger RCTs (du Bois et al., 2003; Ozols et al., 2003). There remain many controversies surrounding the current first-line chemotherapeutics used in this disease. Firstly, there are differing opinions over whether combination chemotherapy with a taxane has superior effects to single agent carboplatin. Secondly, if combination treatment is the first choice, which taxane is appropriate to use has also been debated. The results of ICON3 concluded that single agent carboplatin can be regarded as reasonable first-line treatment in ovarian cancer (ICON Group, 2002), although there was an early trend towards overall survival benefit in those treated with combination therapy. Regarding the choice of taxane, "The Scottish Randomised Trial in Ovarian Cancer" (SCOTROC) compared docetaxel-carboplatin with paclitaxel-carboplatin in first-line treatment. Although there was no overall survival benefit of the docetaxel combination over paclitaxel, both were associated with acceptable toxicities. Docetaxel-carboplatin was associated with significantly more myelosuppression but significantly less neurotoxicity and symptom scoring favoured docetaxel. Therefore, the authors concluded that a combination of carboplatin with docetaxel may be an appropriate chemotherapeutic agent in chemonaive ovarian tumours (Vasey et al., 2004). In addition, there is current debate over whether current standard intravenous (IV) carboplatin-paclitaxel delivery should be replaced with the intraperitoneal (IP) delivery of chemotherapy. IP delivery of drugs, specifically cisplatin, has been examined in several trials for optimally debulked Stage III ovarian cancer (Alberts et al., 1996; Armstrong et al., 2006; Markman et al., 2001). However, although advantages in overall and progression-free survival have been demonstrated, the merits of using this remain uncertain due to issues with efficacy, quality of life and toxicity of IP regimes versus standard IV carboplatin-paclitaxel.

Although a large number of studies have established carboplatin-paclitaxel as standard first-line treatment, current chemotherapeutic regimes used in both first- and second-line treatment will produce differing responses due to the heterogeneous nature of this disease. Therefore, stratification of current and more novel therapies in patients are now required to improve the poor overall response rate in patients.

1.2 Potential mechanisms of drug resistance in ovarian cancer

A first-line chemotherapy regimen of platinum and taxane will achieve response rates of more than 80% in advanced ovarian malignancy, with an overall complete response in 40-60% of patients (Greenlee et al., 2001). Despite treatment advances though, most patients become drug resistant and relapse within a median progression-free survival period of 18 months (Greenlee et al., 2001). Drug resistance accounts for treatment failure and demise in more than 90% of patients with advanced malignancy, and this is related to numerous causative factors (Agarwal and Kaye, 2003). Both intrinsic and acquired drug resistance is encompassed in ovarian cancer (Balch et al., 2004), but most clinical studies have concentrated on tumour characteristics at presentation, rather than in relapsed disease (Teodoridis et al., 2004). Intrinsic mechanisms of resistance occur in approximately 20% of women, and is clinically defined in those who fail to respond to first line chemotherapy (platinum-refractory disease), have stable disease as a best response following first line treatment or have a short treatment-free interval of less than 6 months (Vasey, 2005). If re-challenged with platinum agents or other second-line chemotherapeutics, these tumours have a poor prognosis (Agarwal and Kaye, 2006).

Intrinsic resistance is influenced by the gene expression of cancer cells prior to treatment (Balch et al., 2004). Acquired drug resistance, however, is seen in patients who initially respond to treatment but then proceed to develop resistance to second-line or subsequent chemotherapeutic regimes. It has been suggested that acquired resistance emerges either from the survival and expansion of drug-resistant subpopulations of cells under selective pressure during tumour evolution (Figure 1A), which is induced by chemotherapeutic measures, or that cancer stem cells can repopulate the tumour environment (Figure 1B) (Agarwal and Kaye, 2003). Stem cells may only comprise a very small proportion of the cells within a tumour, but they are naturally chemoresistant through their relative quiescence, i.e. due to spending most of their time in G0, and therefore avoid the toxicity of the initial chemotherapy regime which will target rapidly dividing cells. In addition they have a capacity for DNA repair and have been found to express high levels of specific ABC drug transporters which may contribute to chemoresistance (Dean et al., 2005). Subsequent relapse due to growth of these stem cells may be chemosensitive initially, but ultimately most patients develop resistance after an initial chemoresponsive history. Therefore, a combination of these proposed models (Figure 1A and 1B) of chemoresistance would seem most likely (Figure 1C).

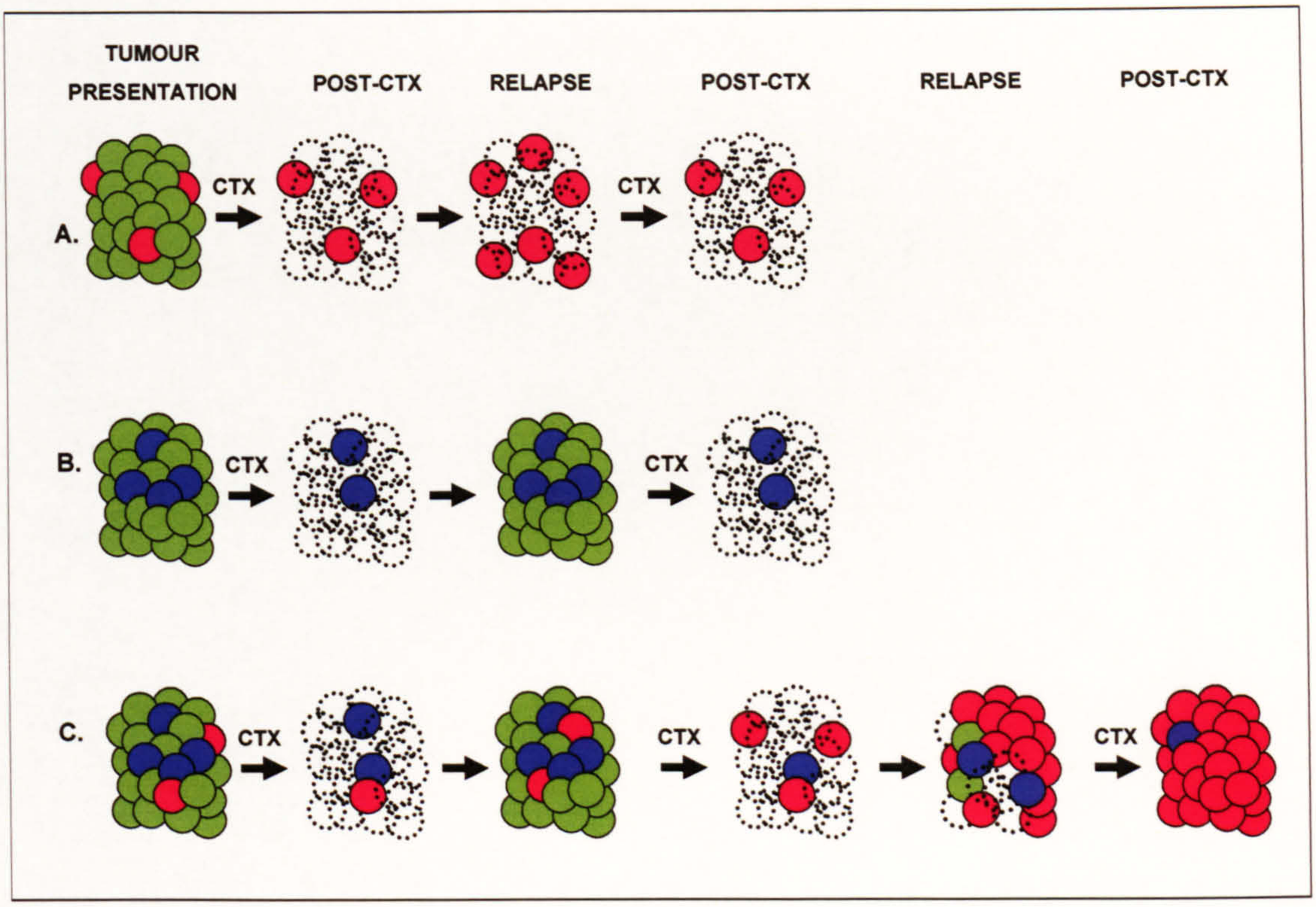


Figure 1 Models of drug resistance in ovarian cancer. (A) Initial response to chemotherapy (CTX) seen due to drug sensitive cells (green) followed by clonal expansion of a subpopulation of chemoresistant cells/regrowth of a chemoresistant progenitor cell population (red). **(B)** Regrowth of a persistent stem cell population (blue) which can be initially chemosensitive. **(C)** Most likely scenario is a combination of (A) and (B) where chemoresistance develops after initial chemosensitive relapse. Illustration modified from Agarwal and Kaye, 2003.

There are diverse possible mechanisms which may be responsible for drug resistance in patients. Firstly, pharmacokinetic variability may exist between patients and will place limitations on aspects such as the first pass metabolism, renal clearance and hepatic metabolism of a drug. In addition, variation in the tumour microenvironment such as hypoxia and altered cell interactions exist and tumour-cell specific mechanisms can contribute (Agarwal and Kaye, 2003). Each of these three possible general mechanisms influence chemotherapeutic response by principally affecting intracellular active drug concentrations, drug-target interaction, target-mediated cell damage, damage-induced apoptotic signaling or the apoptotic effector machinery.

Various pathways within a cell are probably altered in clinical drug resistance due to polygenic gene expression changes (Glasspool et al., 2006) which will affect multiple genes involved in key pathways. A number of specific biochemical pathways and gene expression patterns have been identified as causes of both intrinsic and acquired resistance

to chemotherapy *in vitro* although the clinical *in vivo* relevance of these remains unclear (Agarwal and Kaye, 2003; Vasey, 2005). Many drugs used can be actively pumped from cells by membrane-based proteins and expression of these proteins can lead to multidrug resistance (MDR) towards numerous anticancer agents (Gottesman et al., 1998). Such proteins, including P-Glycoprotein (MDR1), can be important in determining drug resistance *in vitro* (Borst et al., 2000). Recent work has also shown that expression of MRP2, a known ABC transporter, is observed in ovarian cancer and confers resistance to cisplatin chemotherapy and can predict clinical outcome (Surowiak et al., 2006). Other previous work has investigated the role of the p53 protein in drug resistance. *p53* gene mutations can be associated with poor response in patients with ovarian cancer who are exposed to high-dose platinum chemotherapy regimes (Righetti et al., 1996). *In vitro* studies have shown that *p53* mutations and acquired platinum resistance are associated with increased sensitivity to taxanes in ovarian cancer cell lines (Cassinelli et al., 2001). However, overall there remains no conclusive evidence *in vivo* that, firstly, individual genetic modulation of genes such as *MDR1* and *p53* leads to acquisition of clinical drug resistance (Glasspool et al., 2006), or that their ability to predict clinical outcome in patients with ovarian cancer has advantages over currently used markers such as the tumour stage and grade. (Agarwal and Kaye, 2003; Hall et al., 2004).

Increased knowledge of how platinum compounds mediate cytotoxicity, i.e. through formation of DNA-platinum adducts and induction of apoptosis, has increased our understanding of potential drug resistance mechanisms (Kartalou and Essigmann, 2001). Chemoresistant tumours most likely evade apoptosis due to deficient proapoptotic and/or enhanced antiapoptotic signaling pathways (Fojo and Bates, 2003). Altered expression of genes involved in key DNA damage response pathways therefore potentially contribute to the drug resistant phenotype (Teodoridis et al., 2004). The advent of microarray-based technologies has identified a myriad of genes whose expression status are altered in acquired drug resistant ovarian tumours (Jazaeri et al., 2005; L'Esperance et al., 2006). There remains very little evidence though that altered expression status of genes by genetic mutations have a key role to play in acquired resistance mechanisms *in vivo* (Glasspool et al., 2006). Therefore, if genetic alterations have little influence over the control of gene expression in drug resistant tumours, there must be alternative aberrant mechanisms controlling the development of this phenotype.

Previous work in our laboratory has focused on characterising the proteins involved in the recognition of damage induced by platinum agents. Mismatch repair (MMR) proteins, including hMLH1 and hMSH2, recognise and repair damaged or mismatched nucleotides

which can result from DNA replicative mechanisms and other repair processes. It has been shown that cisplatin resistance can be associated with loss of DNA MMR activity in ovarian cancer cell lines (Anthoney et al., 1996; Brown et al., 1997; Drummond et al., 1996). Complete loss of hMLH1 protein expression in cisplatin resistant cell lines was observed with no apparent loss of the *hMLH1* gene (Brown et al., 1997). Loss of hMLH1 protein expression following chemotherapy was shown in ovarian cancer (Strathdee et al., 1999; Watanabe et al., 2001), and this was associated with an epigenetic alteration known as CpG island (CGI) methylation at the *hMLH1* locus (Strathdee et al., 1999). Chapter 1 will now examine the contributory effect of CGI methylation in the pathogenesis of ovarian cancer and its phenotypic consequences. It is now widely recognised that this epigenetic mechanism can transcriptionally repress genes involved in multiple biological pathways in ovarian cancer. Furthermore, co-selection of genes affected by these epimutations can subsequently affect its biological properties, including the propensity to influence drug resistance.

1.3 DNA methylation and epigenetic gene regulation

The term "epigenetics" (greek meaning, "upon" genetics) can be defined as a stable, heritable change in gene expression which is retained during mitosis and/or meiosis. It does not involve a change in the primary base sequence, but instead is stored in the distribution of the modified base 5-methylcytosine, which has previously been aptly described as "the fifth base" (Costello and Plass, 2001). DNA methylation is the only known epigenetic modification of human DNA and results in the enzymatic transfer of a methyl group from the methyl donor S-adenosylmethionine (SAM) to the carbon-5 position of cytosine bases in DNA (Bird, 2002). This almost exclusively occurs at the sequence motif 5'-CpG-3' which are known as CpG dinucleotides (Teodoridis et al., 2004), although non-CpG methylation in mammals has also been reported (Ramsahoye et al., 2000). An illustration of this chemical modification is shown in Figure 2.

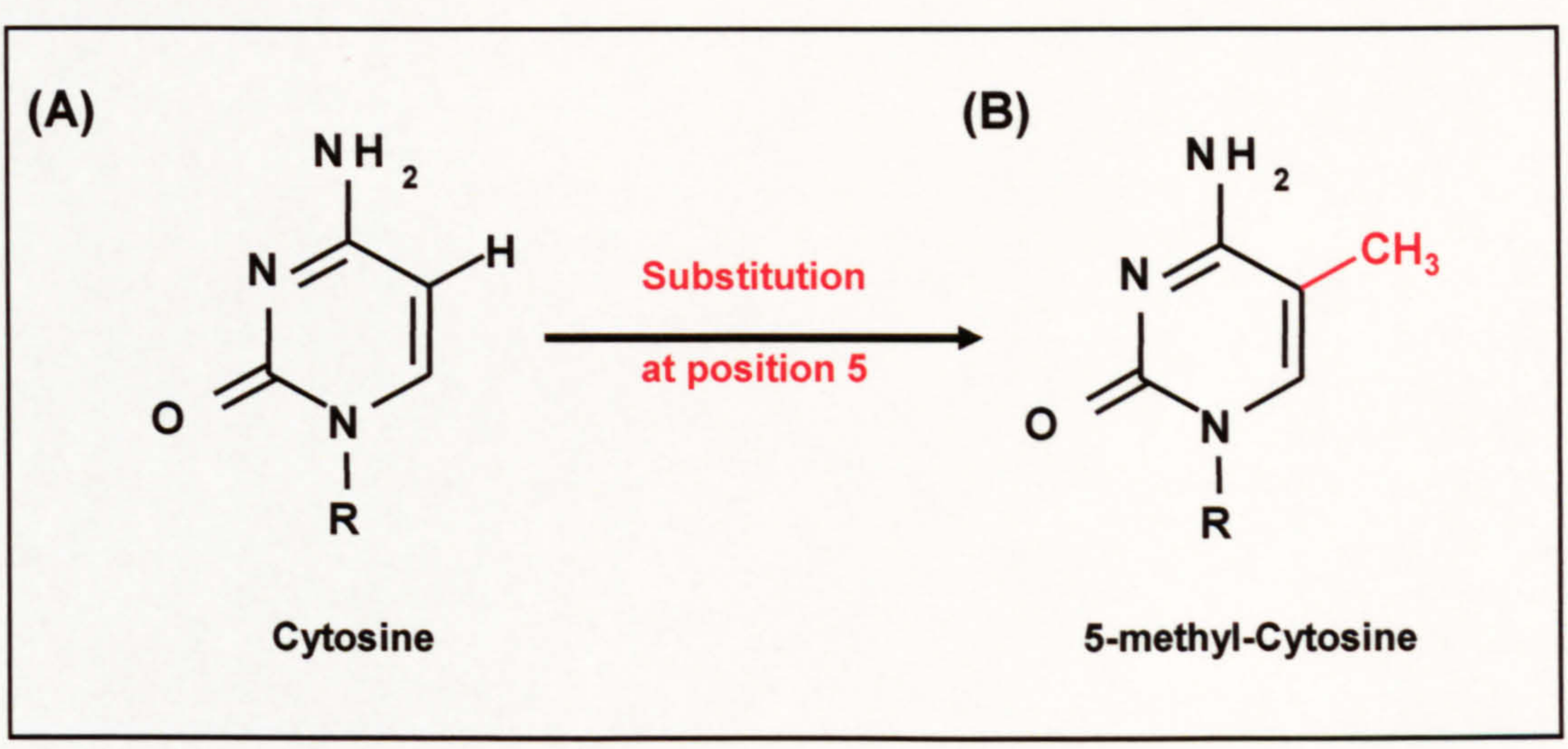


Figure 2 Chemical modification of cytosine methylation. (A) the chemical structure of the base cytosine. **(B)** the chemical structure of 5-methylcytosine following enzymatic transfer of a methyl (CH_3) group.

Over recent years, CpG methylation and its consequences have become more fully understood (Teodoridis et al., 2004). The family of enzymes which catalyse the transfer of a methyl group from the donor molecule, SAM, to a cytosine ring are known as the DNA methyltransferases (DNMT). Several distinct physiologically active members have been cloned and characterised so far in mammalian cells including DNMT1, DNMT3a and DNMT3b (Bird and Wolffe, 1999; Hendrich and Bird, 2000). The main role of DNMT1 is thought to be the post-replicative maintenance of DNA methylation patterns (Leonhardt et

al., 1992), which specifically involves the reinstatement of fully methylated sites from initially hemi-methylated DNA substrates in daughter cells (Chuang et al., 1997; Pradhan et al., 1999). DNMT1 has been shown to bind to proliferating cell nuclear antigen (PCNA), an auxiliary factor of DNA replication, during S phase via a specific binding domain (Chuang et al., 1997; Mortusewicz et al., 2005) consistent with a function in maintaining methylation patterns. This enzyme, though, is additionally able to *de novo* methylate DNA substrates in cancer (Jair et al., 2006). In contrast to this enzyme, DNMT3a and DNMT3b are involved in initiating methylation patterns during early embryogenesis, a process which is known as *de novo* methylation (Okano et al., 1999; Okano et al., 1998). Both DNMT3a and DNMT3b show a preference to unmethylated DNA *in vitro* (Okano et al., 1998), however, there is also data to suggest that both DNMT3a and DNMT3b can maintain methylation patterns in the embryonic stem cells of mice (Chen et al., 2003b). In addition, the DNMT3-like protein, DNMT3L, has also been identified which is inactive as a DNMT *per se*, but is essential for the establishment of germ line DNA methylation and stimulates *de novo* methylation by DNMT3a and DNMT3b (Chen et al., 2005b). DNMTs and their role in the methylation of DNA are vital for mammalian development. Homozygous knockout of DNMT1 or DNMT3b in mice has been shown to be embryonically lethal and DNMT3a knockout mice die at four weeks old (Li et al., 1992; Okano et al., 1999). Mice with reduced expression levels of DNMT1 show genomic hypomethylation and have an increased likelihood of developing lymphomas although they have a lower incidence of other tumour types (Gaudet et al., 2003; Laird et al., 1995). Additionally, DNMT1 overexpression or complete loss of this results in loss of genetic imprinting and lethality of the embryo (Biniszkiewicz et al., 2002; Li et al., 1993).

In humans, patterns of DNA methylation are first established during gametogenesis. However, after fertilisation, dramatic waves of methylation changes are observed. The paternal genome has been shown to be actively demethylated in mitotically active zygotes, followed by a passive and selective loss of DNA methylation continuing into the morula stage (Santos et al., 2002). After implantation, DNA methylation patterns become re-established and are maintained through subsequent cell divisions (Gaudet et al., 2004). The primary role of DNA methylation in normal adult tissues is thought to be the maintenance of transcriptionally silent repetitive DNA elements in the genome (Walsh et al., 1998), which includes sustaining satellite DNA and parasitic elements. This allows the specific targeting of transcription factors to important genomic sites (Bird and Wolffe, 1999; Strathdee and Brown, 2002). There is an overall depletion of CpG dinucleotides spanning the genome and this is presumably because methylated cytosine residues are mutagenic due to spontaneous deamination of 5-methylcytosine to thymine (Bird, 1996; Chan et al.,

2001). In contrast to this, there are small, unequally distributed distinct stretches of DNA (500 base pairs (bp) - 2 kilobases (kb) in length) which are rich in CpG dinucleotides. These clusters of CpGs are known as CpG islands (CGIs), and these frequently co-localise within and around the promoter regions of mammalian genes (Jones, 2002). It is estimated that there are around 30,000 CGIs within the human genome and approximately 50-60% of all genes contain a CGI (Costello and Plass, 2001). In contrast to the rest of the genome, these CGIs remain largely unmethylated in normal tissue (Bird, 1986) regardless of the transcriptional state of the gene. However, it is now apparent that methylation of CGIs is important in X chromosome inactivation in females (Heard et al., 1997; Weber et al., 2005), and in genomic imprinting where promoter methylation of either the paternally or maternally inherited allele is associated with its transcriptional repression (Bartolomei and Tilghman, 1997). There have also been reports of some non-imprinted autosomal CGIs which are methylated in normal cells, and that this mechanism may be important in the establishment and control of cell-type-specific expression of genes. This was initially described for the *maspin* gene (Futscher et al., 2002) but has more recently been shown in several other genes including *MCJ* (Strathdee et al., 2004), *14-3-3σ* (Oshiro et al., 2005) and *HOXA5* (Strathdee et al., 2007). One study has reported that this epigenetic mechanism is most likely relatively rarely involved in the control of cell-type-specific expression of genes in normal tissues (Yamada et al., 2004), although others dispute this (Song et al., 2005).

The two major mechanisms which define the epigenome of a cell are DNA methylation and histone modifications. Modulation of chromatin structure is essential for the regulation of gene expression, but it remains unclear whether DNA methylation is the initial silencing event or whether it is a consequence of earlier chromatin-remodelling events leading to changes in gene expression. Several proposals have been suggested to explain the mechanism by which DNA methylation may cause transcriptional repression of genes. Historically, the suggestion was simply that DNA methylation could physically deter the binding of transcription factors to their binding sites in the promoters of genes and inhibit gene transcription this way. This was shown to affect the binding of several important transcription factors including AR-2, E2F and NFκB (Tate and Bird, 1993). Recent authors though have described an alternative more generally applicable mechanism by which this repression may occur, which establishes a link between DNA methylation and higher order chromatin structure (Bird and Wolffe, 1999; Tyler and Kadonaga, 1999), as illustrated below in Figure 3.

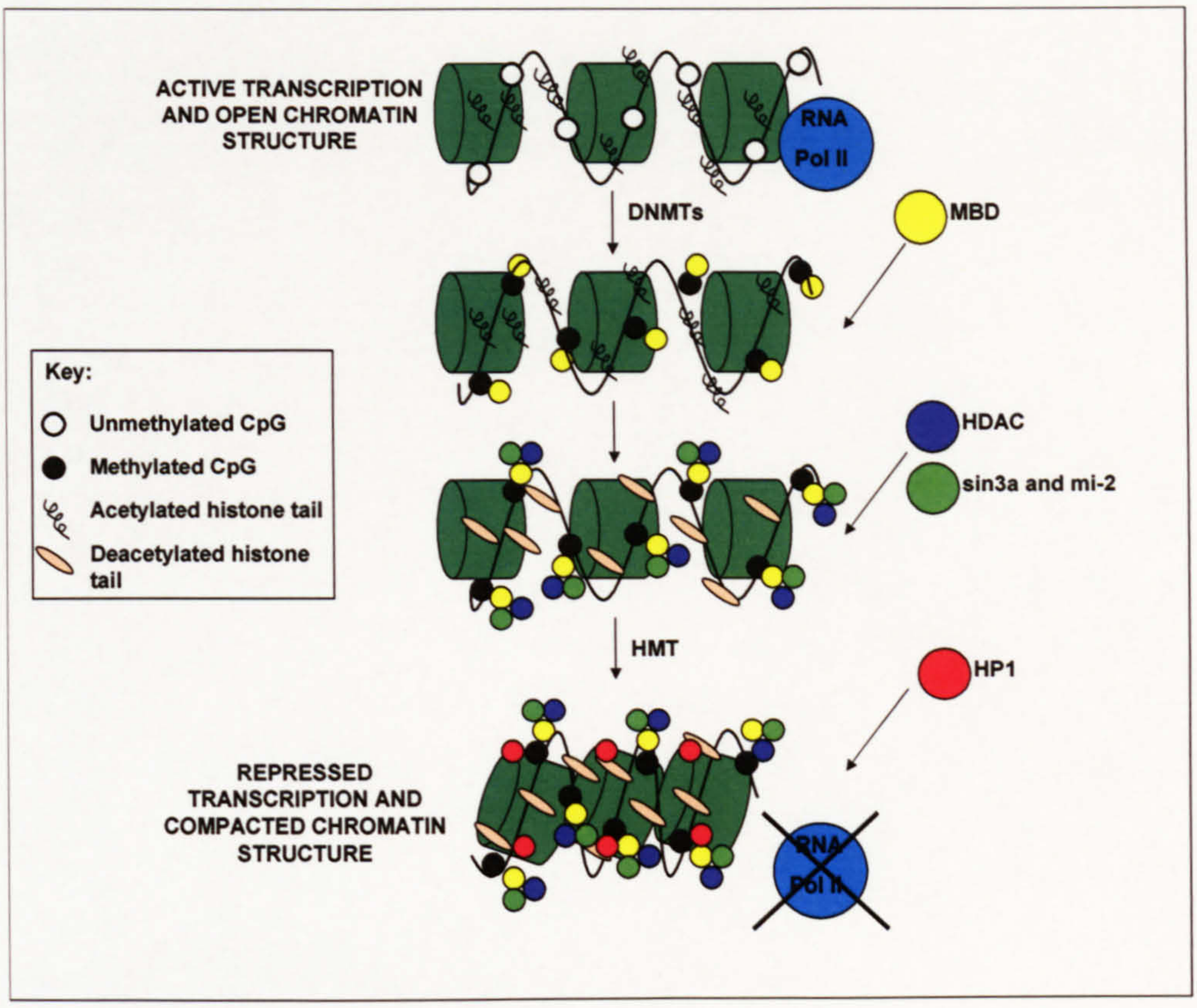


Figure 3 Epigenetic mechanism of transcriptional repression and chromatin remodeling. Active transcription is associated with an open chromatin structure, acetylated histones and unmethylated CpGs (white). RNA polymerase (RNA Pol II) and transcription factors can access and transcribe the gene. DNMTs methylate CpGs (black) and bind methyl binding domain, MBD, proteins (yellow). Subsequent recruitment of histone deacetylases, HDAC, (blue) and chromatin remodeling proteins, sin3a/mi-2, (green) leads to remodeling of chromatin and deacetylation of histone tails. Histone methyltransferases, HMTs, methylate lysine residues, allowing binding of heterochromatin protein 1, HP1, (red) to chromatin.

Initial work in the last decade brought together a link between DNA methylation and gene silencing. In experimental systems, it was shown that promoter methylation does not cause silenced transcription until chromatin-remodelling proteins are recruited to the region (Kass et al., 1997), and further work identified the chromatin-remodelling proteins with which methylcytosine-binding proteins associate (Jones et al., 1998; Ng et al., 1999; Wade et al., 1999). Methylated DNA can recruit a family of methyl-binding domain (MBD) proteins which all share a common MBD motif. Several members of this family (MeCP2, MBD2 and MBD3) are able to associate with protein complexes involving histone deacetylases (HDAC1 and HDAC2) and chromatin remodelling proteins (sin3a and mi-2). Normally, in transcriptionally active genes, lysine residues on the N-terminal tails of the core histones (H2A, H2B, H3 and H4) are acetylated (Marks et al., 2001). However,

following association with these proteins, deacetylation of these histone tails occurs and leads to a tighter binding between positively charged lysine residues of histones and the negatively charged phosphodeoxyribose backbone of the DNA. This tighter binding reduces accessibility of DNA for transcription factors. MBD proteins also engage histone methylases (HMTs) which results in the methylation of lysine 9 of histone 3 (H3-K9) and binding of the heterochromatin protein 1 (HP1) which is involved in maintenance of a transcriptionally silenced state (Bannister et al., 2001; Lachner et al., 2001). Additional recent work has revealed that MBD1 associates with a complex containing H3-K9 specific methyltransferase activity (Sarraf and Stancheva, 2004), providing another possible link between DNA and histone methylation. An alternative mechanism though could be that methylation of DNA occurs after the formation of a closed chromatin state. In mammals, DNA methyltransferases interact with H3-K9 methylases (Fuks et al., 2003; Lehnertz et al., 2003), and loss of H3-K9 methylation in knockout embryonic stem cells decreases DNMT3B-dependent CpG methylation at major centromeric satellites (Lehnertz et al., 2003). H3-K9 methylation and suppression of $p16^{INK4A}$ can occur before CpG methylation which may mean therefore that DNA methylation is secondary to histone modification in gene silencing (Bachman et al., 2003), and that only genes repressed by other mechanisms are then subject to CpG methylation (Bird, 2002; Mutskov and Felsenfeld, 2004). It has also been suggested that DNA methylation could subsequently reinforce the repressed chromatin structure (Szyf, 2003). In addition, there are also more novel findings which suggest a communication between DNA methylation and other histone modifications, including H3-K27me3 and H4-K20me3 (Fraga et al., 2005b). There is really only a partial understanding of the molecular interplay between these epigenetic modifications, and the mechanisms which underlie this intimate link between DNA methylation and histone modifications remain under intense scrutiny.

The importance of DNA methylation patterns in humans, and the cause and possible consequences of disruption to this epigenetic mechanism have been studied intensely over the last decade (Robertson, 2005). During development, a number of congenital malignancies are characterised by abnormal DNA methylation. These include immunodeficiency, centromeric region instability, facial anomalies (ICF) syndrome, which has been linked to mutations in *DNMT3B* (Xu et al., 1999), and imprinting disorders such as Beckwith-Wiedemann and Prader-Willi syndromes (Robertson, 2005). DNA methylation aberrations have also been linked to the phenotypic disconcordance that can be later identified in monozygotic (MZ) twins. Interestingly, it is not possible to distinguish epigenetic differences between MZ twins at an early age (Fraga et al., 2005a), but older MZ twins show striking differences in respect to 5-methylcytosine content and this is

emphasised in those who are separated for longer periods of time (Fraga et al., 2005a). This indicates that environmental factors may have influence over the epigenome. There is also evidence to suggest that global 5-methylcytosine levels can be affected by nutritional status including dietary deficiencies in folate and methionine, and that these may indeed contribute to alterations in the DNA methylation content (Pogribny et al., 1995; Pogribny et al., 2004). An age-dependent increase in methylation is observed in some histologically normal tissues (Ahuja et al., 1998; Issa et al., 1994), but perhaps the most significant and frequently studied association to date has been that aberrant methylation changes are observed frequently in many human cancer types.

1.4 DNA methylation alterations in cancer

Cancer is now recognised as being both a polygenic and polyepigenetic disease. DNA methylation patterns are profoundly altered in human cancer (Robertson, 2005), and this was first demonstrated in the genome of cancer cells which were found to be hypomethylated in comparison to normal tissues (Feinberg and Vogelstein, 1983). In cancer, this genome-wide hypomethylation is mostly due to loss of methylation from repetitive elements in the genome (Yoder et al., 1997) and results in genomic instability. Concomitantly, *de novo* methylation of CGIs around the promoter region of genes is observed in cancer development correlating with transcriptional repression of genes. This is the most well characterised epigenetic alteration in neoplastic cells (Jones and Baylin, 2002). An epigenetic comparison of normal and cancer tissues is illustrated below in Figure 4.

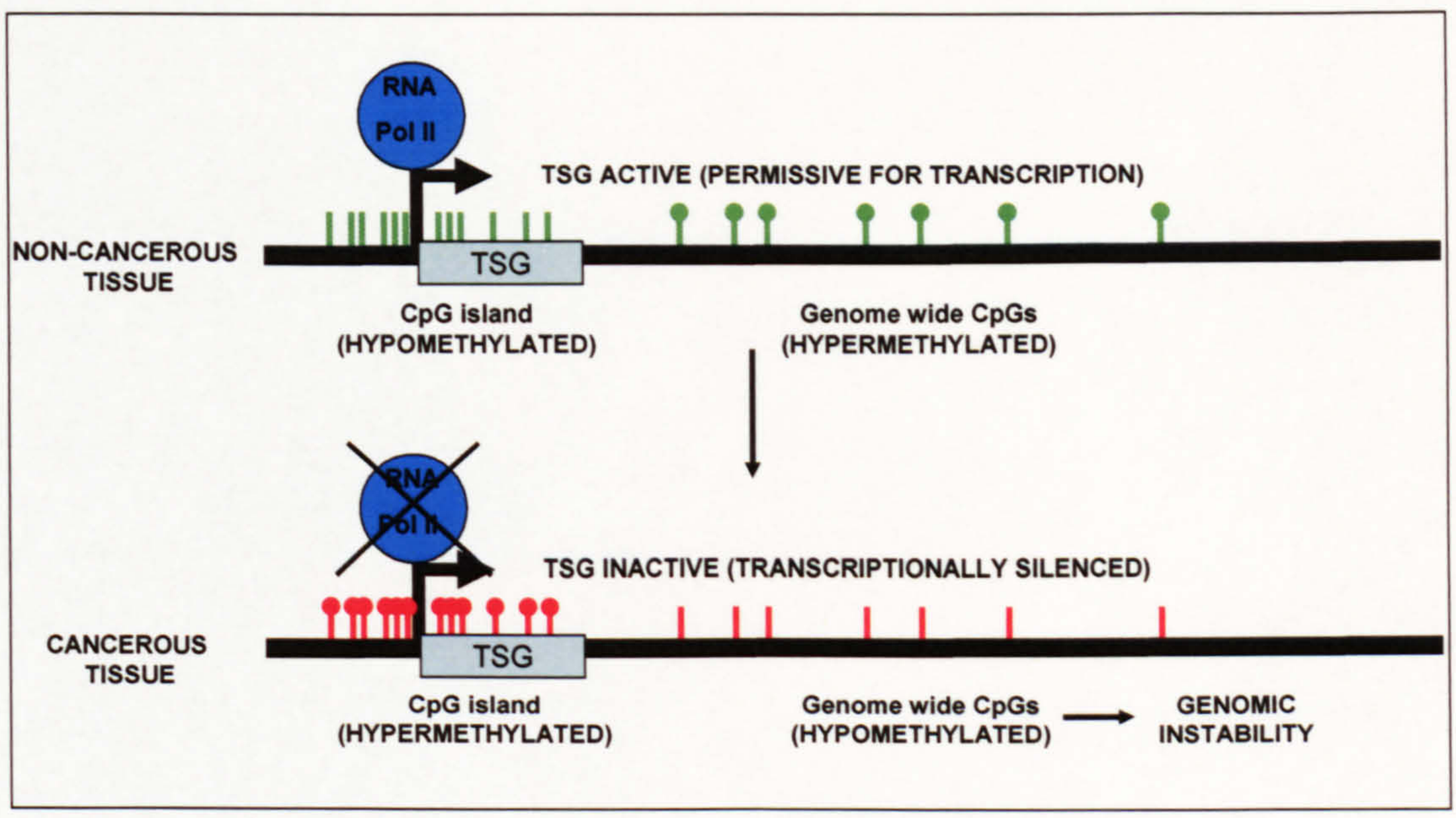


Figure 4 DNA methylation and cancer. A representation of a region of DNA in non-cancerous (top; green) and cancerous (bottom; red) tissues showing the differences in DNA methylation in the two phenotypes. In non-cancerous tissue, genome wide hypermethylation of CpGs (closed green circles) and an actively transcribed tumour suppressor gene (TSG) is associated with a hypomethylated CGI (green lines). In cancerous tissue, the opposite is seen with genome wide hypomethylation (red lines) leading to genomic instability, and CGI hypermethylation (closed red circles) contributing to transcriptional silencing of a TSG.

Despite the fact that we know a great deal regarding the alteration of transcriptionally active euchromatin into a repressive heterochromatic state, the mechanisms by which CGIs remain protected against methylation in normal cells, but subsequently lose this protective barrier in cancer and become hypermethylated are yet to be elucidated. It has been proposed that during tumourigenesis, the segregation of the epigenome into unmethylated and methylated regions may be destroyed leading to a spread of heterochromatin (Turker and Bestor, 1997). This process has been shown for the oestrogen receptor gene in association with aging (Issa et al., 2001; Issa et al., 1994), and although the exact mechanism is unclear, the protective barriers against CGI methylation may be more vulnerable in aging cells and therefore increase cancer "risk" in individuals. It has also been suggested that *de novo* methylation may be "seeded" in exonic CGIs and subsequently spread into the promoter region of genes in cancer (Nguyen et al., 2001). Interestingly, it has been proposed that this exonic methylation seen could be an age-related phenomenon or representative of a preneoplastic lesion. It is also unclear what dictates the specific CGI-associated genes which will be epigenetically altered in different cancer types and why other CGIs are protected from methylation (Frigola et al., 2006). Altered methylation has previously been considered to locally silence discrete genes in cancer cells but recent work has challenged this concept by showing that long-range epigenetic silencing of genes may exist in cancer (Frigola et al., 2006) which hypermethylates neighbouring genes and causes global gene silencing through chromatin remodeling activities. Recent evidence has also argued that genes which are methylated in cancers may be vulnerable to aberrant DNA hypermethylation and epigenetic silencing during tumour initiation and progression because of alterations in chromatin structure in stem or progenitor cells, including dimethylated H3K9 and trimethylated H3K9 (Ohm et al., 2007; Widschwendter et al., 2007).

CGI methylation and subsequent transcriptional silencing occurs at least as often as genetic alterations in tumour suppressor genes (TSGs) in cancer (Herman and Baylin, 2003; Jones and Baylin, 2002). According to Knudson's "two-hit" hypothesis, in carcinogenesis, loss of function of both alleles of a gene is required for malignant transformation (Knudson, 2001). The first hit is most often mutation of a critical gene (e.g. TSG), followed by loss of the wild-type allele through deletion or loss of heterozygosity (LOH). Aberrant promoter methylation offers an alternative reversible mechanism to inactivate key tumour suppressor genes in cancer. For instance, it can constitute the initial hit in many cancers with subsequent mutations or deletions eliminating the second allele. Furthermore, hypermethylation of both alleles has been noted in tumours in the absence of genetic aberrations of a given gene (Herman and Baylin, 2003; Jones and Baylin, 2002).

On average, 600 CGIs are aberrantly methylated in tumours although this is dependent on tumour type and particular histological subtype (Costello et al., 2000). Hundreds of genes have the potential to be regulated by CGI methylation, affecting many properties of a tumour during its development. It has been postulated that in order for a cancer to develop, most malignancies need to develop "hallmarks" such as evasion of apoptosis, insensitivity to antigrowth signals, limitless replicative potential, self-sufficiency in growth signals, sustained angiogenesis and tissue invasion/metastasis (Hanahan and Weinberg, 2000). The role of DNA methylation and its ability to affect properties such as these has been widely reported in many cancers, including ovarian cancer. Previous work has shown that aberrant CGI methylation can affect a number of key genes involved in multiple biological pathways in FIGO Stage III and IV ovarian tumours (Strathdee et al., 2001). Epigenetic changes that confer these types of traits could be selected for during tumourigenesis (Teodoridis et al., 2004). Examples of genes which have been shown to become epigenetically silenced in many cancers including ovarian tumours and could affect the hallmarks of cancer development are shown below in Table 1.

KNOWN GENE REGULATORY FUNCTION	CONSEQUENCE OF EPIGENETIC SILENCING	EXAMPLE OF GENE SILENCED	REFERENCE
APOPTOSIS	PERTURBED APOPTOTIC MECHANISM	APAF-1 **CASP8** DAPK **DcR1*** Fas $p14^{ARF}$* p73* **RASSF1A*** **TMS1***	(Furukawa et al., 2005) (Teitz et al., 2000) (Balana et al., 2003) (Shivapurkar et al., 2004) (Hopkins-Donaldson et al., 2003) (Hashiguchi et al., 2001) (Strathdee et al., 2001) (Yoon et al., 2001) (Terasawa et al., 2004)
REPLICATION AND PROLIFERATION	LIMITLESS REPLICATION AND PROLIFERATION	**PTEN*** **SFRP-1*** SOCS-3	(Yang et al., 2006) (Takada et al., 2004) (He et al., 2003)
CELL ADHESION	TISSUE INVASION / METASTASIS	**OPCML***	(Sellar et al., 2003)
DNA REPAIR	IMPAIRED DNA REPAIR	**BRCA1*** FANCF* **MGMT*** **hMLH1***	(Strathdee et al., 2001) (Taniguchi et al., 2003) (Dhillon et al., 2004b) (Strathdee et al., 2001)
DETOXIFIES DRUGS	IMPAIRED DETOXIFICATION	**GSTP1***	(Makarla et al., 2005)
CELL CYCLE	IMPAIRED CELL CYCLE REGULATION	**p16*** P21	(Hashiguchi et al., 2001) (Roman-Gomez et al., 2002)
METHYLATED IN OVARIAN CANCER	UNKNOWN	**HIC1** **MINT25**	(Strathdee et al., 2001) (Strathdee et al., 2001)

Table 1 Examples and biological consequences of genes which are epigenetically silenced In tumours. Genes highlighted in bold print are those reported to be methylated in ovarian tumours by Teodoridis et al, 2005. *indicates studies which have reported methylation of these genes specifically in ovarian tumours (and references shown). All other genes mentioned have been reported to be methylated in other tumour types, but not specifically ovarian cancer. Other genes which have been reported to be methylated in ovarian cancer are shown, although their specific function remains unknown.

