DNA methylation changes associated with acquired platinum resistance in ovarian cancer.

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A thesis submitted to Imperial College London in fulfilment of the requirements for the degree of Doctor of Philosophy

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September 2011

Abstract

Despite high responses to initial chemotherapy most patients with ovarian cancer (OC) relapse and inevitably die from their disease. Aberrant DNA methylation is frequently seen in ovarian tumours and may provide biomarkers of clinical outcome or insight into mechanisms of chemoresistance.

We firstly performed Differential Methylation Hybridisation (DMH) to identify loci that gained methylation between 34 matched cisplatin sensitive and resistant OC tumour cell lines. Differentially methylated loci identified were further validated by Methylation Specific PCR (MSP) and bisulphite pyrosequencing. Selected loci were further investigated for association with clinical outcome in primary OC tumour samples and matched tumour samples from patients' pre- and post- chemotherapy. Frequent increased methylation of a CpG island at the *NR2E1* gene was identified in this experiment. Increased methylation correlated with decreased gene expression and could be reversed following treatment with a demethylating agent. Increased methylation at *NR2E1* was observed between matched pre- and post- treatment tumour pairs.

A novel biostatistical method, methylation linear discrimination analysis (MLDA), was next used to identify differentially methylated loci in sensitive and resistant A2780 human ovarian cell lines. Eight of nine loci identified were validated by MSP. A locus at the *SP5* gene was further investigated by pyrosequencing and found to show a very high level methylation in most cell lines and ovarian tumours. Increased methylation correlated with decreased gene expression and this could be reversed using decitabine treatment. Knockdown of *SP5* expression caused increased apoptosis.

DMH was next used to identify loci that gained methylation between 3 *in vivo* derived matched sensitive and resistant cell lines. *KIAA1383*, a gene of unknown function, was identified and methylation shown to correlate with response to chemotherapy and progression-free survival (PFS) in patients with OC. Over-expression was found to attenuate the response to cisplatin, in the PEA2 cell line, as measured by cell cycle analysis.

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Acknowledgements

Firstly I wish to thank all those in the Epigenetics group at the Beatson labs in Glasgow and especially Catriona, Liz, Alison, Kim and Jens for their initial help getting things started. At Imperial I again had help from lots of people but especially Wei who has invested a huge amount of time in assisting me with statistical analyses. Nadine, Constanze, James and all of the epigenetics group were very supportive and great fun to work with. Euan and Michelle provided the *in vivo* cell lines and were always there for advice along with Seb and Art.

I spent a great couple of months at the ICR in Sutton working with Sian, Jenny and Ale and really appreciate all their help and support. I am sincerely grateful to Jenny who is completing any experiments relevant to this project. Stan Kaye and Hani Gabra provided support in setting up my new funding at Imperial with Ovarian Cancer Action and I enjoyed my time in the clinic with Hani, Sarah, Rohini and Roshan. Jim Paul and Sharon Harden at the Clinical Trials Unit in Glasgow helped with initial statistics and I am also very grateful to all the patients who have provided samples over the years. Ros Glasspool in Glasgow was always on hand for advice and support. Most importantly I must thank Bob for all his support and supervision – both North and South of the border; without him none of this would be possible.

Finally I wish to acknowledge my family. My parents have provided constant support and encouragement over the years and more recently my parents-in-law, have made writing up and working with a two-year-old achievable... but most especially Alan, my ever patient and supportive husband made the move to London and back again relatively painless... and last but not least our wee jumping bean Sasha, who brings a smile to my face every day... and since my viva on 22nd June little Rory has appeared (1st July 2011) – he is good as gold and has been very patient while I do my corrections!

Declaration

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

Dr. Janet Graham

Dedication

This thesis is dedicated to Lucy, my beautiful and courageous sister-in-law, who was taken from us all too soon on the 1st September 2011. My thoughts are with Pete, James and Elizabeth always, x.

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Abbreviations

Abbreviation	In full
A	Adenine
BLAT	Blast-like alignment tool
BLAST	Basic local alignment search tool
BP	Base pair
C	Cytosine
CGH	Comparative genomic hybridisation
CGI	CpG Island
CIMP	CpG Island methylator phenotype
CMV	Cytomegalovirus
COBRA	Combined bisulphite restriction analysis
CR	Complete response
CSC	Cancer stem cells
DAC	Decitabine
DMH	Differential methylation hybridisation
DMSO	Dimethyl sulfoxide
DNA	Deoxyribose nucleic acid
dNTP	Deoxyribonucleotide triphosphate
EOC	Epithelial ovarian cancer
FDR	False discovery rate
FIGO	International Federation of Gynaecology and Obstetrics
FISH	Fluorescent in situ hybridisation
G	Guanine
GST	Glutathione S transferase
HGS	High grade serous
HRT	Hormone replacement therapy
HSP	Heat shock protein
IP	Intraperitoneal
IV	Intravenous
IVM	In vitro methylated
K	Thousand
LGS	Low grade serous
LINE	Long interspersed nucleotide element
LRP	Lung resistance related protein
M	Methylated
mi RNA	MicroRNA
MLDA	Methylation linear discrimination analysis
MMR	Mismatch repair
MSP	Methylation specific PCR
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
n(OSE)	(Normal) ovarian surface epithelium

N/ PBMC	Normal/ Peripheral blood mononucelocite/whole male genomic DNA
NER	Nucelotide excision repair
OCP	Oral contraceptive pill
OGT	Oxford gene technology
OS	Overall survival
PAM	Prediction analysis microarrays
PCR	Polymerase chain reaction
PD	Pharmacodynamic
PD	Progressive disease
PFI	Platinum free interval
PGP	P glycoprotein
PK	Pharmacokinetic
PR	Partial response
PS	Performance status
(q)RTPCR	(quantitative/ real time) Reverse Transcription PCR
RNA	Ribose nucleic acid
RNAi	RNA interferance
SAM	Significance analysis microarrays
SD	Stable disease
SDS	Sodium citrate
SDS	Sequence detection analysis
si RNA	Small interfering RNA
SNP	Single nucelotide polymorphism
SSC	Sodium dodecyl sulfate
SWOG	Southwest Oncology Group
T	Thymine
t RNA	Transfer RNA
TAH BSO	Total abdominal hysterectomy and bilateral salpingoophorectomy
TBE	Tris borate EDTA
TCGA	The cancer genome atlas
TSG	Tumour suppressor gene
U	Uracil

Introduction

1.1 Overview of the management of epithelial ovarian cancer

Around 130 women are diagnosed with ovarian cancer, in the UK, every week. It is the most lethal of the gynaecological tract malignancies and the fourth most common cause of cancer death in women. Despite advances in research and treatment the incidence continues to increase while mortality has remained stable (except for younger patients where a small improvement has been observed) (http://info.cancerresearch.org/cancerstats).

Approximately 80% of patients are diagnosed at over 50 years of age and most known risk factors appear to be hormonally driven. These include nuliparity, infertility, use of postmenopausal Hormone Replacement Therapy (HRT), obesity and endometriosis (Modugno, Ness et al. 2004) (Calle, Rodriguez et al. 2003). Breastfeeding, tubal ligation and the use of the oral contraceptive pill (OCP) are protective and interestingly the protective effects of the OCP are sustained for up to 25 years after stopping (Whittemore, Balise et al. 2004) (Modugno, Ness et al. 2004). In addition 10-15% of patients have a germline mutation in *BRCA1* or *2* and these younger patients have an increased risk of ovarian cancer in addition to other cancers (most commonly breast cancer but also cervical, uterine, pancreatic and colon cancers for females with *BRCA1* mutations, and breast, pancreas, stomach, gallbladder, bile duct and melanoma for females with *BRCA2*) (Thompson and Easton 2002; Kadouri, Hubert et al. 2007).

Ovarian cancer is a heterogeneous disease with the different histological subtypes behaving differently biologically and showing differential responses to standard and more experimental treatments. The serous subtype is the most common of the epithelial ovarian cancers (EOC), accounting for approximately sixty percent of epithelial cancers, but others include clear cell (approximately ten percent), mucinous (less than five percent) and endometrioid tumours (fifteen percent) (Lalwani, Prasad et al. 2011). In addition, tumours of germ cell origin account for approximately twenty percent of ovarian masses and five percent of cancers. (Kaku, Ogawa et al. 2003).

EOC tumours are graded I-III with grade I being the most differentiated/ least aggressive and grade III being the least differentiated/most aggressive. Recently there has been a move to classify tumours only as high or low grade and eliminating the intermediate Grade II, and serous cancer is increasingly considered as two distinct types depending on Grade: 'High Grade Serous (HGS)' and 'Low Grade Serous (LGS)' (Mishra and Crasta 2010). HGS make up approximately fifty five percent of tumours and are characterised by very frequent mutations in the *TP53* gene (Singer, Stohr et al. 2005; Kobel, Reuss et al. 2010; Mishra and Crasta 2010), while LGS, which account for five percent of tumours, lack *TP53* mutations, but have more frequent mutations in *KRAS* and *BRAF* (Singer, Oldt et al. 2003). This is summarised in table 1 below (Lalwani, Prasad et al. 2011).

In addition, the various subtypes are now collectively divided into type I and type II. Type I tumours include LGS, mucinous and low grade endometriod tumours and type II tumours describe HGS, high grade endometriod and undifferentiated variants. Type I tumours present as large ovarian masses, are often early stage at diagnosis and

generally follow an adenoma to carcinoma step wise progression with a relatively good prognosis. In contrast type II tumours are more aggressive, originate outwith the ovary e.g. the fallopian tube, and along with a nearly 100% rate of *p53* mutations have a high rate of BRCA dysfunction and chromosomal instability. They present late and are associated with poor prognosis. These findings were recently reviewed by Lalwani et al and are integrated into table 1 below (Lalwani, Prasad et al. 2011):

Table 1: Summary of the key observations related to the different histological subtypes (Lalwani, Prasad et al. 2011).

Subtype	Suspected precursor	Dysregulated genes	Salient features
LGS cancer 5%	Borderline ovarian tumours Serous cyst adenomas	KRAS BRAF	Median age 60 Bilateral Peritoneal metastases commonly Platinum 'resistant'
HGS cancer 55%	Fallopian tube	BRCA p53 (approaching 100%) PTEN	Median age 55 Bilateral Early peritoneal metastases Platinum sensitive
Mucinous <5%	Borderline ovarian tumours Mucinous cyst adenomas Display an adenoma to carcinoma progression	KRAS HER2 p53	Median age 45 >90% unilateral Often large
Endometriod 15%	Borderline ovarian tumours Endometriosis (40%) Display an adenoma to carcinoma progression	CTNNB1* PTEN* PIK3CA* p53** BRCA1** PIK3C1**	Median age 50 80% unilateral 30% stage I Platinum 'sensitive'
Clear cell 10%	Borderline ovarian tumours Endometriosis (50%) Display an adenoma to carcinoma progression	HNFIβ PTEN	Median age 60 60% unilateral 40% stage I Platinum 'resistant'

LGS Low grade serous, HGS high grade serous * dysregulated in LG subtype, ** dysregulated in HG subtype

Controversy surrounds the origin of ovarian cancer; historically all subtypes were thought to arise from ovarian surface epithelium (OSE) but more recently it has been argued that at least some of the subtypes, particularly HGS, may arise in the fallopian tube or sites of endometriosis or from the peritoneal lining itself (Auersperg, Wong et al. 2001; Bell 2005; Jarboe, Folkins et al. 2008; Kurman and Shih Ie 2008).

Staging is performed at the time of definitive surgical resection (usually total abdominal hysterectomy and bilateral salpingoophorectomy (TAH BSO), omentectomy and pelvic washings). Stage I tumours describe ovarian cancer limited to one or both ovaries with Stage IC used if ascites is present or the capsule ruptures at the time of surgery. Stage II disease is confined to gynaecological organs, Stage 3 disease to other abdominal organs and lymph nodes and Stage IV describes metastases to lungs or other viscera, for example, liver, spleen or brain. Although local spread onto the outer surface of the liver is common, parencymal/intrahepatic liver metastases (and other visceral metastases) are rare. Ovarian cancer is unique in its pattern of spread, which tends to be localized to the peritoneum rather than more distant metastases, and patients most often die of this local infiltration, causing for example bowel obstruction, as opposed to more distant metastases (Janczar S, Graham JS et al. 2009).

The biology underpinning these distinct epithelial phenotypes (i.e. serous, clear cell, endometriod) is less well defined than in other cancers, for example breast cancer, where the gene expression profile is beginning to be used predictively and prognostically to drive research and treatment choices, and it is generally accepted that no gene expression profile, as yet, has surpassed using the known historical risk factors; age at diagnosis, stage and grade of the tumour, amount of residual disease

and histological subtype (Cannistra 2004), (Bristow, Tomacruz et al. 2002). Many groups are working on this though and in the last couple of years much larger, more adequately powered studies, have begun to emerge, including the work of Tothill and colleagues who identified six subtypes; two of which clearly highlighted groups of patients with significantly worse survival - and they were able to validate this in an independent array (Tothill, Tinker et al. 2008).