In addition to the evidence that specific genes become methylated and silenced, there is also some evidence to support the concept that clusters of CGIs can become co-methylated in cancer, giving rise to a "CpG island methylator phenotype" (CIMP). This idea was originally described in colorectal cancer (Toyota et al., 1999a) but has now been proposed in a variety of tumour types including ovarian cancer (Strathdee et al., 2001). However, there remains controversy over whether the CIMP exists at all (Anacleto et al., 2005; Yamashita et al., 2003), and if it does, whether it may exist as gradual distributions of methylation (Eads et al., 2001; Rashid et al., 2001) rather than as a categorical state (CIMP+/CIMP-). The most recent definition of CIMP, using an unbiased genome-wide method of analysis, has given the strongest evidence to support the existence of a distinct subset of tumours with a methylator phenotype (Weisenberger et al., 2006). The CIMP was originally thought to be due to a general defect in the methylation machinery but it remains

unclear whether there is an underlying biological mechanism for the concurrent methylation changes seen in multiple tumour suppressor genes in cancer (Issa, 2003). One such biological mechanism underlying the increased methylation which has been described is overexpression of DNMTs. This has been shown recently in the context of overexpression of DNMT1 (Nakagawa et al., 2005; Peng et al., 2006). Changes in expression of DNMTs due to genetic factors may potentially be a cause for the frequent methylation seen in those tumours defined as having a CIMP (De Marzo et al., 1999). It has been shown that a (-149 C>T) single nucleotide polymorphism (SNP) can affect the transcription of DNMT3b. An association of this SNP and methylation levels in tumours including lung and ovarian type may exist (Shen et al., 2002; Teodoridis et al., 2005). Most recently, a genetic influence has also been described in a distinct subset of colorectal tumours which almost all had *BRAF* mutations (Weisenberger et al., 2006). In addition, factors that are known to be associated with methylation of CGIs including aging (Ahuja et al., 1998; Issa et al., 1994), chronic inflammation (Hsieh et al., 1998; Issa et al., 2001), and infective factors including viral infections e.g. Epstein-Barr Virus (EBV)-associated gastric carcinoma (Kang et al., 2002; Osawa et al., 2002) and bacterial infections e.g. *Helicobacter pylori* dependent mucosa associated lymphoid tissue (MALT) lymphoma (Kaneko et al., 2003) may all potentially influence the methylation profile of tumours and act in concert with genetic factors (Bjornsson et al., 2004) to propensiate a CIMP.

1.5 The impact of DNA methylation on drug resistance mechanisms in ovarian cancer

1.5.1 DNA methylation and intrinsic drug resistance

Drug resistance remains a major problem in the successful treatment of patients with ovarian cancer. Most chemotherapeutic drugs used in patients with this disease target stress on rapidly dividing cells, ultimately leading to cell death through apoptotic mechanisms. Specifically, platinum-based drugs form inter- and intra-strand crosslinks following their incorporation into DNA (Kartalou and Essigmann, 2001). These adducts are recognised by the MMR system leading to apoptosis and cell death in tumours (Agarwal and Kaye, 2003). Taxanes stabilise tubulin, causing defective spindle formation, G2/M arrest and subsequent apoptosis (Dumontet and Sikic, 1999). Drug resistant tumour cells often possess perturbed proapoptotic and/or antiapoptotic cellular mechanisms (Balch et al., 2004). Consequently, the mechanisms by which chemotherapeutic agents will lead to tumour regression are altered and therefore this leads to changes in the response to important drugs. Genes involved in key DNA damage response pathways including control of the cell cycle, DNA repair and apoptotic signalling can frequently become methylated and silenced in cancer (Teodoridis et al., 2004), leading to such defective mechanisms in cancer cells. These epigenetic changes may confer opposing effects on the intrinsic sensitivity of ovarian tumours to drugs by modulating the apoptotic response. Methylation-mediated epigenetic silencing of genes involved in DNA repair (including *BRCA1*, *MGMT* and *FANCF*) may increase chemosensitivity of tumours. In contrast, this type of epigenetic silencing in proapoptotic genes (including *hMLH1* and *APAF-1*) may aid in chemoresistance mechanisms (Esteller et al., 2000a; Glasspool et al., 2006; Soengas et al., 2001; Taniguchi et al., 2003). Key genes involved in the DNA damage response pathway which have the potential to be epigenetically silenced in ovarian cancer are illustrated in Figure 5. There have been many reports of genes which are downregulated by epigenetic mechanisms in ovarian cancer and examples of these have been previously shown in Table 1. However, it is important to decipher from the myriad of epigenetically silenced gene signatures, which of these will be important predictive markers of disease response to treatment.

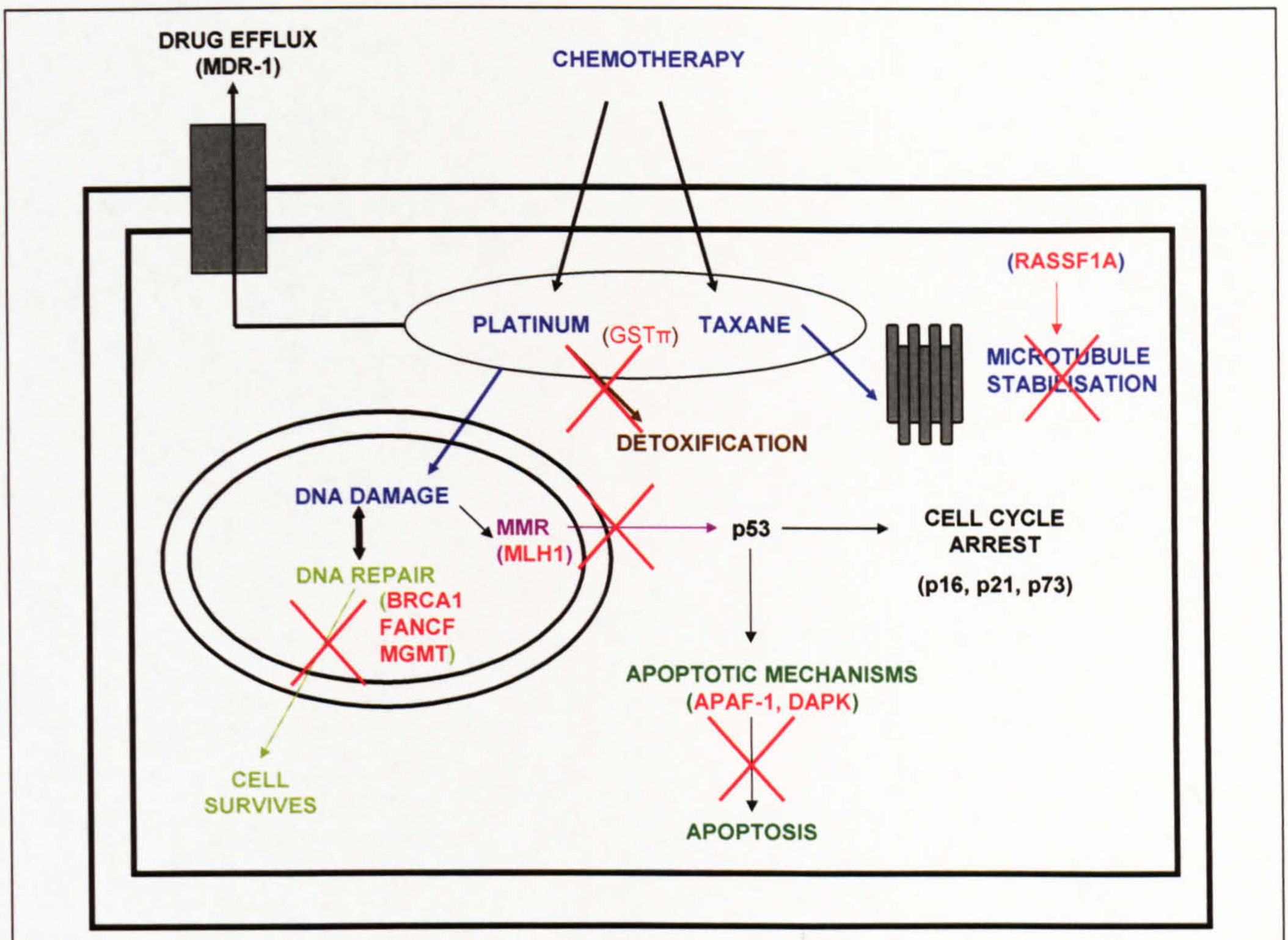


Figure 5 Epigenetic silencing of key genes in ovarian cancer drug resistance. Methylation of genes (shown in red) involved in key DNA damage response pathways in an ovarian cancer cell. Methylation of genes involved in the DNA repair pathway (pale green), including *BRCA1*, *FANCF*, *MGMT*, or drug detoxification (brown), including (*GSTP1*), could enhance chemosensitivity by blocking the ability of cell to survive. Methylation of genes involved in apoptotic mechanisms (dark green), including *MLH1*, *APAF-1* and *DAPK* could enhance cancer cell survival.

BRCA1 deficiency in mammary epithelial cells and breast cancer cell lines has been linked to sensitivity to cisplatin and other DNA damaging agents *in vitro* (Sgagias et al., 2004; Tassone et al., 2003). However, although frequent methylation of *BRCA1* has been reported in ovarian cancer (Esteller et al., 2000b; Ibanez de Caceres et al., 2004), there have been no additional reports suggesting that this is an important predictive marker of chemosensitivity in this disease.

MGMT (O^6-methylguanine-DNA methyltransferase) removes methyl groups as well as larger adducts from the O^6 position of guanine. The alkylation of DNA by alkylating agents at this position of guanine is associated with the formation of mutations in DNA (Gerson, 2004). However, MGMT removes these groups and inhibits cancer cell death induced by such agents. CGI methylation of *MGMT* has been frequently reported in cancers including ovarian, glioma and colorectal cancer (Dhillon et al., 2004b; Esteller et al., 1999a; Esteller

et al., 2000d). This mechanism of epigenetic silencing of *MGMT* has been shown to be associated with response in other tumour types, including patients treated with temozolomide in combination with radiation (Hegi et al., 2004). In addition, hypermethylation of the *MGMT* promoter also correlated with increased survival of patients with diffuse large B-cell lymphoma after chemotherapy (Esteller et al., 2002).

GSTπ belongs to a group of enzymes which detoxifies carcinogens and therefore reduces the ability of these types of compounds to damage DNA (Hayes and Strange, 2000). *GSTP1* is frequently methylated in many tumour types including prostate and ovarian cancer (Makarla et al., 2005; Perry et al., 2006). Epigenetic silencing of *GSTP1* may improve chemosensitivity by preventing detoxification of chemotherapeutic agents. The contribution of this enzyme family to chemoresistance has been shown *in vitro* (Perquin et al., 2001).

Epigenetic alteration of another gene, *FANCF*, involved in the DNA repair complex containing BRCA1 and BRCA2 has been reported in ovarian cancer (Taniguchi et al., 2003). In ovarian cancer cell line models, methylation of *FANCF* was associated with increased cisplatin sensitivity, however, *FANCF* was shown to become demethylated and re-expressed during the acquisition of drug resistance in ovarian cancer (Taniguchi et al., 2003). The contribution of this gene to acquired drug resistant mechanisms though remains debatable.

Methylation of pro-apoptotic genes may be a causative factor in drug resistance in patients. Genes involved in this type of apoptotic response have been shown to be methylated in ovarian cancer (Strathdee et al., 2001). The DNA MMR protein, hMLH1, is one of the most well characterised proteins involved in recognising platinum-induced damage (Papouli et al., 2004). Methylation of *hMLH1* has been reported in many tumour types including ovary (Geisler et al., 2003; Strathdee et al., 2001), colon (Cunningham et al., 1998) and stomach (Kitajima et al., 2003). Evidence has shown that hMLH1 is required for the engagement of apoptosis and that loss of hMLH1 protein expression could be a contributing factor to platinum resistance (Anthoney et al., 1996; Brown et al., 1997; Drummond et al., 1996). Coupling to cell death may not occur when expression of hMLH1 is lost because of reduced attempts at processing O^6-methylguanine or DNA lesions being bypassed during replication (Karran and Hampson, 1996; Moreland et al., 1999).

RASSF1A is another example of a pro-apoptotic gene which is frequently methylated in ovarian tumours (Yoon et al., 2001). This gene has been shown to be methylated through a

"seeding effect" where methylation spreads from the first exon into the promoter region (Yan et al., 2003). In this study, this spreading of methylation observed appeared to be associated with tumour progression in breast cancer. RASSF1A binds to tubulin and leads to the stabilisation of microtubules (Liu et al., 2003). This is the target of taxane-based chemotherapy, and therefore, methylation-induced silencing of *RASSF1A* could abrogate this effect.

In mammalian cells, nonreceptor-mediated apoptosis occurs predominantly via the assembly of a cytochrome c-dependent apoptosome complex containing caspase-9 and apoptotic protease-activating factor-1 (APAF-1) (Adams and Cory, 2002). Dysfunctional activity of this apoptotic mechanism has been implicated in ovarian cancer and drug resistance (Liu et al., 2002). Methylation of *APAF-1* may be associated with chemoresistance in other cancer types including leukaemia and melanoma (Fu et al., 2003; Soengas et al., 2001). Treatment with a DNMT inhibitor was shown to reverse methylation and both restore sensitivity of leukaemic cells to UV light-induced apoptosis (Fu et al., 2003), and increase sensitivity of melanoma to doxorubicin (Soengas et al., 2001).

It is evident therefore that aberrant epigenetic alterations in key genes may alter drug response in patients and enable the clinical prediction of outcome following chemotherapy. Defined groups of genes can become concordantly methylated in ovarian cancer (Strathdee et al., 2001; Wiley et al., 2006) which supports the concept of the CIMP, and epigenetic gene silencing in drug resistance has recently been defined as polygenic (Glasspool et al., 2006). Therefore, large scale genome wide analysis will enable the identification of important gene signatures which are predictive of disease response to treatment. A recent study has shown that patients with late-stage ovarian tumours can be clustered into two distinct groups based on differences in CGI methylation using the high throughput global technique, differential methylation hybridisation (DMH) (Wei et al., 2002). Tumours from the group of patients with shorter PFS after chemotherapy displayed higher concurrent CGI methylation. This study indicates that distinct hypermethylated CGIs may be important prognostic markers in ovarian cancer. Other recent studies have also shown that CIMP+ tumours may be associated with worse prognosis/prediction in many cancers including neuroblastoma (Abe et al., 2007) and gastric cancer (An et al., 2005).

1.5.2 DNA methylation and acquired drug resistance

The majority of clinical studies into drug resistance have examined tumour characteristics in chemonaive tumours rather than at the time of relapse (Teodoridis et al., 2004). This allows for the identification of intrinsic resistance markers of chemoresistance in ovarian cancer. However this does not divulge the pathways or enhance our understanding of the key genes which are potentially selected for during acquired chemoresistance mechanisms. Changes in DNA methylation may occur throughout cancer progression, although these changes may not directly impact on the phenotype of an ovarian tumour until it is challenged with chemotherapy. Chemonaive tumours are heterogeneous consisting of subpopulations of cells with varying degrees of chemosensitivity. It has been suggested that chemotherapy may exert a selective pressure on epigenetically silenced drug sensitivity genes present in small subpopulations of cells and/or may be due to regrowth of a cancer stem cell population in the tumour cell environment. These can then lead to acquired resistance alone or in combination and a chemoresistant tumour cell population will regrow (Agarwal and Kaye, 2003). Therefore, analysis of tumour at relapse will allow for the identification of these selected subpopulations of cells.

Studies of *in vitro* models in ovarian cancer have shown that both methylation patterns (Wei et al., 2003) and methylation of genes individually, such as *hMLH1* (Strathdee et al., 1999), can be selected for during chemotherapy. A study in breast cancer showed that reduced expression of the hMLH1 protein in breast tumour following neo-adjuvant chemotherapy in comparison to chemonaive tumour samples was associated with worse survival in patients (Mackay et al., 2000). The difficulty in obtaining tumour samples at relapse from patients with ovarian cancer though has undoubtedly impacted on the lack of studies examining clinical acquired resistance for this disease. Recently though, the use of plasma DNA to detect methylation differences in patients with ovarian cancer has been investigated successfully (Gifford et al., 2004). Methylation of the *hMLH1* CGI was examined in blood samples from patients with EOC in both chemonaive and relapse samples. Acquisition of *hMLH1* methylation following chemotherapy was predictive of poor overall survival in patients with this disease (Gifford et al., 2004). Therefore, CGI methylation would appear to be a driving force behind the loss of *hMLH1* expression in cancer and other genes may be concomitantly methylated. Identification of these polyepigenetic changes in plasma may therefore provide important epigenetic signatures during treatment, and allow enrichment for patients who will benefit greatly from epigenetic treatments that lead to reversal of chemoresistance (Lyko and Brown, 2005).

There is now an increasing wealth of evidence to suggest that epigenetic alterations in stem cells may be inherently linked to drug resistant properties in tumours (Feinberg et al., 2006). The high incidence of relapse attributable to multidrug resistance and the many histological phenotypes indicative of multipotency suggests that there may be a stem cell-like aetiology to ovarian cancer. Stem cells may only comprise a very small proportion of the cells within a tumour and be relatively quiescent, therefore avoiding the toxicity of the chemotherapy regime which will target rapidly dividing cells (Agarwal and Kaye, 2003). Survival and regrowth of a cancer stem cell population has been suggested to be involved in acquired drug resistance (Dean et al., 2005). Acquisition of methylation post-chemotherapy could represent survival, growth and differentiation of cancer stem cells which were present in the original tumour prior to treatment with chemotherapy (Feinberg et al., 2006). A recent study has identified and characterised side populations of cancer stem-like cells from mouse ovarian cancer cell lines (Szotek et al., 2006). These side populations were found to form measurable tumours sooner than other populations, and also responded less well when treated with doxorubicin (Szotek et al., 2006). In addition, the aggressive nature of EOC has also been attributed to cancer stem cell properties (Bapat et al., 2005).

1.6 DNA methylation as an early diagnostic marker in ovarian cancer

Despite advances in our understanding of the molecular manifestations of ovarian cancer, long-term survival rates have remained relatively static over the last three decades (Barnholtz-Sloan et al., 2003). Current modalities which are used to detect ovarian cancer have limited success i.e., physical, radiological [Ultrasound (US) and Computed Tomography(CT)] and biochemical (CA-125) assessment (Bourne et al., 1993; Sato et al., 2000; van Nagell et al., 2000), and the efficacy of developing a screening test for this insidious and extremely aggressive malignancy remains unproven (Rosenthal et al., 2006). Novel molecular markers and methods which allow the early detection of ovarian cancer do have the potential to impact on the clinical outcome of this disease and improve the current poor survival rates. The efficacy of a biomarker assay will be determined by its sensitivity and specificity. For population-based screening approaches, these are precisely defined. The clinical sensitivity of a biomarker refers to the proportion of subjects with confirmed disease who test positive, whereas its specificity refers to the proportion of healthy control subjects who test negative for the biomarker being used (Pepe et al., 2001). Early detection of ovarian cancer will require a high degree of sensitivity (75%) and a particulary high specificity (99.6%) to achieve an acceptable positive predictive value (Bast, 2003; Jacobs and Menon, 2004). Hypermethylation of CGIs is a potentially attractive marker for detecting this neoplasm, and detection of these changes have been proposed previously as a potential early diagnostic tool in cancer (Esteller, 2003).

In order to use a biomarker in a clinical setting, it has to be readily detected in easily accessible surrogate body sources such as plasma. Cancer-specific methylation patterns can be detected in free DNA released from dead cancer cells (Jahr et al., 2001; Sidransky, 2002), and this free DNA is thought to be released from apoptotic or necrotic tumour cells (Jahr et al., 2001). It has already been shown by several groups that CGI methylation can be detected in plasma with the same characteristic changes as are found in the corresponding tumour (Esteller et al., 1999b; Gifford et al., 2004; Ibanez de Caceres et al., 2004; Weaver et al., 2006), and that this is therefore a promising novel biomarker.

Additionally, numerous genes have been shown to be hypermethylated in cells isolated from other bodily fluids including urine, serum, sputum and stool of cancer patients (Cairns, 2004; Dulaimi et al., 2004; Sidransky, 2002; Wang et al., 2006b). In the case of ovarian cancer, these changes have been detected with high specificity, thus demonstrating

their potential use as a diagnostic tool (Chang et al., 2002). In contrast to cancerous tissues, CGIs in normal tissue are rarely methylated (Bird, 1986; Hendrich and Bird, 2000), including peripheral blood mononuclear cells (PBMC) DNA (Toyota et al., 2001). The development of exquisitely sensitive PCR-based techniques, in particular methylation-specific PCR (MSP) (Herman et al., 1996) and fluorescent-based equivalents (Gifford et al., 2004), have enabled scientists to detect aberrant methylation of specific genes in easily obtainable samples with a small amount of tumour-derived DNA present, such as plasma (Ibanez de Caceres et al., 2004). This is feasible because these types of PCR-based assays can detect 1 methylated allele in 1,000 unmethylated alleles (Herman et al., 1996). Epimutations, specifically CGI methylation, invariably occur in the same region of a gene promoter (Baylin et al., 2000), which is in contrast to genetic mutations which can often occur in a variety of genetic positions. This enables high-throughput analysis of these types of epigenetic changes.

DNA methylation has merits over other assays which rely on the detection of RNA or protein markers, for several reasons (for review see Levenson, 2004). Firstly, RNA and protein analytes are less stable than DNA and this instability leads to a requirement of more specific precautions for sample collection, storage and analysis, which can prove technically challenging in a clinical setting. DNA in clinical samples does not degrade as easily and can be isolated from frozen or paraffin-embedded tissues and then easily amplified using PCR techniques (Crisan and Mattson, 1993; Lehmann and Kreipe, 2001). DNA methylation itself can be qualitatively assessed using PCR in comparison to both protein and RNA measurements which rely on quantitative measures and further comparison to controls. This quantification of RNA or protein becomes more complex in heterogeneous clinical tissue samples. In contrast, DNA methylation analysis will give a categorical answer and is a cancer-specific biomarker (Laird, 2003).

Tumour heterogeneity in ovarian cancer means that groups of potentially methylated markers may increase the sensitivity and specificity of such a biomarker in this type of cancer (Levenson, 2004). There is very little data on the identification of groups of methylated CGIs in the plasma or serum of patients with early stage ovarian cancer. Recently though, one group have encouragingly identified methylation of a group of six genes, including *BRCA1* and *RASSF1A*, in early stage tumours with a high degree of sensitivity and specificity (Ibanez de Caceres et al., 2004), which was not evident in matched normal/benign controls. *BRCA1* or *RASSF1A* was methylated in 68% of ovarian tumour samples, but by additionally examining one of four other genes (*APC*, $p14^{ARF}$, $p16^{INK4A}$ and *DAPK*), 100% of tumour samples were methylated. Encouragingly, 95% of

early stage tumours had detectable levels of methylation in at least one of six genes, confirming that hypermethylation is a marker of early stage disease. Matched serum showed identical patterns of methylation to the tumour in 83% of samples. This is an important study because it shows that ovarian cancer can be detected in serum with a specificity of 100% and sensitivity of 83%. Further studies to identify other useful markers may improve the sensitivity of the test.

In addition to improving early diagnostic and chemotherapeutic strategies, we also need better models to investigate early stage disease. Recent work indicates that epigenetic alterations may be initiating events in the expansion of cells in preneoplastic lesions (Baylin and Ohm, 2006; Feinberg and Tycko, 2004). However, although epigenetic alterations contribute to the pathogenesis of ovarian cancer, the influences of these alterations as initiation events in this type of cancer have been difficult to study. Methylation of specific genes alongside coordinated genetic hits has been shown to potentially drive the development of a cancer (Chen et al., 2004; Chen et al., 2005a). Additionally, multiple epigenetic hits have been shown to be potential early events in pre-cancerous lesions/cells prior to genetic alterations (Derks et al., 2006; Mei et al., 2006; Pijnenborg et al., 2006). This epigenetic silencing in early disease may even addict cancer cells to further mutations and increase the likelihood of tumour progression (Baylin and Ohm, 2006). Further to this, methylation in premalignant breast and colorectal tissue has been suggested to represent a field defect, perpetuating further neoplastic change (Shen et al., 2005; Yan et al., 2006). Methylation has also been previously shown to be associated with recognised stress such as inflammation (Hsieh et al., 1998) or chronic injury related to infective causes (Maekita et al., 2006). A complex series of epimutations may occur when ovarian surface epithelial cells are placed under stress. Recently, a group have addressed the timing of all of these types of events using a mouse model of cultured mouse ovarian surface epithelium to understand the chronological epigenetic events which may begin at an early stage of neoplasia in the ovary (Roberts et al., 2005).

There is also recent opinion to suggest that epimutations of stem cells may be the initiating progenitor event in tumourigenesis (Feinberg et al., 2006). This has been suggested to occur in three steps involving epigenetic interference of stem cells, an initiating mutation event and thereafter genetic and epigenetic plasticity of cells (Feinberg et al., 2006). The concept of an initiating epimutation in stem cells can be supported by previous work. Tumour-related growth has been shown to be stable but reversible *in vitro* (Lotem and Sachs, 2002) and DNA methylation is inherited through cell division (Lorincz et al., 2002). Recent cloning experiments have also shown that the blastocyst cloned from a melanoma

nucleus can differentiate into multiple cell types and that the phenotypic properties of cancer are reversible, suggesting a largely epigenetic code (Hochedlinger et al., 2004). Also, a small subpopulation of stem cells in a brain tumour can propagate a cancer phenotype when consecutively transmitted from mouse to mouse (Singh et al., 2003).

1.7 Methods of detecting DNA methylation and epigenetic alterations in cancer

There are a wide variety of methodologies which can be applied to obtain DNA methylation data. The detection of this epigenetic alteration is based on the ability to differentiate between cytosine and 5-methylcytosine in the DNA sequence. There are three principal approaches to detect this difference in methylation. These involve: (1) the use of the chemical modification of DNA with sodium bisulphite, (2) digestion of DNA with either a methylation-sensitive or -insensitive restriction enzyme, or (3) immunoprecipitation of 5-methylcytosine to distinguish any methylated fractions. The study of DNA methylation changes has been at the forefront of epigenetic research since the initial application of sodium bisulphite conversion of genomic DNA in conjunction with PCR amplification and sequencing (Frommer et al., 1992). Sodium bisulphite conversion relies on the differential deamination of cytosine to uracil without affecting 5-methylcytosine content as shown below in Figure 6. This conversion will produce a difference in DNA sequence, which depends on the sequence's original methylation status.

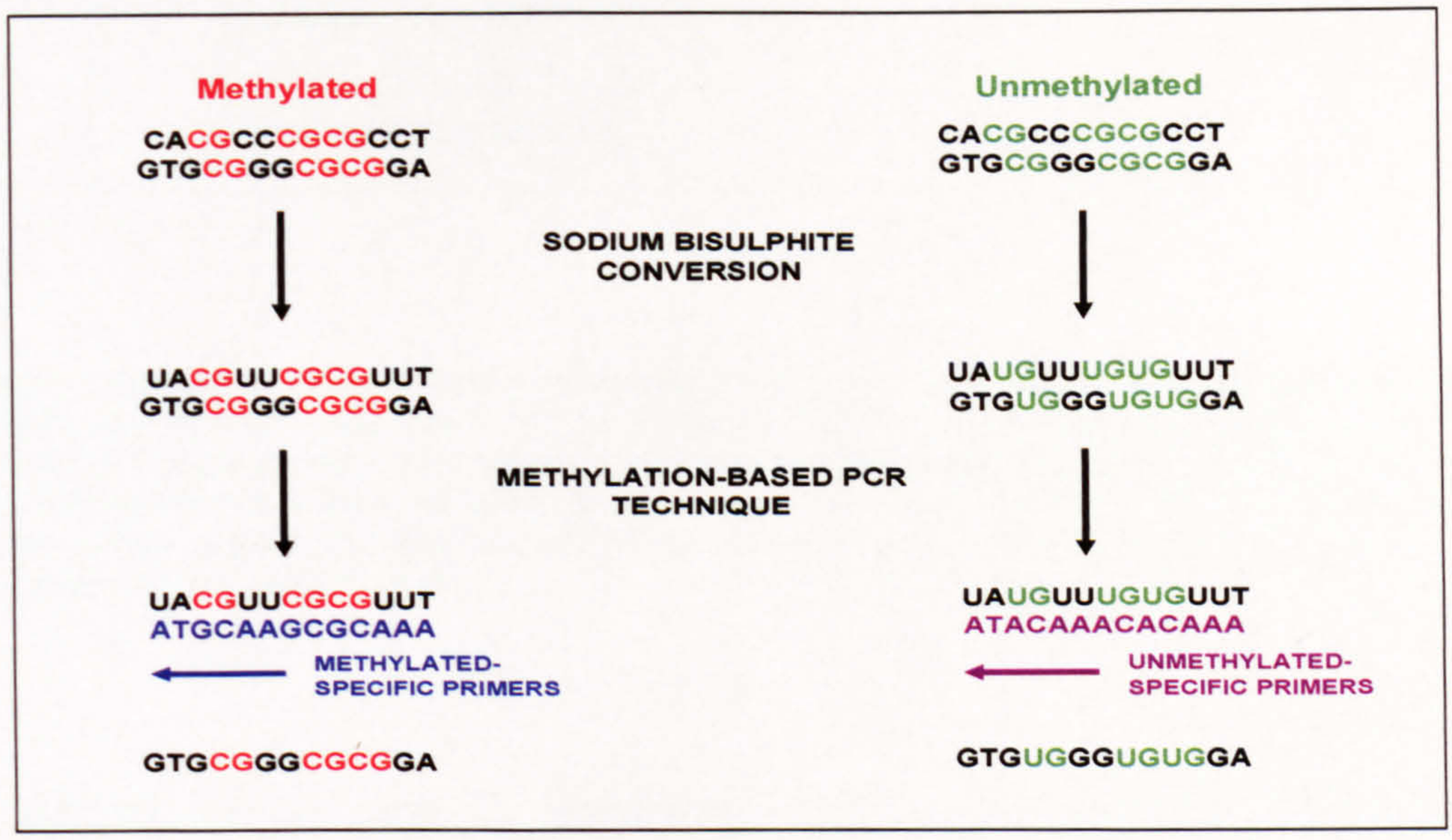


Figure 6 Principles of sodium bisulphite conversion and subsequent PCR-based analysis of DNA. DNA is modified with sodium bisulphite in a methylation-dependent way prior to PCR amplification. Methylated cytosines (red) remain unconverted, whereas unmethylated cytosines are converted to uracils (green), resulting in a difference in DNA sequence. The converted strands of DNA are no longer complementary, and amplification of either strand (top strand shown above) requires primers which are specific for the methylated sequence (blue) or the unmethylated sequence (purple).

Many DNA methylation assays will involve at least one PCR step. The difference in sequence which is apparent following sodium bisulphite treatment can be used to design PCR primers which will either amplify a region depending on its methylation status or alternatively amplify a pool of unmethylated and methylated products as shown below in Figure 7.

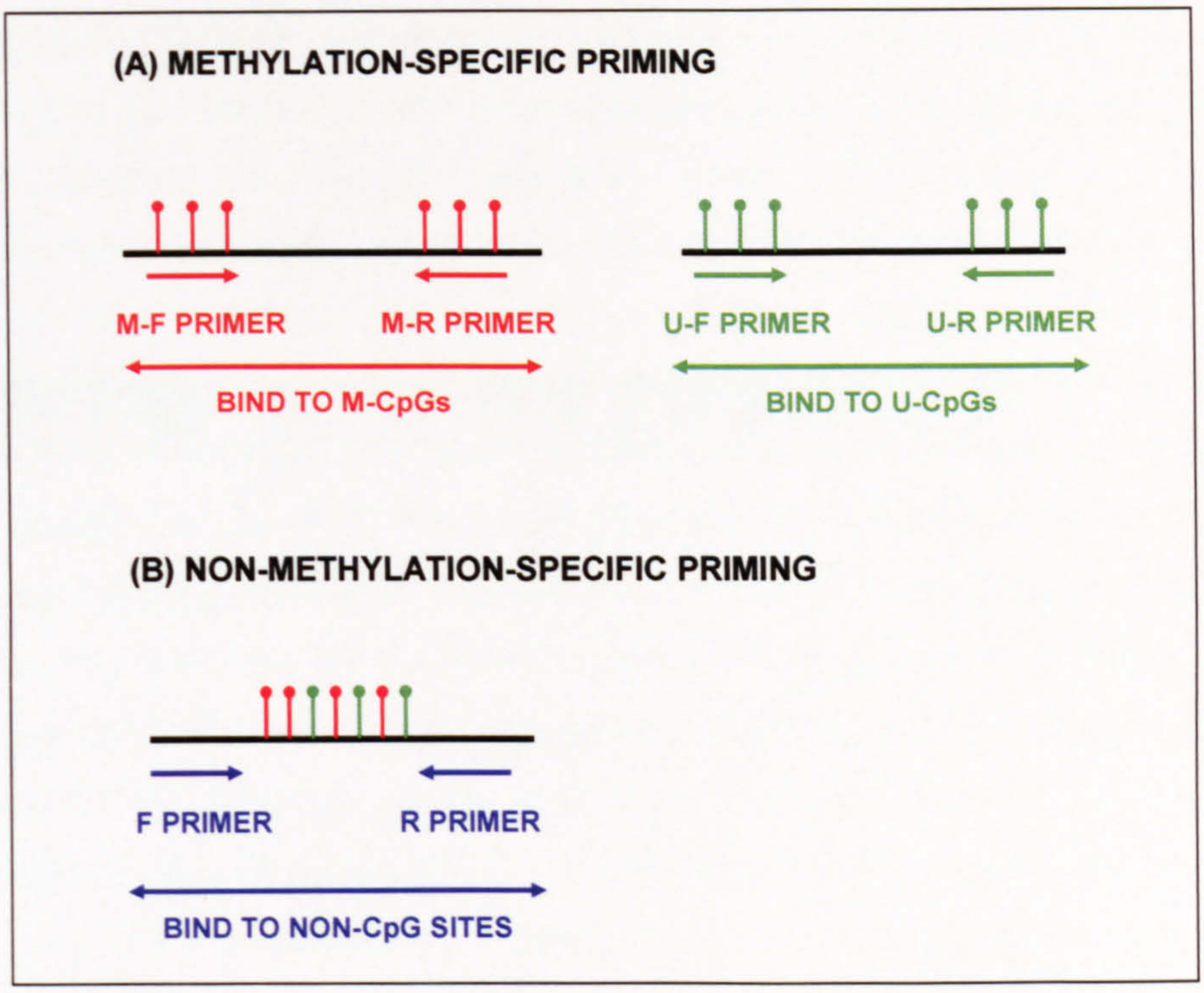


Figure 7 Design of primers following sodium bisulphite conversion of DNA. **(A)** Methylation-specific amplification using forward (F) and reverse (R) primers designed to anneal to a sequence with either complementary methylated (M) CpGs or unmethylated (U) CpGs. **(B)** Alternatively, non-methylation-specific priming of either M or U sequences can be attained using F and R primers which attach outwith the methylation-differential part of the sequence and will amplify DNA regardless of its methylation status.

Established methods using the initial bisulphite conversion of DNA described above include MSP (Herman et al., 1996), bisulphite sequencing (Frommer et al., 1992), combined bisulphite restriction analysis (COBRA) (Xiong and Laird, 1997) and, more recently, pyrosequencing (Ronaghi et al., 1996). MSP is a highly sensitive, qualitative technique, which can detect a small proportion of methylated alleles in a heterogeneous sample, and has been the most widely accepted method of analysing CGI methylation for many years (Derks et al., 2004; Herman et al., 1996). It uses primers which are designed to

anneal either to the methylated or unmethylated sequence (Figure 7A) and has had a significant bearing on our understanding of epigenetics events in many types of cancer including ovarian (Shames et al., 2007). This is a reflection of its accessibility and ease of use in many laboratories (Laird, 2003), which is of considerable importance when considering the application of a method. Although MSP remains a common assay used to detect new epigenetic markers in cancer, it is acknowledged that this technique will only produce a result as categorical information (either methylated or unmethylated). Bisulphite sequencing and COBRA provide more quantitative information using PCR-primers which do not cover any potentially methylated CpG sites (Figure 7B). Amplification generates a pool of products with variable methylation states. COBRA relies on a methylation-sensitive restriction enzyme digest to provide a quantitative assessment of the methylation status of individual CpG sites but is not suited to multiplex reactions. Bisulphite sequencing has the disadvantage of being very labour intensive, and has now been widely replaced with a bisulphite sequencing technique known as Pyrosequencing (Ronaghi et al., 1996; Ronaghi et al., 1998) which can quantitate CpG methylation at individual sites following bisulphite treatment (Tost and Gut, 2006). This sequencing by synthesis based technique involves the luminometric detection of pyrophosphate following sequential single nucleotide incorporation. Pyrosequencing allows the detection of up to 10 CpG sites spanning an 80-nucleotide stretch in a single run (Tost and Gut, 2006). The main advantages of this new technology over traditional sequencing methods are that it is a very quick and efficient quantitative method, it uses a PCR product to directly obtain information, without the requirement of cloning and multiple sequencing reactions, and it can be run in multiplex fashion.

The second approach, which detects methylation using enzyme-based differences, historically involved Southern Blot analysis (Reed et al., 1996), but more recently there has been a surge of interest in developing techniques which can examine genome-wide epigenetic alterations in cancer. Restriction landmark genomic scanning (RLGS) is one such enzyme-based technique which has been used to assess global CGI methylation in tumours (Costello et al., 2002). The study of global DNA methylation alterations at the CGI level can also be achieved using CGI arrays. Differential methylation hybridisation (DMH) was the first technique of this kind which was used to assemble an array-based DNA methylation assay (Huang et al., 1999; Yan et al., 2001), and has been successfully used to detect such alterations in breast and ovarian cancer.

The third approach uses anti-methylcytosine antibodies to enrich for methylated sequences. This relatively new technique is known as methylated DNA immunoprecipitation

(MeDIP), and has been used in conjunction with a comparative genomic hybridisation (CGH) microarray (Weber et al., 2005). This allows simultaneous assessment of methylation status and copy number.

Analysis of methylation on a global scale using robust high throughput platforms still represents a significant challenge. Most recently, methylation profiling has involved the use of novel technologies such as universal bead arrays (Bibikova et al., 2006), promoter-associated methylated DNA amplification DNA chip (PMAD) (Fukasawa et al., 2006), and quantitative assays such as bio-COBRA (COBRA coupled with the Agilent 2100 Bioanalyser platform) (Brena et al., 2006) and COMPARE-MS (combination of methylated-DNA precipitation and methylation-sensitive restriction enzymes) (Yegnasubramanian et al., 2006).

There are limitations in any of the methodologies described which means that no single method is unanimously better than another to examine methylation changes. Methods vary in how sensitive they are to detect methylation differences, their ability to quantitate methylation and their potential application in different tissues and preparations (e.g. paraffin-embedded versus snap frozen tissue) (Laird, 2003). Additionally, the potential to make comparisons and interpret different datasets originating from a variety of methodologies is difficult. For instance, many studies will use variable conditions for the same methodologies, such as altered PCR cycling numbers, which can change the threshold of a positive result e.g. investigation of CGI methylation of *FANCF* (Taniguchi et al., 2003; Teodoridis et al., 2005). Studies may also amplify different promoter regions of a gene which could lead to discrepancies in results obtained depending on methylation status. Techniques which rely on restriction enzyme digests (e.g. COBRA, RLGS and DMH) are limited by the sites available for methylation-sensitive enzymes and the digest efficiency, as incomplete digestion could lead to false positive results. Additionally, care must be taken in the interpretation of data from CGI libraries used in microarray-based experiments as more than 80% of all CGIs are not related to genes and are not likely to be involved in the regulation of gene expression (Takai and Jones, 2002).

Ultimately, the scientist's method of choice will depend on how the technology is to be applied (Laird, 2003), but carefully validated data using an independent method will be of utmost importance. The application of these novel tools in clinical research for genome-wide analysis of methylation will be critical in ovarian cancer. It will not only allow the identification of novel methylation targets, but will also enable assessment of the effectiveness and safety of regimes which can reverse methylation and identify patients for

whom it may be advantageous to treat with novel epigenetic therapies (Lyko and Brown, 2005).

1.8 The therapeutic implications of DNA methylation

Aberrant CGI hypermethylation requires an active mechanism for its maintenance during cell proliferation in cancer. Therefore, this type of epimutation is pharmacologically reversible by small molecule inhibitors, which makes it an attractive focus for the development of novel epigenetic therapies in cancer (Egger et al., 2004; Yoo and Jones, 2006). *In vitro* work has shown that double inactivation of DNMT1 and DNMT3b in a colon cancer cell line reduces cell growth and reverses global methylation of previously dormant tumour suppressor genes (Paz et al., 2003; Rhee et al., 2002). Inhibition of this family of enzymes in cancer could lead to suppression of the growth of a tumour or increase the ability of tumour cells to undergo apoptosis induced by chemotherapeutic agents and hence overcome drug resistance (Teodoridis et al., 2004). The DNMT family of enzymes are recognised as potential targets for the development of epigenetic therapies. Inhibitors of these enzymes represent the most widely studied demethylating agents in phase I-III clinical trails (Lyko and Brown, 2005). Several small molecule drugs which are nucleoside analogues of 2'-deoxycytidine have been studied. These potent inhibitors of DNA methylation include the prototype 5-azacytidine (5-azaC) and the deoxyribose analogue of 5-azaC, known as 5-aza-2'-deoxycytidine (DAC, Decitabine). These are incorporated into the DNA and trap DNMTs during movement of the replication fork (Lyko and Brown, 2005). This leads to passive demethylation of nascent DNA and re-activation of epigenetically silenced tumour suppressor genes. 5-aza-C and DAC have been used to inhibit DNMTs and reverse methylation in tissue culture work for many years (Brown and Plumb, 2004). In the clinical setting, DNMT inhibitors have been studied most intensely with regard to treatment of haematological malignancies (Issa et al., 2004; Lubbert, 2000). The originally described DNMT inhibitor 5-azaC (Vidaza®) was first approved by the Food and Drug Administration (FDA) for use in myelodysplastic syndrome (Kaminskas et al., 2005), and more recently, 5-aza-2'-deoxycytidine (Decitabine, Dacogen®) has also gained FDA approval for use.

The use of these types of nucleoside analogues in clinical trials have been hindered by their innate cytotoxic side effects including thrombocytopenia and neutropenia. These side effects are most likely due to cytotoxicity associated with drug incorporation into DNA independent of their DNA-hypomethylating value (Esteller, 2005). Indeed, the *in vitro* effect of decitabine at higher concentrations has been shown to produce less differentiation and more cytotoxicity (Taylor and Jones, 1979). Higher doses of these DNA-demethylating drugs have cytotoxic actions which are independent of their ability to cause

hypomethylation, but if the dose is lowered, they are more dependent on their role to re-express epigenetically silenced genes. Therefore, it may be more appropriate to use lower dose scheduling as shown for haematological malignancies (Issa et al., 2004) which will reduce myelosuppressive side-effects. However, although these drugs have shown promise as single demethylating agents in haematological malignancies, their use in the treatment of patients with solid tumours remains disappointing (Glasspool et al., 2006). The disparity seen between these different types of malignancy may be due to differing pharmacokinetic and pharmacodynamic mechanisms in solid and haematological malignancies. Therefore, to improve use in solid malignancies, combination with currently used cytotoxics or more novel epigenetic therapies may be best.

The re-expression of genes following the use of a demethylating agent is not finite, and following a period of time, genes will become silenced again by methylation (Bender et al., 1999). The reactivation of multiple tumour suppressor genes could enhance the action of other chemotherapeutic agents used within a certain time frame, thereby increasing apoptosis in cells induced by DNA damaging agents and overcoming drug resistance (Teodoridis et al., 2004). Decitabine has been shown to cause demethylation and re-expression of the *hMLH1* gene (maximal effect at day 9) which sensitises drug resistant tumour xenografts grown in nude mice to a range of cytotoxic chemotherapeutic drugs, including carboplatin (Plumb et al., 2000). This re-sensitisation shown to conventional cytotoxic drugs may be particularly useful in tackling the management of patients with drug resistant ovarian cancer. Crucial histone modifications in the promoter regions of genes silenced through methylation have also been shown (Fahrner et al., 2002; Kondo et al., 2003). The combination of an HDAC inhibitor and a demethylating agent have been shown to enhance re-expression of epigenetically silenced genes compared to the use of either drug alone (Cameron et al., 1999; Gore et al., 2006). Together, these drugs can also lead to enhanced sensitivity to chemotherapeutics in cell lines and increase the antitumour effect seen (Boivin et al., 2002).

If novel epigenetic agents are to be used in a clinical setting, stratification of patient populations will be required. Methylation of particular genes, such as the DNA repair genes, may bestow enhanced chemosensitivity in some patients, and therefore it may be inappropriate to treat these patients with agents which will reverse the methylation status and reduce their sensitivity. Concurrently, some patients may be chemosensitised with epigenetic therapies (Teodoridis et al., 2004). Additionally, it has also been shown that genes with metastasis-related functions such as synuclein-γ (Gupta et al., 2003) can become epigenetically upregulated through hypomethylation and subsequently

overexpressed in aggressive tumour types. Therefore, in order to identify appropriate patients, robust technology and better pharmacodynamic endpoints will be required (Lyko and Brown, 2005).

1.9 Specific aims and approaches of this research project

The specific aims of this research project:

- To investigate if methylation patterns in primary EOC can predict response to chemotherapy.

- To identify DNA methylation markers for acquired cisplatin chemoresistance in an ovarian cancer model system.

- To investigate if aberrant DNA methylation is an early event in ovarian tumourigenesis and if it has potential use as an early detection marker in plasma.

The approaches used to reach these aims:

- 24 CGIs were analysed in a group of late stage EOCs (FIGO Stage III/IV). Association of methylation with response was analysed by grouping of genes according to their biological function.

- DMH was performed on a panel of 16 ovarian cancer cell lines (6 cisplatin-sensitive and 10 cisplatin-resistant). 2 CGIs close to known genes were analysed in EOC specimens.

- 15 CGIs were analysed in a group of early stage EOCs (FIGO Stage I/II) and the most frequently methylated CGIs were analysed in plasma.

Chapter 2

Materials and Methods

2 Materials and Methods

2.1 General equipment

Casy-1 haemocytometer	*Scharfe System*
CR422 centrifuge	*Jouan*
Gilson Pipettes	*Anachem*
Heating block Dri-Block DB 2A	*Techne*
Incubator (37°C)	*Genlab*
Innova 4000 incubator shaker	*New Brunswick Scientific*
Microcentaur microcentrifuge, MSE and EBA12	*Hettich Zentrifugen*
Microwave	*Sanyo*
Nanodrop ND-1000 spectrophotometer	*LabTech*
PCR workstation	*Labcaire*
Rocking Table	*Luckham*
Set of scales PM 300	*Mettler*
Tetrad DNA Engine PTC 225	*MJ Research*
Vortex Whirlmixer	*Fisons*
Water bath SUB36	*Grant*

2.2 General chemicals

All chemicals were of the highest quality available and were supplied by Sigma unless otherwise stated.

2.3 General glass and plasticware

Bijous (5ml)	*Bibby-Sterilin Limited*
Eppendorf tubes (1.5ml)	*Eppendorf AG*
Falcon tubes (15ml and 50ml)	*Becton Dickinson Labware*
Glass pipettes (5, 10, 25ml)	
Pasteur pipettes	

Sterile Pipette filter tips (10, 20, 200, 1000µl)	*Greiner bio-one*
Universals (20ml)	*Bibby-Steriline Limited*
Universal containers (30ml and 100ml)	*Sterilin*

2.4 Patient samples and characteristics

Tumour samples were collected from chemonaive patients undergoing cytoreductive surgery for suspected ovarian cancer. Matched blood samples were collected from a number of patients in the prospective "DNA Methylation Study" at the time of admission to hospital for surgery. Only those patients with pathologically confirmed EOC were included in the analysis. Ethical approval for all samples collected was obtained from relevant authorities and samples were collected according to Medical Research Council operational and ethical guidelines on "Human tissue and biological samples for use in research". All tumour and separated blood samples were stored at -70°C until required for analysis. Pathology reports, including histological subtype and grade, were obtained where possible.

Reasonably complete clinical data sets were available for the following clinical factors: FIGO stage at diagnosis, age, performance status (PS) and size of residual disease at primary surgical procedure. These data were collected prospectively through the Clinical Trials Unit. Stage was categorised using FIGO criteria into early (Stage I/II) versus late (Stage III/IV), age was categorised on the median value, PS was classified as 0, 1 or 2/3 and residual disease as ≤2cm or >2cm.

PFS was defined as the time from first chemotherapy or date of entry onto trial (within 6 weeks of surgery) until date of second line chemotherapy or progression or cancer related death. Progression was defined as either a ≥25% increase in size of at least one measureable lesion, worsening previously evaluable disease, recurrence of a previously successfully treated lesion or appearance of a new lesion as measured on CT scan. Overall survival (OS) was calculated from the date of first chemotherapy or date of entry onto trial until the date of cancer related death. Response to chemotherapy was measured in all patients that had evaluable disease i.e had measurable disease following cytoreductive surgery prior to chemotherapy. This was done anonymously, blinded to the methylation status of each patient and response was defined by modified Southwest Oncology Group (SWOG) criteria (Vasey et al., 2004). Patients who were evaluable for response to

chemotherapy were classified into two groups: responders were those with complete response (CR) or partial response (PR) and non-responders were those with stable disease (SD) or progressive disease (PD).

2.5 DNA extraction from ovarian tissue samples

2.5.1 Materials

100% ethanol

Microdismembrator II *B.Braun*

Mortar and pestle

Phenol and chloroform:isoamyl alcohol (24:1)

3M sodium acetate

2.5.2 Recipe

Lysis Buffer

0.3M sodium acetate (pH 8.0)

0.5% SDS

5mM EDTA

50µg/ml proteinase K

2.5.3 Method

Genomic DNA was isolated from ovarian tissue samples by crushing frozen samples with a mortar and pestle. Samples were then powdered with a microdismembrator II, lysed in 10ml of lysis buffer and shaken overnight at 37°C. Proteins were extracted with phenol and chloroform:isoamyl alcohol (24:1) and DNA was then precipitated in 1:10 volume of 3M sodium acetate and 2 volumes of 100% ethanol.

2.6 Separation of plasma and PBMCs from whole blood

2.6.1 Materials

EDTA vacutainers	*Greiner Bio-one*
Histopaque-1077	*Sigma-Aldrich*

2.6.2 Method

Two EDTA vacutainers per patient containing 10ml whole blood were received and processed within 2 hours and accurately logged with time/date of sample collection. Plasma was separated by centrifuging an EDTA vacutainer at 1500xg at 20°C for 10 minutes, aspirating the supernatant into a 15ml Falcon tube and then repeating the centrifugation step under the same conditions. The plasma was then aliquoted into 2 labelled 1.5ml centrifugation tubes and frozen at -70°C. To separate PBMCs, 3ml of whole blood was layered onto 3ml of Histopaque-1077 in a 15ml Falcon tube and centrifuged at 400xg for 30 minutes at room temperature with the centrifuge brake off. The upper phase was discarded and the opaque interface containing the PBMCs was transferred to a fresh 15ml Falcon tube and suspended in 10ml of PBS (see Chapter *2.17.2* for PBS recipe). This was then centrifuged at 250xg for 10 minutes at room temperature with the centrifuge brake low (-2) and the cell pellet was resuspended in 5ml of PBS and centrifuged again at 250xg. The cell pellet was then resuspended in 0.5ml of PBS and stored as above for plasma.

2.7 Extraction of DNA from plasma and PBMCs

2.7.1 Materials

Ethanol	
QIAamp DNA Blood Mini Kit (Kit size 50)	*Qiagen*
RNase A (100mg/ml)	*Qiagen*

2.7.2 Method

DNA was extracted and purified using the QIAamp DNA Blood Mini Kit according to manufacturer's instructions. Buffer AW1, AW2 and QIAgen protease were prepared according to the manufacturers instructions and all centrifugation steps were performed at 6000xg at room temperature unless otherwise stated below. 1ml plasma/0.5ml PBMCs were thawed and PBMCs were spun down and resuspended in 200µl PBS. Samples were lysed and ethanol precipitated by adding 20µl/4µl of RNase A and 125µl/25µl of QIAgen Protease to the plasma and PBMCs respectively. 1ml/200µl of Buffer AL was added and samples were incubated at 56°C in a water bath for 10 minutes and briefly centrifuged. 1050µl/210µl of 100% ethanol was added, pulse vortexed and briefly centrifuged to complete the precipitation step. The precipitated material was bound to a QIAamp spin column and centrifuged for 1 minute at high speed in a benchtop-centrifuge (filtrate discarded). Samples were washed in 500µl of Buffer AW1 and centrifuged for 1 minute (filtrate discarded). 500µl of Buffer AW2 was added and centrifuged for 3 minutes at full speed (filtrate discarded) and then centrifuged at full speed for a further 1 minute. DNA was eluted by adding 200µl of Buffer AE, incubating at room temperature for 5 minutes and then centrifuging at 6000xg for 1 minute. The eluted sample was pipetted into the same spin column and incubated for 5 minutes and centrifuged for 1 minute as before. To complete the elution, this was repeated five times for plasma and twice for PBMCs. Following extraction of DNA from ovarian tissue samples and blood products, the concentration of each specimen was determined using the Nanodrop® ND-1000 spectrophotometer. The "Nucleic Acid" application module was selected from the software package. 1µl of DNA was pipetted onto the sample pedestal to ensure that the liquid sample column was formed and the light path was completely covered by the sample. This accurately and reproducibly measures DNA concentrations by utilising the 0.2mm pathlength to calculate the absorbance and then converts this into a specific concentration.

2.8 Sodium bisulphite modification of extracted DNA

2.8.1 Materials

β-Mercaptoethanol	*Sigma*
CpGenome™ DNA Modification Kit	*Chemicon International*

dH_2O	
EDTA	*Fishers*
Ethanol	*Hayman*
NaOH pellets	*Fishers*
Sterile H_2O for injection	*B Braun*
Tris Base	*Melford*

2.8.2 Recipes

β-Mercaptoethanol/H_2O

β-Mercaptoethanol	1µl
dH_2O	20ml

3M NaOH

NaOH pellets	1g
dH_2O	8.3ml

20mM NaOH / 90% Ethanol (freshly prepared for each experiment)

100% Ethanol	900µl
dH_2O	93.4µl
3M NaOH	6.6µl

TE

10mM Tris

0.1mM EDTA

pH 7.5

Per modification: Reagent I, supplied with kit (make up fresh each time)

Reagent I	227mg
dH_2O	571µl (vortex)
3M NaOH	20µl (vortex)

Per modification: Reagent II, supplied with kit (can be stored in dark for 6 weeks.) Do not use if reagent II has turned yellow.

Reagent II	1.35g
β-mercaptoethanol/H_2O	750µl

2.8.3 Method

Sodium bisulphite modification is based on the selective deamination of unmethylated cytosines to uracils whereas methylated cytosines remain unchanged. This chemical reaction converts a difference in methylation into a difference in sequence.

1µg of genomic DNA was modified with sodium bisulphite using the CpGenome DNA Modification Kit according to the manufacturer's instructions in a Category 1 environment. 1µg of DNA was denatured at an alkaline pH by mixing with 2µl of Reagent IV and 7µl of 3M NaOH in a 1.5ml centrifugation tube and bringing the total volume to 100µl with sterile H_2O. Samples were incubated at 50°C for 10 minutes and following this, 550µl of freshly prepared Reagent I was added to each sample and incubated at 50°C for 16-20 hours. This reagent contains HSO_3^- which causes sulphonation and hydrolytic deamination of unmethylated cytosines. 5µl of Reagent III (a micro-particulate carrier) and 750µl of Reagent II were added to the reaction and incubated at room temperature for 10 minutes, which allowed the beads to bind to the DNA (in the presence of Reagent II). Samples were then centrifuged at 5000xg for 10 seconds leading to formation of bound DNA in pellet form and the supernatant was discarded. The bound DNA was then desalted by washing in 1ml of 70% ethanol three times. After the supernatant from the third wash was removed, the samples were centrifuged at top speed for 2-3 minutes and the remaining supernatant was removed. The conversion to uracil was completed by alkaline desulphonation which involved incubating samples in 50µl of 20mM NaOH at room temperature for 5 minutes and further desalting by washing twice in 90% ethanol. The remaining supernatant was removed and the cell pellets were air-dried for 20 minutes at room temperature. DNA was eluted from the carrier by incubating in 40µl of TE at 55°C for 15 minutes. The samples were then centrifuged at high speed for 2-3 minutes and the supernatant was pipetted to freshly-labelled eppendorf tubes. This modified DNA was stored at -20°C until required for MSP or other methylation-related experiment.

2.9 Verification of successful bisulphite modification using PCR

Incomplete bisulphite modification can lead to false positive results using MSP (Rand et al., 2002). Therefore it is important to avoid using incompletely modified DNA samples as

these could result in an overestimation of methylated cytosines. In order to address this problem, successful bisulphite modification of the DNA was verified before proceeding to MSP by amplifying a DNA sequence that contains cytosines with COBRA primers (for details, see Table 3). These primers do not contain CpG sites and will only give an amplified product if the cytosines in the original sequence have been successfully converted to uracils, irrelevant of its methylation status. For this purpose, a promoter region of the *CALPONIN* gene (sequence as shown in Table 3) was amplified with every modified DNA sample. Samples that did not give a band of similar intensity were considered unmodified or incompletely modified and the modification reaction was repeated for those samples. The materials and methods used for this are described in Chapter *2.10.1* and *2.10.2*.

2.10 Methylation Specific PCR (MSP)

2.10.1 Materials

dH_2O	
dNTPs 0.2mM	*Applied Biosystems*
70% Ethanol (to wash down workstation)	
Fast Start Taq DNA Polymerase Kit	*Roche*
Human Genomic Male DNA	*Promega*
In Vitro Methylated DNA (IVM)	*Chemicon*
Mineral Oil	*Sigma*
Oligonucleotides (detailed below in Table 2)	*TAGN*
Semi-skirted 96 well PCR plate (0.2ml)	*Abgene*
Sterile H_2O for injection	*B Braun*
Microseal A Film	*MJ Research*

2.10.2 Method

MSP is a qualitative PCR-based technique which is highly sensitive and has the potential to detect small subpopulations of methylated sequences (Herman et al., 1996). It is used to detect a sequence corresponding to a particular methylation state (either methylated or unmethylated) in extracted bisulphite modified genomic DNA. Primers were specifically

designed to utilise the sequence differences between methylated and unmethylated DNA resulting from sodium bisulphite treatment.

To avoid contamination of the amplification reaction (Kwok and Higuchi, 1989), all MSP related equipment and consumables were stored in a pre-PCR environment in the Category 1 room. The MSP reactions were set up in a vertical laminar airflow in a pre-PCR Category 1 environment (PCR workstation). All MSP reactions were carried out on an MJ Research, Tetrad DNA Engine PTC 225.

For all MSP assays, a series of positive and negative controls plus H_2O blank were run simultaneously with each reaction. Undiluted IVM DNA and serial dilutions (1:5, 1:10, 1:20) of this in male whole blood genomic DNA were used as positive controls and a negative control of male whole blood genomic DNA was used.

Reaction mixes were prepared in a PCR workstation which was washed down with 70% ethanol spray prior to use. All tubes and tips were exposed to UV light prior to commencing the experiment. A master mix with the appropriate primer sets was then prepared before addition of DNA. MSP master mixes were made using reagents from the Fast Start Taq DNA Polymerase kit which were thawed and vortexed prior to use except Taq polymerase was kept at -20°C until required. MSP reactions were performed in 96 well PCR plates using 1µl of modified DNA, 150ng of each forward and reverse primer, 0.2mM dNTPs and 1U Faststart Taq in a total volume of 25µl. The H_2O blank control consisted of 24µl master mix and 1ul of sterile H_2O in place of DNA. Specific reaction mixes, primers and cycling conditions are described below.

A master mix for one reaction contained:

Fast Start 10x Buffer	2.5µl
dNTPs	0.5µl
Forward sequence primer	0.5µl
Reverse sequence primer	0.5µl
$MgCl_2$ 25mM	1-4µl, depending on primer conditions
Fast Start Taq DNA Polymerase	0.2µl (add immediately prior to use)
Sterile H_2O	To 24 µl

Wells were sealed with microseal film and reactions were run on a Tetrad DNA Engine PTC 225. Each MSP reaction underwent an initial denaturation and enzyme activation step at 95°C for 5 minutes, followed by 35 amplification cycles of 95°C for 30 seconds (unless otherwise indicated), appropriate annealing temperature for 30 seconds and elongation at 72°C for 30 seconds. This was followed by a final extension step at 72°C for 5 minutes as below.

MSP conditions were as follows:

Initial Denaturation Step of 5mins at 95°C then 35 cycles of:		
Denaturation	30s	95°C
Anneal	30s	Temp. dependent on primer
Elongation	30s	72°C
Following these 35 cycles, final elongation step of 5 mins at 72°C		

2.10.3 MSP oligonucleotides and cycling conditions

Primer sequences and conditions for MSP are shown below in Table 2.

Gene	**Primer Sequence** *Forward (F) / Reverse (R) 5'→3'*	*Product Size (bp)*	*Annealing Temp (°C)*	*[Mg^{2+}] mM*
APAF1	**F:**TTTCGGGTAAAAGGGATAGAATTAGA **R:**TATAACGCCCTTCCCCCGACGACG	140	63	2
BLU	**F:**TTCGTGGGTTATAGTTCGAGAAAGCG **R:**AACGAATTAACCGCGCCTACGC	157	61	2
BRCA1	**F:**GAGTTTCGAGAGACGTTTGG **R:**AATCTCAACGAACTCACGCC	176	63	3
CASP8	**F:**TAGGGGATTCGGAGATTGCGA **R:**CGTATATCTACATTCGAAACGA	321	53	2
DAPK	**F:**GGATAGTCGGATCGAGTTAACGTC **R:**CCCTCCCAAACGCCGA	98	60	2
DcR1	**F:**TTACGCGTACGAATTTAGTTAAC **R:**CATCAAACGACCGAAACG	127	55	2
FancF	**F:**TTTTTGCGTTTGTTGGAGAATCGGGTTTTC **R:**ATACACCTCAAACCGCCGACGAACAAAACG	153	65	2
Fas	**F:**GAAAGGGTAGGAGGTCGGTTTTCGAG **R:**CACTCTTACGCGAAATCAAAAACGAACTCA	269	65	2
GSTpi	**F:**AGTTGCGCGGCGATTTC **R:**GCCCCAATACTAAATCACGACG	140	61	2
HIC1	**F:** TCGGTTTTCGCGTTTTGTTCGT **R:** AACCGAAAACTATCAACCCTCG	95	60	2
MINT25	**F:** GCGAAAGCGAAAGTCGTTCG **R:** CCCAACGCACATAACGAACC	213	58	2
MGMT	**F:** TTTCGACGTTCGTAGGTTTTCGC **R:** GCACTCTTCCGAAAACGAAACG	81	58	2
MLH1	**F:** ACGTAGACGTTTTATTAGGGTCGC **R:** CCTCATCGTAACTACCCGCG	115	64	2
OPCML	**F:** GCGCGGTGCGGGTTTATTTTC **R:** TCCCGATACCGCCTCGAAACGAACG	135	61	2

P14	**F:**GTGTTAAAGGGCGGCGTAGC **R:**AAAACCCTCACTCGCGACGA	122	62	2
P16	**F:** TTATTAGAGGGTGGGGCGGATCGC **R:** GACCCCGAACCGCGACCGTAA	150	65	2
P21	**F:** TAGTACGCGAGGTTTCGGGATC **R:** AACTAACGCAACTCAACGCGAC	197	58	2
P73	**F:**GTTCGCGGTGTTTTTTCGCG **R:**AATACCTACCCAACGCTACG	315	62	1
PTEN	**F:**TTAGGGTTGGGAACGTCGGAG **R:**CAACAACCAAAAACCTAACAACGACGACAA	227	61	2
RASSF1A	**F:** CGAGAGCGCGTTTAGTTTCGTT **R:** GATTAAACCCGTACTTCGCTAA	192	52	2
SFRP-1	**F:**CGTATTTTAGTTTTGTAGTTTTCGG **R:**CCCCCGACCAATAACG	163	64	2
SOCS-3	**F:**TTTTGTGGATTTTACGGTCGT **R:**GAAAAACTAATCCCGAATCGAA	134	57	2
Survivin	**F:**TCGGTATATTTCGCGTCGTTTC **R:**AAACCGAACAATCTCACCCGCT	280	61	2
TMS1	**F:** TTGTAGCGGGGTGAGCGGC **R:** AACGTCCATAAACAACAACGCG	191	65	2
5D4$^{(M)}$	**F:**ATATAGAGTAAAAAGCGACGTTCGT **R:**ACTTTTAAACTTACCCAACCTCGA	112	57	2
5D4$^{(U)}$	**F:**GATATAGAGTAAAAAGTGATGTTTGT **R:**ACTTTTAAACTTACCCAACCTCAAA	113	52	2
119A6$^{(M)}$	**F:**TCGTAGCGATAGGTATAAAGTTACG **R:**AAAAAAACGACCAAATCCGA	100	55	2
119A6$^{(U)}$	**F:**TGTAGTGATAGGTATAAAGTTATGG **R:**AAAAAAAACAACCAAATCCAAA	100	55	2
41D9$^{(M)}$	**F:**CGTATTAGTTTATTTATTATTATCGG **R:**TACCTAACTAAATTTCTACTACGCT	102	60	2
41D9$^{(U)}$	**F:**TTTGTTGGTAGATGGATTTTAGAGTG **R:**AATAAATAAACTAATACAAAATCACC	99	52	2
66G6$^{(M)}$	**F:**GTTCGGGAGAGTTTTTGATAGTC **R:**AAACAAATTACCTAATAAAAACGAA	115	58	2
66G6$^{(U)}$	**F:**TTGGGAGAGTTTTTGATAGTTGT **R:**AAAACAAATTACCTAATAAAAACAAA	114	57	2

Table 2 MSP oligonucleotides and cycling conditions Primers amplify methylated $^{(M)}$ DNA sequences unless otherwise stated. Primers which are specific for the unmethylated sequence are indicated $^{(U)}$. Primers were all designed using MethPrimer (Li and Dahiya, 2002) (http://www.urogene.org/methprimer/).

2.11 Fluorescent MSP and CEQ fragment analysis

2.11.1 Materials

Beckman CEQ 8000 Genetic Analysis System	*Beckman Coulter*
CEQ Sample Loading Solution (SLS), 6ml	*Beckman Coulter*
GenomeLab DNA Separation Capillary Array	*Beckman Coulter*
GenomeLab Separation Gel LPA-1, 10ml	*Beckman Coulter*
GenomeLab Sequencing Separation Buffer	*Beckman Coulter*
Mineral oil (supplied with size standard)	*Beckman Coulter*

Oligonucleotides (WellRed fluorescent tag)	*Sigma*
Sample Microtiter 96 well PCR plates	*Beckman Coulter*
Size standard 400	*Beckman Coulter*
96-well plates non-sterile	*Beckman Coulter*

2.11.2 Method

Fluorescent MSP was performed on bisulphite modified DNA extracted from plasma and PBMCs as described above in Chapter *2.10*. Primers used (OPCML, RASSF1A, HIC1 and $5D4^{(M)}$) and cycling conditions were as shown in Table 2, labelling each forward primer with a WellRed Fluorescent tag.

Fragment analysis of fluorescently tagged PCR was performed with the Beckman CEQ 8000 Genetic Analysis System. This automatically filled the capillary array with a patented linear polyacrylamide (LPA) gel, denatured and loaded the samples, applied a voltage program and analyzed the data. A dye signal level of ≥ 5000 was taken as a positive value. Any level less than this was interpreted as a signal consistent with background noise.

2.12 Combined bisulphite restriction analysis (COBRA)

2.12.1 Materials

BSA	*New England Biolabs*
10x NEBuffer 2 or 4 (detailed below in Table 4)	*New England Biolabs*
Oligonucleotides (detailed below in Table 3)	*TAGN*
QIAquick PCR purification kit	*Qiagen*
Restriction enzymes (detailed below in Tables 3 and 4)	*New England Biolabs*

2.12.2 Method

COBRA was performed to confirm selective data obtained by MSP (Xiong and Laird, 1997). COBRA is a quantitative PCR-based technique which measures the methylation state of individual cytosines in bisulphite modified genomic DNA using primers which amplify DNA irrespective of its methylation state (ie do not contain CpG sites) followed by restriction digest of the product. PCR was carried out as per Chapter *2.10.2* but included

45 cycles of amplification to obtain a stronger product which could be detected following the restriction enzyme digestion step.

After amplification, PCR products were purified using the QIAquick PCR purification kit according to the manufacturer's instructions. All centrifugation steps were carried out at 15700xg. DNA was bound to the QIAquick column supplied with the kit, washed and eluted in 30µl of elution buffer.

The eluted product was digested with a restriction enzyme which cut specific sites whose sequence depended on the methylation state of the unmodified DNA. 10x NEBuffer was required for each restriction endonuclease and supplemented with 100µg/ml BSA when required as detailed below in Table 4. A master mix for one reaction contained:

Eluted product	14µl
10x buffer	2µl
Restriction enzyme	1µl
100x BSA	0.2µl
Sterile H_2O	To 20µl

Digestion was performed on a heating block at 37°C for 2 hours. Following digestion, products were separated on a 2% agarose gel as described below in Chapter *2.13*.

2.12.3 COBRA oligonucleotides and restriction digestion conditions

Gene	**Primer Sequence** *Forward (F) / Reverse (R) 5'→3'*	*Product Size (bp)*	*Annealing Temp (°C)*	*[Mg^{2+}] Mm*	*Restriction enzyme*
BRCA1	**F:**TTTTTTTTGTTTTTTTTATTTTTTGATT **R:**TATCTAAAAAACCCCACAACCTATC	193	59	4	Fnu4HI (U)
Calponin	**F:** GGAAGGTAGTTGAGGTTGTG **R:** CCCAAACTCAAAACTCTAACCTAAC	333	63	3	Not used
HIC1	**F:**TAGTTGGAAAATTTTTTTTTAAGTTTG **R:**AATTACCCCAATTAAAAAAAATAATAC	443	53	2	Hpy118III (M)
MLH1	**F:**GTGAAGGAGGTTAYGGGTAAG **R:**ATACTTAACACTTCTCAAACTCCTCC	354	55	2	BsaHI (M) / Fnu4HI (M)
OPCML	**F:**GTTTTTTTTGTAGGGGAAGT **R:**CAACAACTCCATCCCTAACC	243	59	2	Fnu4HI (M)
RASSF1A	**F:**GTGGGTAGGTTAAGTGTGTTGT **R:**CAACTCAATAAACTCAAACTCCC	438	58	1	BsiEI (M)
SFRP1	**F:** YGTATTTTAGTTTTGTAGTTTTYGG **R:** CCCCCRCCAATAACR	163	53	2	BsiEI (M)
TMS1	**F:**GTTTGGGGTTTTAATTTAGAGGTTT **R:**TCAACTTAAACTTCTTAAACTCCTC	288	57	2	BsiEI (M) / Fnu4HI (M)/ Hpy118III (M)

Table 3 COBRA oligonucleotides and cycling conditions. (M) and (U) indicate that the restriction enzyme cleaves a sequence resulting from modified methylated and unmethylated DNA respectively. The nomenclature of IUPAC is used. Restriction enzymes were mapped using NEBcutter2 (http://tools.neb.com/NEBcutter2) and primers were designed, as before, using Methprimer (Li and Dahiya, 2002).

Enzyme	**NEBuffer**	**BSA**	**Restriction enzyme cut site**
Fnu4HI	4	No	5'….GC/NGC….3' 3'….CGN/CG….5'
Hpy118III	4	Yes	5'….TC/NNGA….3' 3'….AGNN/CT….5'
BsaHI	4	Yes	5'….GR/CGYC….3' 3'….CYGC/RG….5'
BsiEI	2	Yes	5'….CGRY/CG….3' 3'….GC/YRGC….5'

Table 4 Restriction enzyme conditions and cutting site. Details of enzyme, NEBuffer, whether supplemented with BSA and restriction site cut. The IUPAC nomenclature is used in the sequence.

2.13 Agarose gel electrophoresis

2.13.1 Materials

Agarose	*Melford*
Amresco Ethidium Bromide solution	*NBS Biologicals*
Boric acid	*Fisher*
dH_2O	
100bp DNA Ladder	*Invitrogen*
EDTA	*Fisher*
Electrophoresis unit and power pack	*Pharmacia*
GeneGenius Bioimaging System	*Syngene*
Glycerol	*Fisher*
Orange G	*Sigma*
Tris Base	*Melford*

2.13.2 Recipes

TBE (5x)

Tris base	108g
Boric acid	55g
0.5M EDTA	40ml

Make up to 2L with dH_2O and then dilute to 0.5x with dH_2O for use in agarose gel electrophoresis.

2% Agarose Gel

0.5x TBE Buffer	100ml
Agarose	2g

Heat in microwave for 2mins until agarose is completely dissolved. Allow to cool to "hand warm" before adding 2 droplets of ethidium bromide solution.

Loading Buffer

Glycerol	10ml
dH_2O	30ml
Orange G	0.25g

2.13.3 Method

After the PCR step, the products were separated through size by agarose gel electrophoresis. 5µl of loading buffer was added to each PCR product and 20µl of this mixture was pipetted into wells of a 2% agarose gel immersed in 600ml 0.5x TBE Buffer. A DNA ladder was run at the same time to confirm that products were of the expected size. Gels were run for 35-45 minutes at 150 volts.

DNA was visualised with UV light using a Syngene GeneGenius Bioimaging System with GeneSnap version 6.03 software. For each primer sequence, samples which were positive displayed visible bands in their corresponding lanes. The MSP assay including samples of unknown methylation status was only regarded as being successful if there was a visible band in the positive control lanes and no visible bands in both the negative control and H_2O blank lane.

2.14 Differential methylation hybridisation (DMH)

In a collaborative study described in Chapter *4.1*, DMH was performed similarly as previously described (Paz et al., 2003), with some modifications. Figure 8 illustrates the method of sample preparation for DMH. Genomic DNA was digested with MseI (TTAA) which has only a few restriction sites within CpG islands. The DNA fragments were then ligated to endlinker oligonucleotides and divided into two equal aliquots. One aliquot was mock-treated, the other aliquot was digested with the methylation-sensitive restriction enzyme McrBC which cuts methylated DNA at the degenerate recognition site $(G/A)^{m}CN_{40\text{-}3000}(G/A)^{m}C$ (Stewart and Raleigh, 1998). PCR amplification was performed with primers binding to the endlinkers. Unmethylated fragments are amplified in both the McrBC digest and the mock-treated aliquot. In contrast, methylated fragments are digested with McrBC but remain intact in the mock-treated aliquot. The amplicons were then labelled with Cy3 or Cy5 and hybridised to the Human CpG 12K Array (Heisler et al., 2005) (University Health Network, Toronto, Canada) overnight. This array is based on a CpG island library containing approximately 12,000 CpG-rich sequences (Cross et al., 1994). Arrays were washed with 1xSSC, 0.1% SDS and 0.2xSSC, rinsed with H_2O and scanned with an Axon GenePix 4000A scanner. GenePix Pro 6.0 was used for image

analysis. Dye swap experiments were performed for all cell lines to ensure quality control and reproducibility. An example of such a dye swap experiment is shown in Figure 16.

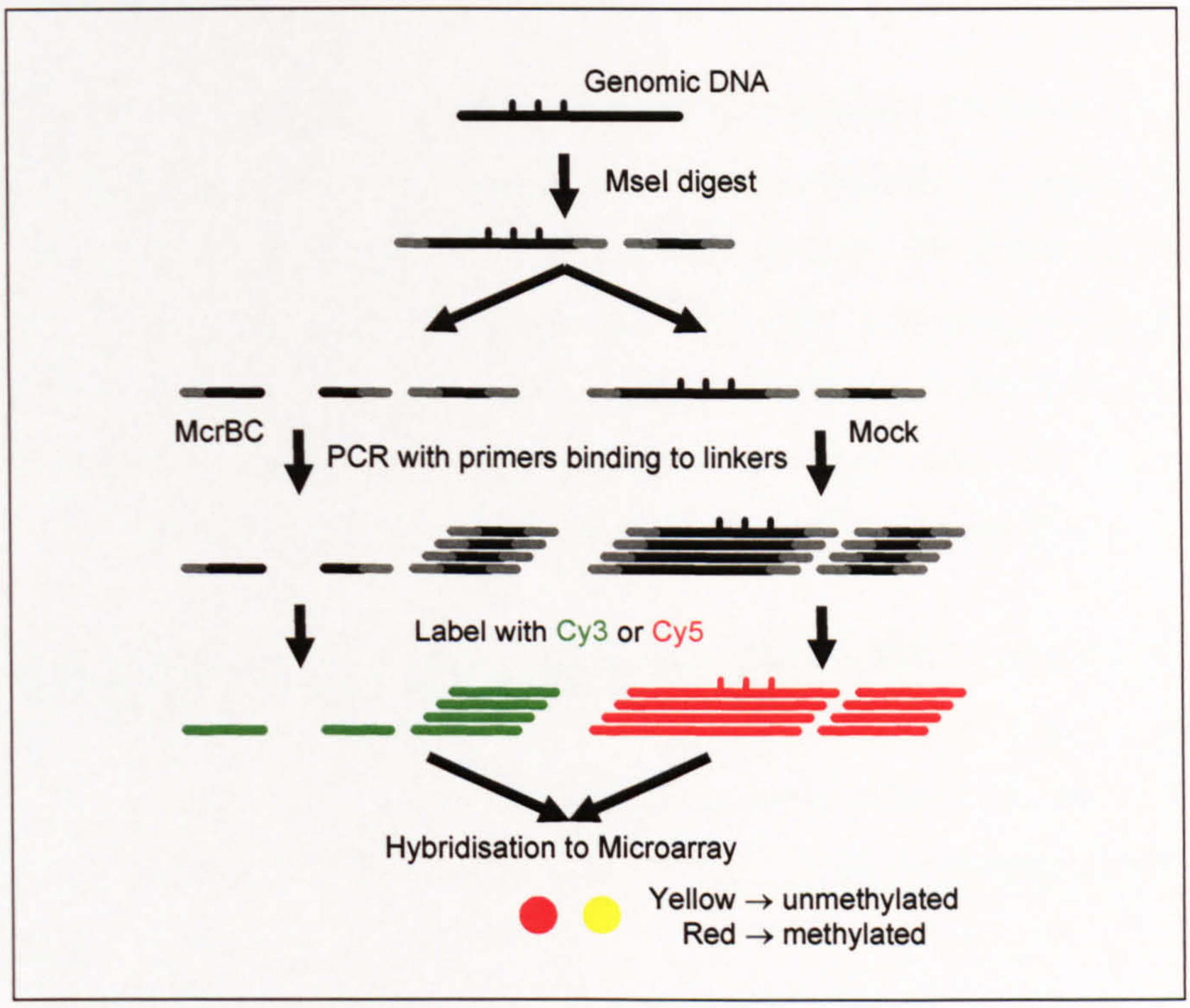


Figure 8 DMH sample preparation. Genomic DNA digested with MseI, ligated to endlinkers and divided into equal aliquots. One mock-treated, the other digested with McrBC, which is a methylation-sensitive restriction enzyme. PCR performed with primers binding to endlinkers. Unmethylated fragments are labelled in both aliquots. Methylated fragments remain intact only in the mock treated aliquot. Amplicons are labelled with Cy3 or Cy5 and hybridised to microarray.

2.15 Sodium bisulphite sequencing

2.15.1 Materials

Ampicillin 100mg/ml stock solution	*Sigma*
Dual Promoter TA Cloning Kit	*Invitrogen*
imMedia Amp Blue sachets	*Invitrogen*
LB medium	*Beatson Institute stores*
QIAquick Gel Extraction Kit	*QIAGEN*
Oligonucleotides (detailed below in Table 5)	*TAGN*
Petri dishes (90mm)	*Sterilin*

SOC Medium	*Invitrogen*
Sterile inoculating loops	*Nunc*

2.15.2 Method

Sodium bisulphite sequencing is a genomic sequencing technique (Frommer et al., 1992) allowing generation of methylation maps with single nucleotide resolution based on bisulphite modification of DNA and sequencing of PCR products. The method involves the subcloning of a PCR product into an appropriate vector and sequencing of the inserts of several individual clones. The resulting final sequence pattern shows that all original cytosines appear as thymines whereas methylated cytosine bases are displayed as cytosines, resulting in quantification of CGI methylation at every CpG position.

Bisulphite modified cell line DNA (from A2780, A2780p6, MCP1, MCP6 and MCP9) were PCR amplified in 25µl reactions. Primer sequences and conditions are shown below in Table 5. PCR reagents and primer concentrations used were as for MSP. The PCR product was extracted and purified from a 2% agarose gel using a QIAquick Gel Extraction Kit as per manufacturer's instructions.

This product was then ligated into a pCR®2.1 vector (supplied with TA Cloning® Kit). This vector is linearized with 3'-T overhangs. The use of *Taq* polymerase in the initial PCR reaction adds a single A to the 3' ends of the PCR product and allows efficient ligation of the PCR insert with the vector due to compatible end. The ligation mixture was incubated overnight at 14°C in 10µl reactions containing 6µl PCR product, 1µl 10x ligation buffer, 2µl pCR®2.1 and 1µl T4 DNA ligase. A negative control of H_2O was included in each sequencing experiment which comprised 6µl H_2O in place of PCR product.