Ninety percent of early stage patients (FIGO stage I/II) (International Federation of Gynaecology and Obstetrics) can be treated with surgery alone although patients with grade 3 or stage 1C or II are often considered for systemic chemotherapy. However due to the nature of the presenting symptoms which can be vague (for example constipation, abdominal bloating, dyspepsia, back ache, urinary frequency, early siety or nausea) approximately 80% present with advanced disease and these patients require chemotherapy in addition to surgery (FIGO Stage III/IV) (Agarwal and Kaye 2003; Cannistra 2004).

Survival is improved for patients who undergo surgery by a specialist surgeon and optimal debulking (no residual disease following surgery) also impacts on survival. 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 more recently it has been suggested that it is patients with less advanced disease who derive maximal benefit from this approach (Crawford, Vasey et al. 2005). For most patients TAH, BSO, omentectomy and pelvic washings is standard but for those with very early disease and or young age a fertility sparing approach is sometimes undertaken.

Adjuvant chemotherapy is offered to most patients with advanced (Stage III and IV cancer) and also to 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 (Winter-Roach, Kitchener et al. 2009). For these patients 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, Brady et al. 2003). There is significant variation in clinical practice but generally most patients are offered carboplatin and paclitaxel for 6 cycles. ICON3 concluded that single agent carboplatin can be regarded as reasonable first-line treatment in ovarian cancer (ICON Group, 2002) and as a result some centres use single agent carboplatin leaving the taxane for use in relapse. Less fit or earlier stage patients are also often offered carboplatin alone. This has the advantage of avoiding the peripheral neuropathy, increased emesis and hair loss of combined treatment but has the disadvantage of additional thrombocytopenia).

It has long been established that taxanes offer a platelet sparing effect when administered along with carboplatin although the mechanism is not understood (Sharma R, Graham JS, manuscript in press). Paclitaxel is generally the taxane of choice although "The Scottish Randomised Trial in Ovarian Cancer" (SCOTROC1) study compared docetaxel-carboplatin with paclitaxel-carboplatin in first-line treatment and found equivalent efficacy (Vasey, Jayson et al. 2004). Patients with advanced disease who respond to chemotherapy but have not had a complete response by 6 cycles are sometimes offered up to 3 additional cycles of chemotherapy. More recent studies, such as CHORUS (ISRCTN 74802813), have investigated the use of neoadjuvant chemotherapy – the rationale being that if maximal debulking has prognostic implications that for some patients – especially those with upper

abdominal disease – the use of up front chemotherapy could improve later surgical results. Interim analysis has shown no disadvantage to proceeding first with chemotherapy and in some centres this has already become established practice for certain patients.

Mucinous tumours show a lower response to chemotherapy than serous tumours and hence confer a poorer prognosis (Pignata, Ferrandina et al. 2008), and are usually only diagnosed when a colorectal cancer has been definitively excluded. A clinical trial randomizing patients with mucinous ovarian cancer to carboplatin and paclitaxel vs. capecitabine and oxaliplatin (an established colorectal regimen) is due to open in the UK (http://www.controlled-trials.com/ISRCTN83438782). Patients with clear cell pathology also show a much lower response to carboplatin and paclitaxel and a Phase III collaborative study, sponsored by CRUK, called CCC1 is currently investigating carboplatin and paclitaxel vs. cisplatin and irinotecan (www.cancerresearchuk.org) in patients with clear cell ovarian cancer. In addition patients with LGS tumours have a higher rate of expression of *BRAF* and *KRAS* and a trial is currently planned to investigate a MEK inhibitor in these patients.

As patients with ovarian cancer tend to die from local spread of their disease as opposed to distinct metastases the role of intraperitoneal (IP) chemotherapy has been examined more recently. Although some controversy surrounds these trials it is generally accepted that although a combination of intravenous (IV) and IP chemotherapy is less well tolerated than a purely IV approach that patients do gain a statistically significant survival benefit. However, although this practice has become standard of care in some countries this is not the case in most of the UK. There are multiple reasons for hesitancy to take up this approach including increased toxicity,

the resource implications (both in terms of insertion of the IP catheters and their subsequent maintenance and complication management) and the belief that in most of the studies like was not compared with like – the patients in the IP arm often received a more dose intense regimen. A number of comprehensive reviews reflecting both sides of the ongoing debate have been published (Armstrong and Brady 2006; Gore, du Bois et al. 2006; Jaaback and Johnson 2006; Petignat, du Bois et al. 2006; Elit, Oliver et al. 2007; Fujiwara, Armstrong et al. 2007; Fung-Kee-Fung, Provencher et al. 2007; Marth, Walker et al. 2007) and current studies aim to reduce toxicity and compare regimens of the same dose intensity.

Patients with germline mutations in *BRCA 1* and *2* have disease which is clinically distinct from the other subtypes - recently a significantly higher incidence of visceral metastases was reported in these patient's (74% of patients compared to 16%) (Gourley, Michie et al. 2009). For patients carrying a *BRCA1* or *2* germline mutation very encouraging results have been seen using PARP inhibitors.

BRCA1 is required for double strand repair, using homologous recombination (HR). PARP inhibitors inhibit Poly ADP ribose polymerase, leading to generation of single strand breaks and replication fork collapse during DNA replication, leading to DNA double strand breaks. Such double strand breaks can be repaired by HR if functional *BRAC1* or *2* is present, but in the absence of HR cells are sensitive to PARP inhibition and die. Thus, although the cell can survive with one or other of these mechanisms intact if both are knocked out simultaneously this has dramatic effects and this is termed synthetic lethality. Additionally, in *BRCA1* or *2* carriers their normal cells are heterozygous (i.e. one mutant and one wild type copy), and have normal HR activity

and therefore normal cells avoid this effect and hence toxicity. In contrast, tumours have lost the wild type allele and have defective HR (Dedes, Wilkerson et al. 2011).

Fong et al recently reported an overall clinical benefit rate of PARP inhibitors in ovarian cancer of 46% (95% CI, 32% to 61%) and median response duration was 28 weeks. There was a significant association between the clinical benefit rate and platinum-free interval across the platinum-sensitive, resistant, and refractory subgroups (69%, 45%, and 23%, respectively). In addition a retrospective analysis of the data indicated an association between platinum sensitivity and extent of olaparib response (radiologic change, p =0.001; CA125 change, p = 0.002) (Fong, Yap et al. 2010). Teodoridis and colleagues have shown that *BRCA 1* and *2* are frequently silenced by methylation in ovarian cancer (Teodoridis, Hall et al. 2005).

There is hope that this therapeutic exploitation could be expanded to patients who have other dysfunctions of their HR/ *BRCA* pathway – so called 'BRCAness' patients. Potential reasons for dysfunction of homologous recombination are thought to include epigenetic hypermethylation of the *BRCA1* promoter (Teodoridis, Hall et al. 2005), somatic mutations of *BRCA1/2* (Foster, Harrington et al. 1996; Geisler, Hatterman-Zogg et al. 2002) or loss of function mutations of other genes important in the pathway (Taniguchi, Tischkowitz et al. 2003). Recently Konstantinopoulos et al reported a gene expression profile capable of identifying such patients (Konstantinopoulos, Spentzos et al. 2010).

A multitude of targeted agents are in clinical trials although their role is not as established as in other cancers - the exception being the use of PARP inhibitors in patients with germline mutations in *BRCA 1* or *2* (Fong, Yap et al. 2010), and perhaps

a further role out to include '*BRCA*ness patients' (Konstantinopoulos, Spentzos et al. 2010) (J. A. Ledermann 2011) .

Currently an adequate screening test for ovarian cancer has not been identified. Although Ca125 is useful in diagnosis and disease monitoring approximately 20% of patients with the disease do not express this marker (Lalwani, Prasad et al. 2011) and in addition because ovarian cancer remains relatively rare within the general population, optimising a test with high enough sensitivity and specificity is difficult. This means that the majority of patients still present with advanced disease and the mainstay of treatment remains surgery and adjuvant chemotherapy.

1.1 **Biomarkers in ovarian cancer.**

No prospective biomarkers, independent for known clinical/pathological markers, have been validated, in epithelial ovarian cancer, to a high enough level as to be clinically useful when attempting to differentiate these heterogeneous responses to treatment in patients (with the exception of identifying patients with germline mutations in *BRCA*). The implication of this is that many patients are given chemotherapy who do not benefit from it – either because they don't need it or because they will not respond to it and this represents a large group of patients receiving toxicity without gain.

Currently the only useful clinical surrogate marker in relapsed disease is the platinum free interval (PFI). This is defined as the time in months from the end of treatment (with a platinum containing regimen) to relapse. Patients who relapse within 6 months are termed platinum resistant, those who never respond platinum refractory and those who relapse after more than one year platinum sensitive. The term partially

sensitive has more recently been used to describe the patients with a PFI of 6 to 12 months. Which of these groups a patient fits into has a significant impact on their projected response to future treatment and their overall survival. Patients who relapse within 6 months have a response to second line platinum based chemotherapy of around 10% whereas those who relapse 24 months following chemotherapy can expect a response of >60% to platinum rechallenge (Gordon, Tonda et al. 2004; Mutch, Orlando et al. 2007; Ferrandina, Ludovisi et al. 2008).

The standard treatment of platinum sensitive patients is rechallenge with platinum +/- a taxane whereas for platinum resistant patients other agents such as liposomal doxorubicin or topotecan are offered – or more recently 'dose dense' platinum based regimens (were treatment is given weekly rather than three weekly). Although dose dense treatment has shown encouraging responses of up to 50% in early phase trials, the impact on overall survival (OS) remains to be tested in a larger randomised setting (Sharma, Graham et al. 2009).

The problem with using the PFI as a surrogate marker is that it tells you the patient will do well or badly according to how quickly they relapse and so it does not help to guide a patient's initial treatment. Most clinicians and researchers would agree that what is really needed is to be able to prospectively predict a patients prognosis and guide the use of adjuvant treatment accordingly (prognostic biomarker) and to be able to stratify particular groups to targeted treatments as a result of the molecular phenotype of their tumour (predictive biomarker). In advanced disease predictive markers are much more needed than prognostic markers - all patients should receive platinum unless it could be predicted that they were resistant to this and should receive an alternative drug. In early stage disease there is a role for prognostic

markers as if the prognosis was exceptionally good this would be an argument for not needing chemotherapy.

In the next subchapter the role of epigenetics, and more especially DNA methylation will be discussed followed by the various mechanisms of drug resistance in ovarian cancer – many of which are epigenetically regulated.

1.2 Epigenetic gene regulation

Epigenetics describe a heritable change in gene expression without a change in the DNA sequence. Abnormal gene regulation, which can be as a result of genetic or epigenetic changes, is central to the initiation and maintenance of cancer and it has been hypothesised that epigenetic mechanisms are the most prevalent driver of tumourogenesis (Sjoblom, Jones et al. 2006). Three main epigenetic modifications exist; DNA methylation, histone modifications (methylation, acetylation, phosphorylation and others) and epigenetic regulation of microRNA's. These will next be discussed in more detail.

1.2.1 DNA methylation

1.2.1.1 Physiological methylation

DNA methylation is the most widely studied epigenetic modification, which occurs in mammalian DNA at CpG dinucleotides, where the hydrogen bond at the fifth position of cytosine becomes methylated (Bird 2002). This is illustrated in Figure 1 below.

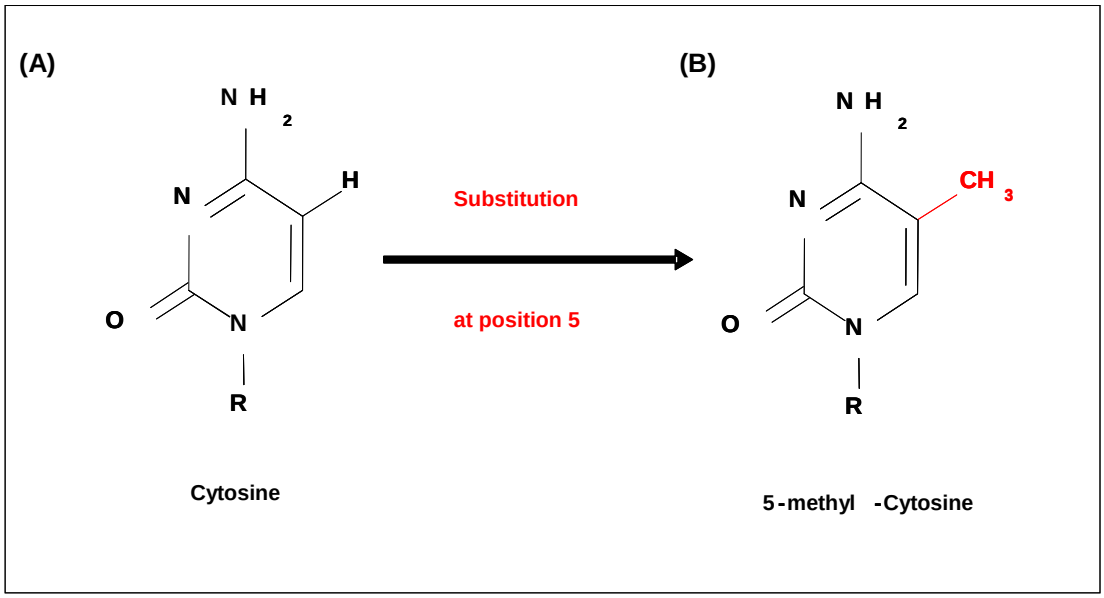


Figure 1. Chemical modification of cytosine methylation. (A) The chemical structure of the base cytosine. (B) the chemical structure of 5-methylcytosine following enzymatic transfer of a methyl (CH3) group (Graham, Kaye et al. 2009).