The ligation reaction mixture was then transformed into competent DH5α *E. coli* (kindly provided by the Beatson Institute Stores). This utilises the ability of the plasmid to be replicated within the dividing bacteria and therefore provide sufficient quantities of plasmid DNA. 40µl of DH5α cells were thawed on ice and added to 2µl of ligation reaction mixture which was then incubated on ice for 30 minutes. This was followed by a heat shock at 42°C for 30 seconds to allow plasmid entry into the bacterium. The mixtures were then placed on ice and 250µl of SOC Medium was added. The resulting mixtures were shaken for 1 hour at 37°C to allow expression of the ampicillin-resistance gene before plating out.

The features of the pCR®2.1 vector are illustrated below in Figure 9. These include an ampicillin resistance gene which allows growth of *E.coli* in ampicillin containing medium and the lac promoter which allows bacterial expression of the lacZα fragment. This fragment encodes for β-galactosidase which is used for blue-white colony screening as described below.

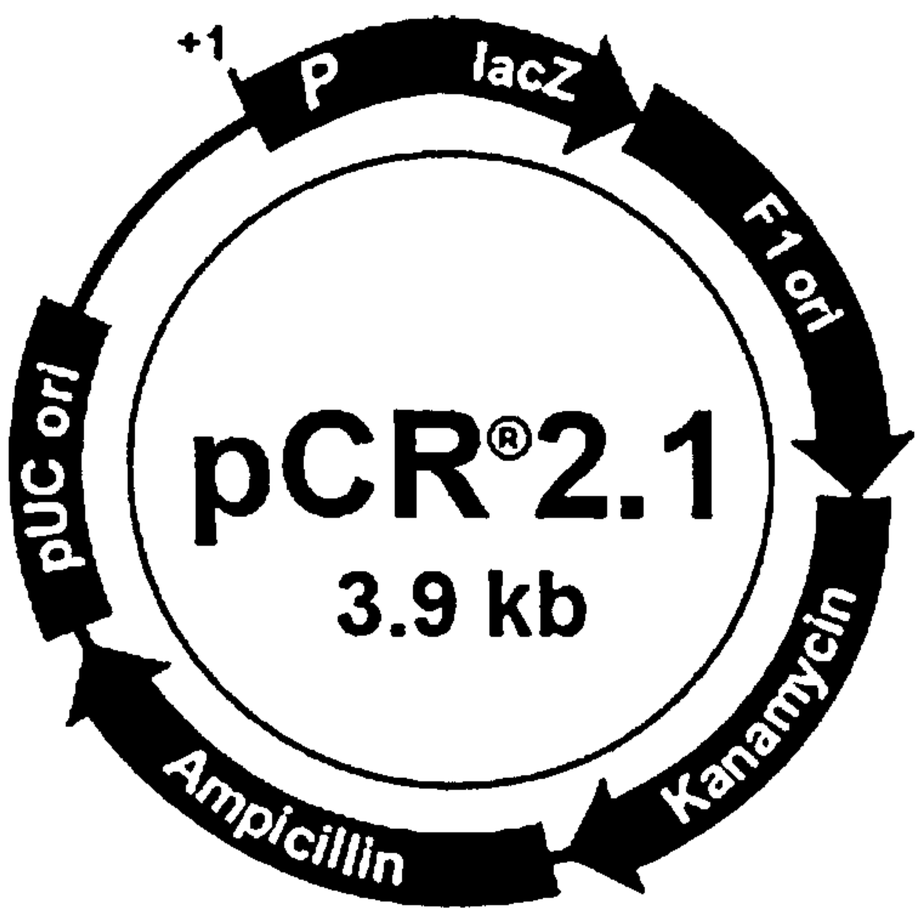


Figure 9 Features of pCR® 2.1 vector. Features include an ampicillin resistance gene, and the lac promoter which permits bacterial expression of the lacZα fragment. This encodes for β-galactosidase which is used for blue-white colony screening.

Agar plates were made up by dissolving an imMedia Amp Blue sachet in 200ml of dH_2O and pipetting out approximately 18ml per 90mm Petri dish once mixture cooled slightly. In addition to ampicillin, nutrients and agar, this media contained both IPTG which induces the *lac* promoter and X-Gal which is cleaved by β-galactosidase yielding a blue product. 75µl of shaken mixture was plated out evenly on plates using a sterilised cell spreader and incubated overnight at 37°C. If transformation was successful, plates yielded both blue and white colonies. Transformants were identified by genetic selection: blue colonies result from cleavage of X-Gal due to LacZα expression but if the PCR product had successfully inserted into the vector, the LacZα gene was disrupted and X-Gal was not cleaved leading to formation of white colonies. White colonies were cultured overnight in the 37°C shaker in 1ml LB media containing 1µl of 100mg/ml ampicillin stock solution. Overnight cultures were then submitted to the Beatson Institute Molecular Services for mini-prep and sequencing. Primers used for sequencing encompassed the T7 promoter within the vector. Methylation density was calculated from at least 10 complete sequences, and the

methylation percentage for each individual CpG was rounded to the nearest increment of 10%.

2.15.3 Bisulphite sequencing oligonucleotides and cycling conditions

Primer Name	**Primer Sequence** *Forward (F) / Reverse (R) 5'→3'*	*Product Size (bp)*	*Annealing Temp (°C)*	*[Mg^{2+}] mM*
LMX1A Seq 1	**F:**TGTTATTGTAAGTTTATTATTTGGGGG **R:**CAATAAATATAAACCCAACAACTCCTAAC	200	61	2
LMX1A Seq 2	**F:**GAAGTTGGAAATTGGTATGAGTTTT **R:**ATACTCCCTTTCCCAACCTAACTAC	379	61	2
LMX1A Seq 3	**F:**AGTTAGGTTGGGAAAGGGAGTATTA **R:**ATTACTTCTCCAAAACTAAAAAAAA	206	55	2
LMX1A Seq 4	**F:**ATTTAGGATTTTGGGTTTTTGTTTT **R:**AACAACCTTACTTACCTAATAAAC	334	57	2
5D4 Seq 1	**F:**TTTTTTTATTTATTTTGTTTTTGGAGTTT **R:**AAAATTCCTCACTAAATTTAATTTCTCC	208	58	2
5D4 Seq 2	**F:**TTGTTGGAGAAATTAAATTTAGTGAG **R:**AATAAAAAAAAATTAAAAAAAAACAAC	333	50	2
5D4 Seq 3	**F:**GTTGTTTTTTTTAATTTTTTTTTATT **R:**AAACTTTATATTTTATTCCTTTAATTTATA	277	55	2
NR2E1 Seq 1	**F:**GTAGGGGATGAGGGTTTTTTTT **R:**CCTCTCTCCAAAATACCAAATAAATT	455	58	2
NR2E1 Seq 2	**F:**ATTTATTTGGTATTTTGGAGAGAGG **R:**ACTAATAATATTAATAAACCTCCAAAC	233	58	2
NR2E1 Seq 3	**F:**GAGTTATATTTTTATATTTATATTTTTTAA **R:**AATCCCCTATAATATCTCCAAAAAC	250	54	2
NR2E1 Seq 4	**F:**GGAGATATTATAGGGGATTTAGTT **R:**CATACAAAATATAAACAATCTCTACC	271	58	2
NR2E1 Seq 5	**F:**TTTGTATGTTTATGTAGAGGGAGAGAT **R:**TCCAACCTACAAAACTCCTAAAC	347	57	2
NR2E1 Seq 6	**F:**ATTTTTGTTTTTTAATATTTTTTTT **R:**CTAACTTACTCATACTAACTATCCC	361	53	2
NR2E1 Seq 7	**F:**GAGTTTTGTAGGTTGGAGGGTAGT **R:**AAAAATCAAACAATCCAAAAACAAC	355	58	2

Table 5 Bisulphite sequencing oligonucleotides and conditions. All primers were designed as before using MethPrimer. 1µl of DNA and 45 cycles of PCR were used in each reaction.

2.16 Pyrosequencing

2.16.1 Materials

1x Annealing Buffer	*Biotage*
Binding Buffer	*Biotage*
Denaturation solution (0.2M NaOH)	*Biotage*
70% ethanol	
Oligonucleotides @ 10µM (detailed below in Table 6)	*TAGN*

PSQ 96 Plate Low	*Biotage*
PSQ 96MA Pyrosequencer	*Biotage*
PSQ 96 Reagent Cartridge	*Biotage*
PSQ 96 SNP Reagent Kit	*Biotage*
Pyrosequencing Thermoplate	*Biotage*
Pyrosequencing Vacuum Prep Workstation	*Biotage*
Streptavidin sepharose HP	*Amersham Biosciences*
Thermofast 96 well semi-skirted PCR plates	*Abgene*
10x Washing Buffer	*Biotage*

2.16.2 Method

Pyrosequencing was used to sequence short regions within specific CGIs and provided quantitative information on levels of methylation at individual CpG sites which had previously been assessed in a qualitative manner using MSP. This high throughput technique involves sequencing by synthesis and detection of hydrolysis of pyrophosphate (PPi) by pyrophosphatase. Pyrosequencing uses sodium bisulphite modified DNA; methylation sites are treated as "C/T SNPs" (methylSNP) with an allele frequency spectrum spanning the entire range (0-100%).

DNA was bisulphite modified and PCR performed, as described previously in Chapter *2.8* and *2.10*, in a total volume of 50µl including 2µl of modified DNA template using 35 cycles of PCR. Either the forward or reverse PCR primer was biotinylated to allow immobilisation to streptavidin coated sepharose beads. 40µl of PCR product was immobilised to sepharose beads and single stranded templates prepared using the Vacuum Prep Workstation in a series of wash steps with 70% ethanol, 0.2M denaturation solution, wash buffer and dH_2O. 10µM sequencing primer was annealed to the template (80°C for 3 minutes) before analysis in the PSQ 96MA Pyrosequencer.

Analysis using the pyrosequencer involves the DNA template and primer complex being incubated with the enzymes DNA polymerase, ATP sulfurylase, luciferase, apyrase and the substrates, adenosine 5' phosphosulphate (APS) and luciferin per sample. dNTPs are added to the reaction and incorporated into the sequencing strand if complementary to the template strand. This is accompanied by release of PPi which is then hydrolysed into ATP in the presence of APS. ATP drives the conversion of luciferin to oxyluciferin which generates visible light which can be detected and translated into a peak by the pyrosequencing software. As the process continues, the complementary DNA strand is

built up and the nucleotide sequence determined from the signal peaks within the pyrosequencing programme. Incorporation of a T at a CpG site indicates unmethylated DNA and incorporation of a C indicates methlyation of that given site. The degree of methylation at individual CpG sites is then analysed using the AQ software.

2.16.3 Pyrosequencing oligonucleotides, cycling conditions and sequence analysed

Sequence name used	Primer Sequence Forward (F) / Reverse (R) / Sequencing (S) 5' – 3'	*Product Size (bp)*	*Annealing Temp (°C)*	*[Mg^{2+}] mM*
5D4	**F:** ATGAATGTGGAGGATGAGATAGTT (5' biotinylated) **R:** CCCAATTTTACAATTCTATTTTCT **S:** CAATTTTACAATTCTATTTT	323	53	2
119A6	**F:** TTTGGAGATATTATAGGGGATTTA **R:** TCCCTCTACATAAACATACAAAA(5' biotinylated) **S:** GGGGTAATGAATTT	288	50	2

Table 6 Pyrosequencing oligonucleotides and conditions. Primers were designed using the Biotage PSQ Pyrosequencing software package.

Sequence name used	Sequence analysed	Number of CpG sites examined
5D4	CTTTYGATAAAATCTYGAYGTCCTAAACCAYGTT AAAAAYGAAYGTA	6
NR2E1	YGGGGATTTTTYGTYGTTGYGTGYGYGGTTTTTTT YGGAAATT	7

Table 7 Sequences and number of CpG sites analysed using pyrosequencing. YG is a potential site of methylation otherwise known as "C/T SNP".

2.17 Cell culture

All tissue culture media was stored at 4°C.

2.17.1 Materials

Cryotubes	*Nunc*
DMSO	*Fisher*
Fetal Bovine Serum (FBS)	*Autogen Bioclear*
L-Glutamine 200mM	*Gibco*
Petri dishes (5cm and 10cm)	*Sterilin*
Penicillin-Streptomycin	*Gibco*
Pipet-aid pipettor	*Drummond Scientific*

RPMI growth medium	*Gibco*
25, 75 and 175cm^2 sterile tissue culture flasks	*Iwaki*
Stericup vacuum-driven filtration System	*Millipore*
NaOH	*Fisher*
6, 24 and 96 well tissue culture plates	*Iwaki*
Trypsin 2.5%	*Gibco*

2.17.2 Recipes

PBS (Phosphate Buffered Saline)

NaCl	137mM
Na_2HPO_4	8.5mM
KCl	44mM
KH_2PO_4	1.4mM

RPMI

RPMI 1640	500ml
L-Glutamine (2mM, final)	5ml
Penicillin/Streptomycin (100mg/ml - optional)	2.5ml
1M NaOH	0.5ml
FBS (10%, final)	50ml

Trypsin

2.5% Trypsin (stock)	20ml
PBS/EDTA*	180ml

*P/E is used because cellular adhesion is in part dependent on the presence of Mg^{2+} which is complexed and effectively removed by EDTA.

2.17.3 Method

Aseptic manipulations were performed using sterile glassware and plasticware in a class II microbiological safety cabinet with vertical airflow. All cell lines were regularly analysed for mycoplasma infection.

Ovarian epithelial cancer cells lines were grown and maintained at 37°C in RPMI 1640 medium supplemented with 10% FBS and 2mM L-Glutamine as monolayers in 25, 75 or 175cm^2 flasks in the presence of 5% CO_2. The cisplatin-sensitive cell lines used in these

experiments were the parental cell line A2780 and 5 clonal derivatives (A2780p3, A2780p5, A2780p6, A2780p13 and A2780p14). The 10 cisplatin-resistant cell lines used were A2780cp70 (Behrens et al., 1987) and MCP1-9 (Brown et al., 1997). In addition, 6 additional cisplatin-resistant cell lines derived from exposing A2780 cells to a single dose of cisplatin (McLaughlin et al., 1991) were used which included C1cis6, C2cis6, C2E3, C3Cis6, C5E4 and C5E4(15).

For the re-expression studies described in Chapter *4.5*, cells were treated with the DNMT inhibitor 5-aza-2'-deoxcytidine (DAC) (Jones and Taylor, 1980) (0.5μM) for 4 days with daily change of medium.

Cell stocks were formed by freezing 10^6 cells in 1ml growth medium with 10% DMSO at -70°C in cryotubes. After 24h, samples were transferred to liquid nitrogen. Cell lines were replaced regularly from frozen stocks to reduce the chances of genetic drift.

2.18 RNA extraction from cell lines

2.18.1 Materials

β-Mercaptoethanol	*Sigma*
DEPC treated H_2O	*Invitrogen*
Ethanol	*Hayman*
QIAshredder spin columns	*Qiagen*
RNeasy Mini Kit	*Qiagen*
RNase-Free DNase Set	*Qiagen*

2.18.2 Method

Total RNA was extracted using the RNeasy Mini Kit spin protocol according to the manufacturer's instructions, with the homogenisation step being carried out using QIAshredder spin columns. All centrifugation steps were carried out at room temperature and at ≥8000xg. DEPC treated H_2O was used in all steps to reduce the probability of RNase contamination. This kit combines the guanidine-isothiocyanate lysis with silica-gel–membrane purification. Samples were lysed by adding 600μl of Buffer RLT to 100μl of cells. Samples were then homogenised using the QIAshredder. 1 volume of 70% ethanol

was added to the lysate to provide ideal binding conditions and mixed by pipetting. The lysate was then loaded onto the RNeasy silica-gel membrane column and centrifuged for 15 seconds. 350µl of Buffer RW1 was added to the column and centrifuged for 15 seconds. RNase-Free DNase Set was used to provide efficient digestion of DNA. The DNase was removed in subsequent wash steps. The lyophilised DNase1 was dissolved in 550µl of RNase-free H_2O and gently inverted to mix. 10µl of this DNase1 stock solution was added to 70µl of Buffer RDD and mixed by gentle inversion. The DNase1 incubation mix was then pipetted directly onto the RNeasy silica-gel membrane and incubated at room temperature for 15 minutes. 350µl of Buffer RW1 was then added to the column and centrifuged for 15 seconds. The column was then placed in a new 2ml collecting tube and 500µl of RPE was added and centrifuged for 15 seconds. A further 500µl of RPE was added to the column and centrifuged for 2 minutes. This centrifugation step was then repeated again in a clean 2ml collecting tube. These steps allow RNA to bind to the column and contaminants are washed away. RNA was eluted twice in 40µl of RNase-free H_2O. RNA was then stored at -70°C until required.

2.19 cDNA synthesis (Reverse Transcription, RT)

2.19.1 Materials

SuperScript First-Strand Synthesis Kit *Invitrogen*

2.19.2 Method

cDNA was prepared according to the manufacturer's instructions by reverse transcribing 5µg of RNA using the Superscript II first strand synthesis system. This kit contains dNTP mix, oligo dT, DEPC treated H_2O, RT buffer, $MgCl_2$ solution, DTT, RNase OUT, RNase inhibitor and superscript II reverse transcriptase (RTase). A no RTase control was included for each sample. Each reaction contained the following:

Ingredients	**+RTase**	**No RTase**
RNA	16µl	8µl
10mM dNTP	2µl	1µl
oligodT	2µl	1µl

This was incubated at 65°C for 5 minutes and then put on ice for at least 1 minute.

A master mix as follows was made up for each no RTase reactions and volumes were doubled for the + RTase reactions:

10X RT Buffer	2µl
25mM $MgCl_2$	4µl
DTT	2µl
RNase OUT	1µl

This reaction mix was incubated at 42°C for 2 minutes and then 2µl of superscript RTase was added to each +RTase reaction at 42°C. This was then incubated at 42°C for 50 minutes, 70°C for 15 minutes and put on ice. RNase was added to each tube (2µl per +RTase; 1µl per no RTase reaction) and incubated at 37°C for 20 minutes. cDNA was then stored at -20°C until required for qRT-PCR.

2.20 Quantitative RT-PCR (qRT-PCR)

2.20.1 Materials

DyNAmo HS SYBR green qRT-PCR kit	*Finnzymes*
Flat cap strips	*Biorad*
Low 96-well white multiplate PCR plate	*Biorad*
Opticon 2 DNA Engine (conditions as for Table 9)	*MJ Research*
RT-PCR oligonucleotides (detailed below in Table 8)	*TAGN*

All other reagents used as described in Chapter *2.10*

2.20.2 Method

qRT-PCR was used to quantitatively evaluate the change in RNA expression levels of *NR2E1* and *LMX1A* in cell lines before and after treatment with Decitabine as described in Chapter *4.5*. qRT-PCR master mixes were made up using the DyNAmo HS SYBR green qRT-PCR kit. The 2x master mix provided contained a hot start version of modified *Thermus brockianus* DNA polymerase, SYBR Green I binding dye, optimized PCR buffer,

$MgCl_2$ and dNTPs in a pre-mixed form. Reactions included 1x SYBR green master mix, 150ng of each forward and reverse primer and 2µl cDNA made up to 25µl with dH_2O. Reaction mixes and plates were prepared in a PCR workstation. A negative control containing H_2O instead of template cDNA was included and a standard curve derived from a range of known concentrations of cDNA was set up in triplate for each sample. The constitutively expressed gene *GAPDH* was used as an internal control to normalise the data and the average value of 3 independent RT-PCR reactions once standardised to *GAPDH* was taken as the concentration of PCR product. Reactions were run on an Opticon 2 DNA Engine according to cycling conditions below in Table 9.

2.20.3 RT-PCR oligonucleotides and cycling conditions

Gene	**Primer Sequence** *Forward/Reverse 5'→3'*	*Genomic Position*	*Product Size (bp)*	*Annealing Temp (°C)*	*$[Mg^{2+}]$ mM/*
GAPDH	F:GTCAAGCTCATTTCCTGGTATG R: GTCTACATGGCAACTGTGAG	Exon 8-9	**214**	**61**	**2**
LMX1A	F:TCATGAACCCCTACACGG R:GGGCTCGGCACCATAA	Exon 9-10	**143**	**61**	**2**
NR2E1	F:ATCAACAAGCCGCATTTTAG R: GCCTCCCTGGTTTCCAG	Exon 1-3	**160**	**65**	**2**

Table 8 RT-PCR oligonucleotides and conditions.

Gene	**Cycling conditions**
GAPDH	**1.** 94°C 15 mins; **2.** 94°C 30 secs; **3.** 63°C 30 secs; **4.** 72°C 30 secs; **5.** 82°C 10 secs; **6.** plate read; **7.** Go to Step 2 x 39 times; **8.** Melting curve 70-93°C
LMX1A	**1.** 94°C 15 mins; **2.** 94°C 30 secs; **3.** 61°C 30 secs; **4.** 72°C 30 secs; **5.** 80°C 10 secs; **6.** plate read; **7.** Go to Step 2 x 42 times; **8.** Melting curve 70-93°C
NR2E1	**1.** 94°C 15 mins; **2.** 94°C 30 secs; **3.** 62°C 30 secs; **4.** 72°C 30 secs; **5.** 80°C 10 secs; **6.** plate read; **7.** Go to Step 2 x 42 times; **8.** Melting curve 70-93°C

Table 9 Cycling conditions for qRT-PCR.

2.21 Statistical methods of analysis

2.21.1 Chi-squared (χ^2) test

The Chi-squared test is a non-parametric test of statistical significance which is used to test associations between categorical variables. The chi-squared statistic is computed from a cross tabulation of the two variables. This goodness-of-fit test compares the observed and expected frequencies in each category (Armitage, 1994). Fisher's Exact Test was used if the smallest expected value was less than 5.

2.21.2 Mann Whitney U Test

This is a non-parametric test to investigate whether two independent samples come from the same population. Mann-Whitney U tests whether two samples populations are equivalent in location. The observations from both groups are combined and ranked, with the average rank assigned in the case of ties (Armitage, 1994).

2.21.3 Unsupervised gene shaving

Gene shaving is based on a dimension reduction method, Principal Components Analysis (PCA), to identify subsets of genes that vary concordantly across samples. This was used in this thesis to identify consistently methylated groups of genes associated with clinical outcome (CIMP). PCA extracts the most important features of the data and re-maps the data into a reduced dimensional space. Weights are allocated to objects depending on their contribution to the projection with the larger the absolute value of the weight, the larger the contribution from that item. Gene shaving uses the dimension reducing capacity of PCA to identify subsets of features with high weights that maximally separate the samples.

2.21.4 The cluster quality R^2 statistic

The R^2 statistic ($R^2 = 100V_B/V_T$) is used to estimate the quality of an identified pattern. The larger the R^2 statistic, the better the separation of the patient population and/or the higher the coherence between selected features. V_B is a measure of variance between samples and V_T measures the total variance of a cluster.

Chapter 3

Methylation analysis of candidate genes during epithelial ovarian cancer development

3 Methylation analysis of candidate genes during epithelial ovarian cancer development

3.1 CGI methylation in late stage ovarian tumourigenesis

The aim of this chapter was to identify markers which are methylated in late stage EOC. These could potentially be translated into clinically useful predictive DNA methylation biomarkers of response to chemotherapy. The methylation status of 24 candidate CGIs (*APAF-1*, *BLU*, *BRCA1*, *CASP8*, *DAPK*, *DCR1*, *FANCF*, *FAS*, *GSTP1*, *HIC1*, *MGMT*, *MINT25*, *MLH1*, *OPCML*, *P14*, *P16*, *P21*, *P73*, *PTEN*, *RASSF1A*, *SFRP1*, *SOCS-3*, *SURVIVIN* and *TMS1*) which had previously been reported to be methylated were examined in a group of 106 late stage epithelial ovarian tumours. The samples obtained were from chemonaive tumours taken at the time of initial cytoreductive surgery. The CGIs overlapped the promoter/1st exon of genes and included genes specifically involved in DNA repair and drug detoxification (*BRCA1*, *FANCF*, *GSTP1* and *MGMT*), regulators of proliferation (*PTEN*, *SFRP1* and *SOCS-3*), regulators of apoptosis (*APAF-1*, *DCR1*, *FAS*, *MLH1*, *P14*, *P73*, *RASSF1A*, *SURVIVIN* and *TMS1*) and genes that had previously been reported to be methylated in ovarian cancer (*HIC1*, *MINT25* and *OPCML*).

Methylation-specific PCR (MSP) was used to assess CGI methylation (see chapter *2.10*). Primers for MSP were designed to amplify methylated sequences whose methylation status had previously been correlated with transcriptional silencing of the corresponding gene in other studies. MSP is a highly sensitive assay (Herman et al., 1996) and has been widely used to analyse CGI methylation patterns in various tumour types. Incomplete bisulphite modification of DNA can lead to false positive results (Rand et al., 2002) therefore stringent controls and scoring criteria were applied in this study. Successful bisulphite modification of the DNA sample was verified using a region of the *CALPONIN* promoter as described in chapter *2.9*. This sequence will only be amplified if the cytosines in the template sequence are converted to uracils (Teodoridis et al., 2005). Samples which did not give a band of similar intensity were considered unmodified or incompletely modified and the modification experiment was repeated for these, as illustrated in Figure 10.

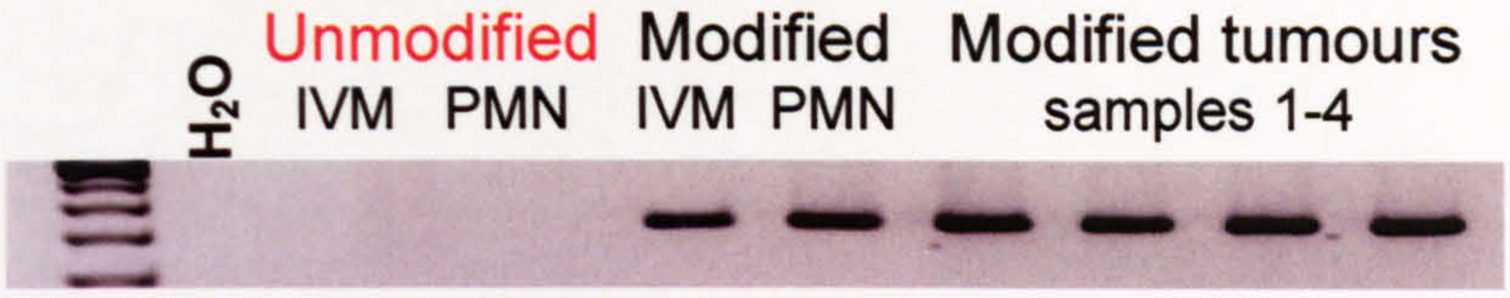


Figure 10 Examples of successful sodium bisulphite modifications. H_2O, reaction without template DNA; unmodified (red) and successfully modified (black) IVM, *in vitro* methylated DNA and PMN, DNA from whole male blood; 4 modified tumour samples.

MSP is not a quantitative assay but by using a stringent modification control as well as including a standard curve of IVM DNA in male genomic DNA dilutions, the results obtained here were semi-quantitative. No PCR product using methylation-specific primers, and hence no evidence of methylation of these CGIs, was observed for normal or immortalised ovarian surface epithelium or DNA extracted from PBMCs. In each experiment, MSP gave a product using IVM DNA and low level methylation signals comparable to <1:10 dilution of IVM DNA into normal unmethylated DNA which were not reproducible were disregarded. A maximum number of 35 cycles of PCR was used in each reaction to avoid amplification of very low intensity signals or low levels of unmodified DNA which could lead to false positive results. All MSP data obtained was repeated on at least two independent reactions and those results which did not correlate were repeated again. Finally, frequently positive MSP results were confirmed using COBRA (Xiong and Laird, 1997), (see chapter *2.12*). This is a quantitative assay measuring methylation at individual CpG residues and allowed verification of MSP scores obtained.

Overall, methylation of at least one gene was observed in 60% of late stage tumours. Frequent methylation was observed for *OPCML*, *DCR1*, *RASSF1A*, *HIC1*, *BRCA1* and *MINT25* (33%, 31%, 26%, 17%, 12% and 12%, respectively), whereas no methylation was observed for *APAF-1*, *DAPK*, *FANCF*, *FAS*, *P14*, *P21*, *P73*, *SOCS-3* and *SURVIVIN*. The remaining genes showed only a low frequency of methylation, <10%. The methylation frequencies of the group of epithelial ovarian tumours examined here are shown below in Table 10 and examples of MSP results are shown in Figure 11.

MSP results were confirmed using COBRA which is an independent quantitative method of assessing methylation at individual CpG residues. 54 MSP results were quantified using COBRA and this covered 7 selected CGIs (*BRCA1*, *HIC1*, *MLH1*, *OPCML*, *RASSF1A*,

SFRP1 and *TMS1*). There was 87% (47/54 samples) concordance between the MSP and COBRA results and examples of this are shown below in Figure 12.

Chapter *5.2* and *5.3* will discuss the analysis and validation of these results as clinically useful predictive biomarkers in ovarian cancer.

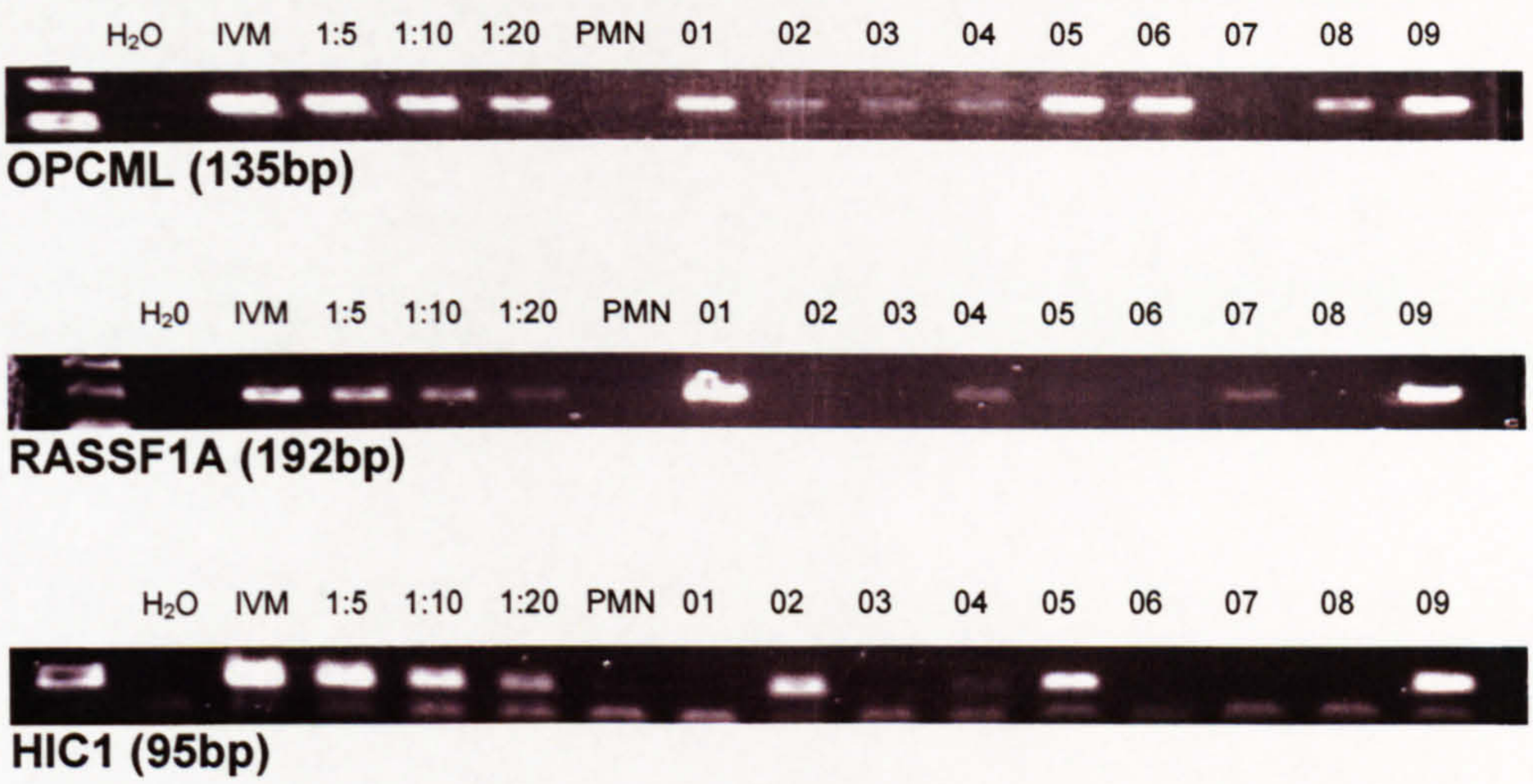


Figure 11 Examples of MSP results. CGI methylation of tumour samples (numbered 01-09) determined at loci indicated (product size). H_2O, reaction without template DNA; IVM, *in vitro* methylated DNA; 1:5, 1:10 and 1:20 serial dilutions of IVM in PMN; PMN, DNA from whole male blood.

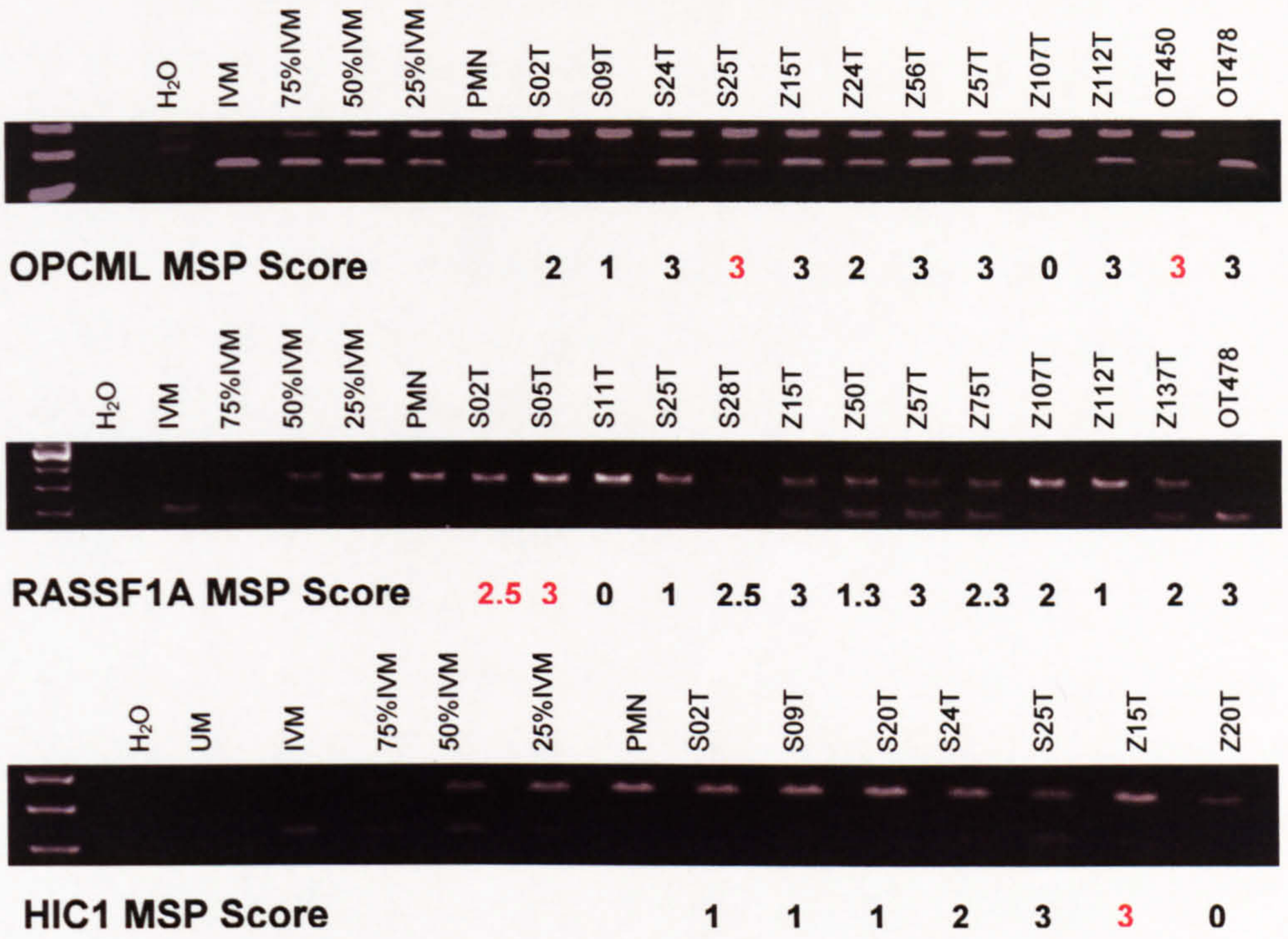


Figure 12 Examples of COBRA results. Quantitative methylation assay using restriction digest confirming MSP results for OPCML, RASSF1A and HIC1. Each MSP score is shown below each COBRA gel image. MSP scores which did not correlate with COBRA are indicated in red. Controls included IVM, *in vitro* methylated DNA; 75% IVM, 50% IVM and 25% IVM dilutions of IVM in PMN; PMN, DNA from whole male blood. Tumour sample numbers indicated above each image.

As discussed in the introduction, it has been shown that clusters of CGIs can become co-methylated in cancers, including late stage ovarian cancer (Strathdee et al., 2001). Groups of genes may exist which can define particular CpG island methylator phenotypes (CIMP) of cancers including colorectal cancer that are independent of histological type (Toyota et al., 1999a). This concept of a CIMP existing in a subset of ovarian tumours was investigated using a bioinformatics approach called unsupervised gene shaving (Chapter *2.21.3*) to identify concordant patterns of methylation that separate the samples into subgroups, in collaboration with Dr. Jacqueline Hall. This was applied to all of the 106 stage III/IV samples and 23 genes. *SOCS-3* was not included, because the CGI associated with this gene was examined following the statistical analysis. The primary aim of this approach was to investigate the coherence of gene methylation and identify patterns of methylation that are non-random in this group of late stage ovarian tumours.

Pattern 1 (Figure 13A) was identified which has a cluster score (R^2) of 0.34 (Chapter *2.21.4*) and accounts for 87.1% of the PCA (Principal Components Analysis) solution. The derivation of Pattern 1 is unlikely to have occurred by chance as shown by the distribution

of the cluster quality R^2 statistic for 200 permutations (Figure 13B). Pattern identification ceased here as the next pattern only accounted for a small proportion of the full PCA solution (<3%). Pattern 1 shows concordant positive methylation between genes and is mostly comprised of frequently methylated genes *OPCML*, *DCR1*, *RASSF1A*, *MINT25* and *HIC1*, but also *SFRP1* which shows less methylation. This group of loci was identified as having a degree of concordance which was unlikely to have occurred by chance (p=0.002) but the cluster quality score (R^2) suggested only a weak degree of concordance between these genes. No CGIs were identified in the pattern to have negative concordance, which the gene shaving approach would have identified if present. One frequently methylated gene which is not present here in the non-random patterns is *BRCA1* suggesting methylation of this gene may occur via a different underlying process or biological selection. This is consistent with a previous observation using different analysis on a different group of Stage III/IV ovarian tumours within our group (Strathdee et al., 2001) which suggested that *BRCA1* had negative concordance with the methylator phenotype defined. The methylation pattern from unsupervised gene shaving was independent of age (p=0.239, N=100) and histological subtype (p=0.247, N=80).

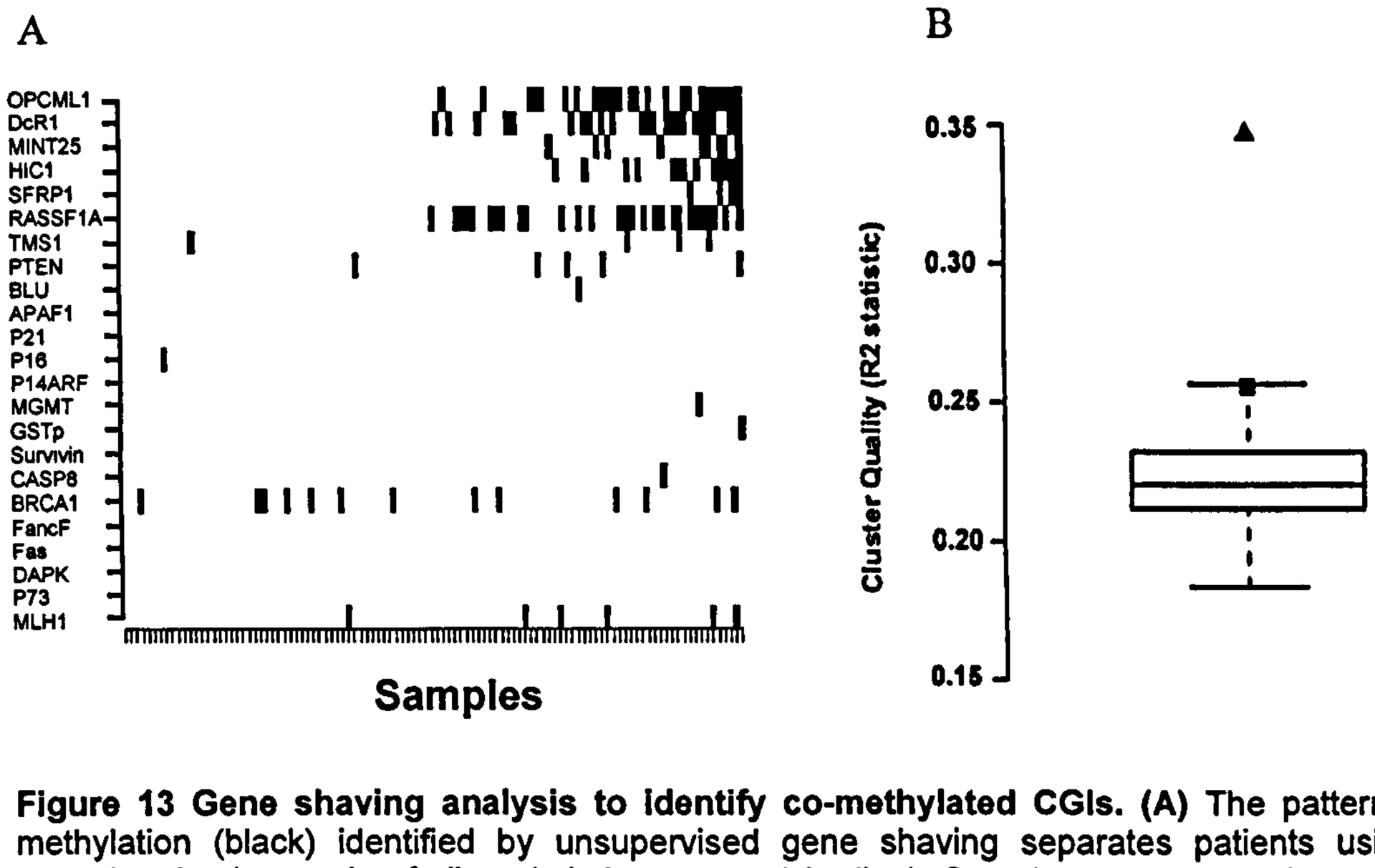


Figure 13 Gene shaving analysis to identify co-methylated CGIs. (A) The pattern of gene methylation (black) identified by unsupervised gene shaving separates patients using a co-methylated sub sample of all methylation events (shading). Samples are ordered from right to left according to the frequency (no. of methylation events per sample) of methylation in the pattern. This cluster explains 87.1% of the equivalent principal components analysis solution (n=106). **(B)** The boxplot shows the distribution of the cluster quality R^2 statistic for 200 permutations. The square signifies the upper confidence interval of this distribution; the triangle shows the R^2 value of pattern 1. A larger R^2 suggests a tighter cluster of coherent genes.