Three to five percent of all cytosines in the human genome are methylated. Approximately 70% of genes have CpG rich regions, called CpG islands (CGI's), often located in the promoter or first exon regions – which under normal conditions are unmethylated but if methylation occurs this can have a profound effect on gene transcription, resulting in gene silencing (Saxonov, Berg et al. 2006). There are estimated to be about 30 000 of such CGIs in the human genome. Various definitions for a CGI exist – in this thesis the Gardener Garden definition is used – this defines a CGI as a region with at least 200 base pairs and with a GC percentage that is greater than 50% and with an observed/expected CpG ratio that is greater than 60% (Gardiner-Garden and Frommer 1987). More recently epigenetic mapping of lung fibroblasts and human embryonic stem cells has uncovered that stem cells show a unique ability to methylate non CG residues and DNMT3A and DNMT3L have been implicated in this process (Costello, Krzywinski et al. 2009).

In contrast to CpG islands, repetitive genomic sequences are heavily methylated and this is thought to have a role in the protection of chromosome integrity, by preventing chromosome instability, translocations and gene disruption through the reactivation of endoparasitic sequences (Esteller 2007). This area has recently provoked increased interest and it is thought that global demethylation is likely to contribute to a large number of genetic changes that are a feature of carcinogenesis (Esteller 2007). As yet it is not clear whether global demethylation influences promoter hypermethylation or vice versa.

Cytosine methylation represents a stable heritable yet reversible mark. Evidence that the well known long term effects of early environmental exposures have an epigenetic component that can be maintained across generations has sparked great interest in the epigenetic field (Costello, Krzywinski et al. 2009). DNA methylation is usually associated with transcriptional repression – either due to inhibition of the binding of factors to their cognate DNA recognition sequences or because it recruits methyl CpG binding proteins (MeCPs and MBDs) together with co-repressor molecules (Guil and Esteller 2009).

DNA methylation patterns are established via DNA methyltransferases (DNMT's). Four mammalian subtypes are known – DNMT1, 2, 3a and 3b (Bird 2002). DNMT1 is classically described as the maintenance DNMT whereas the others are thought to induce *de novo* methylation (Bestor and Ingram 1983; Li, Bestor et al. 1992). DNMT1 mediates the reinstatement of fully methylated sites from initially hemi-methylated substrates in daughter cells and consistent with this it has been shown to bind to proliferating cell nuclear antigen during S phase (Leonhardt, Page et al. 1992). In fact it seems DNMT1 is not just a maintenance enzyme but also has the ability to induce

de novo methylation in cancer by interacting with proteins such as transcription factors (p53, STAT3, HP1), histone modifiers (HDAC1, HDAC2) and ligands (DAXX) to specifically repress targeted genes (Cheung, Lee et al. 2009) .

DNMT3a and b are thought to show a preference for unmethylated DNA and are involved in initiating methylation patterns during embryogenesis including embryonic stem cells, in mice (Cheung, Lee et al. 2009). A further DNMT known as DNMT3L is essential for the establishment of germ line DNA methylation and stimulates *de novo* methylation by DNMT3a and b. These two enzymes are clearly of fundamental importance as knock outs for DNMT1 cause mice to die at 4 weeks and homozygous knockout of DNMT1 and 3b is embryonically lethal (Li, Bestor et al. 1992).

There have been several reports associating increased levels of DNMT1 with solid tumours including pancreatic, gastric, cholangiocarcinomas and colon cancers (Etoh, Kanai et al. 2004; Peng, Kanai et al. 2005; Zuo, Luo et al. 2008). Zuo found that co-transfection with antisense eukaryotic expression plasmids of DNMT1 and DNMT3b gene and single transfection with antisense eukaryotic expression plasmid of DNMT1 gene could suppress the growth and proliferation of QBC-939 (cholangiocarcinoma cell line), block the cell cycle at G1 phase and increase apoptosis, resulting in a smaller tumour in the subcutaneous tissue of nude mouse. The suppressing biological effect of co-transfection was stronger than single transfection with antisense DNMT1 and single transfection with antisense eukaryotic expression plasmid of DNMT3b gene had no effects on the biological characteristics of QBC-939 (Zuo, Luo et al. 2008).

DNMTs interact with EZH2 (a Polycomb Group protein) (Vire, Brenner et al. 2006) to tri-methylate histone H3 at the lysine at position 27 from the carboxy tail (H3K27me3). EZH2 is required for H3K27 trimethylation, which is associated with a repressive chromatin state and transcriptional silencing, and so DNMTs modulate gene repression at both the DNA and histone level (Shen, Liu et al. 2008).

DNA methylation is required for the maintenance of a variety of cell types and is involved, along with transcription factors, in programming cells to adopt different phenotypes. It regulates the genome by influencing gene expression and chromosome structure and by suppressing transposons (Costello, Krzywinski et al. 2009) , and other potentially harmful viral and parasitic sequences (Liu, Wylie et al. 2003).

In humans two waves of global demethylation are observed. Soon after fertilization the highly methylated gametes are actively demethylated (termed reprogramming). Then in the gamete further demethylation occurs at different times in male and female genomes. In males this is believed to be an active process whereas in the female it has been proposed to be a passive process, involving the dilution of methylation secondary to a lack of DNMT1 as the DNA replicates (Cheung, Lee et al. 2009). Both sexes then regain methylation during implantation and DNMT3 has been implicated here. DNA methylation is also responsible for silencing of the second X chromosome in females (Santos, Hendrich et al. 2002) (so they do not have twice as much expression of genes).

In addition, DNA methylation is important in genomic imprinting where promoter methylation of either the paternal or maternal allele is associated with transcriptional repression (Bartolomei and Tilghman 1997). Imprinted genes are protected from the first wave of global demethylation and this ensures proper monoallelic expression of

imprinted genes which are necessary in early embryogenesis (Cheung, Lee et al. 2009). Methylation has also been implicated in cell type-specific gene expression. *MCJ* (Strathdee, Davies et al. 2004), *HOXA5* (Strathdee, Sim et al. 2007) and *MASPIN* (Futscher, Oshiro et al. 2002) have all been shown to be regulated in this way.

1.2.1.2 Pathological methylation

Several congenital malignancies have been linked to abnormal DNA methylation. Immunodeficiency Centromeric Region Instability Facial anomalies (ICF) syndrome is associated with mutations in DNMT3B (Xu, Bestor et al. 1999) and Beckwith-Wiedemann and Prader-Willi syndromes are both associated with abnormal imprinting as a result of aberrant DNA methylation (Robertson 2005).

In ageing and cancer the usual patterns of methylation tend to reverse, with the coding CpG sites becoming hypomethylated (Issa 1999; Ahuja and Issa 2000; Issa 2000; Liu, Wylie et al. 2003; Richardson 2003), while promoter CGI's become hypermethylated. The genome wide hypomethylation is thought to cause genomic instability and re-expression of harmful viral genomic sequences and oncogenes, whereas the promoter hypermethylation can result in silencing of genes.

This is illustrated in Figure 2 below:

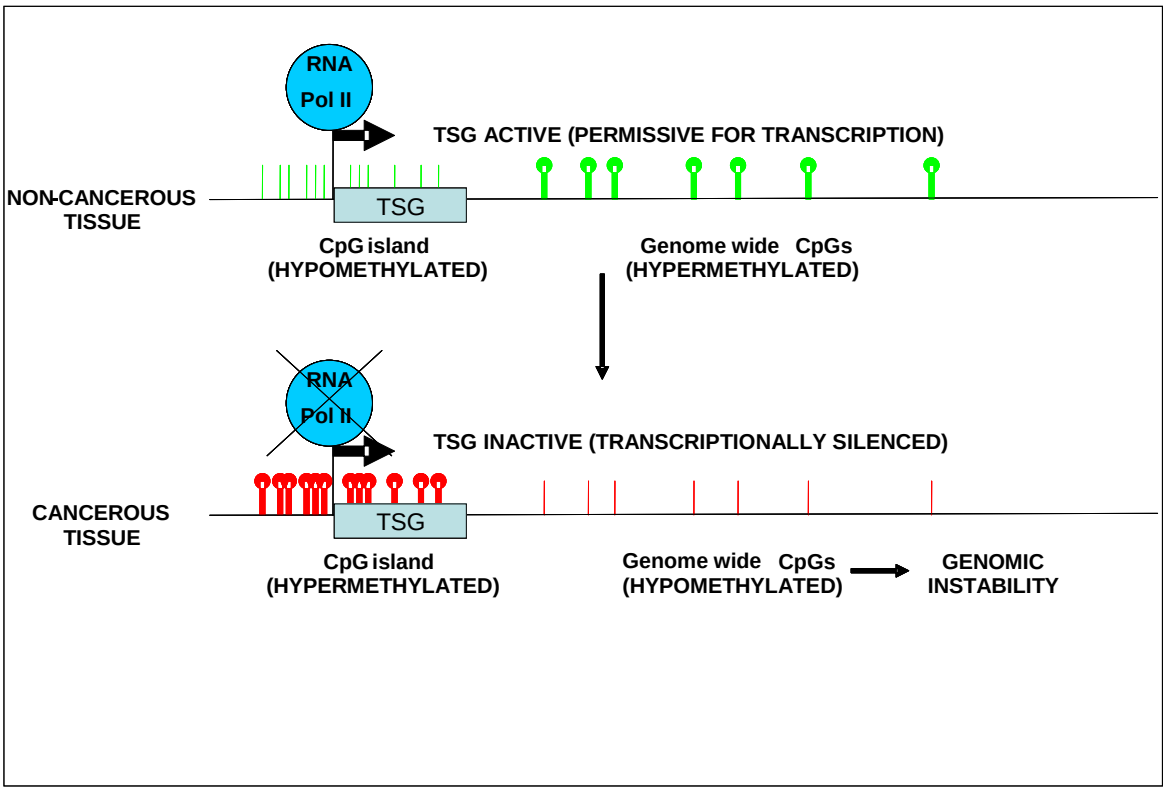


Figure 2. DNA methylation and cancer. A representation of a region of DNA in non-cancerous (top; green) and cancerous (bottom; red) tissues showing the differences in DNA methylation in the two phenotypes. In non-cancerous tissue, genome wide hypermethylation of CpG's (closed green circles) and an actively 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 (Graham, Kaye et al. 2009).

It has been proposed that distinct subsets of tumours have a marked propensity for promoter CpG island DNA methylation and associated gene silencing. This has been shown most convincingly for colorectal cancer, where it is termed CIMP (CpG island methylator phenotype), but is also proposed for other solid tumours. Issa and colleagues initially observed a group of promoters whose methylation was associated with proximal colon cancers showing mucinous differentiation and a high frequency of micro satellite instability (MSI) (Toyota, Ahuja et al. 1999). Yasmashita contested the existence of such a phenotype and that gradually acquired epigenetic changes could supersede genetic mutations (Yamashita, Dai et al. 2003). Following this a

study by Laird and colleagues strongly suggests that CIMP does exist and that it is closely associated with colon cancers exhibiting BRAF mutations and MSI (Weisenberger, Siegmund et al. 2006).

A similar phenotype, consisting of genes associated with DNA damage response, has been proposed for ovarian cancer (Strathdee, Appleton et al. 2001) (Teodoridis 2005), although the genes concordantly methylated are different from that seen on the methylator phenotype associated with colon cancer. This study determined the methylation frequencies of 24 CGI's of genes associated with DNA damage responses or with ovarian cancer in 106 advanced stage EOCs. Frequent methylation was observed for 6 genes including *BRCA1*. Unsupervised gene shaving identified a non-random pattern of methylation for 5 of these genes. Methylation of at least one of the three genes associated with DNA repair/ detoxification was associated with an improved response to chemotherapy and the study supported the hypothesis of a methylator phenotype in EOC. Examples of individual genes or pathways that have been shown to be regulated by DNA methylation are discussed in Chapter 1.3.

In addition to promoter DNA hypermethylation and global hypomethylation various corresponding histone modifications are key to organising nuclear architecture and subsequent gene transcription and DNA methylation and histone modification have been shown to closely influence each other in order to regulate gene expression. It is thought that chromatin alterations can determine gene expression and that subsequent methylation changes 'lock' the pattern of expression; rather like a gate and a lock.