Gene	Chromsome alignment / MSP product distance from TS* (bp)	Function	MSP primer Reference	Methylation Frequency	
				Stage III	Stage IV
APAF-1	12q23.1 / +34 to +173	Apoptosis	(Fu et al., 2003)	0	0
BLU	3p21.3 / +972 to +1128	Unknown	(Agathanggelou et al., 2003)	1.2 (1/80)	0
BRCA1	17q21 / -80 to +96	DNA repair	(Strathdee et al., 2001)	8.8 (7/80)	23.1 (6/26)
CASP8	2q33 / +536 to +856	Apoptosis	(Teitz et al., 2000)	1.2 (1/80)	0
DAPK	9q34.1 / +4 to +101	Apoptosis	(Balana et al., 2003)	0	0
DCR1	8p21.3 / +27 to +153	Apoptosis	(van Noesel et al., 2002)	28.1 (16/57)	38.9 (7/18)
FANCF	11p14.3 / +280 to +432	DNA repair	(Taniguchi et al., 2003)	0	0
FAS	10q23.3 / -260 to +9	Apoptosis	(Hopkins-Donaldson et al., 2003)	0	0
GSTP1	11q13.2 / +29 to +168	Drug detoxification	(Jeronimo et al., 2001)	1.2 (1/80)	0
HIC1	17p13.3 / -230 to -135	Unknown	(Dong et al., 2001)	10.5 (6/57)	38.9 (7/18)
MGMT	10q26.3 / +26 to +106	DNA repair	(Balana et al., 2003)	0	3.8 (1/26)
MINT25	22q11 / +38 to +250	Unknown	(Strathdee et al., 2001)	12.3 (7/57)	11.1 (2/18)
MLH1	3p21.3 / -476 to -361	DNA repair/ Apoptosis	(Strathdee et al., 1999)	5 (4/80)	7.7 (2/26)
OPCML	11q25 / from -500**	Ovarian tumour suppressor	(Sellar et al., 2003)	36.8 (21/57)	22.2 (4/18)
P14	9p21 / +201 to +322	Apoptosis	(Esteller et al., 2000c)	0	0
P16	9p21 / +192 to +341	Cell cycle	(Herman et al., 1996)	0	3.8 (1/26)
P21	6p21.3 / -142 to +55	Cell cycle	This thesis	0	0
P73	1p36.3 / -166 to +149	Apoptosis	(Strathdee et al., 2001)	0	0
PTEN	10q23.3 / +925 to +1151	Proliferation	This thesis	8.6 (5/58)	0
RASSF1A	3p21.3 / -65 to +127	Apoptosis	(Honorio et al., 2003)	26.2 (21/80)	26.9 (7/26)
SFRP-1	8p11.2 / +2 to +164	Proliferation	This thesis	5.2 (3/58)	5.6 (1/18)
SOCS-3	17q25.3 / +205 to +338	Proliferation	This thesis	0	0
Survivin	17q25.3 / -31 to +249	Apoptosis	This thesis	0	0
TMS1	16p11.2 / -44 to +147	Apoptosis	(Conway et al., 2000)	5.1 (3/59)	5.6 (1/18)

Table 10 Methylation frequencies in late stage epithelial ovarian tumours. Methylation frequency % (number methylated / total number of samples) of each candidate gene examined in FIGO Stage III/IV epithelial ovarian tumours. Chromosome alignment, *amplified MSP product distance up(-)/down(+) stream from predicted transcriptional start site (TS) shown in bp, gene function and MSP primer reference given. **according to Sellar *et al* (2003).

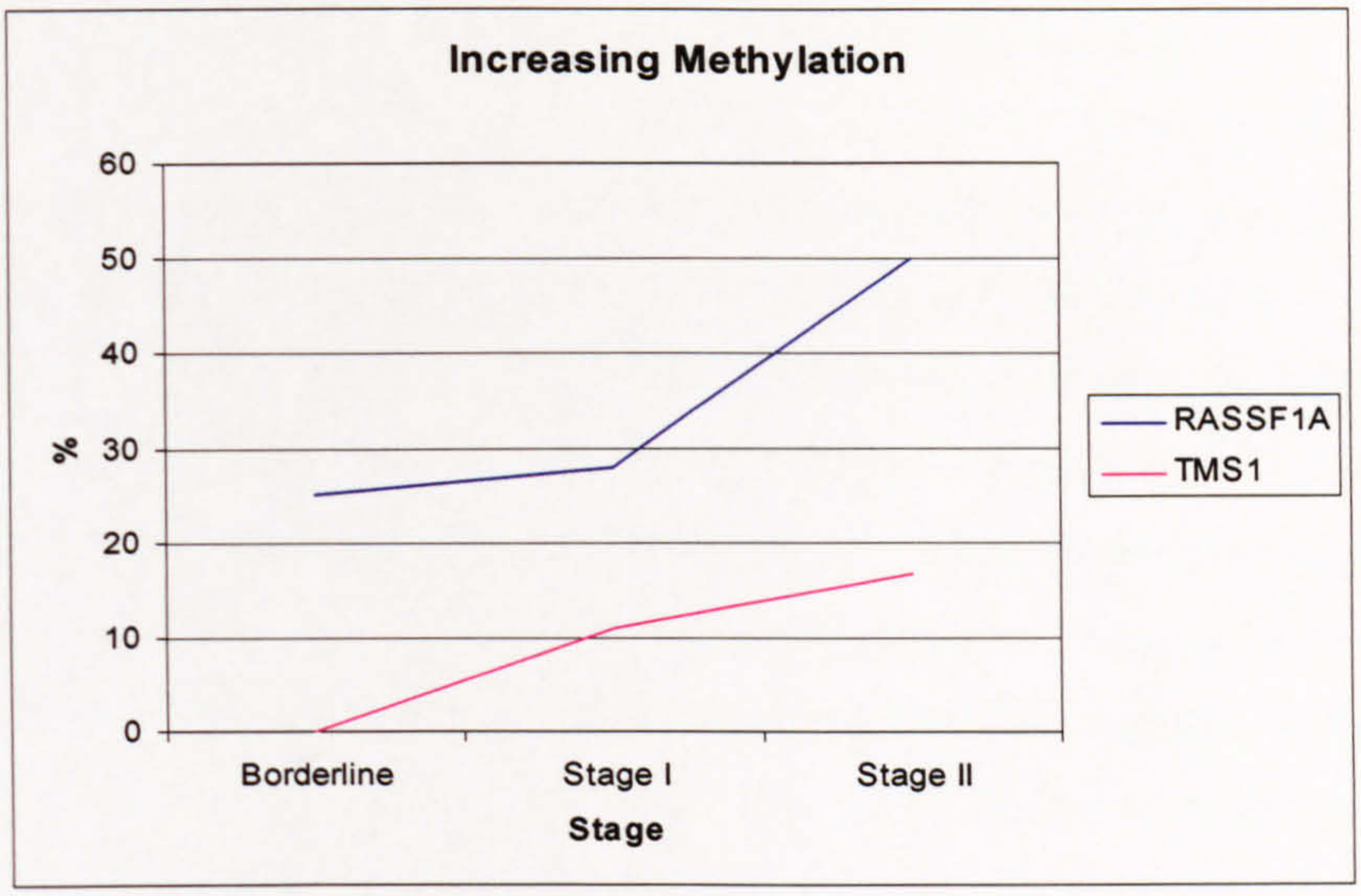
3.2 CGI methylation in early stage tumours

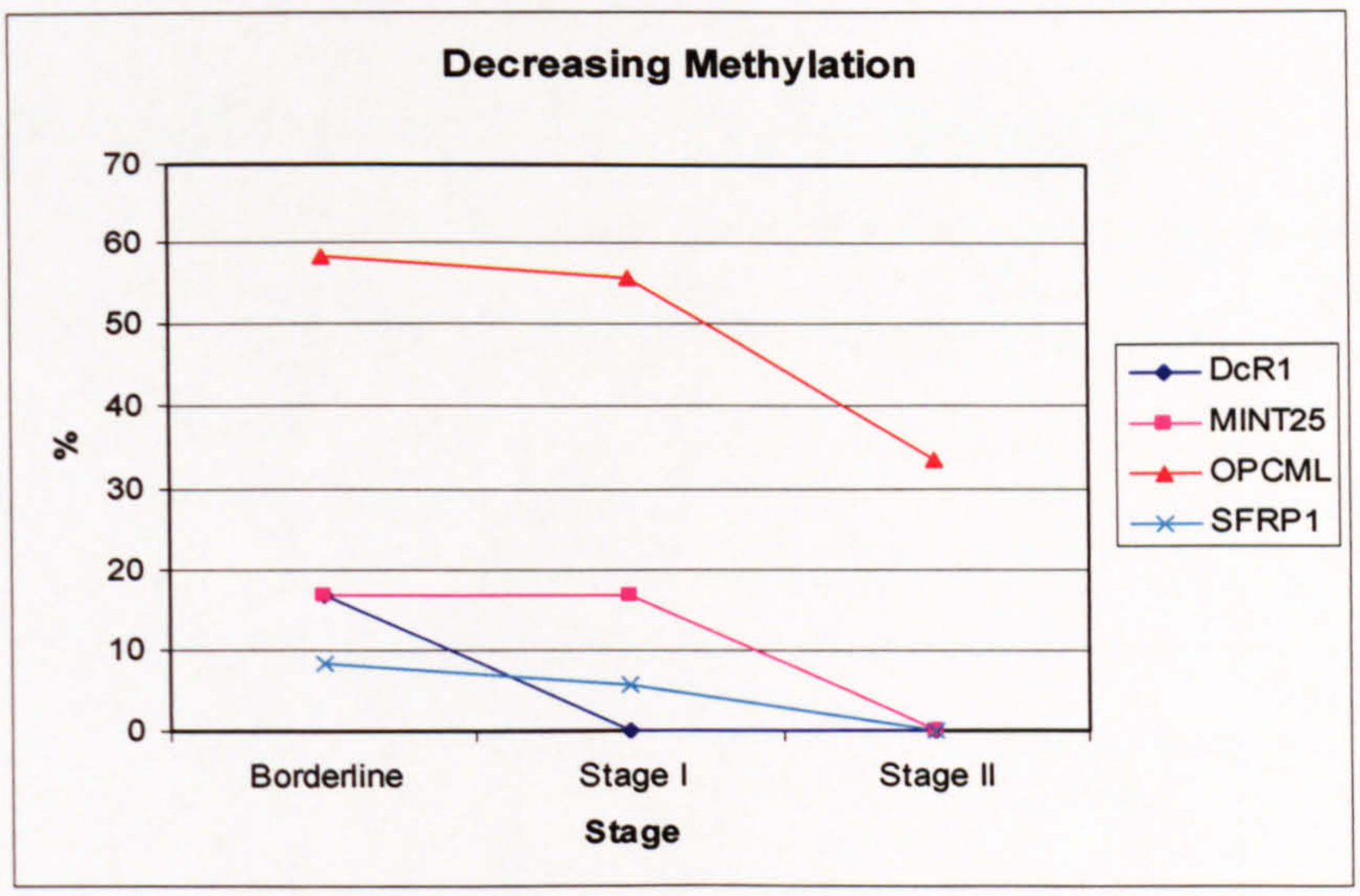
The aim of this chapter was to identify candidate CGIs which are methylated in early stage EOC and uncover CGI methylation changes which occur during disease progression. Previous reports have suggested that epigenetic aberrations including methylation events occur more frequently as a cancer develops (Guo et al., 2006; Jones and Baylin, 2002; Mehrotra et al., 2004).

The methylation status of 14 of the candidate CGIs examined in chapter *3.1* (*APAF-1*, *BLU*, *BRCA1*, *DCR1*, *HIC1*, *MGMT*, *MINT25*, *MLH1*, *OPCML*, *P16*, *P21*, *RASSF1A*, *SFRP1* and *TMS1*) were examined in a group of 36 early stage epithelial ovarian tumours as shown below in Table 11. The tumours comprised 12 borderline, 18 stage I and 6 stage II epithelial ovarian tumours and the same acceptance criteria for MSP as detailed in Chapter *3.1* were used again.

Overall, methylation of at least one gene was observed in 78% of early stage tumours. Specifically, 75% of borderline tumours, 83% of stage I tumours and 67% of stage II tumours showed methylation of at least one gene. Frequent methylation of *OPCML*, *RASSF1A*, *HIC1* and *MINT25* (53%, 31%, 22% and 14% respectively) was observed in this group of early stage tumours. Taken together, 64% of tumours were methylated in at least one of 3 genes (*OPCML*, *RASSF1A* and *HIC1*). The remaining genes showed either a low frequency of methylation, <10% (*DCR1*, *P21*, *SFRP1*, *TMS1*), or no methylation (*APAF-1*, *BLU*, *BRCA1*, *MGMT*, *MLH1* and *P16*).

As expected, methylation frequency of some genes increased with advancing stage of early disease (*RASSF1A* and *TMS1*). Early stage disease included borderline, stage I and stage II tumours. Unexpectedly, however, this pattern was reversed in other genes where methylation frequency decreased as early stage disease progressed (*DcR1*, *MINT25*, *OPCML* and *SFRP1*). For the remaining genes, no general trend (*HIC1* and *P21*) or no change in methylation (*APAF-1*, *BLU*, *BRCA1*, *MGMT*, *MLH1* and *P16*) was observed. These trends are illustrated in a panel of three graphs shown below in Figure 14 and Table 11.





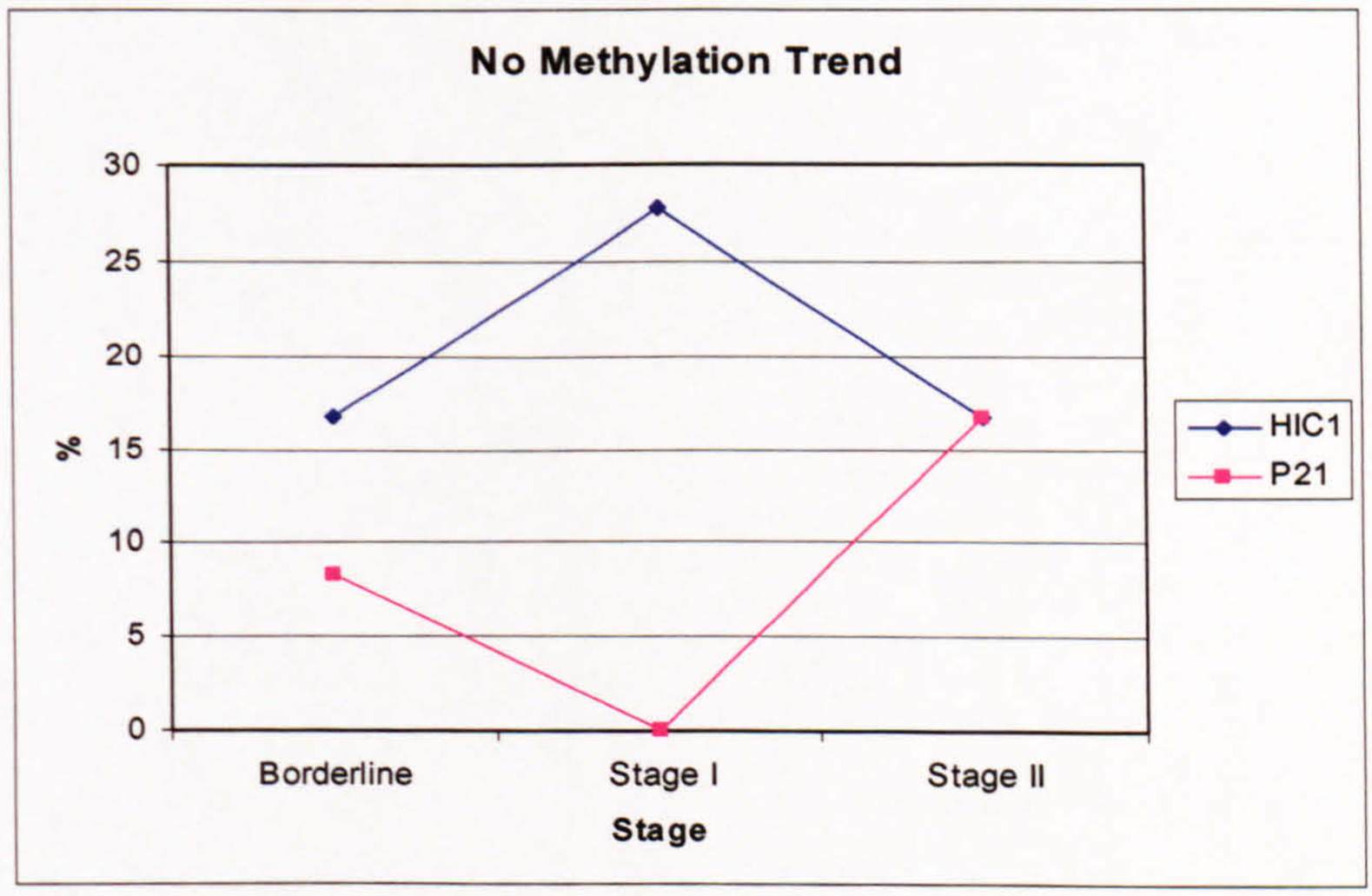


Figure 14 Methylation trends of genes observed in early stage ovarian tumours. As early stage ovarian tumours became more advanced, a trend of increasing frequency of methylation was observed for *RASSF1A* and *TMS1*. Decreasing frequency of methylation was observed for *DcR1*, *MINT25*, *OPCML* and *SFRP*. No methylation trend was observed for *HIC1* and *p21*. For absolute numbers of methylated genes, see Table 11.

A reduction in methylation frequencies with advancing tumour stage (early vs. late stage disease) was observed for some genes (*OPCML*, 53% vs. 33% and *RASSF1A*, 31% vs. 26%) whereas for other genes, we observed increased methylation frequencies for late stage disease (*BRCA1*, 0% vs. 12%, *DCR1* 6% vs. 31% and *MLH1*, 0% vs. 6%). Fisher's Exact Test was used to determine if these differences observed for each CGI with advancing stage of tumour were statistically significant. Methylation of *BRCA1* (p=0.039) and *DCR1* (p=0.003) both significantly increased with stage when comparing late stage with early stage disease. MSP bands were of variable intensity for some CGIs examined in both early and late stage disease which may indicate tumour heterogeneity within a sample (e.g. *HIC1*). In contrast, some CGIs showed bands of similar intensity in all tumour specimens examined, as shown above in Figure 11.

Gene	Methylation Frequency			
	Borderline	Stage I	Stage II	Stage III/IV
APAF-1	0	0	0	0
BLU	0	0	0	0.9 (1/106)
BRCA1	0	0	0	12.3 (13/106)
DCR1	16.7 (2/12)	0	0	30.7 (23/75)
HIC1	16.7 (2/12)	27.8 (5/18)	16.7 (1/6)	17.3 (13/75)
MGMT	0	0	0	0.9 (1/106)
MINT25	16.7 (2/12)	16.7 (3/18)	0	12 (9/75)
MLH1	0	0	0	5.7 (6/106)
OPCML	58.3 (7/12)	55.6 (10/18)	33.3 (2/6)	33.3 (25/75)
P16	0	0	0	0.9 (1/106)
P21	8.3 (1/12)	0	16.7 (1/6)	0
RASSF1A	25 (3/12)	27.8 (5/18)	50 (3/6)	26.4 (28/106)
SFRP-1	8.3 (1/12)	5.6 (1/18)	0	5.3 (4/76)
TMS1	0	11.1 (2/18)	16.7 (1/6)	5.2 (4/77)

Table 11 Methylation frequencies in early stage versus late stage epithelial ovarian tumours. Methylation frequency % (number methylated / total number of tumours) of each candidate gene examined in borderline, and FIGO Stage I-IV epithelial ovarian tumours.

3.3 CGI methylation in non-tumour tissue

The methylation status of the 24 CGIs previously examined in the tumour samples in Chapter *3.1* and *3.2* were analysed in 4 normal ovarian surface epithelium (nOSE) and 3 immortalised ovarian surface epithelium (iOSE) samples. No methylation signal was detected in any of these samples.

16 specimens were obtained from biopsies of normal tissue immediately adjacent to the ovarian tumour and included tissue next to 3 borderline tumours, 8 stage I tumours and 5 stage II tumours. Overall, 63% of these normal samples showed methylation of at least one of the 14 CGIs analysed in early stage tumours. Specifically, methylation of *HIC1*, *DCR1*, *APAF1*, *OPCML* and *RASSF1A* (50%, 38%, 6%, 6% and 6% respectively) was observed in these adjacent normal epithelial tissues. However, methylation within the corresponding tumour was not always observed as illustrated in Figure 15. In addition, methylation of *BLU*, *BRCA1*, *MGMT*, *MINT25*, *MLH1*, *P16*, *P21*, *SFRP-1* and *TMS1* was not observed in adjacent normal tissue, although methylation of *MINT25* and *TMS1* was noted in 13% and 19% of the corresponding tumour samples respectively.

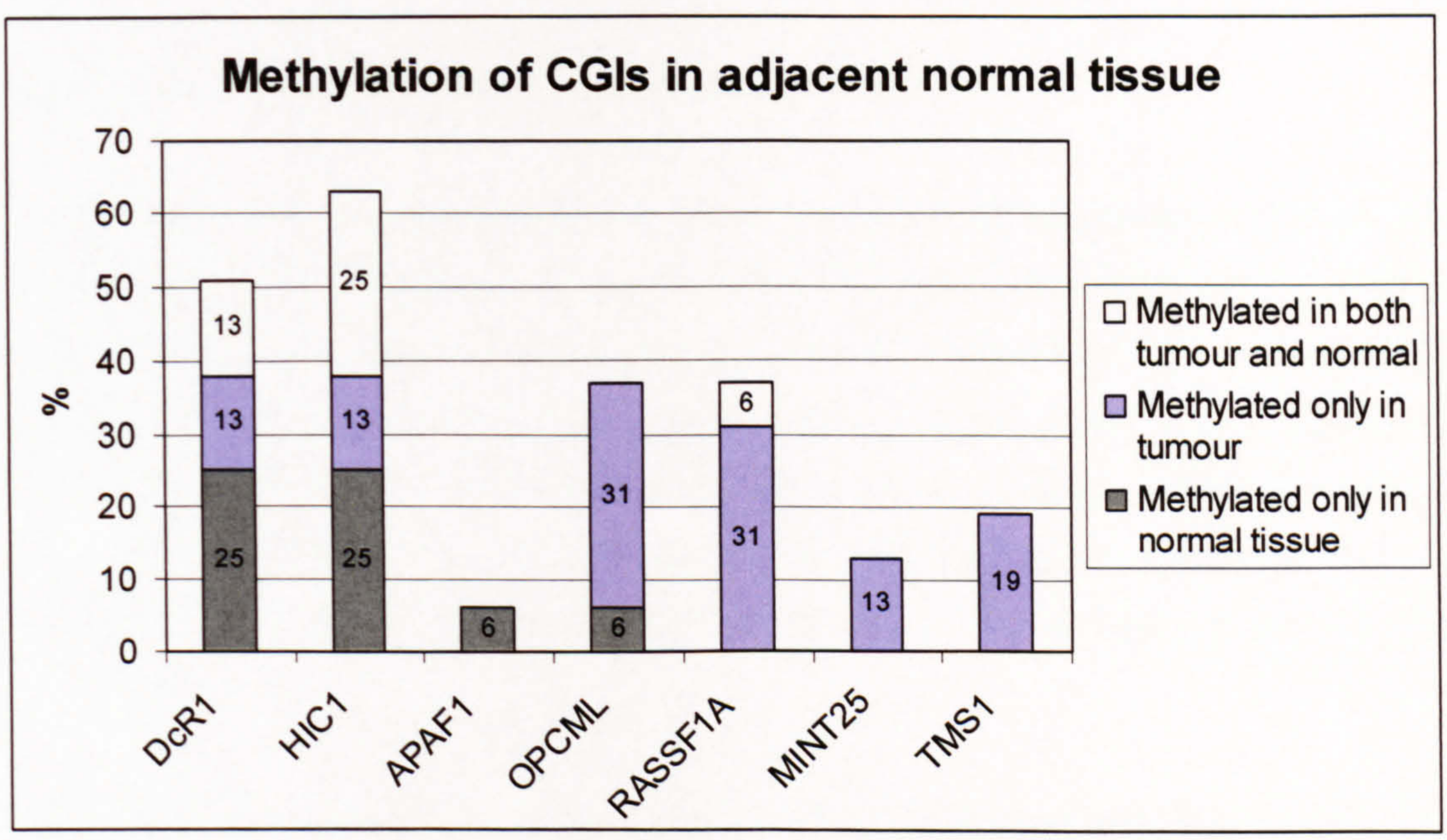


Figure 15 Methylation of CGIs in adjacent normal tissue. Percentages of methylation observed in normal tissue adjacent to tumours. Grey bar: methylation only detected in normal adjacent tissue but not in corresponding tumour sample; Blue bar: methylation only detected in tumour samples, White bar: methylation in both normal adjacent tissue and matched tumour.

3.4 Discussion

The acquisition of aberrant CGI methylation is a now a widely accepted hallmark of cancer and it has been previously shown that multiple CGIs can become methylated in late stage epithelial ovarian tumours compared to normal ovarian surface epithelium (Strathdee et al., 2001; Wei et al., 2002). In this Chapter, MSP and COBRA methods were firstly used to analyse methylation of 24 candidate CGIs (*APAF-1*, *BLU*, *BRCA1*, *CASP8*, *DAPK*, *DCR1*, *FANCF*, *FAS*, *GSTP1*, *HIC1*, *MGMT*, *MINT25*, *MLH1*, *OPCML*, *P14*, *P16*, *P21*, *P73*, *PTEN*, *RASSF1A*, *SFRP1*, *SOCS-3*, *SURVIVIN* and *TMS1*) in 106 late stage III/IV epithelial ovarian tumours (Teodoridis et al., 2005) and 4 normal ovarian surface epithelium samples. Further to this, the methylation status of 14 of these candidate loci (*APAF-1*, *BLU*, *BRCA1*, *DCR1*, *HIC1*, *MGMT*, *MINT25*, *MLH1*, *OPCML*, *P16*, *P21*, *RASSF1A*, *SFRP1*, *TMS1*) were examined in early stage disease (12 borderline, 18 stage I and 6 stage II) and matched non-tumour tissue samples. The CGIs examined were linked to genes biologically involved in the cellular responses to DNA damage. This included genes involved in DNA repair/drug detoxification, control of cell cycle, apoptosis, proliferation, and those which have been previously shown to be frequently methylated in ovarian cancer.

Aberrant DNA methylation in ovarian carcinogenesis

A comparison between the methylation percentages reported in this study for the late stage tumours and previous publications show similar CGI methylation frequencies in ovarian tumours for *BRCA1* (Baldwin et al., 2000; Esteller et al., 2000b; Strathdee et al., 2001), *CASP8* (Strathdee et al., 2001), *DAPK* (Terasawa et al., 2004), *DCR1* (Shivapurkar et al., 2004), *HIC1* (Strathdee et al., 2001), *MINT25* (Strathdee et al., 2001), *MLH1* (Strathdee et al., 2001), *P16* (Brown et al., 2001; Marchini et al., 1997; Strathdee et al., 2001; Wong et al., 1999), *P73* (Chen et al., 2000), *SFRP1* (Takada et al., 2004) and *SOCS-3* (Sutherland et al., 2004).

The methylation frequencies observed for *FANCF*, *OPCML* and *TMS1* in the late stage tumours (0%, 33.3% and 5.2% respectively) were not in agreement with previous reports in ovarian cancer. *FANCF* had originally been reported to be methylated in 21% of ovarian tumours (Taniguchi et al., 2003). The MSP cycling number used in the aforementioned study was 45 cycles compared to this study which used 35 cycles for the results obtained above and this may have accounted for the observed differences in methylation frequencies. A frequency of *FANCF* methylation (24%), similar to that of Taniguchi *et al*,

was reported in a more recent study (Dhillon et al., 2004a). However, the tumours examined were of the granulosa sub-type and this difference in histological type may contribute to the percentage differences seen here. *OPCML* methylation frequency had previously been reported in 83% of ovarian tumours (Sellar et al., 2003) which is the highest reported frequency of aberrant CGI methylation in ovarian malignancy to date. However, the stage of tumour examined was not shown in this previous study. The results of this thesis show that methylation in the group of borderline and early stage tumours was higher than that seen in late stage disease (58.3% and 50% versus 33.3% respectively). It may be that different stage proportions were examined in the study by Sellar *et al.* leading to higher frequencies (ie more early stage tumours) reported. An alternative reason for differences seen may be that *OPCML* methylation varies with histology. Histological data available for the late stage group of tumours in the work here showed variability in methylation frequencies. Specifically, 0% and 33% in clear cell and mucinous tumours, respectively. The decrease in methylation frequency seen with tumour progression may be due to loss of the methylated *OPCML*-allele, but this is unlikely here because in the previous study lower frequencies of methylation for those tumours without LOH was observed, indicating preferential loss of the unmethylated allele (Sellar et al., 2003). Methylation of *TMS1* was reported previously in 19% (Terasawa et al., 2004) and 40% (Akahira et al., 2004) of ovarian cancers but frequency was variable between histological types which may account for the differences seen in this current study.

Overall, CGI methylation was a frequent event in early stage disease (78%), and specifically, methylation of *OPCML*, *RASSF1A* and *HIC1* were frequently observed in borderline and early stage ovarian tumours (53%, 31% and 22% respectively). One previous study reported that *RASSF1A* was frequently methylated in 11/17 Stage I ovarian tumours (Ibanez de Caceres et al., 2004), but there have been no other studies examining CGI methylation of these specific genes in early stage ovarian cancer. Similar methylation frequencies of these CGIs were reported in the group of late stage tumours examined (Teodoridis et al., 2005) and this compares favourably with previous reports examining methylation of *HIC1* (Strathdee et al., 2001) and *RASSF1A* (Yoon et al., 2001). However, it is important to note that these reports have examined late stage disease and are therefore not directly comparable with the results for the early stage group of tumours.

Different methylation events may be important when considering the initiation, progression and phenotype of ovarian tumours. The identification of frequent methylation events occurring at an early stage of carcinogenesis supports the concept that *HIC1*, *RASSF1A* and *OPCML* may constitute tumour suppressor genes whose inactivation may

favour tumour development (Dammann et al., 2000; Hoff et al., 2000; Sellar et al., 2003) and provide a driving force in the formation of a tumour at an early stage. Perhaps early epigenetic changes in cells could lead to a tendency to genetic and epigenetic alterations that further advance the neoplastic process and "addict" cells to various oncogenic pathways (Baylin and Ohm, 2006). It is interesting to consider that there may be tumour-specific methylation events leading to the initiation and development of tumours and what those may be. In keeping with the concept of methylation of *HIC1*, *OPCML* and *RASSF1A* being initiating epigenetic events, methylation of *HIC1* has been shown to cooperate with the genetic disruption of *p53* in mouse knockout models and drive the development and progression of cancer (Chen et al., 2004). More recent work has demonstrated the functional cooperation between HIC1 and p53 and how these are homeostatically regulated in a feedback loop through the histone deacetylase, SIRT1 (Chen et al., 2005a). Actively transcribed HIC1 forms a repressive complex with SIRT1, which leads to acetylation of p53, allowing p53 to *trans*-activate *HIC1*. However, if *HIC1* is epigenetically silenced, *SIRT1* is upregulated which causes deacetylation and inactivation of p53. This initial epigenetic silencing of the *HIC1* gene may ultimately allow cells to survive DNA damage and promote the development of tumours (Chen et al., 2005a).

A recent report examining methylation of *RASSF1A* in childhood gliomas reported frequent methylation in tumours and widespread CGI methylation across the entire CGI which indicates that this epigenetic alteration may be an early event rather than a late clonal event (Hamilton et al., 2005). *RASSF1A* is thought to be responsible for *Ras*-dependent growth inhibition due to its proapoptotic function (Vos et al., 2000), since elimination of Ras inhibits apoptosis induced by transient transfection of *RASSF1A* into 293-T cells. *RASSF1A* has also been shown to induce cell cycle arrest by inhibition of cyclin D1 accumulation, thereby preventing G_1/S-phase cell cycle progression (Shivakumar et al., 2002). A recent study has shown a high prevalence of *RASSF1A* promoter methylation in both endometrial tumours and pre-cancerous hyperplastic tissues (Pijnenborg et al., 2006) whereas mutation of *K-RAS* and *B-RAF* were infrequent. A further study in colorectal cancer has examined the timing of methylation of a group of genes, including *RASSF1A*, and has shown that promoter methylation of these genes was present in early adenomas without mutations or chromosomal alterations (Derks et al., 2006), suggesting that this may be an early event in cancer development. Therefore, inhibition of expression of *RASSF1A* through methylation may be an early initiating event in the carcinogenic process which is independent of genetic mutations. This epigenetic silencing would lead to a loss of the proapoptotic and cell cycle-suppressive functions of *RASSF1A* and promote tumour development.

Opioid binding protein/cell adhesion molecule-like gene, *OPCML*, is a member of the IgLON family (LSAMP, OPCML/OBCAM) and is likely to play a role in cell adhesion and cell-cell recognition (Schofield et al., 1989). *OPCML* has the functional characteristics of a tumour suppressor gene in an ovarian cancer cell line *in vitro* and *in vivo* when xenografted into nude mice and is frequently inactivated by allele loss and CGI promoter methylation in sporadic ovarian cancer (Sellar et al., 2003). Reduced expression of other members of the IgLON family (*LSAMP* and *NEGRI*) have also recently been shown in sporadic EOC (Ntougkos et al., 2005), and epigenetic silencing has been shown to be accountable for this reduced expression in *LSAMP* (Chen et al., 2003a). However, there is still a necessity to understand the function of *OPCML* and other members of the IgLON family in the normal ovary, and how methylation of this family of genes may be related to the malignant phenotype. The molecular mechanism leading to the epigenetic inactivation of *OPCML* in EOC remains largely unknown but it has been shown that activation of the oncogene *RAS* may play an important role in epigenetic inacitivation of *OPCML* in EOC (Mei et al., 2006). This *RAS*-mediated epigenetic silencing of *OPCML* was shown in immortalized human ovarian surface epithelial cells and may therefore represent an early event in ovarian carcinogenesis, which would be in keeping with the results described in this thesis. *RAS*-induced hypermethylation of genes involved in key apoptotic pathways, cell cycle, DNA repair and differentiation has been reported previously (Alcock et al., 2002; Contente et al., 1999; Guan et al., 1999; Peli et al., 1999). These may be early events which are important in the development and progression of a tumour. *OPCML* contributes to cell adhesion properties and the epigenetic loss of this gene may have important biological consequences. Adhesion molecules can trigger intracellular signaling which may then alter proliferative changes. In addition, silencing of *OPCML* could play an important role at a later stage of ovarian carcinogenesis when propensity for metastatic peritoneal spread becomes important. In this case, the methylation events that a tumour cell accumulates during its history may not always contribute to the tumour phenotype until a later stage of development.

Methylation of *BRCA1* was not observed in early stage tumours which is similar to a previous report (Wang et al., 2004) where *BRCA1* hypermethylation was not detected in benign or borderline ovarian tumours. Some studies have reported methylation of *BRCA1* in ovarian tumours but either did not mention tumour stage or analysed late stage disease (Geisler et al., 2002; Hilton et al., 2002; Strathdee et al., 2001). One group have speculated that *BRCA1* methylation may be an early event in ovarian tumourigenesis (Baldwin et al., 2000) which is in contrast to the results here. No methylation of *APAF-1*, *BLU*, *MGMT*, *MLH1* and *P16* was observed in borderline or early stage ovarian tumours which is

comparable to previous findings in ovarian tumours (Strathdee et al., 2001) and the results above for late stage disease (Teodoridis et al., 2005). The results in this thesis would suggest that methylation of these six loci are not early events during the development and progression of ovarian carcinogenesis. Three of these genes (*BRCA1*, *MGMT* and *MLH1*) are involved in DNA repair and were methylated in late stage tumours, suggesting that epigenetic changes in these DNA repair genes do not significantly contribute to the early stages of sporadic ovarian tumourigenesis. Impaired DNA repair and genomic instability may be considered to be "hallmarks of cancer" (Hanahan and Weinberg, 2000). The findings here suggest that epigenetic silencing of *BRCA1*, *MGMT* and *MLH1* are late events in ovarian cancer. There may be alternative mechanisms and genes affected, which have not been investigated within the scope of this work, but which may contribute to genetic instability. Alternatively, this instability may not be a frequent event during initiation and early development of a tumour.

An alternative explanation for the methylation differences seen between early stage and late stage epithelial ovarian tumours may be that these are independent, separate disease entities. There remains continued controversy over whether benign, borderline and malignant ovarian tumours are part of a continuous spectrum of disease or if they arise *de novo* (Cvetkovic, 2003). Perhaps this is not always a disease which progresses stepwise from a very early lesion to late stage metastatic cancer. The methylation differences such as those seen above could influence the phenotypic behaviour of the tumour itself and determine its metastatic potential.

Methylation events in non-tumour adjacent tissue

Methylation of *HIC1*, *DCR1*, *APAF-1*, *OPCML* and *RASSF1A* (50%, 38%, 6%, 6% and 6% respectively) was observed in normal adjacent non-tumour tissue. In the case of *RASSF1A*, methylation in the non-tumour tissue could be due to tumour cell infiltration since the matched tumour also showed *RASSF1A* methylation and MSP can detect as little as 0.1% methylated sequence (Herman et al., 1996). However, it is unlikely that this alone can explain the observed methylation frequency of *HIC1*, *DCR1*, *APAF-1* and *OPCML* in non-tumour tissue. Firstly, if methylation in adjacent normal tissue is due to tumour cell infiltration, methylation should also occur in the matched tumour which was not observed in the majority of cases. Secondly, if this is the case, the ranks of methylation frequencies should be similar in tumour and non-tumour matched tissues, with *OPCML* and *RASSF1A* methylation being more frequent in non-tumour tissue than *HIC1* or *DCR1* which is not the case. Perhaps though, the methylation differences seen are due to tumour heterogeneity in

ovarian cancer. It is possible that multiple biopsies of the ovarian tumour samples would yield more information on methylation status than one individual biopsy and corroborate the findings in non-tumour tissue, but again this would seem unlikely to be the main issue here.

An alternative explanation is that methylation of these genes in normal tissues could represent a premalignant epigenetic lesion which is a mediator of a "field defect" in these tissues, although the origin of this field defect remains unknown (Yan et al., 2006). Although perhaps not transforming on their own, epigenetic inactivation of specific genes due to methylation may then be permissive for acquisition of additional genetic and epigenetic changes which ultimately lead to cancer growth. This concept has been shown in sporadic colorectal cancer (Shen et al., 2005) and it has been suggested that detection of this epigenetic lesion may be useful in the risk assessment for colorectal cancer although the idea of a field defect in patients with colorectal adenomas has been disputed by others (Rashid et al., 2001). However, a more recent study examining normal adjacent breast tissue suggested that there is indeed a localised field of *RASSF1A* hypermethylation in both tumours and associated normal tissues, and that premalignant epigenetic changes spreading out from the epicentre of the tumour may be more widespread than currently thought (Yan et al., 2006). Aberrant CGI methylation has previously been observed in normal tissue samples adjacent to several cancers (Eads et al., 2001; Florl et al., 2004; Kanaya et al., 2003; Leung et al., 2001; Yu et al., 2002). In addition, a recent study showed defined epigenetic changes in the stromal cells surrounding breast cancers and suggested that these may play a role in maintaining the cellular microenvironment during breast cancer development (Hu et al., 2005). Previous studies have reported absence of methylation in non-malignant tissues adjacent to ovarian cancer or normal ovarian tissues for *DCR1* and *RASSF1A* (Shivapurkar et al., 2004; Yoon et al., 2001). However, methylation of *HIC1* has been detected in normal breast ductal tissue, normal bone marrow samples and normal cerebellum (Fujii et al., 1998; Lindsey et al., 2004; Melki et al., 1999). Methylation of *HIC1* has also been reported in 19% of non-malignant ovarian tissues from patients undergoing surgery for benign gynaecological disease (Rathi et al., 2002). Therefore, although we did not observe methylation in nOSE or iOSE, the possibility of *HIC1* methylation in nOSE cannot be excluded here.