Adult cancers may derive from stem or early progenitor cells. Ohm et al found that in embryonic stem cells, certain genes are held in a 'transcription-ready' state mediated

by a 'bivalent' promoter chromatin pattern consisting of the repressive mark, histone H3 methylated at Lys27 (H3K27) by polycomb group proteins, plus the active mark, methylated H3K4. The authors hypothesized that cell chromatin patterns and transient silencing of these important regulatory genes in stem or progenitor cells may leave these genes vulnerable to aberrant DNA hypermethylation and heritable gene silencing during tumour initiation and progression (Ohm, McGarvey et al. 2007).

Embryonic stem cells rely on Polycomb group proteins to reversibly repress genes required for differentiation. Widschwendter et al demonstrated that stem cell Polycomb group targets are up to 12-fold more likely to have cancer-specific promoter DNA hypermethylation than non-targets, supporting a stem cell origin of cancer in which reversible gene repression is replaced by permanent silencing, locking the cell into a perpetual state of self-renewal and thereby predisposing to subsequent malignant transformation (Widschwendter, Fiegl et al. 2007).

1.2.2 Histone modifications

Histones are the protein moiety around which DNA is packaged within chromatin and DNA methylation at CGIs is associated with chromatin being in a repressive state for gene transcription. Nucleosomes form the basic repeating subunit of chromatin and consist of around 147 base pairs of DNA wrapped around a histone octamer that is formed by four histone partners – H3, H4, H2A and H2B (Kouzarides 2007). In general condensed chromatin (heterochromatin) mediates transcriptional repression, whereas transcriptionally active genes are in areas of open chromatin (euchromatin) (Kouzarides 2007).

Extending out of the nucleosome are charged amino-terminal histone tails which are subjected to a huge number of potential post-translational modifications (Kouzarides 2007). At least eight modifications have been observed of which the most common are methylation, acetylation and phosphorylation. Modifications have previously been detected in more than sixty different residues and in addition the situation is more complex because lysines can be mono-, di- or trimethylated and arginines can be mono- or dimethylated (Kouzarides 2007). Acetylation of lysine residues is catalyzed by histone acetyltransferases (HATS) resulting in a more open chromatin whereas deacetylation is performed by HDACs and associated with transcriptional repression. Some of the most common activating and repressive marks are shown in table 2 below.

Table 2 Examples of histone marks caused by methylation and acetylation.

Histone Residue	Histone Modification	Histone Residue	Histone Modification
Activating		*Repressive*	
H3 K4	M	H3 K9	M
H3 K9	A		
H4 K5	A	H3 K27	M
H4 K8	A		
H4 K12	A	H4 K20	M
H4 K16	A		

H histone, K lysine, M methylation, A acetylation

Methylated DNA can recruit methyl binding domain proteins (MBD proteins) and these are able to associate with protein complexes involving histone deacetylases (HDAC's) and chromatin remodelling proteins (sin3a and mi-2). As illustrated in Figure 3, transcriptionally active genes have an open structure with acetylated lysine residues. In order for transcriptional repression histone deacetylation occurs which leads to a closed tighter binding between the positively charged lysines and the

negatively charged DNA backbone. This tighter binding makes it more difficult for transcription factors to bind.

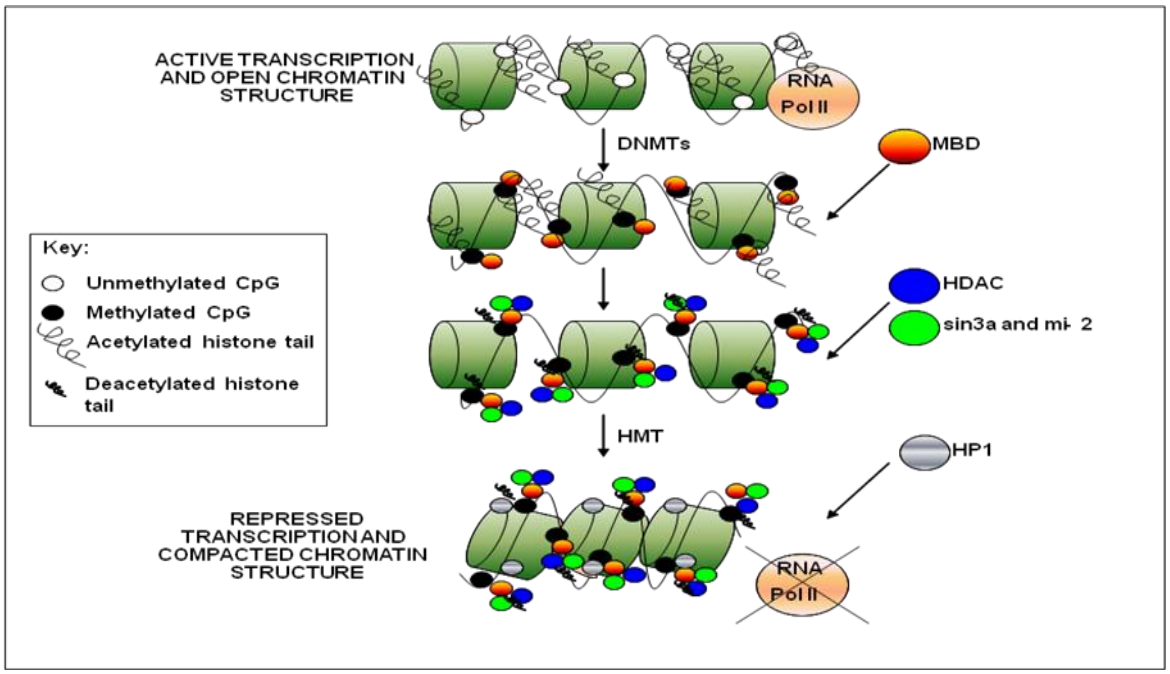


Figure 3. Epigenetic mechanism of transcriptional repression and chromatin remodelling. Active transcription is associated with an open chromatin structure, acetylated histones and unmethylated CpG's (white). RNA polymerase (RNA Pol II) (peach) and transcription factors can access and transcribe the gene. DNMTs methylate CpG's (black) and bind methyl binding domain, MBD, proteins (orange). Subsequent recruitment of histone deacetylases, HDAC, (blue) and chromatin remodelling proteins, sin3a/mi-2, (green) leads to remodelling of chromatin and deacetylation of histone tails. Histone methyltransferases, HMTs, methylate lysine residues, allowing binding of heterochromatin protein 1, HP1, (grey) to chromatin (Graham, Kaye et al. 2009).

As stated, the methylation of DNA is usually connected to certain histone modifications and this sets a mark for the binding of methyl-binding domain proteins, which act in a chromatin-repressive mode (Chapman-Rothe Nadine 2009). As an example of the interaction between the various histone modifications, the histone position H3K9 is a site of both acetylation and methylation. Deacetylation of H3K9 is required for methylation to occur, which is then a repressive epigenetic mark. Trimethylation of H3K9 results in the recruitment and binding of the transcriptional repressor, heterochromatin protein HP1. HP1 binding to the methylated H3 forms a positive feedback loop, mediating the propagation of heterochromatin over wide chromosomal ranges. Thus, covalent modification of the histone tails directly affects

higher order chromatin structure and thereby offers a mechanism of epigenetic gene activation or silencing (Graham, Kaye et al. 2009).

1.2.3 MicroRNA

The most recent area of interest has been of the role of microRNAs (miRNAs) in epigenetic regulation. MiRNA's are short pieces of non-coding, single stranded RNA, 21-25 nucleotides long, which can also control genes expression (He and Hannon 2004; Chapman-Rothe Nadine 2009). They were first identified in *C elegans*, where Lin-4 was shown to have a key role in development, and have since been shown to be highly conserved amongst species (Pasquinelli, Reinhart et al. 2000) Most miRNAs bind to the target 3' untranslated region (UTR) and act as translational repressors (Bartel 2004). An additional role has been characterised whereby they have shown to be components of the RNA interference pathway (RNAi). Here they behave in a similar manner to siRNAs although there are some differences; miRNAs, especially those in animals, usually exhibit imperfect base pairing to a target and inhibit the translation of many different mRNAs (messenger RNA) with similar sequences. In contrast, siRNAs typically base-pair perfectly and induce mRNA cleavage only in a single, specific target (Pillai, Bhattacharyya et al. 2007).

MiRNA profiling has been performed in ovarian cancer and has been shown to successfully differentiate ovarian tumours from normal ovary. It has also been able to define sub groups with significantly different survival times. MiRNAs could potentially be a rich source of epigenetic markers, like DNA methylation. Exosomes, generated from tumour cells and circulating in the blood, have been shown to contain miRNA reflecting that expressed in the original tumour, representing a potential

surrogate marker (Taylor and Gercel-Taylor 2008). In addition, miRNAs may play a role in regulating cisplatin response as miR-214 has been shown to negatively regulate PTEN at the 3' UTR and this particular miRNA is upregulated in many ovarian tumours. Interestingly this effect could be reversed using AKT inhibitors confirming the link between miR-214, the AKT pathway and platinum resistance (Yang, Kong et al. 2008).

As discussed, there is thought to be a complex interaction between DNA methylation, histone modifications and more recently microRNA, which is not fully understood - and in the case of DNA methylation it is likely that small changes in lots of genes rather than substantial methylation of an individual candidate is clinically relevant. This means that the ability to detect small real changes as opposed to false positives remains a big challenge if we are to be able to translate from the bench to the bedside, using high throughput studies such as methylation and expression arrays. For the purpose of this thesis I will concentrate solely on the role of DNA methylation at the gene promoter in cancer and more specifically its role in acquired drug resistance.

1.3 **Drug resistance in ovarian cancer**

For the majority of patients the standard of care remains extensive surgery +/- carboplatin and paclitaxel. Although approximately 80% respond to initial treatment, 5 year survival remains poor at approximately 40% and drug resistance is thought to cause treatment failure and death in more than 90% of patients with metastatic disease (Greenlee, Hill-Harmon et al. 2001; Agarwal and Kaye 2003).

The 20% of patients who essentially never respond to chemotherapy are defined as having intrinsic drug resistance and for the rest who initially respond and then relapse the term acquired drug resistance is used. For this group less frequent and shorter duration of responses are consistently seen following re-treatment (Vasey 2005). The mechanisms underlying intrinsic and acquired drug resistance are thought to be different.

There are two main hypotheses for how acquired drug resistance occurs and this is one of the most controversial parts of ovarian research in current times. One theory is that ovarian cancer is derived from different clones and that over time a selective pressure is applied, via chemotherapy, whereby the chemosensitive clones disappear and the chemoresistant clones are left – survival of the fittest.

The alternative hypothesis is that 'cancer stem cells' (CSC) exist and that chemotherapy kills off the standard cancer cells but the cancer stem cells are not targeted effectively (Agarwal and Kaye 2003). Cancer stem cells must be capable of self renewal and able to form cells which differentiate into multiple different cell types. In addition they must be highly tumorogenic and often the test of whether a subpopulation is tumour initiating is their ability to cause tumours from a very low cell number. The two hypotheses are shown in Figure 4 below:

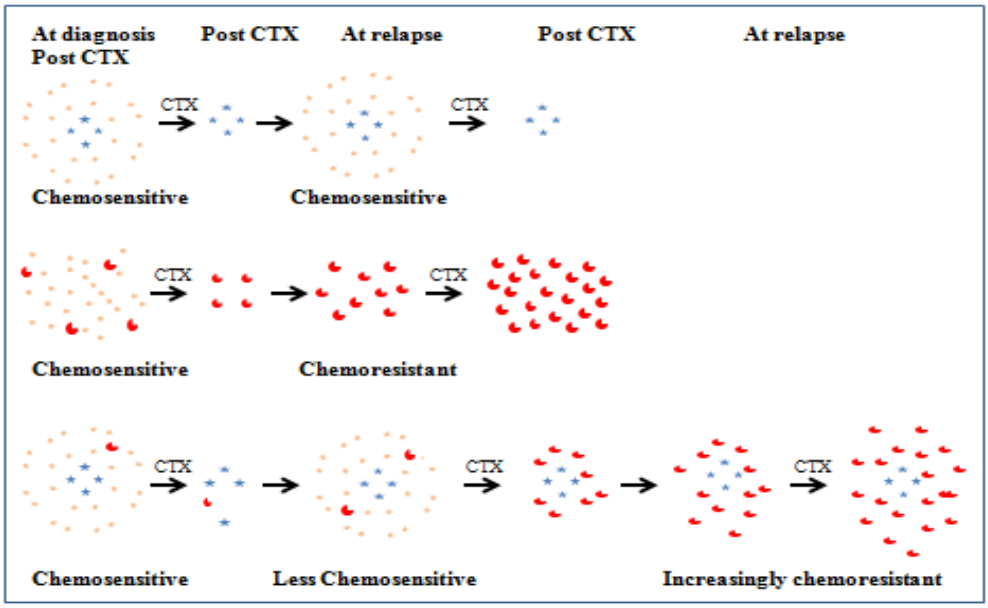


Figure 4. Models of drug resistance in ovarian cancer. (a) Regrowth of a persistent stem cell population (peach) which can be initially chemosensitive. (b) Initial response to chemotherapy (CTX) seen due to drug sensitive cells (peach) followed by clonal expansion of a subpopulation of chemoresistant cells/regrowth of a chemoresistant progenitor cell population (red). (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 (Agarwal and Kaye 2003).

The underlying cause of acquired drug resistance was recently investigated by Cooke et al. In order to investigate this in high-grade serous (HGS) ovarian cancers, the authors first analysed cell line series derived from three cases of HGS carcinoma before and after platinum resistance had developed (PEO1, PEO4 and PEO6; PEA1 and PEA2; and PEO14 and PEO23) (see chapter 2.17.4). They then performed analysis using 24-colour fluorescence in situ hybridisation (FISH) and SNP array comparative genomic hybridisation (CGH) and showed mutually exclusive endoreduplication and loss of heterozygosity events in clones present at different time points in the same individual. This implies that platinum-sensitive and -resistant disease was not linearly related, but shared a common ancestor at an early stage of tumour development. Array CGH analysis of six paired pre- and post-neoadjuvant treatment HGS samples from the CTCR-OV01 clinical study did not show extensive

copy number differences and this suggested that one clone was strongly dominant at presentation. Their data demonstrated that cisplatin resistance in HGS carcinoma develops from pre-existing minor clones but that enrichment for these clones is not apparent during short-term chemotherapy treatment (Cooke, Ng et al. 2010).

Tumour sustaining cells were first identified in leukaemia cells (CD34+/CD38-) by Bonnet and colleagues (Bonnet and Dick 1997). In solid tumours sub populations with stem cell properties were first demonstrated in breast cancer (CD 44+/24-) (Al-Hajj, Wicha et al. 2003) and more recently in ovarian cancer among others (glioma, melanoma, prostate, colon, lung, head and neck and pancreatic cancer)(Collins, Berry et al. 2005; Fang, Nguyen et al. 2005; Kim, Jackson et al. 2005; Dalerba, Dylla et al. 2007; Li, Heidt et al. 2007; Prince, Sivanandan et al. 2007).

Cancer initiating cells in ovarian cancer were first proposed in 2005 by Bapat and colleagues (Bapat, Mali et al. 2005). They identified a subgroup of cells that could grow in spheroid culture, in a similar manner to that found in ascites (Burleson, Casey et al. 2004), and when injected into mice could form tumours similar to the original tumour. These cells expressed CD117 (Natali, Nicotra et al. 1992). Szotek *et al* one year later demonstrated a subpopulation, using their dye efflux ability, which were highly tumorogenic compared to the cells that had retained the dye (Szotek, Pieretti-Vanmarcke et al. 2006). These cells also expressed CD117.

More recently Zhang *et al* demonstrated a distinct sub population in ovarian cancer that fulfilled all the criteria of a tumour sustaining cells – they were highly tumorogenic, showed increased chemoresistance and up regulation of stem cell and

chemoresistance associated genes as demonstrated by RTPCR. *OCT4, NESTIN, NANOG, SCF, NOTCH-1, BMI-1* and *ABCG2* were examined in two tumour samples. All genes showed a clear increase in expression in the ovarian cancer initiating cells (OCIC's) grown under stem cell selective conditions as compared to the parental bulk of cancer cells or OCIC's grown in differentiated conditions (withdrawal of growth factors and addition of 10% FBS). *OCT4, NESTIN* and *NANOG* are established stem cell markers necessary for embryogenesis, neurigenesis and haemopoiesis (Loh, Wu et al. 2006) (Mohle and Kanz 2007) (Wiese, Rolletschek et al. 2004). The Notch pathway was shown to be important by Hopfer et al (Hopfer, Zwahlen et al. 2005).

Tumour sustaining cells possess several of the characteristics that promote chemoresistance such as expression of membrane efflux transporters, enhanced DNA repair and low mitotic index hence the addition of ABCG2 to the RTPCR profile (Sharom 2008). Zhang used CD117 and additional CD44+ selection to define the stem cell population (Zhang and Rosen 2006). The main controversy surrounds the origin of these cells – i.e. do cancer stem cells originate from normal stem cells or do they arise within the tumour itself somehow acquiring this phenotype.

In addition to the debate as to whether drug resistance is selected for during chemotherapy as a result of the emergence/ persistence of drug resistant clones or CSC's a wide variety of mechanisms for acquired drug resistance have been investigated *in vitro*. These can broadly be divided into pharmacokinetic (PK) and pharmacogenomic (PD) variation relating to the individual patient and changes in the tumour itself and its microenvironment.

1.3.1 Intrapatient variation

Patients have varying first pass metabolism, ability to convert prodrugs to their active metabolite and varying rates of hepatic metabolism and renal clearance. It is thought that some patients are more able to efficiently efflux active drug from their cells. In addition silencing of drug sensitivity genes, TSGs and genes involved in for example DNA damage response (as cisplatin induces DNA damage) are likely to affect chemosensitivity and hence resistance. Currently most chemotherapies are dosed using the patient's surface area (a measure proportional to their height and weight) but it is clear from the huge variation in toxicity that patients get from chemotherapy that this is over simplistic. If a biomarker could be optimized that predicted the dose necessary for a patient to obtain the maximal biological effect with minimal toxicity this would be of huge utility.

1.3.2 Key processes and pathways implicated in drug resistance

1.3.2.1 Drug efflux

Cancers often develop resistance to multiple drugs and this is termed multi drug resistance. The ABC transporter proteins consist of several members - P-glycoprotein (PGP) (Baekelandt, Holm et al. 2000), multidrug resistance protein (MRP1 or ABCB1) (Marsh, King et al. 2006; Green, Soderkvist et al. 2008; Johnatty, Beesley et al. 2008; Grimm, Polterauer et al. 2010), lung resistance-related protein (LRP) and breast cancer resistance protein (BCRP or ABCG2) (Dou, Jiang et al. 2010). *LRP*

expression has been shown to confer a poorer prognosis in squamous lung cancer and acute myeloid leukaemia (AML) (Zhou, Zittoun et al. 1995) and to predict a poorer response to treatment in AML and ovarian cancer (Izquierdo, van der Zee et al. 1995). These proteins are all involved in drug efflux – i.e. they result in more of the cancer drug being excluded from the cancer cell and interestingly, more recently, this property has been utilized to identify 'side populations' thought to represent cancer stem cells; as this is thought to be one of their key attributes.

Various single nucleotide polymorphisms (SNPs) have been investigated in drug efflux proteins in ovarian cancer although results have been conflicting. Bosch et al demonstrated that ovarian cancer patients homozygous for the C1236T SNP in *ABCB1* showed a 25% decrease in the clearance of docetaxel compared to wild-type or heterozygous however Nakajima and colleagues could not reproduce this in their population. And in contrast Nakajima found the C3435T SNP in *ABCB1* to be associated with paclitaxel clearance whereas Bosch had not found this (Bosch, Huitema et al. 2006), (Nakajima, Fujiki et al. 2005). Equally inconsistent results have been seen when predicting response to chemotherapy (Johnatty, Beesley et al. 2008).

PGP is normally expressed at detectable levels in organs including the colon, adrenal gland, kidney and liver. The *MDR1* gene encodes PGP and is thought to be over-expressed in about 50% of cancers (Hochhauser and Harris 1991). In solid tumours the most research relating to this protein has been in breast cancer. High PGP levels in post-treatment samples correlate with poor outcome but it was unclear what the mechanism for this was (Trock, Leonessa et al. 1997). In osteosarcoma and colon cancer uncertainty also exists about the correlation between PGP over expression and drug efflux and there is some suggestion that *PGP* may also confer increased

aggressiveness (Linn and Giaccone 1995; Eid, Bodrogi et al. 1996; Oka, Fukuda et al. 1997).

1.3.2.2 Drug inactivation

In addition to the pumps themselves, others have also investigated the level of activating and detoxifying enzymes present within cells. The Glutathione S-transferases (GSTs) are a group of detoxifying enzymes that are involved in cisplatin and doxorubicin metabolism, among others. Multiple isoenzymes exist of which GST π is the predominant one in ovarian cancer (Ikeda, Sakai et al. 2003; Cao, Shen et al. 2007).

GSTP1 is frequently methylated in prostate and ovarian cancer and epigenetic silencing may increase chemosensitivity by preventing detoxification of chemotherapy (Hayes and Strange 2000). Conflicting studies have been published in ovarian cancer with some laboratories demonstrating a correlation between the isoenzyme expression and poorer response to chemotherapy, overall survival, and increased residual tumour at the end of chemotherapy (Green, Robertson et al. 1993) - whilst others have not been able to reproduce this (Tanner, Hengstler et al. 1997).

1.3.2.3 DNA repair, damage signaling and apoptosis

As outlined in Chapter 1.1 the platinum family of drugs are the cornerstone chemotherapies in ovarian cancer. Originally cisplatin was utilized but more recently carboplatin was shown to be at least as efficacious and less toxic (Darcy, Tian et al. 2007). These drugs exert their effects by forming DNA-platinum adducts, resulting in apoptosis (Kartalou and Essigmann 2001). DNA is repaired when these DNA adducts

are recognized and removed via the nucleotide excision repair (NER) pathway. As most patients initially respond to treatment but subsequently relapse aberration of the DNA repair pathways is thought to be a critical mechanism of acquired drug resistance.

ERCC1 is a nucleotide excision repair gene, which is hypothesized to have a role in ovarian cancer although conflicting studies exist. It encodes a DNA repair protein that forms part of the NER pathway. GOG 158 was a large randomized Phase III which reported in 2000. It compared cisplatin and a long infusion of paclitaxel with carboplatin and a short infusion of paclitaxel. One of the secondary endpoints of this study was the correlation between mRNA expression of *ERCC1* and PFS and OS in peripheral blood leukocytes and no link was seen (Darcy, Tian et al. 2007).

Most of the other clinical studies have investigated the role of SNPs in *ERCC1* and response to treatment or prognosis. GOG-172, a randomized Phase III study of IP and IV cisplatin and paclitaxel vs. IV administration only, for the treatment of newly diagnosed, stage III ovarian cancer included a secondary endpoints which investigated whether the codon 118 polymorphism in *ERCC1* was associated with prognosis. This particular SNP was not significantly associated with disease progression or death although other studies have suggested that there is a correlation between *ERCC1* SNPs and both PFS and OS in ovarian cancer (Krivak, Darcy et al. 2008). Steffenson et al though found that in ovarian cancer, *ERCC1* codon 118 SNPs predicted response to chemotherapy but did not impact on survival. The main criticism leveled at the SNP studies to date is that most have been too small and therefore not adequately powered to answer these questions definitively.

Mismatch repair (MMR) is another key DNA repair pathway; it is required for engagement of apoptosis (Anthoney, McIlwrath et al. 1996; Drummond, Anthoney et al. 1996) and loss of MMR results in microsatellite instability which can be detected in plasma DNA. *MLH1* is part of this pathway and its gene expression can be silenced by methylation. Such methylation has been demonstrated in 40% of ovarian tumours and is more frequent in samples from recurrence (compared with the primary tumour). Gifford et al have shown that a high proportion of EOC patients in the SCOTROC1 trial acquire methylation of *MLH1*, as detected in plasma DNA, following chemotherapy and that this correlates with poorer survival in ovarian cancer (Gifford, Paul et al. 2004).

MLH1 methylation can be reversed using decitabine (dac) and this results in reversal of drug resistance in human tumour xenografts (Plumb, Strathdee et al. 2000) hence showing the potential of methylation as both a biomarker and a potentially drugable target in ovarian cancer. Although these pre-clinical results suggest that demethylating agents could enhance tumour sensitivity to platinum based chemotherapy via re-expression of *MLH1* especially in patients with ovarian cancer who have relapsed following chemotherapy this has not been proven prospectively in a clinical trial, as yet.

FANCF is another protein, belonging to the same family as *BRCA2*, which is known to have a role in modulating DNA repair. Gene expression can be affected by mutation or methylation and this results in increased susceptibility to DNA cross linking agents such as cisplatin (D'Andrea 2003). Taniguchi showed that *FANCF* was methylated in EOC cell lines and that this correlated with lack of mono-ubiquitinated *FANCD2* and hypersensitivity to cisplatin and mitomycin C (Taniguchi, Tischkowitz

et al. 2003). This is an example of the double-edged sword of demethylating agents, as in this case there is a risk that demethylating drugs could actually result in decreased response to chemotherapy.

Numerous components of the apoptosis pathway have been implicated in cisplatin resistance, including *BCL2, BAD, BAX* and *XIAP* although a direct link to acquired drug resistance in ovarian cancer remains to be established for most. *RASSF1A* is frequently methylated in ovarian cancer (Yoon, Dammann et al. 2001) and has been associated with disease progression in breast cancer (Yan, Shi et al. 2003). *RASSF1A* binds to tubulin resulting in microtubule stabilization and as the taxanes are the second key group of drug in ovarian cancer this is likely to be of clinical relevance. Heat shock protein (HSP) 90 inhibitors, which target this pathway, are currently being tested in clinical trials in solid tumours (Powers and Workman 2006) .