Another possible explanation of the findings in non-tumour adjacent tissue may also involve the idea of a cancer stem cell population. There is an increasing body of evidence that suggests the cellular and molecular events surrounding the initiation of tumour development are fuelled by mutation of cancer stem cell-like cells (Marx, 2003; Singh et

al., 2003) and that this small subpopulation of stem cells can maintain the cancer phenotype (Singh et al., 2004) in solid tumours. This view has now been extended to encompass the idea that early epigenetic alterations of stem cells may substitute for genetic mutation and be the initiating factor in the carcinogenic process and evolve in normal tissue before the recognised tumour arises (Feinberg et al., 2006). The methylation of non-tumour adjacent tissue in this study which occurs even in the absence of epigenetic change in the corresponding tumour may represent an epigenetic disruption of progenitor cells which leads to cancer through a stepwise process, setting the scene for further epigenetic alterations later. These initial epigenetic events may lead to a polyclonal population of cells which have potential for neoplastic change. If this is the case, then the goal will be detection of these pre-neoplastic epigenetic lesions (further discussed in Chapter 5), defining risk and ultimately treating patients with epigenetic therapies before the tumour mass develops. The caveat is the current technical difficulty in isolating and identifying these cancer stem cells from solid tumours (Hill, 2006).

Potential epigenetic changes within normal ovarian surface epithelium

Although no methylation of the 24 candidate CGIs was detected in 4 nOSE, it would be intriguing to examine a greater number of nOSE samples from female patients with differing clinical histories and pathological diagnoses. There may be many factors which lead to methylation in ovarian surface epithelium. However, the difficulty is obtaining OSE cells and maintaining them in culture, because they are very fragile and easily disrupted at the time of surgical removal while handling the ovary. It has previously been proposed that ovulatory rupture and repair predispose the OSE to mutations (Fathalla, 1971). Therefore, decreasing the number of ovulatory cycles in a female's reproductive lifetime using the oral contraceptive pill (OCP) may potentially reduce the lifetime risk of developing ovarian cancer. It would be interesting to examine the OSE from patients with an OCP history versus those without, allowing us to investigate differences in the methylation status of these tissues.

The effect of aging can potentially alter the epigenetic environment within nOSE. It was firstly reported that a *NotI* restriction site within exon 1 of the oestrogen receptor was methylated in normal colonic mucosa in association with aging (Issa et al., 1994). Findings such as this led to the established idea that some CGIs are methylated in association with aging in normal tissues (Waki et al., 2003). These changes in the epigenetic environment of nOSE associated with the aging process may then increase the risk of malignancy and this has previously been proposed (Chen et al., 2005a).

When examining nOSE from patients, it is also potentially important to consider inflammatory conditions which may alter the epigenetic status of tissues. Chronic inflammation has been shown to be associated with increased methylation. For instance, normal-appearing colonic mucosa of patients diagnosed with ulcerative colitis is associated with increased methylation of the $p16^{INK4a}$ promoter (Hsieh et al., 1998). It has recently been proposed that NFκB may be a key modulator in the link between chronic inflammation and cancer (Dobrovolskaia and Kozlov, 2005; Zaenker, 2006). Further to this, the integration of NFκB cell signaling by proinflammatory cytokines and chemokines with epigenetics has recently been proposed (Vanden Berghe et al., 2006), linking the spectrum of inflammatory changes with epigenetics and cancer risk. The ovary can be subject to a variety of inflammatory insults during its reproductive lifetime including polycystic ovarian syndrome (PCOS), oophoritis secondary to acute or chronic pelvic inflammatory disease (PID) and endometriosis.

Any of these conditions could potentially increase methylation in the nOSE. Further nOSE analyses are essential to avoid using, for example, age-dependently methylated sequences as a tumour marker.

The potential existence of CIMP

It has been shown that concordant promoter hypermethylation of multiple genes, which is known as the "CpG island methylator phenotype" (CIMP), may exist in gastric and colorectal carcinomas (An et al., 2005; Toyota et al., 1999a; Toyota et al., 1999b; Toyota et al., 2000) although there has been some debate regarding the validity of CIMP (Anacleto et al., 2005). However, a recent study using an unbiased genome-wide method of analysis has given the strongest support of its existence to date (Weisenberger et al., 2006). One previous study has examined the concept of CIMP in EOC and suggested that potentially two groups of CIMP-positive tumours may exist (Strathdee et al., 2001). In this previous work, one group exhibited methylation of *HIC1*, *MINT25*, *MINT31* and *P73*, and the second group showed susceptibility to *BRCA1* methylation. The concept of concordant methylation in EOC was addressed in this study using a bioinformatics approach called gene shaving (Hastie et al., 2000) which objectively identifies coherent methylation patterns. A non-random pattern of methylation was observed for CGIs including *OPCML*, *DCR1*, *RASSF1A*, *MINT25*, *HIC1* and *SFRP1* (Teodoridis et al., 2005) which supports the existence of CIMP. This non-random pattern observed could potentially be due to either a specific defect driving active co-methylation of certain genes or through a more passive mechanism. A possible explanation may be that aberrations in the DNMT enzyme

machinery may result in co-methylation of certain genes (De Marzo et al., 1999). There is also modest evidence to suggest that DNMT overexpression or functional polymorphisms are related to CGI methylation in cancer (Teodoridis et al., 2005). The analysis presented shows that a single group of genes were identified to be concordantly methylated. Of the identified loci in this study, only *MINT25* has previously been found to be associated with the CIMP by other authors (An et al., 2005; Kusano et al., 2006; Toyota et al., 1999a), although not in ovarian cancer. This statistical method may represent a new way of identifying genes which are concordantly methylated. Methylation of *MINT25* may be common to the CIMP and allow identification of further genes which are concordantly methylated with *MINT25* and are part of a methylator phenotype in a variety of tumour types. However, it remains unclear what the biological process driving this concordance is. It is possible that the concordant methylation of these 6 genes reflects a difference in methylation propensity or a selective advantage and this will most likely affect more genes than the candidates examined here. Genome wide screening methods in ovarian cancer (Wei et al., 2002) will ultimately identify further methylated loci which have potential to be defined within a CIMP category. This may aid in gathering further evidence regarding whether CIMP exists (1) as a definitive biological lesion, (2) within a spectrum of multiple random epigenetic events or (3) as a combination of both, as the biological significance of the CIMP remains unknown.

Chapter 4

Identification of novel DNA methylation markers of acquired chemoresistance in ovarian cancer

4 Identification of novel DNA methylation markers of acquired chemoresistance in ovarian cancer

4.1 Analysis of a CGI library to identify potential novel chemoresistance markers in ovarian cancer

The initial aim of this part of the project was to identify and characterise novel DNA methylation markers for acquired chemoresistance in ovarian cancer. These could then potentially help identify clinically relevant mechanisms of acquired resistance which would impact on detection of relapsed, chemoresistant disease in patient subgroups and allow stratification of chemotherapy regimes to individual patients. In a collaborative effort with Dr. Tim H.-M. Huang (Ohio State University, Columbus, Ohio) and Dr. Jens Teodoridis (University of Glasgow), a CGI microarray was analysed using Differential Methylation Hybridisation (DMH) for DNA sequences whose methylation state discriminate between cisplatin-sensitive and cisplatin-resistant ovarian surface epithelial cancer cell lines. DMH was used to detect genome wide changes in CGI methylation in DNA from 16 well characterised, matched ovarian cancer cell line models including the cisplatin-sensitive parental cell line A2780, 5 cisplatin-sensitive clonal derivatives (A2780p3, A2780p5, A2780p6, A2780p13 and A2780p14) and 10 cisplatin-resistant cell lines (A2780cp70, MCP1-9). The resistant cell lines were generated by repeated exposures of the A2780 cell line to cisplatin (Behrens et al., 1987; Brown et al., 1997). A schematic description of this method is given in chapter *2.14*. An example of a DMH microarray experiment and a dye-swap for the A2780 cell line is shown below in Figure 16.

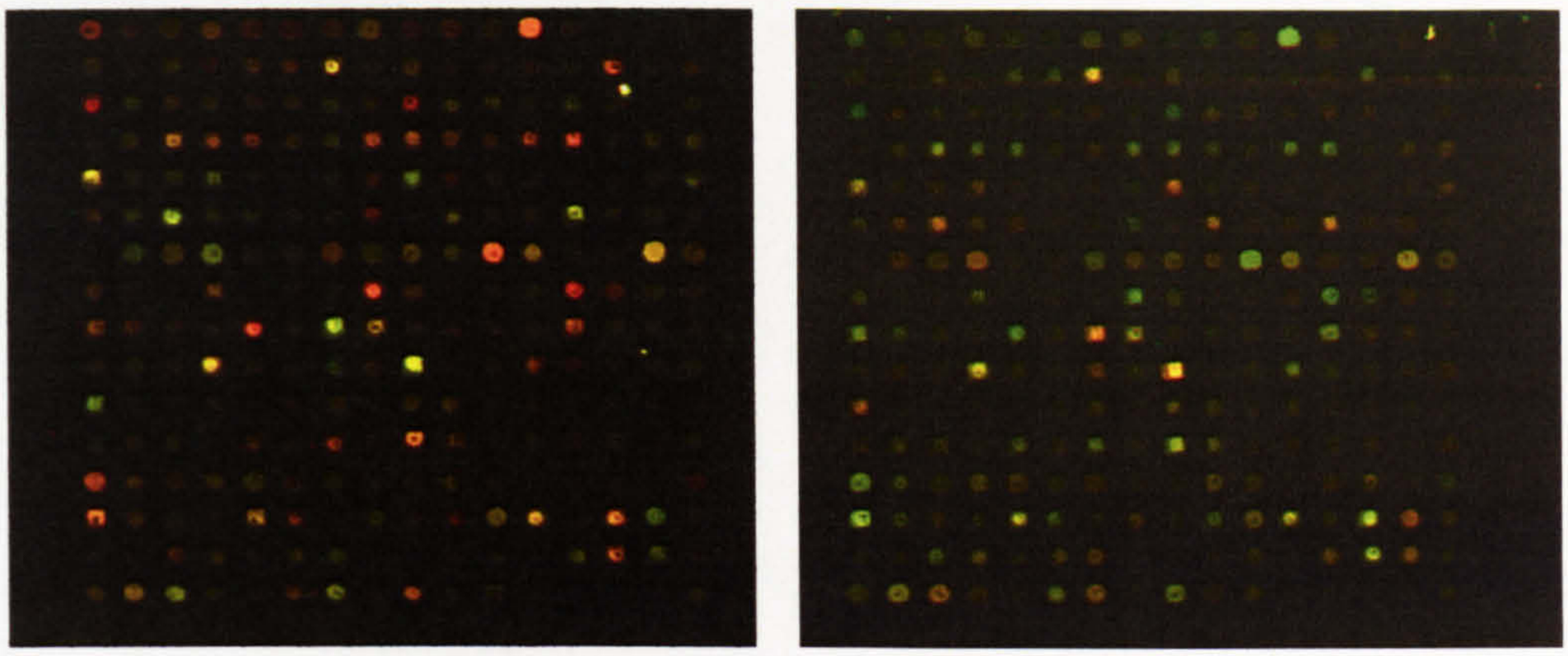


Figure 16 Example of a DMH microarray experiment for the cell line A2780. left: McrBC digested DNA was labelled green (Cy3), mock-digested DNA was labelled red (Cy5); right: dye swap experiment.

Signal intensities were corrected for background intensity and after removal of weak (equal or less than the average plus two standard deviations of background signals) and frequently missing signals from each sample data set, Prediction Analysis for Microarrays (PAM) (Tibshirani and Efron, 2002) was applied to the microarray data using PAM 2.0 for Excel. The results are obtained by gradually removing those sequences which are creating noise by increasing a threshold estimated by cross validation. PAM identified a set of 13 sequences whose methylation states optimally discriminate between cisplatin-sensitive and cisplatin-resistant cell lines. The sequences are shown below in Table 12 and these included 119A6, 66G6, 41D9, 5D4 and 123D9 which are described in more detail later in this chapter. Two sequences did not map to CGIs, one sequence mapped to the 3' region of *AHNAK* and two sequences could not be aligned to the genome. CGIs mapping to *LMX1A* and *SOX12* were identified independently twice. Therefore, DMH identified putative methylation of CGIs located within or in proximity to 7 known genes as shown below in Table 12. Within the listed sequences below, a higher rank indicates a stronger discriminatory sequence.

PAM Rank	Resistant score	Sensitive score	Microarray ID[1]	Chromosome	CGI[2]	Distance from TS[3] (bps)	Gene symbol
1	0.1739	-0.2675	119A6	6q21	Yes	0	NR2E1
2	0.1708	-0.2627	66G6	9q22.32	Yes	-	-
3	0.1141	-0.1756	42D9	no alignment	-	-	-
4	0.0919	-0.1415	41D9	12q13.12	Yes	+82	WNT1
5	0.0872	-0.1342	5D4	1q23.3	Yes	+2021	LMX1A
6	0.0644	-0.0991	6D4[4]	1q23.3	Yes	+2021	LMX1A
7	0.0551	-0.0848	55F8	7q36.3	Yes	-	-
8	0.0476	-0.0732	80H5	no alignment	-	-	-
9	0.0199	-0.0306	39E1	16p13.3	No	+6300	-
10	0.0158	-0.0243	123D9	20p13	Yes	-1055	SOX12
11	0.0115	-0.0177	109A6	11q12.3	Yes	+102006	AHNAK
12	0.0027	-0.0042	122D9[5]	20p13	Yes	-1055	SOX12
13	0.0004	-0.0006	51H8	3p14.2	No	-1033	ZNF312

Table 12 Ranking of DNA sequences which discriminate cisplatin-sensitive and cisplatin-resistant cell lines identified by Prediction Analysis for Microarray (PAM). [1]http://data.microarrays.ca/cpg/searchsingleclones.htm [2]CpG islands are defined as "stretch of DNA of at least 200bp long with at least 50% GC content" (http://data.microarrays.ca/cpg/faq.htm) [3]TS: Predicted transcriptional start site [4]Sequence identical to 5D4. [5]Sequence identical to 123D9.

4.2 Validation of identified sequences using MSP

It was important to validate the results of the DMH analysis using an independent method to detect CGI methylation. The methylation states of these sequences in the cisplatin-sensitive and cisplatin-resistant cell lines were therefore firstly examined using MSP, as described previously in Chapter 3. MSP was performed on the sequences within or immediately upstream of known genes for the top-ranking sequences. 66G6 was included because PAM analysis identified it as the second-strongest individual discriminator between cisplatin-sensitive and cisplatin-resistant cell lines (Table 12). The results which are illustrated in Figure 17 clearly show that no methylation was detectable for either of the two top-ranking sequences, 119A6 or 66G6, in cisplatin-sensitive cell lines but in contrast there was strong methylation in cisplatin-resistant cell lines. For 5D4 and 41D9, weak methylation signals were observed in some of the cisplatin-sensitive cell lines but an overall distinct increase was still observed for these candidate loci. No methylation was detected for 123D9 using several different MSP primer sets and therefore this sequence was removed from further analysis although it should be noted that this was in keeping with the PAM results where it was the weakest discriminator. Although no methylation for 123D9 was detected using MSP, this method will only detect methylation within the primer binding sites, i.e. at specific CpG sites, and there may still be changes in methylation in other regions of 123D9 that are detected by DMH. The MSP results for 119A6, 66G6, 5D4 and 41D9 confirm and corroborate the PAM analysis of the DMH data. In addition to the cell lines described above, a further 6 cisplatin-resistant cell lines (C1cis6, C2cis6, C2E3, c3cis6, C5E4 and C5E4(15)) were included in the MSP validation (Figure 17) which were derived by exposing A2780 cells to a single high dose of cisplatin (McLaughlin et al., 1991). Methylation of 119A6, 66G6, 41D9 and 5D4 was not as frequent in these cell lines compared to the multiply selected cisplatin resistant cell lines but there was still clear evidence of acquisition of methylation of the sequences analysed by MSP following a single cisplatin exposure.

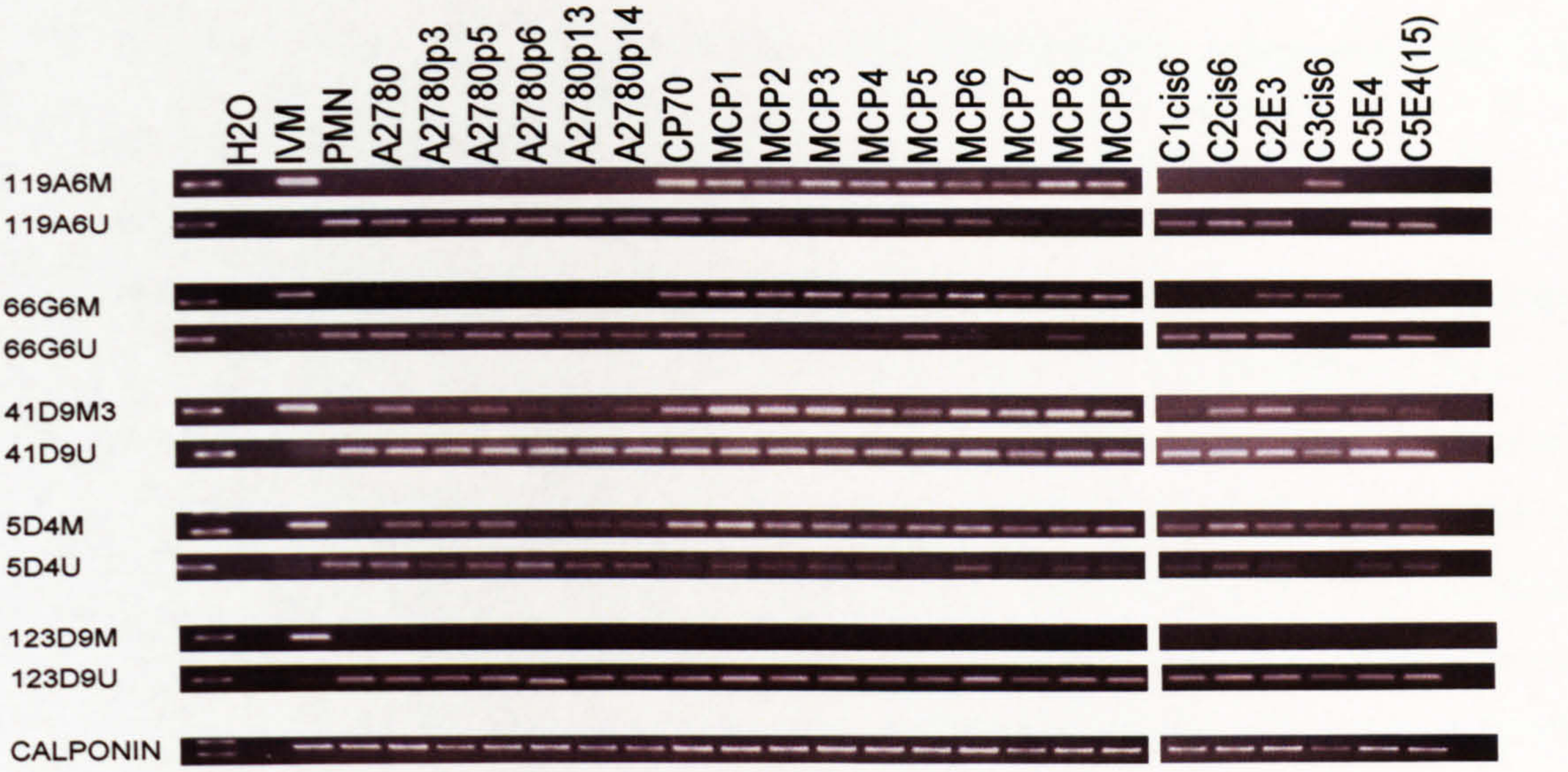


Figure 17 MSP validation of DMH results. Validation using MSP of 119A6, 66G6, 41D9, 5D4 and 123D9 in the cell lines analysed by DMH as well as 6 additional cisplatin resistant cell lines, C1Cis6-C5E4(15). (M), methylated primer set and (U), unmethylated primer set. H_2O, reaction without template DNA; IVM, *in vitro* methylated DNA; PMN, DNA from whole male blood. Amplification of bisulphite modified *CALPONIN* promoter used as a control for successful bisulphite modification, as described previously.

4.3 Methylation frequencies of identified sequences in epithelial ovarian tumours

Methylation of 119A6, 66G6, 5D4 and 41D9 showed a distinct increase in chemoresistant versus chemosensitive cell lines as shown above in Figure 17. The methylation status of these four sequences was therefore next analysed using MSP in DNA from 199 primary epithelial ovarian tumours which included 16 early stage (I/II) and 183 late stage (III/IV) tumours to identify if similar changes could be seen in *in vivo* as *in vitro*, as methylation events in tumours may not necessarily mimic those seen in cell lines. The tumours analysed were comprised of 125 samples from the prospective DNA Methylation Study which were collected frozen from chemonaive tumours undergoing cytoreductive surgery for primary ovarian cancer, and 74 samples from the retrospective study described in Chapter 3. 119A6 and 5D4 were methylated in 12.6% (25/199) and 61.8% (123/199) of the ovarian tumour samples respectively as shown below in Table 13. Methylation frequencies for 119A6 and 5D4 were higher for early versus late stage disease with 119A6 being methylated in 18.8% (3/16) versus 12% (22/183) and 5D4 being methylated in 75% (12/16) versus 60% (111/183) respectively. In contrast, no methylation was identified for 66G6 and 41D9 in 55 of the primary epithelial ovarian tumours, as shown below in Figure 18. This would suggest that methylation of 66G6 and 41D9 is a rare event in primary ovarian tumour specimens. 66G6 and 41D9 were therefore removed from further analysis as rare methylation in primary ovarian tumour samples would reduce their potential usefulness as clinical markers of this disease, although it can not be excluded that methylation of these genes could be selected for during chemotherapy since only chemonaive tumours were examined.

	Methylation frequency of identified sequences			
Samples (N)	**119A6**	**5D4**	**66G6**	**41D9**
Retrospective stage I and II (16)	18.8 (3/16)	75 (12/16)	-	-
Retrospective stage III and IV (58)	10.3 (6/58)	58.6 (34/58)	-	-
Prospective stage III and IV (125)	12.8 (16/125)	61.6 (77/125)	0 (0/55)	0 (0/55)
Overall ovarian tumours (199)	12.6 (25/199)	61.8 (123/199)	0 (0/55)	0 (0/55)

Table 13 Methylation frequencies of identified sequences in epithelial ovarian tumours. Methylation frequency % (number methylated/total number of samples)

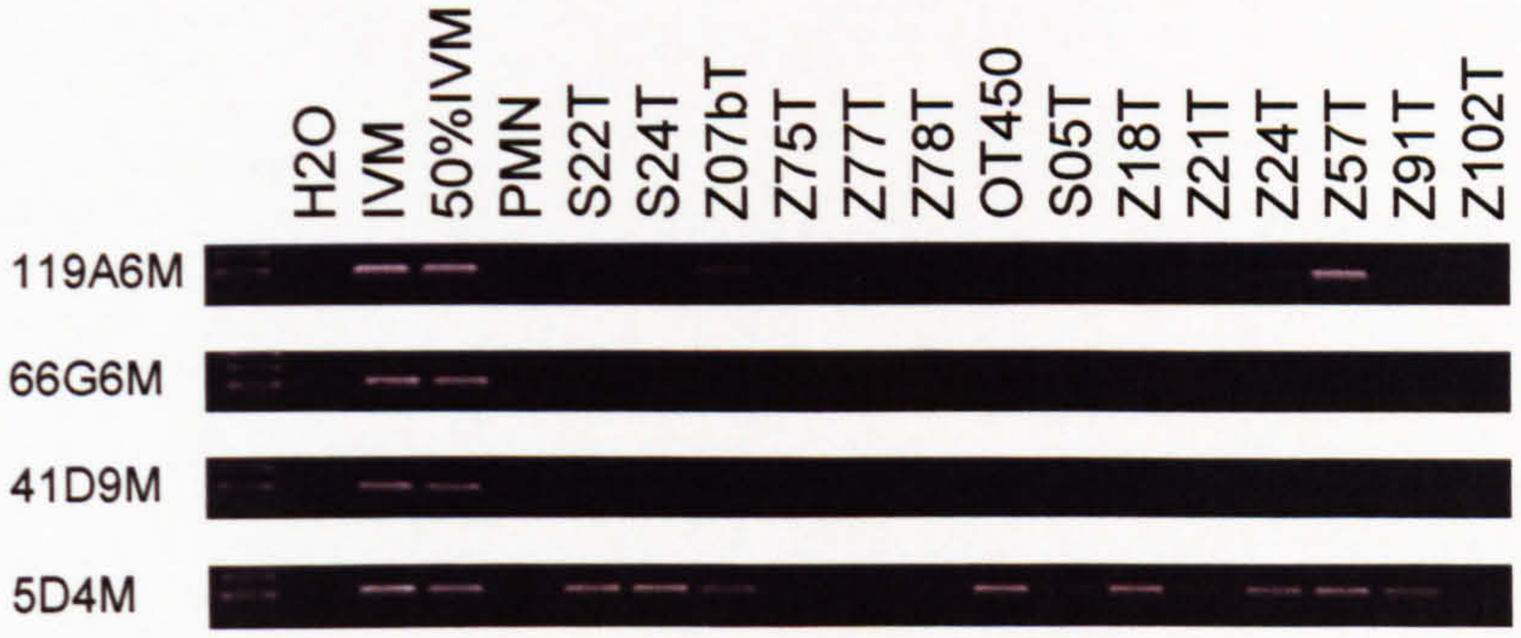


Figure 18 Examples of MSP for 119A6, 66G6, 41D9 and 5D4 in primary epithelial ovarian tumours. Controls included as per Figure 11 including 50% IVM, dilution of 1:2 *in vitro* methylated DNA into PMN. M, methylated primers shown.

Methylation of 119A6 or 5D4 was not detected in 4 normal ovarian surface epithelium (nOSE 1-4) DNA samples. In addition, for the 3 immortalised ovarian surface epithelial (iOSE 1-3) samples analysed, no methylation was detected for 119A6 but 2 out of 3 of the samples (iOSE samples 1 and 3) showed methylation at 5D4 as shown below in Figure 19. In combination, the very frequent methylation seen in primary ovarian epithelial tumours (61.8%) and iOSE (67%), but not mortal OSE, may suggest that methylation of 5D4 is an early event in the development of ovarian cancer.

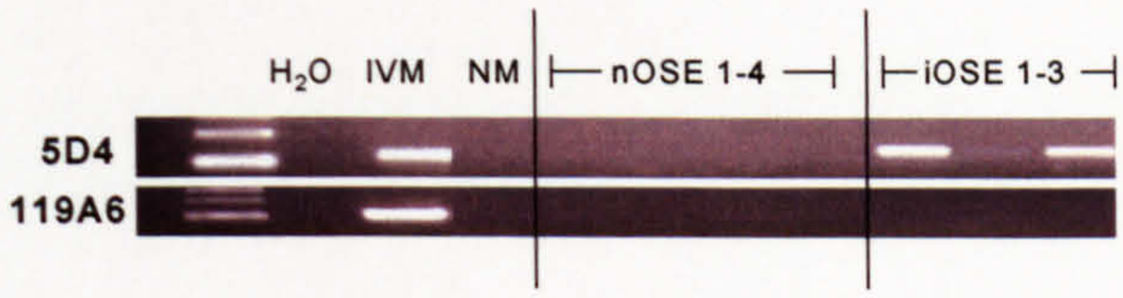


Figure 19 MSP: 5D4 and 119A6 in ovarian surface epithelial samples. Controls included as above. 4 normal mortal ovarian surface epithelium samples (nOSE) and 3 immortalised ovarian surface epithelial samples (iOSE) shown. Methylation identified in iOSE samples 1 and 3 for 5D4.

As described above, the initial DMH study identified 119A6 and 5D4 as being sequences whose methylation states could discriminate cisplatin-selected from parental cell lines. In the first instance, MSP was used to analyse a set of 12 matched pairs of ovarian tumours biopsied from patients both before and after platinum-based chemotherapy (samples kindly provided by the University Medical Centre Groningen, The Netherlands). Residual tumour that was present after completion of chemotherapy was considered to be indicative of cisplatin-resistance where the tumour may be expected to be enriched for resistant cells and analysis of this allowed a direct comparison with the *in vitro* model described above in the validation set. Using MSP, methylation of 119A6 was increased in 33% (4/12) of paired tumours post-chemotherapy (Figure 20: paired samples 1, 2, 5 and 12) which is in keeping with the idea of 119A6 being a marker of chemoresistance. Histological analysis showed that the percentage of tumour cells in all paired samples were almost equal (except paired samples 4 and 5) which would indicate that the obvious increase in methylation seen in 119A6 post-chemotherapy is not due to a quantitative increase in tumour cells following treatment or simply enrichment of tumour cells after chemotherapy. In contrast to 119A6 though, there was no consistent change in methylation of 5D4 following chemotherapy when examined with MSP. Frequent methylation was seen in both pre- and post-chemotherapy samples for this sequence (Figure 20: paired samples 1, 2, 4, 5, 8, 11 and

12). This is in keeping with the overall frequent methylation seen previously in primary epithelial ovarian tumours for 5D4 but not with the theory that 5D4 may be a marker of chemoresistance as was originally hypothesised.

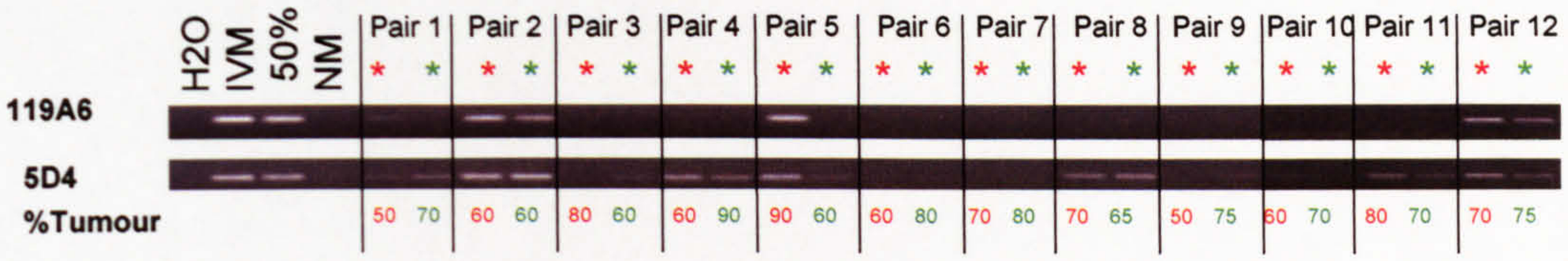


Figure 20 Methylation changes in chemonaive versus residual disease. Methylation of 119A6 can increase in residual disease after chemotherapy(*) compared to chemonaïve(*) tumours whereas methylation of 5D4 is similar in both. Tumour percentage by histological analysis is shown (% Tumour).

However, it was important to quantitatively analyse the methylation differences between the paired samples. Pyrosequencing technology was used to address this for both 119A6 and 5D4. As shown below in Figure 21 and 22, 7 and 5 individual CpG sites within the MSP product sequences for 119A6 and 5D4 respectively were analysed using pyrosequencing technology, (for method, see Chapter *2.16*), for each of the 12 paired samples. Background CpG methylation was determined by pyrosequencing of DNA from male whole blood, as shown in Figures 21B and 22B (dotted line). This allowed normalisation of each result to this background level of methylation seen in whole male blood (unmethylated control) and eliminated any "noise". For 119A6 and 5D4, there was a background "noise" level of 3% and 10.3% respectively. 119A6 pyrosequencing showed a quantitative strong increase in methylation over the 7 CpG sites in 4/12 (33.3%) paired samples following chemotherapy (pairs 1, 3, 4 and 5) as shown in Figure 21. 5D4 pyrosequencing showed a quantitative strong increase in methylation over the 5 CpG sites in 3/12 (25%) paired samples following chemotherapy (pairs 4, 5 and 10) as shown in Figure 22. The quantitative increase in methylation seen for each of the CpG sites which were pyrosequenced for 119A6 and 5D4 are tabulated below in Table 14.

(A) 119A6: % CpG methylation at each loci		CpG 1	CpG 2	CpG 3	CpG 4	CpG 5	CpG 6	CpG 7
Pair 1	Chemonaive	0	10.1	7.9	3.3	0	2.4	0
	Residual disease	12.5	50.2	20.2	17.5	17.4	2.1	56.2
Pair 3	Chemonaive	0	0	3.9	0	0	0	0
	Residual disease	8.2	36.5	38.6	3.5	30.1	25.1	45.9
Pair 4	Chemonaive	0	22.4	6.8	13.6	13.6	0	0.5
	Residual disease	16.8	41.7	28.3	24.3	30.1	14.3	24
Pair 5	Chemonaive	0	7.4	2.3	2	0	0	7.3
	Residual disease	27	42.8	36.4	31.1	33.9	23.6	45.4

(B) 5D4: % CpG methylation at each loci		CpG 1	CpG 2	CpG 3	CpG 4	CpG 5
Pair 4	Chemonaive	12.7	11.7	7.1	15.6	37.8
	Residual disease	19.9	11.8	5.0	30.9	76.4
Pair 5	Chemonaive	6.6	10.7	6.3	12.3	15.7
	Residual disease	52.1	50.9	43.7	53.2	63.2
Pair 10	Chemonaive	4.1	9.1	3.1	8.4	17.9
	Residual disease	19.3	27.7	6.2	23.2	41.8

Table 14 Pyrosequencing CpG methylation percentage at each individual CpG site. Paired samples which showed a quantitative overall increase in methylation in residual disease compared to chemonaive tumours for **(A)** 119A6 and **(B)** 5D4. Values for each individual CpG site are shown.

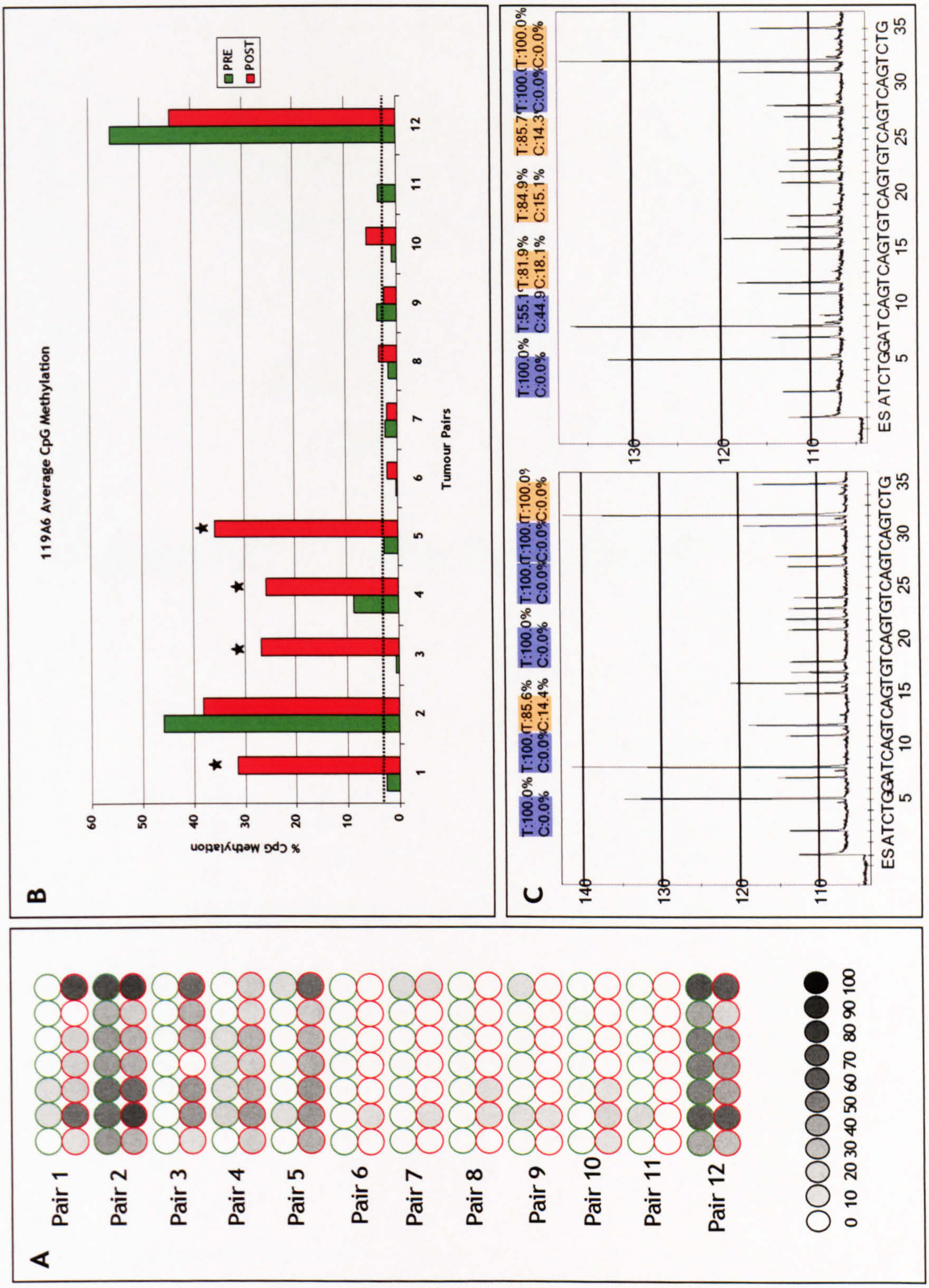


Figure 21 Pyrosequencing analysis of 119A6 in 12 paired chemonaive tumours (green) and residual disease following chemotherapy (red). (A) Methylation average (%) of 7 individual CpG sites from at least 2 independent experiments. Grey scale depicts % of methylation. **(B)** Average CpG methylation over 7 sites for each matched pair. Dotted line indicates background methylation in DNA from whole male blood of 3%. *Increase in methylation seen in residual disease samples 1, 3, 4 and 5. **(C)** Examples of pyrograms from pyrosequencing analysis of matched Pair 1 of chemonaive (left) and residual disease (right) ovarian tumours.

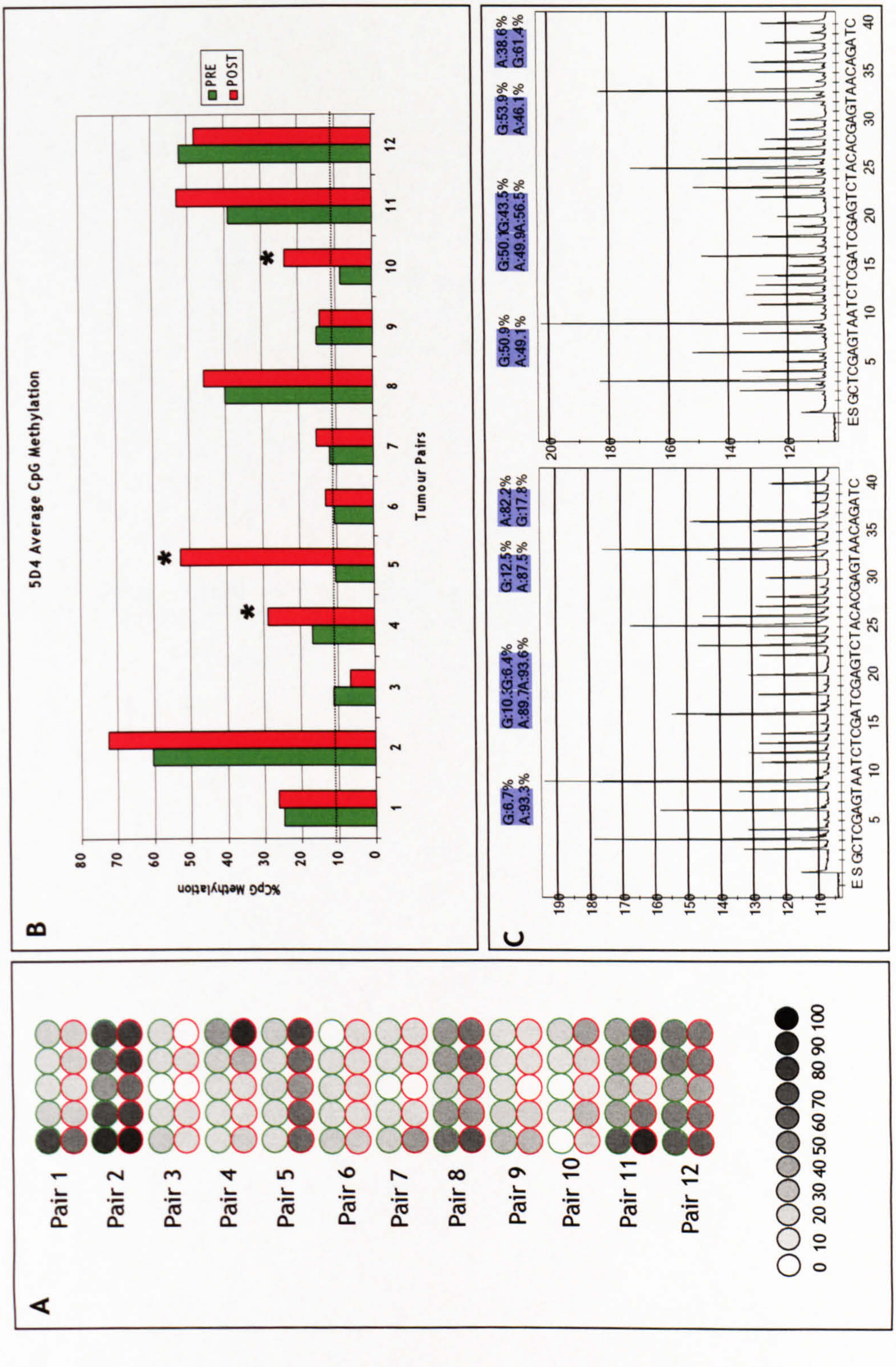


Figure 22 Pyrosequencing analysis of 5D4 in 12 paired chemonaive tumours (green) and residual disease following chemotherapy (red). (A) Methylation average (%) of 5 individual CpG sites from at least 2 independent experiments. Grey scale depicts % of methylation. **(B)** Average CpG methylation over 5 sites for each matched pair. Dotted line indicates background methylation in DNA from whole male blood of 10.3%. *Increase in methylation seen in residual disease samples 4, 5 and 10. **(C)** Examples of programs from pyrosequencing analysis of matched Pair 5 of chemonaive (left) and residual disease (right) ovarian tumours.

In summary, the findings above would suggest that 119A6 and 5D4 may be important novel DNA methylation markers of acquired chemoresistance due to the acquisition of methylation in both the paired residual tumours and cell lines. In addition, the 5D4 locus is very frequently methylated in chemonaive primary ovarian tumours and iOSE specimens, and therefore in addition to its potential role as a marker of acquired resistance, it may also be a marker for the early detection of ovarian cancer. To further strengthen their potential role as biomarkers, methylation of 5D4 or 119A6 is not observed in nOSE and this suggests that these sequences are preferentially methylated in tumour compared to non-tumour ovarian surface epithelium. This is a crucial finding when considering a methylated locus as a potential clinical marker.

4.4 Bisulphite sequencing of 119A6, 5D4 and *LMX1A*

As discussed in Chapter *3.1*, MSP is not an assay which can be used to quantitatively measure density of CGI methylation. For this reason, bisulphite sequencing was used to compare the differences in methylation density between the cisplatin-sensitive and cisplatin-resistant cell lines, and corroborate the MSP findings. The identification of specific regions which showed quantitative differences in methylation could then be used to identify specific CpG sites for Pyrosequencing analysis in the matched tumour samples. 119A6 maps to the 1st exon /transcriptional start site of the human gene encoding for *NR2E1*, a nuclear receptor, and it is located within a CGI (Figure 23). 5D4 is 2kB downstream of the transcriptional start site of the human gene which encodes for *LMX1A*, a LIM box transcription factor, but importantly it maps to a CGI that extends into the promoter region of *LMX1A*, as shown below in Figure 23.

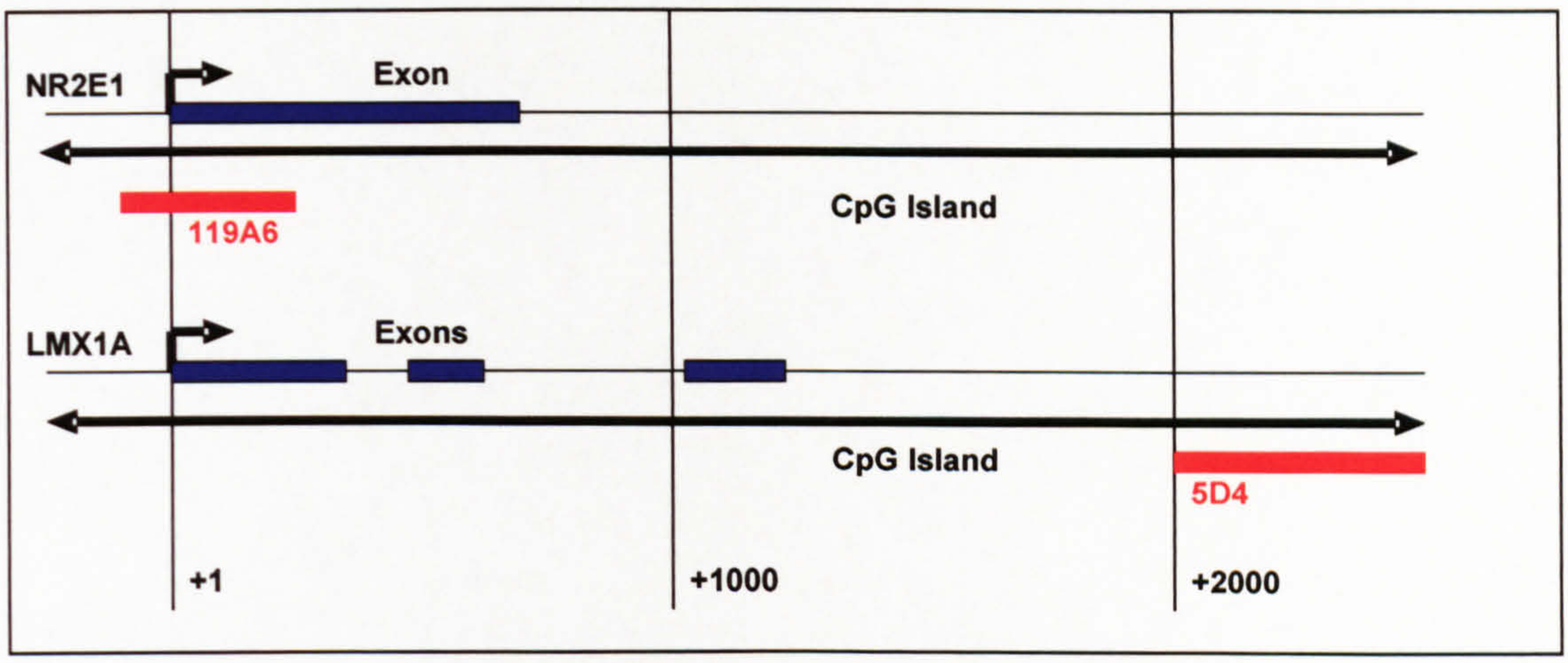


Figure 23 Genomic positions of 119A6 and 5D4. The positions of 119A6 and 5D4 in relation to the predicted transcriptional start sites of *NR2E1* and *LMX1A*, respectively.

Sodium bisulphite sequencing of 5 cell lines (A2780, A2780p6, MCP1, MCP6 and MCP9) described in the MSP validation experiment above was performed to quantify the DNA methylation status of 3 regions: 119A6 (within the CpG island of *NR2E1*), 5D4 and the region which overlaps the transcriptional start site of *LMX1A*. The latter region was analysed to see if the methylation status at 5D4 is associated with the methylation status of the DNA region around the predicted transcriptional start site of *LMX1A*. The cell lines examined were 2 cisplatin-sensitive cell lines (A2780 and A2780p6) and 3 cisplatin-resistant cell lines (MCP1, MCP6 and MCP9). 119A6 was sequenced from 797bp

upstream of the predicted transcription start site of *NR2E1* (sequence length 1494bp) and quantified the methylation status of 102 CpGs in this region. *LMX1A* was sequenced from 595bp upstream of its predicted transcription start site (sequence length 971bp) and quantified methylation of 82 CpGs. The region surrounding 5D4 was also examined which was 2021bp downstream of the predicted transcription start site of *LMX1A* (sequence length 610bp) and included 36 CpGs.

As described in Chapter *2.15*, successful transformants (white colonies) were selected by genetic selection using blue:white colony screening as shown in Figure 24 and submitted for sequencing. Complete sequencing of ≥10 colonies was included for each region and the methylation density of each individual CpG was rounded to the nearest increment of 10%.

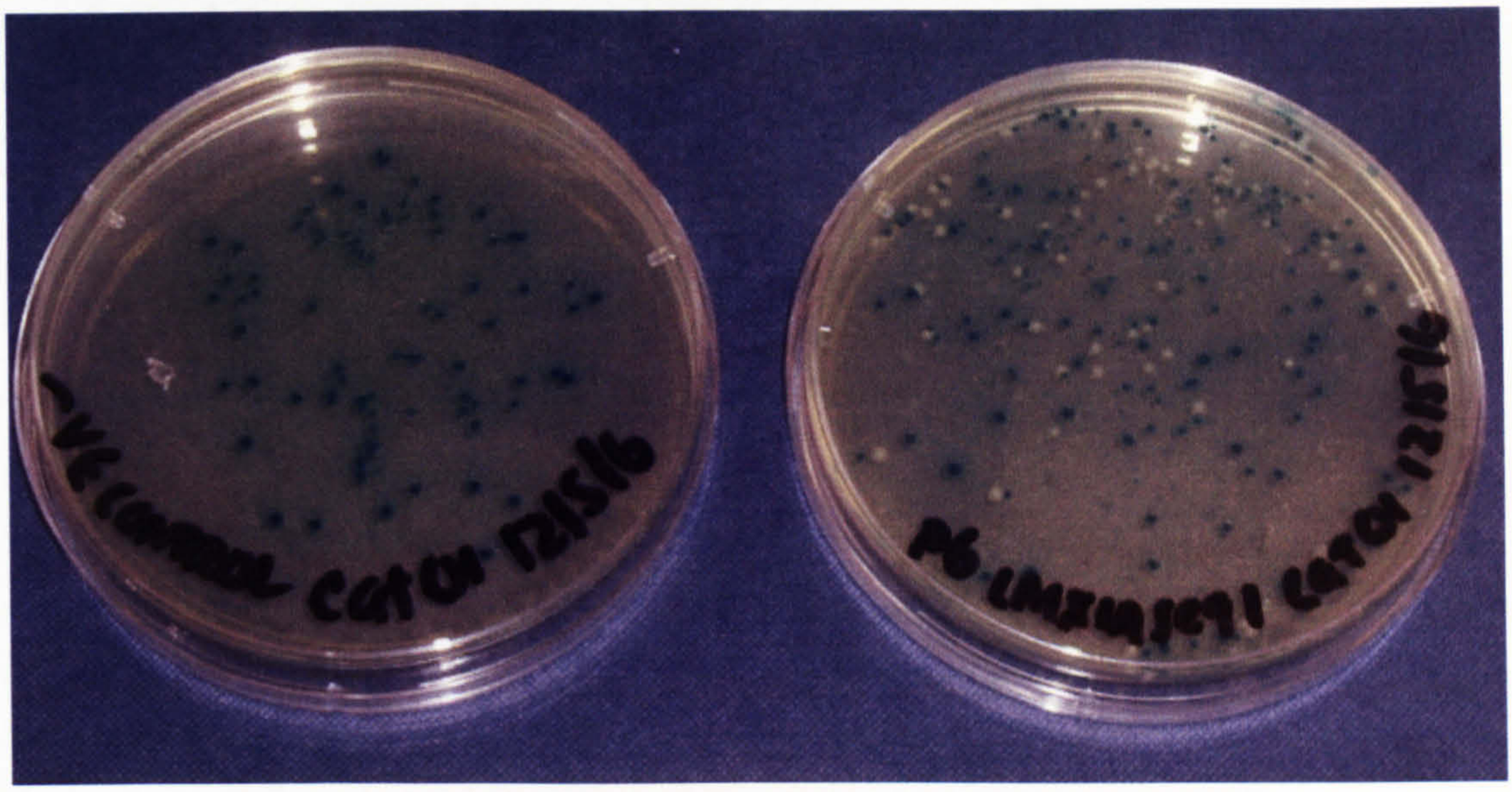


Figure 24 Blue;white colony screening for sodium bisulphite sequencing. This screening method relies on disruption of the LacZα gene resulting in uncleaved X-Gal and white colony formation in successful transformants. Left plate (–ve control plate): only blue colonies grown. Right plate: both blue and white colonies grown.

The bisulphite sequencing results of the 3 loci examined are shown below in Figure 25. Bisulphite sequencing showed distinct changes in the overall methylation density of all 3 loci when comparing cisplatin-sensitive versus cisplatin-resistant cell lines which further confirms the DMH and MSP results. Sequencing of *NR2E1* confirmed the differences observed in the validation MSP experiment (Chapter *4.2*). There was dense methylation of CpG sites in the cisplatin-resistant cell lines compared to low levels of methylation in the

cisplatin-sensitive cell lines but the bisulphite sequencing results showed that the density of methylation was not distributed evenly throughout the DNA regions examined. The methylation differences were more pronounced in the region flanking the predicted transcriptional start site of *NR2E1*. The methylation differences were even more apparent downstream of the transcriptional start site of *NR2E1* where methylation increased in the cisplatin-resistant cell lines. Average methylation densities of 119A6 were 5.6% in the cisplatin-sensitive cell lines which increased to 53.7% in the cisplatin resistant cell lines. In both the 5D4 and *LMX1A* sequences, these differences were certainly not as obvious. For *LMX1A*, stronger methylation was seen in the cisplatin-resistant versus cisplatin-sensitive cell lines although there was a region downstream of the transcriptional start site (CpG 52-82) where the differences in methylation density were less pronounced. 5D4 showed similar results in that although more methylation was seen in resistant cell lines, there were not the same distinctive differences as seen in the *NR2E1* sequence. Average methylation densities of 5D4 and the region overlapping the predicted transcriptional start site of *LMX1A* were 18.1% and 14.1% respectively in the cisplatin-sensitive cell lines which increased to 59.1% and 33.5% in the cisplatin resistant cell lines respectively.

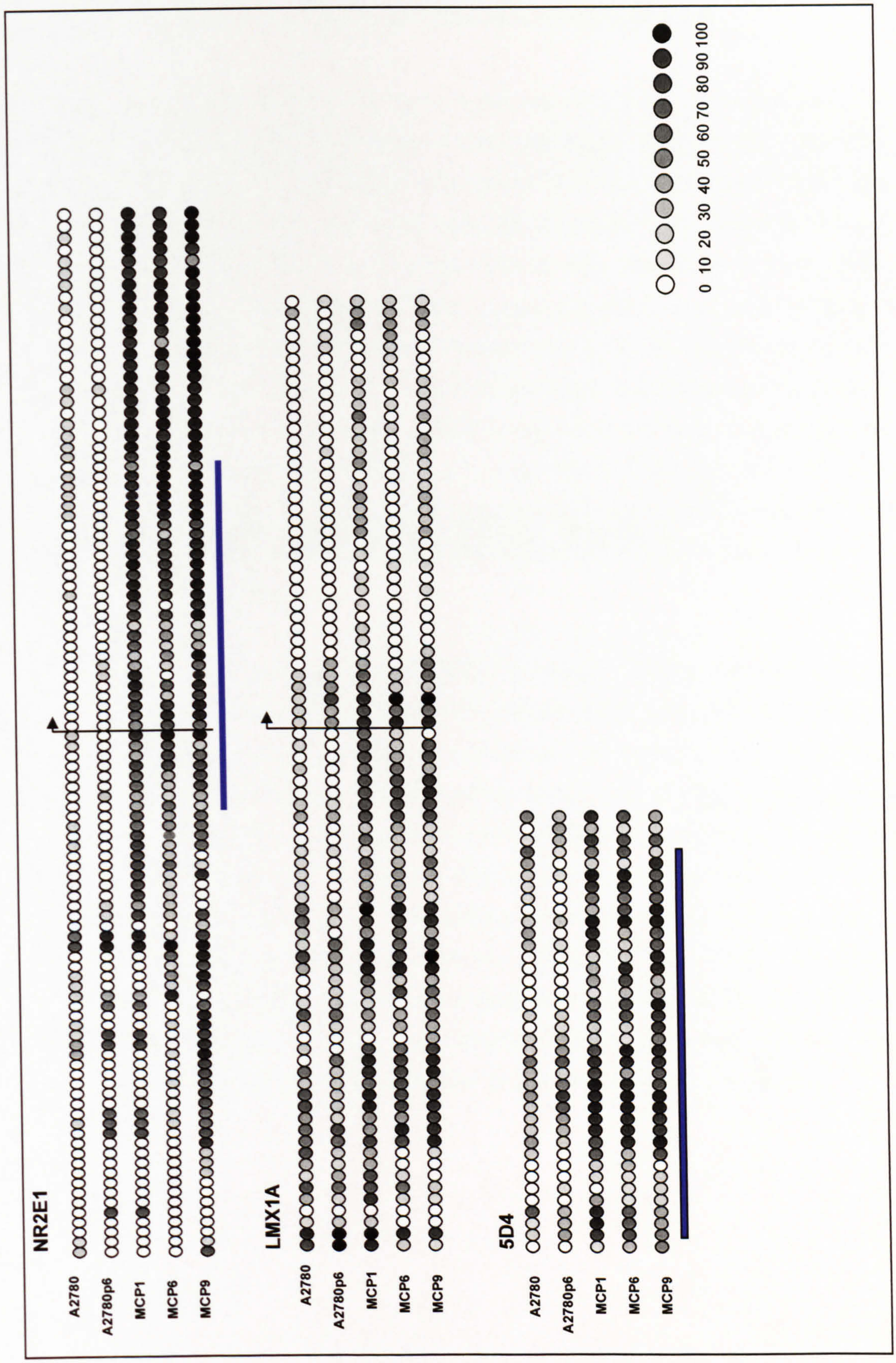
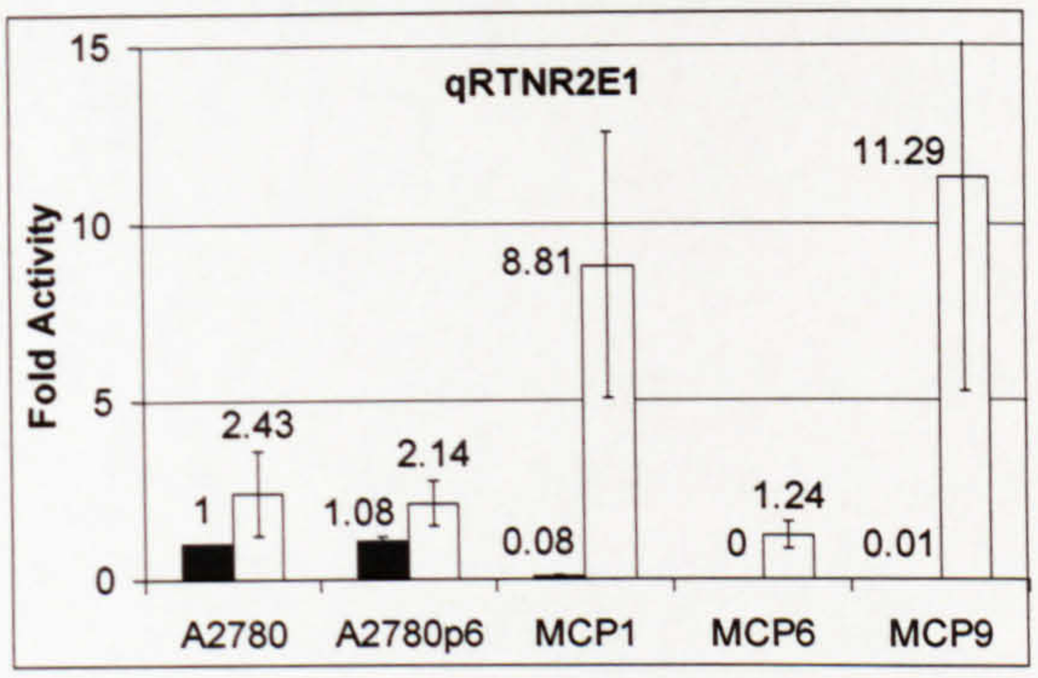


Figure 25 Bisulphite sequencing in ovarian cancer cell lines. Sodium bisulphite genomic sequencing of A2780, A2780p6, MCP1, MCP6 and MCP9 to quantitatively assess methylation densities of the loci 119A6 (top panel), the region which overlaps the transcriptional start site of *LMX1A* (middle panel) and 5D4 (bottom panel). Grey scale depicts % of methylation. Transcriptional start site of *NR2E1* and *LMX1A* shown by arrows. DMH locus shown in blue.

4.5 Expression profiling of *NR2E1* and *LMX1A*

CGI methylation within promoter regions of genes has been shown to be associated with transcriptional repression of the reciprocal gene (Bird and Wolffe, 1999). The RNA expression levels of *LMX1A* and *NR2E1* were quantified in the cisplatin-sensitive cell lines A2780 and A2780p6, and the cisplatin-resistant cell lines MCP1, MCP6 and MCP9 using qRT-PCR (see Chapter *2.20*), in collaboration with Dr. Jens Teodoridis. As shown below in Figure 26, increased methylation of 119A6 (mapping to *NR2E1*) and 5D4 (mapping to *LMX1A*) in cisplatin-resistant cell lines (MCP1, MCP6 and MCP9) compared to cisplatin-sensitive lines (A2780 and A2780p6) was associated with reduced expression levels of both *NR2E1* and *LMX1A*, respectively. qRT-PCR was used to quantitatively examine the expression levels by measuring mRNA levels in the five cell lines for both *NR2E1* and *LMX1A* before and after treatment with the demethylating agent decitabine (DAC). This was calculated successively in three individual experiments to verify values obtained, as shown in the graphs on Figure 26.

Figure 26 shows that treatment of MCP1, MCP6 and MCP9 with the demethylating agent DAC led to re-expression of *NR2E1* and *LMX1A* in these cell lines. qRT-PCR confirmed that *NR2E1* mRNA levels in the cisplatin-resistant cell lines (MCP1, MCP6 and MCP9) were reduced by at least 92% in comparison to the mRNA level of *NR2E1* in the cisplatin-sensitive cell line, A2780. This suggested that methylation of *NR2E1* was associated with reduced mRNA expression in cisplatin-resistant cell lines. The differences in the expression levels of *LMX1A* following treatment with decitabine are less pronounced if comparing the cell lines A2780 and A2780p6 with MCP1, MCP6 and MCP9. This may be because 5D4 and the region which flanks the predicted transcriptional start site of *LMX1A* already show methylation even in the cisplatin sensitive cell lines. However, after treatment with decitabine, an obvious increase in expression of *LMX1A* is observed.



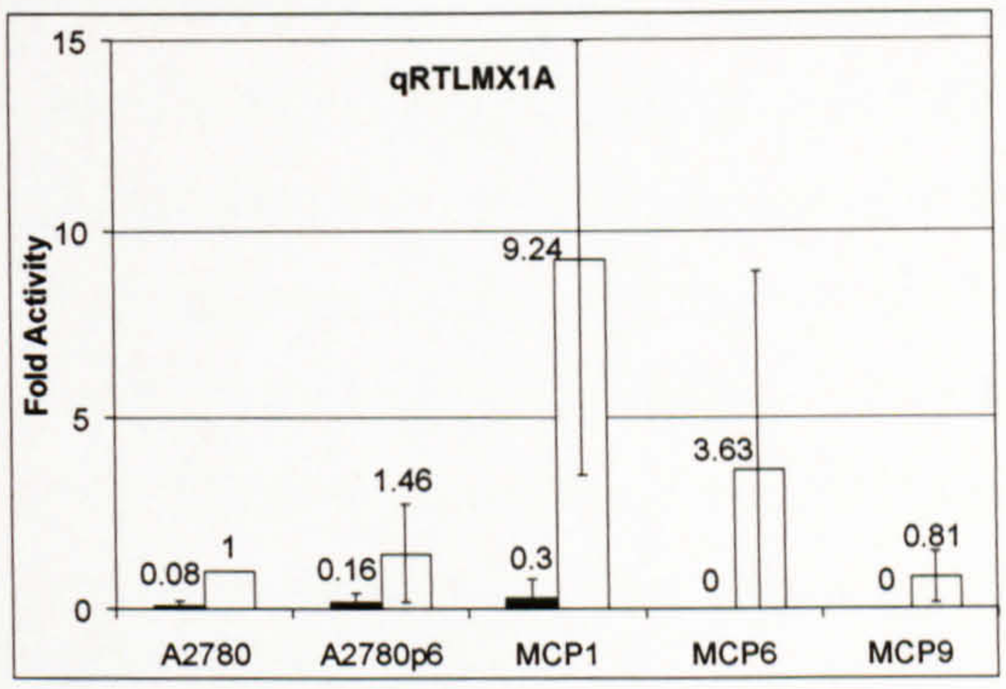


Figure 26 Quantitative RT-PCR values for *NR2E1* and *LMX1A*. qRT-PCR was performed with primers amplifying *NR2E1* (left) and *LMX1A* (right) cDNA. *GAPDH* qRT-PCR was used for normalisation and values are from 3 independent experiments. Values represent averages ± standard deviation. Filled bars: untreated cells, open bars: DAC-treated cells. (DAC = Decitabine)

4.6 Discussion

Successful treatment of patients with advanced ovarian malignancy lies not only in the surgical and medical management of the initial presenting tumour mass, but also in the management of acquired drug resistance in the relapsed patient (Vasey, 2005). Identifying markers of chemoresistance will enable clinicians to optimise chemotherapeutic regimes to individual patients and personalise therapy. Further to this, identification of novel CGI methylation markers may allow enrichment for patients who will benefit greatly from epigenetic treatments that lead to reversal of chemoresistance (Lyko and Brown, 2005). DMH was used as a discovery approach to identify genome-wide differences in CGI methylation in a panel of 6 cisplatin-sensitive and 10 cisplatin-resistant well characterised, matched, human ovarian cancer cell lines. PAM (Tibshirani and Efron, 2002) identified 13 sequences whose methylation states optimally discriminate between cisplatin-sensitive and cisplatin-resistant cell lines, and the validation using MSP which was carried out on five of these sequences (119A6, 66G6, 41D9, 5D4 and 123D9) agreed with the PAM analysis. 119A6 and 66G6 showed a strong methylation signal in cisplatin-resistant versus cisplatin-sensitive cell lines. 41D9 and 5D4 showed weak methylation signals in some of the cisplatin-sensitive cell lines but an overall increase was still observed in the resistant lines. Methylation of 123D9 could not be detected and this may reflect differences in the DMH and MSP methodologies. However, this sequence has little discriminatory power according to PAM analysis. In the validation work, 41D9 and 66G6 showed no methylation signal in primary ovarian tumours. Therefore, the resulting work in Chapter 4 focussed on 5D4 and 119A6 as potential markers of cisplatin resistance.

5D4 was methylated in 61.8% of primary ovarian tumours overall, and interestingly, frequent methylation was observed in both early (75%) and late stage (60%) disease. This not only indicates that 5D4 is frequently methylated in ovarian cancer but also it can be detected frequently in early stage carcinogenesis (stage I and II), which has implications for its use in early detection, as applied and discussed in Chapter 5. The high methylation frequency was further emphasised when comparing results with those of Chapter 3. In this previous study, MSP identified aberrant methylation changes of 24 candidate CGI loci in a group of epithelial ovarian tumours. It was important to use the same stringent acceptance criteria for the MSP method in order to directly compare both studies. 5D4 methylation frequency in Stage I-IV disease was higher than that of *OPCML* (37.4%) (Teodoridis et al., 2005), and is comparable to one of the highest reported methylation frequencies in the literature for ovarian cancer (Sellar et al., 2003). In agreement with the above findings,

frequent methylation of the CGI of *LMX1A* has been observed by others. *LMX1A* has been shown to be differentially methylated in HCT116 wild-type versus DNMT1/DNTMT3b knockout (DKO) cells (Hu et al., 2005; Paz et al., 2003). Colorectal adenoma and carcinoma showed methylation of this CGI (42% and 55% respectively) (Paz et al., 2003) and a potential role for CGI methylation of *LMX1A* in tumourigenesis was suggested, but not in drug resistance. Methylation of this CGI has also been reported in myeloid leukaemic cell lines using a novel profiling method (Gebhard et al., 2006) which is one of the largest published lists of potentially methylated genes. Encouragingly, there was no methylation identified in mortal ovarian surface epithelium samples but interestingly, methylation was seen in 66.7% of immortalised ovarian surface epithelium samples. This further emphasises that 5D4 may play a role as an early event in the initiation of ovarian cancer due to these changes which are seen in immortalised cells. Methylation of *LMX1A* was also shown to be associated with transcript loss (Paz et al., 2003). This is in keeping with the qRT-PCR results here where methylation of 5D4 is associated with transcriptional silencing.

5D4 maps to a CGI which extends into the promoter region of a human gene known as *LMX1A*. This gene encodes a LIM-box transcription factor involved in roof plate formation and brain development during murine development (Millonig et al., 2000). In chick developing spinal cord, LMX1A has been shown to induce expression of Wnt1 (Chizhikov and Millen, 2004). This is interesting because in the DMH screen, methylation of 41D9, mapping to a CGI located at the *WNT1* promoter, was identified in the cisplatin-resistant cell lines. Alterations in the canonical Wnt signalling pathway have been implicated in the pathogenesis of a variety of tumour types (Duan et al., 2006; Simon et al., 2005). Overexpression of β-catenin has been suggested to play a role in ovarian cancer (Rask et al., 2003; Wang et al., 2006a) although the specific molecular alterations of the Wnt pathway which are involved remain to be elucidated. Epigenetic silencing of *LMX1A* could be an early event which may influence other epigenetic and genetic pathways, causing a tumour to addict itself to the oncogenic Wnt pathway (Baylin and Ohm, 2006; Weinstein, 2002). The methylation changes seen in drug-resistant cell lines also suggest that alterations of the Wnt pathway may play a role in drug resistance mechanisms. Normally, Wnt1 overexpression favours tumour development, but the results of this thesis suggest that lack of methylation of *LMX1A* and *Wnt1* are associated with chemosensitivity. A similar situation exists in the BRCA1 pathway, where inactivation of its components (BRCA1, FANCF) favour tumourigenesis but are associated with increased chemosensitivity (Taniguchi et al., 2003). It may be that LMX1A may be sufficient but not necessary for Wnt1 expression (i.e. overexpression of LMX1A will lead to an increase in

Wnt1 expression, but a reduction in expression of LMX1A through epigenetic mechanisms will not reduce Wnt1 expression). Potentially, it could be envisioned that other transcription factors compensate for the loss of LMX1A at the *WNT1* promoter and maintain its expression, contributing to tumour development and/or chemoresistance. Epigenetic silencing of *LMX1A* could then be involved in tumourigenesis and factors relating to chemoresistance via a Wnt1 independent mechanism. LMX1A also induces *MSX1*, which in turn interacts with the p53 tumour suppressor and inhibits tumour growth by induction of apoptotic mechanisms (Park et al., 2005). It has been shown that overexpression of *MSX1* in OVCAR3 ovarian tumour cells inhibits cell proliferation by increasing the length of the G1 phase of the cell cycle (Park et al., 2001). This further emphasises the potential of this pathway to suppress growth of ovarian tumour cells and its role in chemotherapy induced apoptosis.

119A6 maps to a CGI overlapping the first exon of the human *NR2E1* gene which encodes an orphan nuclear receptor and was methylated in 12.6% of primary ovarian tumours. This gene is the human homologue of murine *TLX*, mapping to a region of common deletion in human lymphoid leukaemia on chromosome 6q21 and may function as a tumour suppressor gene (Jackson et al., 1998). The results above show that methylation of the 119A6 locus is associated with transcriptional repression of the associated gene. This is the first report of an epigenetic mechanism regulating the transcriptional status of this nuclear receptor although methylation of other nuclear receptors including retinoic acid receptor alpha2 and IL2 have been reported previously (Farias et al., 2002; Misawa et al., 2005).

5D4 and 119A6 represent potential novel loci for the study of chemoresistance markers in ovarian cancer. 5D4 and 119A6 showed an increase in methylation of 25% (3/12) and 33.3% (4/12) respectively when comparing post-chemotherapy to chemonaive tumour samples. This makes them promising epigenetic markers of cisplatin-resistance in relapsed disease. This increase in methylation at the time of relapse has also been reported for other markers such as *hMLH1* (Gifford et al., 2004).

The relationship between the abnormal expression of transcription factors and drug resistance has been the focus of various studies and emerging evidence suggests that drug resistance in cancer can arise from elaborate gene expression of multiple transcription factors. For example, inhibition of *PAX2* (which is frequently overexpressed in renal carcinoma) with antisense-cDNA can enhance cisplatin-induced apoptosis in renal carcinoma cells (Hueber et al., 2006), activation of *NFκB* can mediate paclitaxel resistance in ovarian cancer cell lines (Mabuchi et al., 2004), overexpression of *Ets-1* results in

increased cisplatin resistance in the ovarian cancer cell line 2008 (Wilson et al., 2004) and overexpression of *BARX2* leads to increased sensitivity against cisplatin in the ovarian cancer cell line PEO1 (Sellar et al., 2002). Currently though, the relevance of these mechanisms to the development of drug resistance in patients remains unclear.

TLX, the murine homolog of NR2E1, is an upstream regulator of *PAX2* (Yu et al., 2000) and suppresses *PAX2* expression in mice. This is intriguing because, firstly, the human neuronal apoptosis inhibitory protein (*NAIP*) gene promoter has been shown to be a downstream target of PAX2 which then suppresses apoptosis in the developing kidney (Dziarmaga et al., 2006). In addition, downregulation of *PAX2* can enhance cisplatin sensitivity (Hueber et al., 2006), and therefore methylation and silencing of *NR2E1* may increase chemoresistance by increasing *PAX2* expression in tumours and inhibiting cisplatin-induced apoptosis through binding to *NAIP*. PAX2 has also been shown to activate *WNT4* gene expression (Torban et al., 2006), and therefore could also be potentially involved in regulatory mechanisms of the Wnt signalling pathway in carcinogenesis, although any involvement in drug resistance mechanisms remains to be investigated. TLX can also regulate *PTEN* and other genes involved in signalling pathways, including TGFβ and MAPK signalling in the mouse retina (Zhang et al., 2006). TLX is required for development of the retina (Yu et al., 2000; Zhang et al., 2006) and brain including the dentate gyrus (Monaghan et al., 1997) and the subventricular zone (Shi et al., 2004). Stem cells are located in these regions and indeed TLX-positive neuronal cells show stem cell-like properties in their ability to self-renew and are multipotent (Shi et al., 2004).

It has previously been proposed that acquired drug resistance following chemotherapy can be due to either the survival and growth of chemoresistant subpopulations initially present in heterogeneous chemonaive tumours, or may be due to cancer stem cells repopulating the tumour environment (Agarwal and Kaye, 2003). Both of these are possibilities in acquired resistance mechanisms, either singularly or in combination. NR2E1 seems to induce cisplatin-induced apoptosis (Dziarmaga et al., 2006; Hueber et al., 2006), and cisplatin-based chemotherapy may select pre-existing subpopulations with epigenetically silenced *NR2E1*. Alternatively, there is increasing interest in the suggestion that epigenetic alterations in stem cells may be inherently linked to many properties seen in tumours, such as drug resistance (Feinberg et al., 2006), and that survival of cancer stem cells may provide them with a role in acquired drug resistance following chemotherapy (Dean et al., 2005). In addition, stem cells have been reported to contribute to the aggressive behaviour of EOC (Bapat et al., 2005). *NR2E1* and *LMX1A* are both expressed in stem or progenitor

cells and are implicated in stem cell maintenance, cell fate determination and differentiation (Burbach and Smidt, 2006; Shi et al., 2004). These stem cells may only comprise a very small proportion of the cells within a tumour and be relatively quiescent, therefore avoiding the toxicity of the chemotherapy regime which will target rapidly dividing cells. Therefore, the acquisition of methylation post-chemotherapy could represent survival, growth and differentiation of cancer stem cells which were present in the original tumour prior to treatment with chemotherapy. However, from the results described, it is not possible to distinguish if drug resistant subpopulations or stem cells contribute to the resistance seen. Considering that both of the genes examined here were firstly detected in a cell culture-based search for methylation markers of chemoresistance, it seems unlikely that stem cell-related processes should occur in such an *in vitro* system. However, other groups have reported the presence of side populations of tumourigenic stem-like cells in cell lines cultured *in vitro* for several years (Kondo et al., 2004; Patrawala et al., 2005). Therefore, the presence of stem cell populations and consequently a role for them in acquired drug resistance in these cell cultures cannot be excluded.

The above findings represent novel diagnostic and therapeutic challenges in ovarian cancer. The clinical implications of these are discussed in Chapter 5.

Chapter 5

Clinical application of aberrant DNA methylation markers in ovarian cancer

5 Clinical application of aberrant DNA methylation markers in ovarian cancer

5.1 Early detection biomarkers: identification of CGI methylation in plasma

Ovarian cancer remains difficult to detect at an early stage with currently available screening methods. Results described in Chapters 3 and 4 showed that CGI methylation can be frequently identified in early stage EOC. 5D4 (2kB downstream of the predicted transcription start site of the transcription factor, *LMX1A*), *OPCML*, *RASSF1A* and *HIC1* were frequently methylated in early stage ovarian tumours (75%, 53%, 31% and 22% respectively). 87.5% of the early stage tumours examined were methylated in at least one of these 4 genes. Therefore, a combination of these methylation markers could potentially impact on the current poor sensitivity levels of detecting early stage disease and therefore improve poor survival rates for patients presenting with ovarian cancer. However, CGI methylation will only be acceptable as a clinical marker of early detection if we can identify the changes described above in easily accessible body fluids, such as plasma extracted from whole blood.

Matched plasma and PBMC DNA samples were examined from patients in the prospective DNA methylation study undergoing cytoreductive surgery for ovarian cancer. This was to see if the same epigenetic changes could be detected in plasma as were seen in corresponding tumour. In an initial feasibility study, thirty matched blood and chemonaive epithelial ovarian tumour samples were analysed and compared for methylation of the four loci which were frequently methylated in early stage disease (5D4, *OPCML*, *RASSF1A* and *HIC1*) with the aim of correlating these with MSP data from the corresponding tumour sample. A gene which was negative for hypermethylation in the tumour DNA was always negative in the matched plasma DNA. The converse was also true, in that when a gene was methylated in plasma, it was also methylated in matched tumour DNA, representing a specificity of 100%. The plasma-positive cases were all sampled from patients with late stage III/IV EOC. However, because only 1/30 samples was from a Stage I ovarian tumour, more early stage samples will require analysis for conclusions to be drawn regarding CGI methylation frequencies in these early stage cancers. Methylation of at least one of the four genes analysed was found in 100% of the 30 ovarian tumour samples, with individual methylation frequencies of 90%, 50%, 33.3% and 20% for *5D4*, *OPCML*, *HIC1*

and *RASSF1A* respectively. Individually, methylation of 5D4, *OPCML*, and *RASSF1A* was observed in 43.3% (13/30), 20% (6/30) and 6.7% (2/30) of the plasma samples respectively. No methylation signal was detected for *HIC1* in any of the plasma samples examined. The sensitivity of detecting methylation of 5D4 alone in plasma from patients whose tumour was methylated at this locus was 48.2%. Overall, the sensitivity of detecting methylation of at least one of the four genes examined was 53.3% (16/30) in the matched plasma samples (i.e. 14 patients showed no methylation in plasma DNA despite showing methylation in tumour). Encouragingly, 10% (3/30) and 3.3% (1/30) of plasma samples were co-methylated in at least 2 or 3 genes respectively. Table 15 shows the methylation status of all loci for matched plasma and tumour samples.

	GENE ANALYSED IN MATCHED TUMOUR (T) OR PLASMA (P)							
	5D4		OPCML		RASSF1A		HIC1	
PATIENT	T	P	T	P	T	P	T	P
1	M	U	U	U	M	U	U	U
2	M	M	U	U	U	U	U	U
3	M	M	U	U	U	U	U	U
4	M	U	U	U	M	M	M	U
5	M	M	M	U	M	U	U	U
6	U	U	U	U	M	U	U	U
7	M	U	M	U	M	U	M	U
8	M	M	U	U	U	U	M	U
9	M	M	M	M	U	U	M	U
10	M	M	U	U	U	U	U	U
11	M	U	U	U	U	U	U	U
12	M	U	M	M	U	U	U	U
13	M	U	U	U	U	U	U	U
14	M	U	U	U	U	U	U	U
15	M	U	U	U	U	U	U	U
16	M	M	M	M	U	U	U	U
17	M	M	M	M	M	M	U	U
18	M	M	U	U	U	U	U	U
19	U	U	M	U	U	U	M	U
20	U	U	U	U	U	U	M	U
21	M	M	U	U	U	U	U	U
22	M	U	M	U	U	U	M	U
23	M	M	M	U	U	U	U	U
24	M	U	M	U	U	U	U	U
25	M	M	M	U	U	U	U	U
26	M	M	M	M	U	U	M	U
27	M	U	U	U	U	U	U	U
28	M	U	M	U	U	U	M	U
29	M	U	M	M	U	U	M	U
30	M	U	M	U	U	U	U	U
% Meth.	90%	43.30%	50%	20%	20%	6.70%	33.30%	0%

Table 15 Methylation status of matched tumour and plasma. Tabulation of methylation status of the loci (5D4, *OPCML*, *RASSF1A* and *HIC1*) for 30 matched tumour (T) and plasma (P) patient samples.