1.3.2.4 Growth factor signaling

The growth factor signaling pathways are the focus of many research groups and much of the interest in developing novel therapies has been channeled towards designing molecules that target these pathways. EGFR (ERBB1) and ERBB2, both cell surface receptor tyrosine kinases, are overexpressed in ovarian cancer (Nielsen, Jakobsen et al. 2004; Zhou, Qiu et al. 2006; Sheng and Liu 2011). They signal via both the MAPK and P13K pathways and activation of these pathways can lead to phosphorylation of BAD and BCL2 inhibiting apoptosis. Overexpression of EGFR and ERBB2 has been associated with cisplatin resistance *in vitro* and poor prognosis in ovarian cancer, *in vivo* (Benedetti, Perego et al. 2008). In addition amplifications of PI3K and activation of AKT have been predicted in approximately one third of patients with ovarian cancer and drugs targeting both these pathways are in

development (Zhang, Zhang et al. 2009). *PTEN*, the frequently dysregulated tumour suppressor gene, is known to regulate AKT signaling (Li, Gao et al.). In addition to inactivation by deletions and mutations it has been shown to be epigenetically regulated, with gene silencing secondary to methylation in lung amongst other cancers (Soria, Lee et al. 2002).

1.4 Summary of key points from the introduction relevant to this project

Epigenetic silencing of genes via DNA promoter methylation is seen in multiple critical genes and pathways associated with ovarian cancer. Epimutations occur more frequently than genetic mutations and they can be detected readily in surrogate tissues such as plasma and ascites (Gifford, Paul et al. 2004). This means that methylation profiling can be utilised to identify key pathways which can then be investigated in more depth, by functional analysis of key genes.

Aberrant DNA methylation can potentially be used to diagnose cancer earlier or to identify groups of patients who may benefit from epigenetic and other targeted therapies(Gifford, Paul et al. 2004). In addition, for genes that acquire methylation during the course of chemotherapy it can highlight pathways and key genes that are important in ovarian cancer pathogenesis (Dai, Teodoridis et al. 2010). Epigenetics is being increasingly implicated in both embryonic and cancer stem cell initiation and maintenance and therefore study of DNA methylation may help to further unravel the answers to cancer initiation and maintenance (Rizzo, Hersey et al. 2011).

It appears that small changes in multiple genes may be of more functional consequence than large changes in one gene and this presents a real challenge when interpreting results from high throughput analyses. In addition, differentiating mutations that are fundamental – so called 'driver (epi) mutations' from ones that are observed but not critical – so called 'passenger mutations' will be the key to using these techniques to increase our knowledge. The field of epigenetics is becoming increasingly complex and the interplay between DNA promoter methylation, histone modifications and micro- and non-coding RNAs is not fully understood.

1.4.1 Thesis hypotheses to be tested

The aim of the experiments outlined in this thesis was to investigate whether increased methylation of genes in drug resistant ovarian tumour cell lines could identify novel biomarkers of acquired drug resistance in EOC. If this was the case one would predict that these candidates would show increased methylation in matched tumour pairs following chemotherapy if *in vitro* resistance correlates with *in vivo* resistance. In addition it could be hypothesised that changes in methylation would cluster to particular key pathways which are required for ovarian cancer initiation and progression. In order for methylation to have a phenotypic effect it would be predicted that an increase in methylation would correlate with a decrease in expression and that this could be reversed using demethylating agents. In order to address these questions bioinformatic approaches were combined with microarray analysis of methylation by differential methylation hybridistion (DMH) in order to identify differentially methylated genes in human ovarian cell line models of acquired drug resistance. Any genes identified in this manner were independently validated in

the laboratory using MSP or pyrosequencing of bisulphite modified DNA – in matched sensitive and resistant cell lines, primary ovarian cancer tumour pairs and matched samples from patients with ovarian cancer, pre- and post- relapse. The correlation between promoter methylation and gene expression was investigated using qRTPCR in cell lines that had been treated with or without decitabine, and in order to assess the phenotypic effect of knockdown or over-expression of key genes short interfering RNA (siRNA) and over-expression following gene reintroduction were performed respectively.

1.4.2 Outline of the aims of the experiments outlined in each individual chapter

The aim of the experiments outlined in chapter 3 was to identify and validate novel DNA methylation markers for acquired drug resistance in ovarian cell lines and evaluate their relevance to acquired resistance in patient samples using prediction analysis for microarrays (PAM). We also aimed to validate pyrosequencing of bisulphite-modified DNA as a better technique for examining candidate loci in the laboratory, than MSP.

In Chapter 4, following a comparison of the A2780 sensitive and resistant cell lines, the loci identified using methylation linear discrimination analysis (MLDA) vs. PAM were characterised in order to assess whether this novel statistical method was useful as a means of detecting differentially methylated loci. Again loci identified biostatistically were characterised in the laboratory by both MSP and pyrosequencing.

In Chapter 5 the main aim of the experiments outlined was to assess whether loci identified by comparing *in vivo* derived cell lines (as opposed to the previous experiments which had all compared *in vitro* derived cell lines) could guide us to more clinically important genes or pathways.

The primary aim of Chapter 6 was to use functional assays to further assess the effect of over- and under-expression of two genes which had been identified in previous chapters. qRTPCR, siRNA knockdown and over expression using whole gene cloning were all utilised.

In work that was performed in parallel to these experiments, in Chapter 7 OGT customised arrays were used to hybridise both cell lines and primary tumour samples, using DMH. The preliminary results of loci identified from these experiments are discussed and in particular the loci which had been identified from the original 12k array, and characterised in earlier chapters of the thesis, were re-examined in this independent data set.

2 Materials and Methods

2.1 Addresses for companies used.

Abgene	Abgene House, Blenheim House, Epsom, Surrey, KT19 9AP
Ambion	Now Applied Biosystems
Amersham Biosciences	Now GE Healthcare Life Sciences; Amersham Place, Little Chalfont, Buckinghamshire, HP7 9NA
Anachem, Lambda	Anachem House, 1&2 Titan Court, Laport Way, Luton, Bedforshire, LU4 8EF
Applied Biosystems	Lingley House, 120 Birchwood Boulevard, Warrington, Cheshire, WA3 7QH
Autogen Bioclear	1 Orchard Place, Nottingham Business Park, Nottingham, NG8 6PX
B Braun	Thorncliffe Park, Sheffield, S35 2PW
Beckman Coulter	Oakley Court, Kingsmead Business Park, London Road, High Wycombe, HP11 1JU
Becton Dickinson (BD)	The Danby Building, Edmund Halley Road, Oxford, OX4 4DQ
Bibby-Sterilin Limited	Parkway Building, Pen - Y - Fan Industrial State, Newport, Gwent, NP11 3EF
Biorad	Bio-Rad House, Maxted Road, Hemel Hempstead, Hertfordshire, HP2 7DX
Biotage GB limited	Distribution Way, Dyffryn Business Park, Ystrad Mynach, Hengoed CF82 7TS
Biotek UK	6 Bull Street, Potton, Bedfordshire SG19 2NR
Chemicon	Now Millipore
Corning	Elwy House, Lakeside Business Village, St. David's Park, Ewloe, Flintshire, CH5 3XD
Dharmacon	Now Thermo Scientific
Drummond Scientific	Distributed in UK by Alpha laboratories; Alpha Laboratories, 40 Parham Drive, Eastleigh,

	Hampshire, SO50 4NU
Eppendorf AG	Endurance House, Vision Park, Histon, Cambridge, CB24 9ZR
Falcon	Now BD
Finnzymes	Now Thermo Scientific
Fisher	Bishop Meadow Road, Loughbororugh, Leicestershire, LE11 5RG
FlowJo	Tree star Inc 340 A Street Suite # 206 - Ashland, OR 97520 USA
Genisphere LLC	2801 Sterling Drive, Hatfield, PA 19440
Genlab	Tanhouse Lane, Riverview Industrial Estate, Widnes, Cheshire, WA8 0SR.
Gibco	Supplied by Invitrogen
Grant	Shepreth, Cambridgeshire, SG8 6GB
Greiner bio-one	Greiner Bio-One Ltd. Brunel Way, Stroudwater Business Park, Stonehouse, GL10 3SX
Hayman	supplied by Fisher
Heraeus	Now Thermo Scientific
Hettich Zentrifugen	Föhrenstr.12, D-78532 Tuttlingen, Germany
Imperial College London	Imperial College London central stores, Hammersmith Hospital Campus, Du Cane Road, London, W12 0NN
Invitrogen	3 Fountain Drive, Inchinnan Business Park, Paisley, PA4 9RF
Iwaki	Now Corning
Labcaire	175 Kenn Road, Clevedon, Somerset, BS21 6LH
Labsystems	Now Thermo Scientific
LabTech	1 Acorn House, The Broyle, Ringmer, East Sussex, BN8 5NW
MMM Medical Equipment UK	Unit 2-3, Gateway Drive Business Park, Gate Way Drive, Yeadon, Leeds (West Yorkshire), LS19 7XY
Lomita	Distributed in UK by VWR
Melford	Bildeston Road, Chelsworth, Ipswich, Suffolk, IP7

	7LE
Mettler	64 Boston Road, Beaumont Leys, Leicester, LE4 1AW
Millipore	Units 3&5 The Courtyards, Hatters Lane, Watford, Hertfordshire, WD18 8YH
MJ Research	1815 Edgewood Street , St. Bruno (Quebec), Canada, J3V 4P1
Molecular devices	660-665 Eskadale Road, Winnersh Triangle, Wokingham, RG41 5TS
Nanodrop	Now Thermo Scientific
NBS Biologicals	14 Tower Square, Huntingdon, Cambridgeshire, PE29 7DT
New Brunswick Scientific	17 Alban Park, Hatfield Road, St Albans, Hertfordshire, AL4 0JJ
New England Biolabs	67 Knowl Piece, Wilbury Way, Hitchin, Hertfordshire, SG4 0TY
Nunc	Now Thermo Scientific
Ohaus	Ohaus Europe GmbH, Heuwinkelstrasse 3, CH-8606 Nänikon, Switzerland (European Headquaters)
Origene	UK distributor Cambridge Biosciences; Munro House, Trafalgar Way, Bar Hill, Cambridge CB23
PAA	Termare Close, Hounstone Business Park, Yeovil, Somerset, BA22 8YG
Perkin Elmer	Chalfont Road, Seer Green, Beaconsfield, Buckinghamshire, HP9 2FX
Pharmacia	Davy Avenue, Knowlhill, Milton Keynes, MK5 8PH
Pharmacia Biotech	Now GE Healthcare Life Sciences; Amersham Place, Little Chalfont, Buckinghamshire, HP7 9NA
Promega	Delta House, Chilworth Science Park, Southampton, Hampshire, SO16 7NS
Qiagen	Qiagen House, Fleming Way, Crawley, West Sussex, RH10 9NQ
Roche	Charles Avenue, Burgess Hill, East Sussex, RH15

	9RY
Sanyo	9 The Office Village, North Road, Loughborough, Leicestershire, LE11 1QJ
Scientific Industries	Distributed in UK by VWR
Sigma	Fancy Road, Poole, Dorset, BH12 4QH
Sterilin	Parkway Building, Pen - Y - Fan Industrial State, Newport, Gwent, NP11 3EF
Syngene	Beacon House, Nuffield Road, Cambridge, CB4 1TF
Thermo Electron Corporation	Unit 5 The Ringway Centre, Edison Road, Basingstoke, RG21 6YH
Thermo Scientific	Stafford House, Boundary Way, Hemel Hempstead, Hertfordshire, HP2 7GE
Trigene	Supplied by Medichem International; PO Box 237, Sevenoaks, Kent, TN15 0ZJ
VWR	Hunter Boulevard, Magna Park, Lutterworth, Leicestershire, LE17 4XN

2.2 **General equipment**

Accuspin micro 17 centrifuge	*Fisher*
Gilson Pipettes	*Anachem, Lambda*
Heating block Dri-Block DB 2A	*Techne*
Incubator (37^{o}C)	*Genlab*
Innova 4000 incubator shaker	*New Brunswick Scientific*
Microcentaur microcentrifuge	*Hettich Zentrifugen*
Microwave	*Sanyo*
Nanodrop ND-1000 spectrophotometer	*LabTech*
PCR workstation	*Labcaire*
Luckham Rocking Table	*MMM Medical Equipment UK*
Set of scales PM 300	*Mettler*
Tetrad 2 peltier thermal cycler	*Biorad*
Genie 2 Vortex	*Scientific Industries*

Water bath SUB36	*Grant*
Electrophoresis power supply EPS600	*Pharmacia Biotech*
Electrophoresis tank	*Fisher*
Magnetic stirrer	*Fisher*
Pyromark MD pyrosequencer	*Biotage*

2.3 General chemicals

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

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

2.5 Patient samples and characteristics

2.5.1 Primary ovarian cancer and residual disease matched pairs

12 pairs of ovarian surface epithelial tumours were obtained from the University Medical Center Groningen (Groningen, The Netherlands), with appropriate ethical approval. Of these, one sample was taken before chemotherapy; the second sample was taken at second look surgery after chemotherapy. All samples were stored frozen

at -70°C. All paired tumour samples were sent to the Paediatric Malignancy Core Facility at Great Ormond Street Hospital for genotyping. They used the Promega Powerplex 16 system (www.promega.com/products/genetic.../str.../powerplex-16-system/) which identifies matching probabilities that fall between 1 in $1.83x10^{17}$ and 1 in $1.41x10^{18}$. Unfortunately two of the relapse pairs were found to not be matched. Samples 55 and 57 had 10 matched and 33 unmatched alleles. Samples 125 and 98 had 11 matched and 32 unmatched alleles. These samples therefore had to be removed from the analysis, limiting the number of samples to a maximum of 10 pairs.