5.2 Predictive biomarkers in ovarian cancer

The majority of the 24 genes examined in Chapter 3 have previously been shown in experimental models to be associated with cellular sensitivity/response to DNA damage (Teodoridis et al., 2005). In order to identify potentially useful predictive markers of disease response to treatment at initial presentation, patterns of methylation were examined in the group of retrospective late stage III/IV ovarian tumours to see if there was any correlation with chemotherapy response. This work was done in collaboration with Dr. Jacqueline Hall. All of the patients had been treated with platinum-based chemotherapy (100%), either cisplatin or carboplatin, and the majority had received a course of taxoid chemotherapy (69%). Only 70 of the 106 patients were suitable for assessment of their response to chemotherapy. For the purpose of this analysis, complete and partial clinical response were combined and compared to stable and progressive disease. The CGIs were grouped according to the biological role of a specific gene, as shown below in Table 16, and examined for associations with clinical response to chemotherapy. It was hypothesised that disruption of a biological pathway at any specific point had the potential to affect the functioning of that cellular response. In addition, grouping genes together also reduced problems of multiple statistical analyses. A group was considered methylated if at least one CGI examined was methylated. Correlation between methylation and response was assessed by the χ^2 test unless the smallest expected value was less than 5, and then Fisher's Exact Test was used (Chapter *2.21.1*). All statistical analysis in Chapter 5 was performed using the SPSS v12.01 software package (SPSS Inc., Chicago, USA).

BIOLOGICAL FUNCTION	GENE GROUPS
APOPTOSIS	*APAF1, CASP8, DAPK, DCR1, Fas, MLH1, P14, P73 RASSF1A, Survivin, TMS1*
OVARIAN TUMOUR SUPPRESSOR	*OPCML*
PROLIFERATION	*PTEN, SFRP1, SOCS3*
DNA REPAIR / DRUG DETOXIFICATION	*BRCA1, FANCF, MGMT, GSTP1*
CELL CYCLE	*P16, P21*
OTHER / UNKNOWN	*BLU, HIC1, MINT25*

Table 16 Genes grouped according to specific biological function.

Methylation of any one of the 24 CGIs examined compared to no gene methylated did not associate with response to treatment (p=0.51, n=64). Methylation of the group of genes involved in regulation of apoptosis (*APAF-1*, *CASP8*, *DAPK*, *DCR1*, *FAS*, *MLH1*, *P14*, *P73*, *RASSF1A*, *SURVIVIN* and *TMS1*) was not significantly associated with response to chemotherapy in those patients that were evaluable (p=0.74). *MLH1* was included in the group of apoptosis-related genes, rather than the DNA repair set, because a lack of *MLH1* has been shown to lead to DNA damage tolerance due to loss of engagement of an apoptotic response (Luo et al., 2004). Methylation of the groups of genes involved in cell cycle control and proliferation (*P16*, *P21*, *PTEN*, *SFRP1*, *SOCS-3*) did not significantly correlate with response (p=0.999). However, methylation of at least one of the group of genes involved in DNA repair and drug detoxification (*BRCA1*, *GSTP1*, *MGMT*) was significantly associated with increased response to chemotherapy (p=0.013, n=70). In the patient group that showed methylation of *BRCA1*, *GSTP1* or *MGMT*, response rate to chemotherapy was 100% compared to a response rate of 62.7% for patients not showing methylation of any one of these genes. The majority of the ovarian tumours examined in the DNA repair group showed methylation of *BRCA1* (85%), but methylation of *BRCA1* alone showed only borderline significance for association with response to treatment (p=0.049, n=70).

The patterns of CGI methylation which are described above may have the potential to predict clinical outcome but adequate power and careful validation of results is important. The validation study of the hypotheses generated here are described below in Chapter *5.3*.

5.3 Validation of predictive biomarkers

The retrospectively collected ovarian tumour samples which have been discussed in previous chapters were obtained from a heterogeneous population of patients who did not always receive comparable chemotherapeutic regimes following cytoreductive surgery. It was important to confirm and validate the observations and hypotheses generated in Chapter *5.2* in a prospective collection of tumours from patients who all received standard first-line platinum based chemotherapy (+/- taxane) following surgery. To achieve this aim, the "DNA Methylation Study" was set up to collect primary epithelial ovarian tumours from a more defined homogenous patient population. Samples were collected at the time of cytoreductive surgery prior to chemotherapy. The precise number of samples which were required depended on the exact details of recruitment rate and the minimum follow-up period of this study. It was assumed that 50% more patients than events were required, which meant having approximately a minimum of 1 year follow-up for the vast majority of patients. The sample size calculations were based on the primary end-point of PFS. Assuming a power of 90%, between 131 and 258 patients were required for this study (hazard ratio 2.0).

The statistical analysis of the MSP results in Chapter *5.2* showed that methylation of at least one of the group of genes involved in DNA repair and drug detoxification (*BRCA1*, *FANCF*, *GSTP1*, *MGMT*) was significantly associated with increased response to chemotherapy (p=0.013, n=70). We tested this in an independent data set using samples from the prospective collected ovarian tumours.

The initial MSP analysis of *BRCA1*, *FANCF*, *GSTP1* and *MGMT* was performed in 125 ovarian tumours which were collected prospectively although, as shown below, not all of these samples were included in statistical analysis. Methylation of *BRCA1* (n=13, 10.4%) and *FANCF* (n=1, 0.8%) were observed but no methylation was seen for either *MGMT* or *GSTP1*. These methylation frequencies observed are comparable to those obtained in the retrospective study, as shown in Table 10, Chapter *3.1*.

77 of the 125 tumours analysed had collected clinical information. 19 of these samples were removed from further statistical analysis because 3 tumours were found to be non-epithelial derived ovarian tumour samples and 16 tumours were of an earlier stage (<Stage III) than those examined in Chapter *5.2*. 58 tumour samples were therefore available for further analysis. Of these 58 patients, 28 patients had unevaluable disease (i.e had no measurable tumour volume following cytoreductive surgery) and 11 patients had some

missing or incomplete clinical data and therefore were excluded which reduced the number to 19 suitable patients. Clearly these numbers are small and the study is underpowered to validate the original findings. However, due to time constraints it was not possible to prospectively accrue sufficient samples, so the data was analysed as it was.

For the purpose of this analysis, complete and partial clinical response were again combined and compared to stable and progressive disease. Those patients with evaluable response data included 12 patients who responded to chemotherapy and 7 patients who did not respond. These results are illustrated below in Table 17.

Outcome	DNA Repair Unmethylated	DNA Repair Methylated	Total
No response	7	0	7
Response	9	3	12
Total (both)	16	3	19

Table 17 Cross tabulation of response to chemotherapy in the prospective validation set.

For several reasons, the 19 patient samples which were evaluable for response were underpowered for any further statistical interpretation. Firstly, there were 28 patients with missing response endpoints due to unevaluable disease prior to chemotherapy. There was also insufficiently mature response data for 11 patients. In addition to these issues, there were also a low number of methylation events in the DNA repair set of genes which made it difficult to assess the data statistically with such small numbers. However, it should be noted that a trend was observed in Table 17 which showed that there were more methylation events in the DNA repair set in responders versus non-responders (3 versus 0), in keeping with the statistically significant findings in the retrospective group.

5.4 Identifying novel markers of acquired resistance in ovarian cancer

The potential to uncover novel DNA methylation markers of acquired chemoresistance in ovarian cancer was investigated in Chapter 4. Two novel markers, 5D4 and 119A6, were identified which could potentially improve detection in the relapsed patient and individualise treatment in subgroups of patients. 199 patient samples were analysed for methylation of these 2 markers. 126 of these had sufficient clinical information for associations with clinical characteristics to be sought and had been treated with platinum +/- taxane therapy as a first line treatment regime. Where it was appropriate, Pearson χ^2 or Fisher's Exact Test were used to examine associations between methylation frequency and stage, grade and histology (details as shown in Table 18). The Mann Whitney U Test was applied to test associations between methylation and age. This uses a range of frequencies rather than specific categories. Methylation of 119A6 and 5D4 was independent of age (119A6: p=0.87 and 5D4: p=0.34, N=125), stage (early vs. late stage, 119A6: p=0.23 and 5D4: p=0.99, N=126) and grade (well/moderately vs. poorly/undifferentiated, 119A6: p=0.65 and 5D4: p=0.13, N=93). Stage was categorised into early versus late because the numbers for each individual stage were low. In spite of the small numbers, for 119A6, there was a slight trend of a reduction in methylation with advancing tumour although this was not statistically significant (p=0.23). Removal of cases with unknown histology showed that the proportion of cases methylated for 119A6 and 5D4 was not significantly different between serous and non-serous histological subtypes (119A6: p=0.68 and 5D4: p=0.75, N=94).

	119A6	5D4	Total (n)
HISTOLOGICAL			
Serous	6 (13%)	29 (64%)	45
Endometrioid	3 (33%)	6 (67%)	9
Clear Cell	2 (25%)	5 (63%)	8
Mucinous	1 (17%)	3 (50%)	6
Other	0	1 (50%)	2
Adenocarcinoma	2 (8%)	15 (63%)	24
Unknown	3 (9%)	17 (53%)	32
TOTAL	**17**	**76**	**126**
TUMOUR STAGE			
Early	4 (25%)	10 (63%)	16
Late	13 (12%)	66 (60%)	110
TOTAL	**17**	**76**	**126**
TUMOUR GRADE			
Well	1 (17%)	2 (33%)	6
Moderate	4 (17%)	14 (61%)	23
Poor/Undifferentiated	4 (6%)	39 (61%)	64
TOTAL	**9**	**55**	**93**
PATIENT RESPONSE			
Response	6 (13%)	28 (62%)	45
No Response	2 (8%)	13 (54%)	24
TOTAL	**8**	**41**	**69**

Table 18 Details of clinicopathological data of 126 tumours examined for methylation of 119A6 and 5D4.

Kaplan Meier survival curves and log rank tests were used to assess associations with PFS (defined as from presentation to first progression) or OS. Methylation of either 119A6 or 5D4 was not associated with PFS or OS (PFS/119A6: p=0.34, PFS/5D4: p=0.47; OS/119A6: p=0.86, OS/5D4: p=0.93). The Kaplan Meier curves are illustrated below in Figure 27.

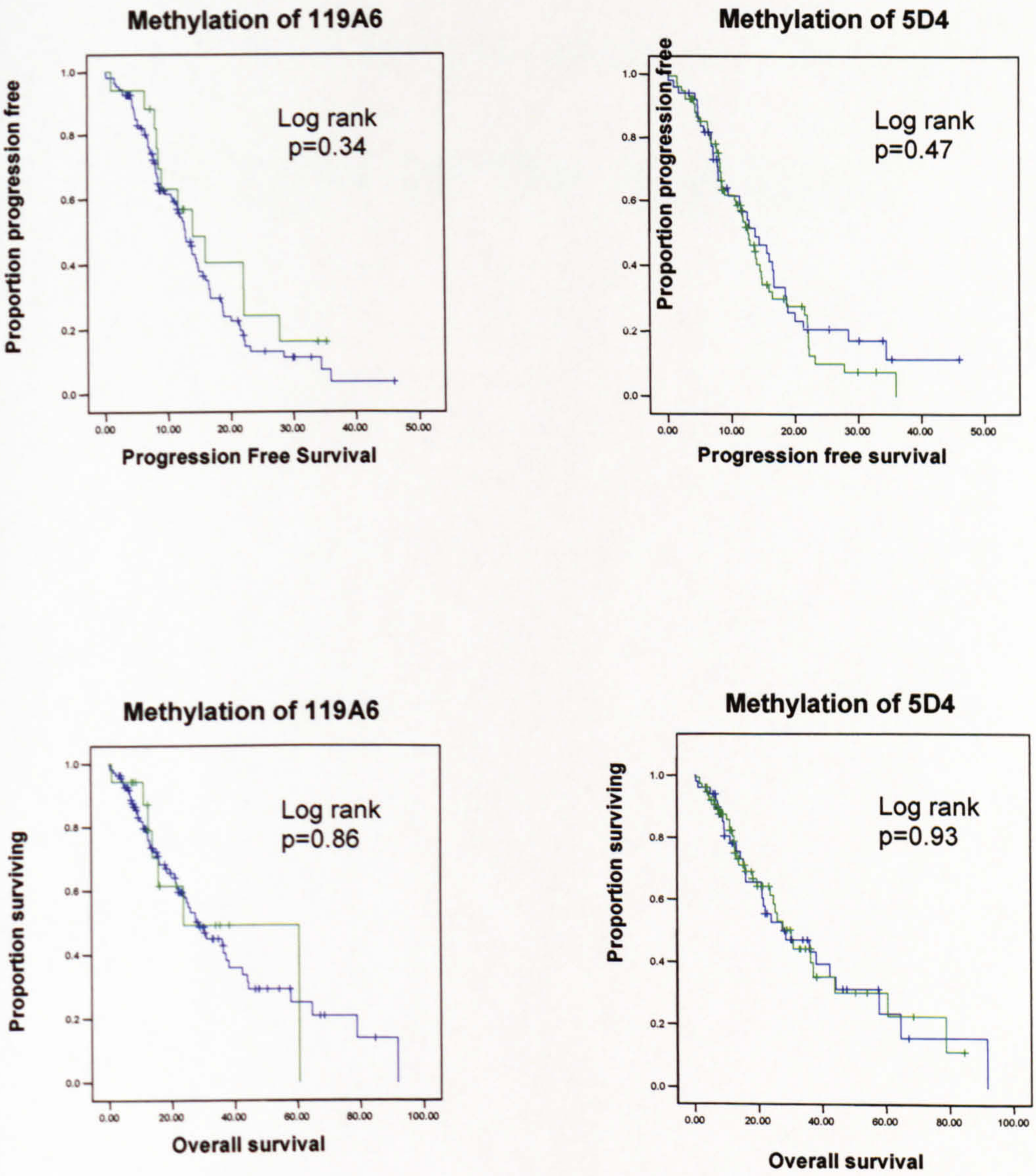


Figure 27 Kaplan Meier survival curves to assess association of 119A6/5D4 methylation with PFS or OS. Methylation of either marker does not associate with PFS/OS. Green line = methylated; Blue line = unmethylated.

Response information was only available for 69 patients as shown below in Table 19. 24 patients were classified as non-responders and 45 were responders. No association was shown between response to chemotherapy and methylation of 119A6 or 5D4 (p=0.54 and p=0.52 respectively). The cross tabulation of this is shown in Table 19.

	5D4 (Methylated)	119A6 (Methylated)	Total
Response	28 (62%)	6 (13%)	**45**
No response	13 (54%)	2 (8%)	**24**
			69

Table 19 Cross tabulation of response to chemotherapy for methylated loci 119A6 and 5D4.

5.5 Discussion

The results in Chapters 3 and 4 illustrate that aberrant CGI methylation is detected at all stages of EOC. It is accepted that this can potentially be used as a diagnostic tool to detect cancer (Esteller, 2003), and the results here show that different groups of methylated CGIs may specifically be useful for early disease detection, prediction of response to chemotherapy and as markers of relapsed disease. It is feasible that as a cancer cell accumulates an increasing number of methylation events over time, these may then potentially impact on the phenotype of the tumour and subsequently affect its ability to respond to chemotherapy. It is also worth considering that heterogeneous tumours may exhibit differing methylation patterns, therefore examining groups of potentially methylated markers may increase the sensitivity and specificity of a biomarker (Levenson, 2004). In order to use CGI methylation as a clinical biomarker, it firstly has to be readily detectable in easily accessible surrogate body sources such as plasma. It has already been shown that CGI methylation can be detected in plasma with the same characteristic changes as are found in the corresponding tumour (Esteller et al., 1999b; Gifford et al., 2004; Ibanez de Caceres et al., 2004; Weaver et al., 2006). Encouragingly, this is highly specific for ovarian cancer (Chang et al., 2002). Methylation analysis is particularly suited to plasma DNA because highly sensitive PCR-based assays such as fluorescent-MSP only require small amounts of DNA. These types of MSP-based assays will potentially be very useful in the clinic to detect changes since hypermethylation of a given gene tends to occur at the same location (i.e. close to the promoter), meaning that one PCR primer set can be used in most patients (Baylin et al., 2000), avoiding the need for multiple different analyses. This is in sharp contrast to DNA mutations in genes such as *p53* (Fliss et al., 2000) which can often involve numerous base alterations at a multitude of positions and therefore are not easily analysed in a high throughput manner. In addition, aberrant CGI methylation which is frequently identified in tumours is not observed in normal tissues (Bird, 1986), including peripheral blood mononuclear cells (PBMCs) and these can be used as a negative control in comparison to tumour DNA in plasma samples (Toyota et al., 2001). DNA is also more stable than mRNA which reduces the technical problems of handling potentially unstable RNA when processing tissue and blood samples.

The potential for early detection methylation biomarkers

The efficacy of screening methods in ovarian cancer remain unproven (Rosenthal et al., 2006) and to date there has been no cost-effective screening strategy available for this

disease (www.figo.org). However, a reliable screening protocol which would specifically identify early stage lesions could improve the dismal survival rates associated with this clinically silent disease (Hickey et al., 1999). The results in Chapter *5.1* have identified CGI methylation as a promising molecular strategy for early detection of ovarian cancer that may be independent of its functional implications. There have been many groups who have identified tumour-specific hypermethylation in surrogate body fluids matched to several early stage tumour types. These include methylation changes in serum from patients with breast cancer (Dulaimi et al., 2004), urine from patients with kidney tumours (Cairns, 2004) and sputum from patients with lung cancer or with a smoking-related risk of this disease (Belinsky et al., 2006; Wang et al., 2006b). However, the results in this thesis are one of the first descriptions of these epigenetic changes in matched early stage ovarian tumours and plasma. Only one previous study which investigated methylation as a frequent early event in ovarian tumours and matched serum, plasma and peritoneal fluid could be identified (Ibanez de Caceres et al., 2004). Frequent CGI methylation of 5D4 (*LMX1A*), *OPCML*, *RASSF1A* and *HIC1* were observed in early stage epithelial ovarian tumours (75%, 53%, 31% and 22% respectively) as shown in Chapters 3 and 4. 87.5% of the early stage tumours examined were methylated in at least one of these genes. These methylated loci were then further investigated in matched plasma and tumour from patients undergoing cytoreductive surgery for EOC. In this initial feasibility study, methylation of at least one of these four genes (5D4, *OPCML*, *RASSF1A* or *HIC1*) was observed in 16/30 of the plasma samples, giving a sensitivity of 53.3%. When methylation was detected in plasma, it was always detectable in the corresponding tumour, representing a specificity of 100%. This suggests a mechanism by which tumour DNA is either directly shed into the circulation or may be due to DNA released following apoptosis in neoplastic cells (Sidransky, 2002), either of which would then lead to the identical methylation changes seen. Hypermethylation of any gene may not have been detected in 46.6% (14/30) plasma samples due to low levels of neoplastic DNA in some patients which are not detectable by Fluorescent-MSP as described in previous work (Ibanez de Caceres et al., 2004). Additionally, previous work in ovarian cancer has shown that there is only a 73% concordance between tumour and plasma methylation (Hickey et al., 1999). Heterogeneity is seen both in individual ovarian tumours and between samples, meaning that CGI hypermethylation will vary in tumours (Rathi et al., 2002; Strathdee et al., 2001), as shown above. Future studies will require analysis of a panel of markers which could include 5D4, *OPCML*, *RASSF1A* and *HIC1*. This will inevitably lead to marker redundancy, but will increase diagnostic coverage and the sensitivity of a screening test. The sensitivity will require to reach at least 75% in order to achieve a positive predictive value of 10% (Bast,

2003). The specificity of this study is 100% since methylation in plasma was only observed when methylation could be detected in the matched tumour, which is in keeping with requirements for the specificity of such a test to be at least 99.6% (Jacobs and Menon, 2004). In addition, the specificity of diagnosis will also benefit from screening a larger number of methylation markers, such as one of those described by Ibanez de Caceres *et al.* (*RASSF1A*, *BRCA1*, *APC*, $p14^{ARF}$, $p16^{INK4a}$ or *DAPK*) and this will potentially reduce the probability of detecting false positives. Although this thesis did not identify many of these genes as being hypermethylated in ovarian cancer, tumour heterogeneity may mean that methylation of these markers will occur in larger sample sets examined. However, from the results of this feasibility study in the plasma sample collection, if this can be confirmed in larger groups of patients, these methylated markers may have potential as a foundation for a larger set of early diagnostic epigenetic markers in EOC.

In an attempt to identify relevant early detection biomarkers, it is important to firstly understand the epigenetic mechanisms which may be implicated in the initiation event(s) responsible for development of an ovarian neoplasm. Currently, it would suffice to say that we know more details about the maintenance of methylation in tumour suppressor genes rather than the initiating events themselves. There have been some recent studies examining the role of methylation in the potential malignant transformation of benign and low malignant potential ovarian tumours to invasive cancers (Makarla et al., 2005; Wiley et al., 2006). In addition, other groups have suggested that epigenetic events most frequently occur during the earliest stages of neoplasia, including the development of precancerous lesions (Feinberg and Tycko, 2004; Yamada et al., 2005). In addition to tumour suppressor genes being epigenetically inactivated in the initiation events of a cancer, there may also be an epigenetic "switch" in gene-imprinting status which could contribute to the early stages of many tumours, which has been suggested for colorectal cancer (Cui et al., 2003; Holm et al., 2005; Sakatani et al., 2005). Recent promising work has shown the use of cultured mouse ovarian surface epithelium in identifying sequential molecular changes, including the epigenetic silencing of tumour suppressor genes, during the development of ovarian cancer from an initial premalignant model (Roberts et al., 2005). Using this novel model, it may be possible to identify other epigenetic events, in addition to the potential markers described in this thesis, which can initiate an EOC and be used, in the long term, as early diagnostic markers of this disease. In addition, there may be specific events which can be seen in the adjacent normal tissue which then put a patient "at risk" of developing an overt tumour. Certainly, results described in Chapter 3 for non-tumour adjacent tissue showed that methylation of specific genes (*HIC1*, *DCR1*, *APAF-1*, *OPCML* and *RASSF1A*) were seen in these samples, and in some cases this was a frequent

event. Perhaps cancer progresses in a step-wise fashion as epigenetic and genetic control of homeostasis is lost, as described for *HIC1* (Chen et al., 2005a). Interestingly, methylation of *HIC1* in non-tumour adjacent tissue was frequently seen in Chapter 3 and may represent a very early event in ovarian carcinogenesis. This type of homeostatic loss of control could lead to the disease progressing through phases including metaplasia, dysplasia, early stage and metastatic disease. If this is an early event, it is curious that this epigenetic event was not detected in the plasma DNA of ovarian cancer patients. It may be that increasing the number of samples analysed will show methylation of this gene in some patients. Overall, understanding the molecular components which initiate epigenetic change and gene silencing could provide the much needed answers as to what markers may be important for risk assessment and early detection in many cancers, including ovarian. This concept will be discussed further in Chapter 6 examining future outlooks which may lead on from this project.

The use of predictive biomarkers

Predictive biomarkers can potentially predict a response to a specific chemotherapy and therefore provide information that will lead to a decision regarding treatment. Ultimately, these markers have the potential to translate into individualised treatment options for patients. Having investigated the CGI methylation of genes, the results obtained were assessed with statistical methods to interpret if this biological process was linked with the clinical outcome of ovarian cancer patients. Recently, large genome-wide studies have focussed on ovarian cancer and have shown that methylation of groups of CGIs could provide important predictive or prognostic signatures in assessing and managing cancer (Wei et al., 2006; Wei et al., 2002). In addition, it has also been suggested that the CIMP may have associations with clinical outcome in cancers including neuroblastoma (Abe et al., 2005; Abe et al., 2006) and colorectal cancer (Van Rijnsoever et al., 2003), although there have yet to be any studies such as these which suggest a link of the CIMP with outcome in ovarian cancer.

The predictive potential of the candidate CGI methylation data in 106 late stage tumours from the retrospective study (Chapter 3) were first examined statistically. Genes were grouped according to biological function although categorising the genes in this way is admittedly oversimplifying the biological process involved here. Methylation of at least one gene involved in DNA repair or drug detoxification (*BRCA1*, *GSTP1* or *MGMT*) was associated with improved response to chemotherapy ($p=0.013$). These genes have previously been shown to be potentially important predictive markers in other cancer

types. MGMT (O^6-methylguanine- DNA methyltransferase) is a DNA repair enzyme which removes methyl groups as well as larger adducts from the O^6 position of guanine. The alkylation of DNA at the O^6 position of guanine is associated with the formation of mutations in DNA (Gerson, 2004). Methylation of *MGMT* has been reported in a variety of cancers including granulosa cell tumours of ovarian origin, head and neck carcinoma and colorectal cancer (Dhillon et al., 2004b; Esteller et al., 1999a; Esteller et al., 2000d). MGMT activity has been shown to be a major mechanism causing resistance to alkylating agents by rapid reversal of adducts formed at the O^6 position of guanine by such chemotherapeutics (Esteller and Herman, 2004). In concordance with the results above, methylation of a CGI in the *MGMT* promoter has been shown to be an independent predictive marker of longer survival for glioblastoma patients treated with temozolomide (Hegi et al., 2004) and hypermethylation of the *MGMT* promoter also correlated with increased survival of patients with diffuse large B-cell lymphoma after chemotherapy (Esteller et al., 2002). The Glutathione-S-Transferases (GST) are a family of metabolic enzymes which detoxify potentially carcinogenic agents by catalysing the conjugation of glutathione to a variety of electrophilic intermediates. This prevents the accumulation of compounds that can lead to damaged DNA (Hayes and Strange, 2000). GSTs might detoxify chemotherapeutic drugs within cancer cells which would contribute to chemoresistance, and this is well established in cell culture models (Perquin et al., 2001). Therefore, silencing of *GSTP1* could increase sensitivity to chemotherapy. *GSTP1* is the most frequently methylated gene in prostate cancer (Perry et al., 2006), although to date, there have still been no studies examining CGI methylation of *GSTP1* and clinical chemosensitivity. BRCA1 aids in the maintenance of genomic integrity and participates in double strand break repair by homologous recombination (Tutt and Ashworth, 2002). Several *in vitro* studies show that the integrity of the BRCA1 pathway is important for tumour response to chemotherapy. It has been shown that BRCA1 deficiency is linked to sensitivity to cisplatin and other DNA damaging agents (Sgagias et al., 2004; Tassone et al., 2003), although the need for further clinical studies of BRCA1 and response to chemotherapy has recently been highlighted (Kennedy et al., 2004). It has been reported that promoter hypermethylation of *BRCA1* is a frequent event in sporadic ovarian tumours (Esteller et al., 2000b; Ibanez de Caceres et al., 2004) and mechanistically, methylation of *BRCA1* could inhibit cells from repairing the damage caused by chemotherapeutic agents leading to an apoptotic response in these damaged cells.

As shown, multiple biological pathways are affected by methylation in individual patients and this will lead to complex patterns in patients with the same phenotype. However, some of these events will not directly influence the outcome in patients. In order to decipher

which methylation events were important and influenced the biological phenotype, a larger homogeneous study was required. The results above were taken from a retrospective study which was excellent for the hypothesis-generating observations above but tumours varied in histological type and had been exposed to various chemotherapies. Therefore, the validation of these results was examined in an independent set of epithelial ovarian tumour samples from a more defined patient population in the prospective "DNA Methlyation Study", funded through the Scottish Gynaecological Cancer Trials Group. The validation result for the potential predictive markers *BRCA1/GSTP1/MGMT* was not statistically significant. However, there was a trend in keeping with the previous results. One reason why these results may not have been significant was because the validation set was significantly underpowered and there had not been enough time for a number of clinical events to occur. To address this question, the study will require further analysis at a later date after allowing more events to accumulate for analysis.

Ultimately, the results described within this subchapter suggest that methylation profiling may be useful to identify individual patients who may benefit from specific current therapies to improve clinical outcome. Also though, and perhaps more importantly, these types of studies may identify those patients who are eligible to enter clinical trials investigating novel epigenetic therapies depending on their methylation profile.

The potential to identify markers of relapsed disease

Biomarkers which identify patients with chemoresistant tumours have the potential to significantly contribute to the optimisation of second-line chemotherapies in the treatment of ovarian cancer. Identification of these markers remains the elusive goal in successfully identifying and treating the patient with relapsed disease. Ultimately, this may lead to the ability to recognise patients who can undergo reversal of chemoresistance with novel epigenetic therapies (Lyko and Brown, 2005). Methylation of the novel markers, 119A6 and 5D4, were shown to be selected for during platinum-based chemotherapy in Chapter 4. Despite this selection during chemotherapy, methylation of 119A6 or 5D4 in chemonaïve ovarian tumours taken at presentation was not shown to be associated with response to chemotherapy, progression free survival or overall survival. Again, a lack of a significant association between methylation patterns and clinical outcome is not evidence for its absence here. There may not have been adequate statistical power here to detect small effects or the examination of these two novel loci in isolation may not be sufficiently sensitive. Indeed, an increase in sensitivity may be achieved by measuring these markers as part of a larger methylation profile (Glasspool et al., 2006). The lack of statistical findings

may have been due to the fact that the samples examined were heterogeneous chemonaive tumours, and to understand the biology of chemoresistance, ovarian tumours/plasma samples from the relapsed patient may be required for this type of analysis. These types of relapse samples have previously been the focus of a study which successfully identified that acquired methylation of the DNA mismatch repair gene, *hMLH1*, is associated with patient survival, rather than the methylation status at presentation (Gifford et al., 2004). Potentially, one could hypothesis that a combination of *hMLH1*, 5D4 and 119A6 may be a useful set of methylation markers for acquired chemoresistance but this will need further assessment with appropriate samples.

Chapter 6

Summary and future outlook of this project

6 Summary and future outlook of this project

6.1 Summary of findings

This project aimed to identify novel methylation patterns associated with early disease detection, prediction of disease response to chemotherapeutics and chemoresistance markers in ovarian cancer. CGI methylation was a frequent event at all stages of EOC. In early stage chemonaive tumours, a set of 3 candidate loci (CGIs linked to the *OPCML*, *RASSF1A* and *HIC1* genes) were methylated for at least one locus in 64% of tumours. Further results revealed that methylation of 5D4, mapping to the novel gene *LMX1A*, was a potential novel epigenetic marker which was very frequently methylated in 75% of early stage ovarian tumours. In combination, 87.5% of the early stage tumours examined were methylated in at least one of these 4 loci. The clinical application of this was investigated by examining matched plasma from chemonaive patients with ovarian cancer with the aim of identifying methylation changes in a relatively non-invasive blood test. Detection of *LMX1A* (5D4) methylation in plasma was found to have a sensitivity of 48.2% and a specificity of 100%. The sensitivity may increase by examining a larger panel of markers.

In late stage chemonaive ovarian tumours, the MSP data was examined to identify if methylation of groups of markers could be correlated with response to chemotherapy. Methylation of CGIs associated with either *BRCA1*, *GSTP1* or *MGMT*, which are involved in DNA repair and drug detoxification, were correlated with an improved response to chemotherapy ($p=0.013$). The validation of these results has not been completed due to the immaturity of clinical response data but it will be important to assess if these relationships can be validated in an independent set of ovarian tumours. Also, in this group of late stage tumours, a non-random pattern of DNA was seen. This demonstrated that there is an underlying biological mechanism which leads to co-methylation of specific genes but the cause of this remains unidentified.

Two novel sequences (5D4 and 119A6) were identified in a genome-wide CGI screen whose methylation status can discriminate cisplatin sensitive and resistance cell lines, and who are also abberantly methylated in ovarian tumours. These novel loci may represent newly discovered epigenetic biomarkers in ovarian cancer which are selected for during drug resistance mechanisms.

6.2 The challenge of identifying early epigenetic markers

The evidence has shown that ovarian cancer is only curable if detected at an early stage. The results in this thesis have indicated that epigenetic methylation events are ubiquitous in early stage ovarian neoplasia and can also be detected in matched non-tumour adjacent tissue. A promising novel marker, 5D4, has been described which has one of the highest reported methylation frequencies in ovarian cancer and potential in early diagnosis. Future research should be targeted at detecting which additional epigenetic events are potential initiating factors in premalignant and early stage cancers. If methylation abnormalities arise early in normal tissues, then comparison between histologically normal ovarian surface epithelium from early stage cancer patients and healthy controls may identify methylated markers which are useful in assessing risk, and ultimately developing a screening test. A single tumour may contain important histological components which could be involved in carcinogenesis and should be examined including inclusion cysts, cortical invaginations of the surface epithelium and stromal abnormalities (Cvetkovic, 2003). These possible precursor lesions may create a tumour-promoting microenvironment and it would be interesting to examine the role of epigenetic change in these lesions. Potential issues will include the difficulty which histopathologists have in identifying these precursor lesions, the fact that normal ovaries are rarely removed due to the current climate of more non-invasive treatment modalities and the rarity of identifying an early stage ovarian tumour itself. Examination of adjacent tissues next to a matched early stage tumour provide some much needed answers regarding the epigenetic events which initiate an ovarian neoplasm such as those described here. Comparison between tumour and equivalent histologically "normal" non-tumour tissue from the same patient may be possible using technologies such as DMH to detect differences. Alternatively, the use of the mouse ovarian surface epithelium (Roberts et al., 2005) may provide an excellent model to identify early epigenetic events.

Identification of a panel of early epigenetic markers, including methylation of 5D4, *OPCML*, *RASSF1A* and *HIC1* could alert the clinician that the patient is at risk of ovarian cancer and lead to the implementation of appropriate treatment at an earlier stage. What will be the appropriate treatment though and when will it be best implemented? Admittedly, the difficulty will be when epigenetic changes are identified but existing technologies used, such as CT scanning, classify the patient as "disease free". The role of methylation, and examining if it has a higher sensitivity than existing methods, will only be determined in longitudinal studies in which patients are followed up to see if they do

indeed develop a recognised malignancy. This could potentially involve patients being recruited to trials and followed up for many years which may be technically unfeasible. It could be that ultimately treatment of "at risk" lesions which show identical epigenetic changes to an occult neoplasm should receive similar management. This could then potentially impact on improving survival from this disease. The caveat, though, is that there is a recognised high morbidity associated with complex pelvic surgery and the use of this procedure in women with "at risk" lesions rather than definitive cancers may lead to surgical-related complications in women, some of whom may have false-positive screening results (Jacobs and Menon, 2004). This may then reduce the benefits of early detection and treatment in women with true-positive results.

Earlier results in this thesis have shown that the use of a non-invasive blood test will be an important tool in future studies examining methylation as an early diagnostic marker in ovarian cancer. It should be noted that this is a very early study but the sensitivity achieved is encouraging for future larger screening studies. There are, however, some potential considerations when applying this type of test to early stage ovarian neoplasia. It has previously been thought that the early detection of lung cancer may be hindered by the fact that DNA from early stage lesions may not be efficiently released into the plasma due to reduced vascularisation of this stage of lung tumour (Belinsky, 2004). The same may be said for premalignant/early stage changes in the ovary which would then reduce the sensitivity of a blood test considerably. However, encouragingly, other groups have shown that a detectable amount of DNA from stage 1a ovarian cancer (ie confined to one ovary) is released into the bloodstream (Hickey et al., 1999). There are also issues with the specificity of such a test. Can we be sure of an ovarian origin for specific methylation changes seen in plasma? Again, one could hypothesis that a larger panel of markers may increase the specificity of the test but this would have to be specific for ovarian cancer if used for diagnosis.

The identification of methylation in bodily fluids also has implications, not only in detection of disease at an early stage, but also in how an ovarian neoplasm is staged. It has previously been proposed that DNA is released directly into the plasma from early stage Ia ovarian tumours (Hickey et al., 1999). However, another pathway may be that DNA from tumour cells shed into the peritoneal cavity could enter the bloodstream through the lymphatics (Hickey et al., 1999). Hickey *et al* have suggested that there is potential to "molecularly upstage" an ovarian cancer if tumour DNA was detected in peritoneal fluid. This is an interesting point if we consider our methylation analysis results. Currently, cytology of peritoneal aspirate is used in conjunction with surgical staging and

histopathological diagnosis of an ovarian tumour to accurately stage a tumour using FIGO (International Federation of Gynaecology and Obstetrics) criteria. Currently, and still under debate by some groups, adjuvant platinum based chemotherapy is used in patients with ≥ Stage 1c disease or in those with high grade earlier stage tumours (Trimbos et al., 2003). Potentially though, if ovarian cancer was molecularly upstaged by detection of methylation in peritoneal fluid, this would perhaps not only increase the use of adjuvant therapy in early stage disease, but also introduce the use of novel epigenetic therapies to target these early methylation events.

6.3 Translating identified epigenetic markers into routine clinical use

This thesis has shown that epigenetic biomarkers can be used to stratify both current chemotherapeutics and potentially novel epigenetic therapies to individual patients depending on their methylation status. The major challenge still remains though that well-designed prospective clinical trials are required to obtain samples at crucial stages of treatment and relapse. These trials need to be carefully designed with well-defined objectives and clinical endpoints. There is a need for new objective measures of clinical outcome. Studies vary in their use of endpoints and how they define these. To explain, survival encompasses other factors which may influence the direct survival of the patient. PFS and OS do not explain the underlying biological mechanism of cell death following chemotherapy. The endpoint of response is used with the aim of encompassing information on chemoresistance but is not quantitative. New quantitative and objective measures of response to chemotherapy may help uncover new biologically relevant markers. Identification of these markers will require genome-wide analysis, such as the microarray experiment described in Chapter 4, to obtain complex data concerning the ovarian cancer epigenome. The difficulties and complexities in statistically analysing the tremendous amount of data obtained from these types of experiments will also have to be addressed. Validation of the hypothesis-generating studies described will be required and this thesis has illustrated that this can only occur after a sufficient number of events have been allowed to accumulate. It is worth noting that the vast majority of published biomarker studies to date have not been validated and this will be a crucial component in the future.

Tumour biopsies from relapsed patients with ovarian cancer are rarely used in studies due to the difficulties in procuring such samples. However, in order to test the hypothesis, that methylation of 5D4 and 119A6 are selected for during chemotherapy and are markers of relapse in ovarian cancer, these are the types of samples will be necessary for analysis. If it is true that chemotherapy positively selects for resistant subpopulations of cells (Agarwal and Kaye, 2003), methylation analysis of tumour biopsies at relapse may shed some light on the methylation status of these subpopulations and their potential role in acquired resistance. Acquired methylation will be magnified in post-chemotherapy samples due to selective pressure. To avoid the problems of inter-patient heterogeneity, matched samples pre- and post-chemotherapy will need to be examined and correlated. One of the problems in acquiring these samples lies in the fact that secondary cytoreductive surgery is rarely performed in the relapsed patient and its use remains controversial (Benedetti Panici et al.,

2007). To date, there has been no randomised trial comparing secondary cytoreductive surgery with second-line chemotherapy in relapsed ovarian cancer and therefore these samples are not readily available as patients most commonly undergo treatments with a secondary cytotoxic agent. Admittedly, surgery for recurrent disease is technically challenging with associated risks of organ damage such as bowel or bladder, and therefore it remains ethically unconsiderable to perform a second laparotomy in order to obtain biopsied tumour from this type of patient. An alternative consideration may be biopsy of relapsed tumour via laparoscopy, however, this will require careful ethical consideration in setting up a clinical trial and appropriate patient consent explaining the risks of the procedure. Otherwise, the collection of plasma at relapse and/or ascitic fluid remains the most likely source of this type of material following chemotherapy. The less invasive techniques involved would most likely increase the number of patients consenting to trials and could also allow investigation of selective mechanisms controlling methylation at different timepoints during treatment. The caveat is that the concentration of DNA in these samples may be low therefore reducing the sensitivity of the test. However, if a greater number of patients are consented to this more non-invasive sampling method, the power of the test may increase.

The functionality of the novel genes relating to chemoresistance described in Chapter 4, *LMX1A* (5D4) and *NR2E1* (119A6), and their potential roles in ovarian tumourigenesis will require further investigation. In addition to examining the mRNA expression levels of *LMX1A* and *NR2E1* shown, it will be important to attempt to correlate these results with protein expression levels in the cisplatin-sensitive and cisplatin-resistant cell lines. This will involve the use of Western Blotting and Immunohistochemistry techniques. Potential functional studies could involve the overexpression and knockdown of these genes *in vitro* to allow determination of their effects on colony formation (*LMX1A*) and drug sensitivity (*LMX1A* and *NR2E1*). Additionally, it was hypothesised in the discussion section of Chapter 4 that methylation-related transcriptional repression of *NR2E1* may influence chemoresistance mechanisms by increasing the expression of the transcription factor, *PAX2*, in tumours. It would be intriguing to examine the expression of *PAX2* using RT-PCR in both chemoresistant cells which show methylation of *NR2E1*, and in relapsed tumours following chemotherapy. Increased expression of *PAX2* should reduce cisplatin-induced apoptosis and lead to chemoresistance. The Wnt signalling pathway and its involvement in the pathogenesis of ovarian cancer remains unclear, but methylation of *LMX1A* and *NR2E1* could potentially affect this pathway in cancer. It would be interesting to look at this pathway in ovarian tumours using a candidate gene approach to determine the influence which methylation may have on associated Wnt-signalling genes. Both of

these novel markers are involved in stem cell maintenance and differentiation and a potential link between epigenetic changes of these genes in stem cell compartments could be investigated using tissue microarray-based approaches (TMAs). Are these eventual markers of chemoresistance methylated in stem cells and then selected for during chemotherapy? Finally, it could be considered if selection of methylation of these novel markers has a role to play in the chemoresistant phenotype of other tumours.

The hypothesis-generating results obtained from the work described in this thesis do require further validation, but the identification of these predictive and chemoresistance markers may influence future translational studies. Potentially, these results could lead to the rational use of epigenetic therapies, such as those described in Chapter 1, which will improve sensitivity to current chemotherapeutics and improve the dismal survival rates associated with this disease. Well-designed large prospective trials are still required though to identify those key genes silenced by epigenetic mechanisms among the multiple potential candidates that can help a clinician make real-time treatment decisions in respect to a particular regimen depending on a patient's epigenetic profile.

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