2.5.2 Primary ovarian cancer and relapsed disease matched pairs

14 pairs of ovarian surface epithelial tumours were obtained from the Department of women and children at Leuven University Belgium, again with appropriate ethical approval. One sample was taken at diagnosis and one at relapse. All samples were stored frozen at -70°C. All paired tumour samples were sent to the Paediatric Malignancy Core Facility at Great Ormond Street Hospital for genotyping and found to be true matches.

2.5.3 Ovarian cancer "retrospective" primary tumour samples

Tumour samples and matching adjacent normal tissue were collected from chemonaive patients who had surgery for suspected ovarian cancer. 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". The

following data were recorded: age at diagnosis, stage and grade of tumour at diagnosis, details of any chemotherapy given and the subsequent response to this in months (chemotherapy at diagnosis or relapse), (responses categorised as complete response (CR), partial response (PR), stable disease (SD), progressive disease (PD)). PFS and OS were also documented for most patients. DNA was extracted from the primary tumours and also from matching adjacent normal where available, and stored anonymously at -70^{o}C. Stage was categorised I-IV using FIGO and age was categorised on the median value.

2.5.4 Ovarian cancer "prospective" primary tumour samples

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. Again, 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^{o}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 Glasgow West of Scotland Cancer Centre 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.

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, Jayson et al. 2004). Patients who were evaluable for response to chemotherapy were classified into two groups: responders were those with complete response or partial response and non-responders were those with stable disease or progressive disease.

For all sample collections PFS was defined as the time from first chemotherapy or entry into a clinical trial (if applicable) 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 was calculated from the date of first chemotherapy or date of entry onto a trial (if applicable) until the date of cancer related death.

2.5.5 Patient characteristics

The primary tumour characteristics are outlined below; mean age was 60 and the majority of tumours were of advanced stage. Known clear cell and mucinous histology was excluded. Table 3a shows the cohort of tumours that were used for analysis of methylation of candidate loci. Table 3b shows the sub-cohort that were

used for DMH using customised arrays (OGT II). Table 3c is the updated table that appeared in the Dai et al publication following reviewers comments re correct pathology for "unknown" samples (Dai, Teodoridis et al. 2010).

Table 3a Characteristics of primary tumour samples (combined cohort).

Clinical Parameters (n=210) (includes test and validation samples)		
Age	Range	(19, 83)
	mean (95% CI), Years	60 (59, 62)
FIGO Stage	Ic	16
	II	7
	III	142
	IV	43
	Missing	2
Grade	G1	61
	G2	29
	G3	76
	Gx	44
Histology	Serous	148
	Endometrioid	20
	Unknown	42
Chemotherapy	Platinum alone	50
	Platinum + Taxane	118
	Other Platinum-based	12
	Taxane	2
	No treatment	6
	Unknown	22
Response	CR (Complete response)	54
	PR (Partial response)	42
	SD (Stable disease)	23
	PD (Progressive disease)	19
	NE (not evaluable)	19
	Missing	53
Overall survival (OS)	Alive/censored	75
	Dead	127
	Missing	8
	Median (95% CI), Years	2.66 (2.13, 3.19)
Progression free survival (PFS)	Not progressed	26
	Progressed	173
	Missing	11
	Median (95% CI), Years	1.25 (1.07, 1.43)

Table from Wei Dai

Table 3b Characteristics of primary tumour samples (OGT II cohort).

Clinical Parameters (n=111)

Age	Range	(32,83)
	mean (95% CI), Years	61 (59,63)
FIGO Stage	Ic	2 (1.8)
	III	85 (76.6)
	IV	24 (21.6)
Grade	G1	4 (3.6)
	G2	16 (14.4)
	G3	71 (64.0)
	Unknown	20 (18.0)
Histology	Serous	42 (37.8)
	Endometriod	8 (7.2)
	Other	35 (31.5)
	Unknown	26 (23.4)
Chemotherapy	Platinum	26 (23.4)
	Platinum + Taxane	67 (60.4)
	Other	18 (16.2)
	Missing	0
Response	CR (Complete response)	27 (24.3)
	PR (Partial response)	20 (18.0)
	SD (Stable disease)	13 (11.7)
	PD (Progressive disease)	8 (7.2)
	NE (not evaluable)	14 (12.6)
	Missing	29 (26.1)
Overall survival (OS)	Alive/censored	37 (33.3)
	Dead	74 (66.7)
	Missing	0
	Median (95% CI), Years	2.52 (1.67,3.37)
Progression free survival (PFS)	Not progressed	9 (8.1)
	Progressed	102 (91.9)
	Missing	0
	Median (95% CI), Years	0.92 (0.76,1.08)

Table from Wei Dai prior to publication (Dai, Teodoridis et al. 2010).

The above is the original table which was submitted for the Dai et al manuscript but following reviewers' comments further work was done to establish the histological diagnosis for unknown cases and this is shown below, in order to provide further information, as it was available at the time of doing the corrections to this thesis. The table below is therefore the one that appeared in the final publication. It includes 159

patients in total (OGT II&III). The results of the OGT III study were not available when this thesis was written up and are therefore not presented.

Table 3c: OGT II: Updated patient characteristics (N=159) (OGT II and III).

Clinical Parameters		**OGT II N=111 (%)**	**OGT III N=48 (%)**
Age	Range	(32,83)	(44,86)
	mean (95% CI), Years	61 (59,63)	64 (61,67)
FIGO Stage	Ic	2 (1.8)	7 (14.6)
	III	85 (76.6)	35 (72.9)
	IV	24 (21.6)	6 (12.5)
Grade	G1	4 (3.6)	4 (8.3)
	G2	16 (14.4)	10 (20.8)
	G3	71 (64.0)	28 (58.3)
	Unknown	20 (18.0)	6 (12.5)
Histology	Serous	102 (91.9)	44 (91.7)
	Endometrioid	8 (7.2)	4 (8.3)
	Unknown	1 (0.9)	0
Chemotherapy	Platinum	26 (23.4)	19 (39.6)
	Platinum + Taxane	67 (60.4)	24 (50.0)
	Other	14 (12.6)	2(4.2)
	Missing	4 (3.6)	3 (6.3)
Response	CR (Complete response)	27 (24.3)	2 (4.2)
	PR (Partial response)	20 (18.0)	5 (10.4)
	SD (Stable disease)	13 (11.7)	3 (6.3)
	PD (Progressive disease)	8 (7.2)	5 (10.4)
	NE (not evaluable)	14 (12.6)	20 (41.6)
	Missing	29 (26.1)	13 (27.1)
Overall survival (OS)	Alive/censored	37 (33.3)	27 (56.3)
	Dead	74 (66.7)	18 (37.5)
	Missing	0	3 (6.2)
	Median (95% CI), Years	2.52 (1.67,3.37)	2.79 (2.18, 3.41)
Progression free survival (PFS)	Not progressed	9 (8.1)	14 (29.2)
	Progressed	102 (91.9)	31 (64.6)
	Missing	0	3 (6.2)
	Median (95% CI), Years	0.92 (0.76,1.08)	1.15 (0.81, 1.49)

The standard parameters which are known to affect PFS and OS were examined in the data set and the results are shown in the tables on the following pages: Table 4a shows the results for PFS in the combined data set (test and validation sets). Table 4b the results for OS in the combined data set (N=210) and table 4c shows the results for PFS and OS in the OGT II examination (Chapter 7) (Dai, Teodoridis et al. 2010).

Table 4a: Progression-free survival analysis

Variable	No. of cases	Disease progression	Median PFS (95% CI), years	HR[1]	95% CI[2]	p value
Stage						<0.001[3]
Stage Ic	16	4	NE	1.00		
Stage II	7	2	NE	0.81	(0.15, 4.42)	0.805[4]
Stage III	142	125	1.08 (0.9, 1.26)	5.32	(1.96, 14.42)	0.001[4]
Stage IV	43	41	1.25 (1.07, 1.43)	5.79	(2.07, 16.24)	0.001[4]
Grade						<0.001[3]
G1	61	47	2.42 (1.93, 2.91)	1.00		
G2	29	21	1.72 (1.02, 2.42)	1.25	(0.74, 2.11)	0.396[4]
G3	76	68	1.00 (0.77, 1.23)	2.14	(1.45, 3.14)	<0.001[4]
Gx	44	37	0.96 (1.07, 1.43)	2.15	(1.39, 3.33)	0.001[4]
Age	210	173	1.25 (1.07, 1.43)	1.02	(1.01, 1.04)	0.004[3]
Chemotherapy						0.045[3]
Platinum	50	44	0.92 (0.47, 1.37)	1.00		
Platinum+Taxane	118	105	1.33 (0.84, 1.83)	0.68	(0.47, 0.96)	0.03[4]
Other	12	12	1.58 (1.55, 1.61)	0.51	(0.26, 1.00)	0.05[4]
Response[4]						0.018[3]
Responders (CR+PR)	96	89	1.42 (1.02, 1.82)	1.00		
Non-responders (PD+SD)	42	39	0.90 (0.63, 1.17)	1.57	(1.08, 2.30)	

1: Hazard ratio; -2: 95% confidence interval of HR; -3: p value of score test; -4: p value of Wald test. Table from Wei Dai.

Table 4b: Overall survival analysis

Variable	No. of cases	Death	Median OS (95% CI), years	HR[1]	95% CI[2]	p value
Stage						0.021[3]
Stage Ic	16	4	NE	1.00		
Stage II	7	1	NE	0.39	(0.04, 3.50)	0.401[4]
Stage III	142	89	2.58 (2.00, 3.16)	2.62	(0.96, 7.14)	0.060[4]
Stage IV	43	32	2.66 (2.13, 3.19)	3.09	(1.08, 8.78)	0.035[4]
Grade						0.103[3]
G1	61	33	2.66 (1.85, 3.47)	1.00		
G2	29	18	2.50 (0.75, 4.26)	1.36	(0.77, 2.43)	0.293[4]
G3	76	49	3.05 (2.39, 3.71)	1.10	(0.71, 1.71)	0.680[4]
Gx	44	27	1.74 (2.13, 3.19)	1.80	(1.08, 3.01)	0.024[4]
Age	210	127	2.66 (2.13, 3.19)	1.03	(1.01, 1.05)	0.001[3]
Chemotherapy						<0.001[3]
Platinum	50	35	1.25 (1.08, 1.42)	1.00		
Platinum+Taxane	118	70	3.00 (2.59, 3.41)	0.468	(0.31, 0.71)	<0.001[4]
Other	12	10	3.58 (1.46, 5.70)	0.335	(0.15, 0.74)	0.007[4]
Response[4]						0.005[3]
Responders (CR+PR)	96	58	3.05 (2.61, 3.49)	1.00		
Non-responders (PD+SD)	42	31	1.83 (0.53, 3.13)	1.87	(1.20, 2.93)	

1: Hazard ratio; -2: 95% confidence interval of HR; -3: p value of score test; -4: p value of Wald test. Table from Wei Dai

Table 4c Univariate analysis of effect of established clinical parameters on survival

Variable	No. of cases	Disease progression (%)	Median PFS (95% CI), years	1-Year PFS Rate (95% CI), %	B	HR[1]	95% CI[2]	p value
Stage								0.182[3]
Stage Ic	2	0	NE	100	NE	NE	NE	NE
Stage III	85	78 (92)	0.96 (0.76, 1.16)	45 (34, 56)	0	1.00		
Stage IV	24	24 (100)	0.76 (0.52, 1.00)	38 (19, 57)	0.31	1.37	(0.86,2.18)	0.184
Histological type								0.139[3]
Serous	102	95 (93)	0.92 (0.72, 1.12)	44 (34, 54)	0	1.00		
Endometrioid	8	6 (75)	1.50 (0.70, 2.30)	63 (29, 97)	-0.61	0.54	(0.24, 1.24)	0.145
Unknown	1	1 (100)	NE	NE	NE	NE	NE	
Grade								0.371[3]
G1	4	4 (100)	0.92 (0.03, 1.81)	50 (0, 100)	0	1.00		
G2	16	13 (81)	0.88 (0.26, 1.50)	47 (22, 72)	0.04	1.04	(0.34, 3.20)	0.946
G3	71	65 (92)	0.92 (0.67, 1.17)	47 (35, 59)	0.03	1.03	(0.37, 2.84)	0.953
Unknown	20	20 (100)	0.67 (0, 1.53)	35 (14, 56)	0.47	1.60	(0.55, 4.70)	0.391
Age	111	102 (92)	0.92 (0.76, 1.08)	41 (31, 51)	0.01	1.01	(0.99, 1.03)	0.181[3]
Chemotherapy								0.087[3]
Platinum	26	25 (96)	0.60 (0.48, 0.72)	31 (13, 49)	0	1.00		
Platinum+Taxane	67	59 (88)	1.07 (0.89, 1.25)	52 (40, 64)	-0.52	0.60	(0.37, 0.96)	0.031*
Other	14	14 (100)	0.92 (0.11, 1.73)	50 (24, 76)	-0.22	0.81	(0.42, 1.56)	0.523
Response[4]								<0.001[5]***
Responders (CR+PR)	47	45 (96)	1.14 (1.03, 1.25)	60 (46, 74)	0.00	1.00		
Non-responders (PD+SD)	21	21 (100)	0.58 (0.79, 1.05)	14 (0, 0.29)	1.25	3.49	(1.97, 6.17)	<0.001[5]***

For legend see footnote at bottom of next

Table 4c cont. Univariate analysis of effect of established clinical parameters on survival N=111, PFS event=102, death=73)

Variable	No. of cases	Death, %	Median OS (95% CI), years	1-Year OS Rate (95% CI), %	B	HR	95% CI	p value
Stage								0.216[3]
Stage Ic	2	0	NE		NE	NE	NE	
Stage III	85	39 (46)	3.00 (2.46, 3.54)	89 (81, 97)	0	1.00		
Stage IV	24	7 (29)	0.67 (0, 1.59)	50 (15, 85)	0.33	1.39	(0.82, 2.36)	0.218
Histological type								0.175[3]
Serous	102	71 (70)	2.25 (1.47, 3.03)	77 (69, 85)	0	1.00		
Endometrioid	8	2 (25)	NE	83 (53, 100)	-0.95	0.39	(0.10, 1.60)	0.191
Unknown	1	1 (100)	NE	NE	NE	NE	NE	
Grade								0.157[3]
G1	4	3 (75)	2.17 (0.47, 3.88)	75 (32, 100)	0	1.00		
G2	16	11 (69)	1.51 (0.45, 2.57)	58 (32,84)	0.56	1.76	(0.48, 6.44)	0.395
G3	71	46 (65)	3.00 (2.29, 3.72)	82 (73, 91)	0.01	1.01	(0.31, 3.30)	0.992
Unknown	20	14 (70)	1.62 (0.94, 2.30)	70 (48, 90)	0.58	1.78	(0.50, 6.34)	0.374
Age	111	73 (66)	2.58 (1.77, 3.39)	71 (63, 79)	0.02	1.02	(1.00,1.04)	0.0625[3]
Chemotherapy								0.002**[3]
Platinum	26	25 (96)	1.25 (0.79, 1.71)	59 (39, 79)	0	1.00		
Platinum+Taxane	67	59 (88)	3.00 (2.67, 3.33)	86 (78, 94)	-0.82	0.44	(0.26, 0.76)	0.003**
Other	14	14 (100)	3.58 (0.05, 7.11)	84 (64, 1.04)	-1.21	0.30	(0.12, 0.72)	0.007**
Response[4]								0.007**[3]
Responders (CR+PR)	47	30 (64)	3.08 (2.51, 3.65)	91 (82, 99)	0	1.00		
Non-responders (PD+SD)	21	16 (76)	1.25 (0.63, 1.87)	71 (51, 91)	0.84	2.32	(1.24, 4.35)	0.009**

B, coefficient of Cox proportional hazard regression model; HR, hazard ratio; CI, confidence interval; NR, not reached; 1Hazard ratio estimated from Cox proportional hazard regression model.2Confidence interval of the estimated hazard ratio. 3p value of Score test. 4 Response was measured by RECIST 1.0; the patients with complete/partial response are the responders to chemotherapy, while the patients with progressive or stable disease are those who did not response to chemotherapy +p<0.1;*p<0.05;**p<0.01. Table taken from Wei Dai (Dai, Teodoridis et al. 2010).

2.6 Sodium bisulphite modification of extracted DNA

2.6.1 Materials

Epitect bisulphite conversion kit	*Qiagen*
Ethanol	*Sigma*
RNA-ase free water	*Qiagen*

2.6.2 Method

Sodium bisulphite modification is based on the selective deamination of unmethylated cytosines to uracils whereas methylated cytosines remain unchanged (Clark, Statham et al. 2006). This chemical reaction converts a difference in methylation into a difference in sequence. The Qiagen epitect bisulphite modification kit was used, as per the manufacturer's instructions using 1000 ng DNA. The final reaction was eluted in 20 µl of elution buffer. This step was repeated once. 40 µl of bisulphite modified DNA was therefore obtained and this could be stored at minus 20°C for at least 12 weeks.

2.6.3 Calponin PCR control to check adequate bisulphite modification

Incomplete bisulphite modification can lead to false positive results using MSP (Rand, Qu et al. 2002). Therefore it is important to avoid using incompletely modified DNA samples as these could result in an overestimation of CpG methylation. In order to address this problem, successful bisulphite modification of the DNA was verified, before proceeding to MSP, using calponin primers. These 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 methylation status. 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 are the same as that for MSP (described in the next section, Chapter 2.6) except, that following PCR amplification, 5 µl of product was ran on an agarose gel as opposed to 20 µl. This was to aid detection of partially modified samples as this could be missed using 20 µl of PCR product as this produced a very bright signal.

2.7 Methylation Specific PCR (MSP)

2.7.1 Brief outline of the technique

Methylation Specific PCR (MSP) is used to detect either the presence or absence of methylation in bisulphite modified genomic DNA. Bisulphite treatment results in all unmethylated cytosines (Cs) being converted to uracils (U's) but methylated cytosines (Cs) are protected from this. Following a subsequent PCR the final sequence of methylated and unmethylated DNA is therefore different and this can be utilised by designing primers which are specific to each. This is illustrated in figure 5 below. The methylated primer should only bind to the methylated sequence and therefore a product only seen on the gel if methylation is present. It is unusual for DNA to be entirely methylated and therefore a sample can show amplification with the unmethylated primer even if there is a high degree of methylation present.

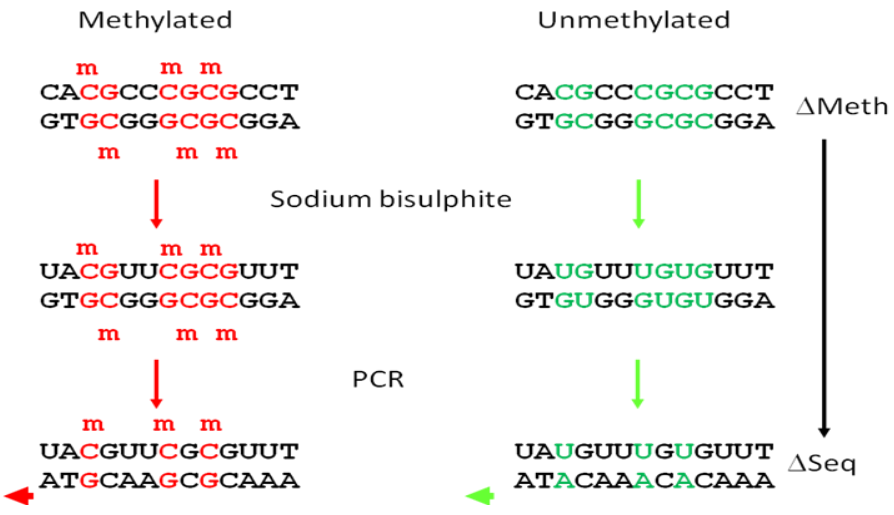


Figure 5. A change in methylation status becomes a change in sequence following sodium bisulphite PCR. CGm, methylated CpG illustrated in red, unmethylated CpG is Green.

MSP is a highly sensitive PCR technique (Herman, Graff et al. 1996) however it is limited by the fact it is only a semi-quantitative assay. The degree of methylation is estimated using serial dilutions of the positive and negative control as illustrated in figure 6 below. This does allow for an estimate of the degree of differential methylation between samples in one experiment but it limits the ability to compare results across experiments or between different clinical centres. A high degree of optimisation is required. For the methylated primer it is important to have a very clear band present in the IVM/ Positive control without any amplification in the negative or water controls. Similarly in the unmethylated primer it is important to see a clear band in the unmethylated control but not in the methylated control or the water.

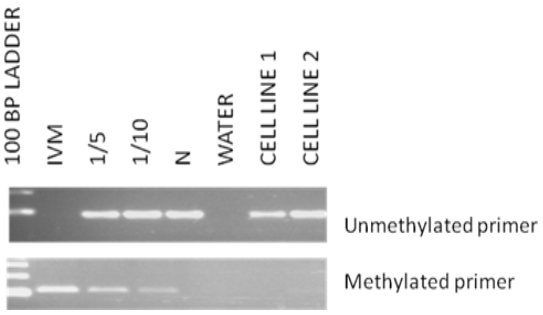


Figure 6. Serial dilutions of controls used for quantification purposes in MSP. 100 BP LADDER 100 base pair ladder, IVM *in vitro* methylated DNA (100% methylated), N, normal whole male genomic DNA, 1/5 20% methylated, 1/10 10% methylated.

2.7.2 Materials

70% v/v Ethanol (to wash down workstation)	*Sigma*
Sterile water (H_2O) for injection	*B Braun*
Fast start Taq DNA polymerase kit	*Roche*
dNTPs 0.2mM	*Applied Biosystems*
Oligonucleotides (detailed below in Table 4)	*Invitrogen*
Human genomic male DNA (N)	*Promega*
In Vitro Methylated DNA (IVM)	*Chemicon*
Semi-skirted 96 well PCR plate (0.2ml)	*Abgene*
Microseal A film	*MJ Research*

2.7.3 Method

All MSP reactions were carried out on a Biorad Tetrad 2 peltier thermocycler. Because of the repeated cycles of PCR used to amplify a PCR product from a small amount of DNA it is important to avoid contamination pre-PCR (Kwok and Higuchi 1989). All PCR reactions were prepared in a PCR work station using a vertical laminar airflow and UV decontamination. For each experiment a water blank and positive and negative controls' were run. Undiluted IVM (representing completely methylated DNA) was serially diluted (1:5 and 1:10) with human genomic male DNA (N), (representing completely unmethylated DNA). Primers were designed that would only amplify the methylated or unmethylated sequence using the methprimer website using a criteria of >100 base pairs, GC content 50% and observed/ expected ratio >60% (http://www.urogene.org.methprimer).

70% ethanol spray was used to wash down the PCR work station before and after each experiment. Pipette, tubes, tips and dH_20 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 the Taq polymerase which was kept at -20^oC until required and not vortexed. 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 1µl of sterile H_2O in place of DNA. This is summarised in table 5 below:

Table 5

MSP: Mastermix for one standard reaction	
Fast Start 10x Buffer	2.5µl
DNTPs	0.5µl
Forward sequence primer	0.5µl
Reverse sequence primer	0.5µl
$MgCl_2$ 25Mm	1-3µl, 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 flm and reactions were run on the Tetrad 2 peltier thermal cycler. Each MSP reaction underwent an initial denaturation and enzyme activation step at 95^{o}C for 5 minutes, followed by 35 amplification cycles of 95^{o}C for 30 seconds (unless otherwise indicated), appropriate annealing temperature for 30 seconds and elongation at 72^{o}C for 30 seconds. This was followed by a final extension step at 72^{o}C for 6 minutes. MSP conditions are illustrated in table 5 below:

Table 6

MSP: cycling conditions.		
Initial Denaturation Step of 5mins at 95^{o}C then 35 cycles of:		
Denaturation	30s	95^{o}C
Anneal	30s	Temp. dependent on primer
Elongation	30s	72^{o}C
Following these 35 cycles, final elongation step of 5 mins at 72^{o}C then 4ºC for 10 minutes.		

2.7.4 MSP oligonucleotides and cycling conditions

Primer sequences and conditions for methylated and unmethylated MSP are shown below in table 7.

Table 7

MSP primer conditions and product size.					
12k Microarray ID/ Gene		**Primer Sequence** ***Forward (F) / Reverse (R) 5'→3'***	***Product Size (bp)***	***Annealing Temp (^oC)***	***[Mg^{2+}] mM***
121D9 *CRABP1*	MF MR UF UR	TAT GAT TAG TGT TTC GAT CGG TTT C GAC CAC TTT CCT CAA CAT AAC GT ATG ATT AGT GTT TGA TTG GTT TTG A TAC AAC CAC TTT CTT CAA CAT AAC A	104 106	60	2
24D3 *SP5*	MF MR UF UR	GGT TTT ATT TTG GGA GGT AGG TAA C ACA CCT CTA CGA AAA ATC CGA GGT TTT ATT TTG GGA GGT AGG TAA T ACA CCT CTA CAA AAA ATC CAA A	96 96	65	2
38D7 *AGBL2*	MF MR UF UR	GTA TGA TAT TTA TTA ATG CGG TTT C CTT CCG AAA AAC TAA ACC TCG GTA TTG TAT TTA TTA ATG TGG TTT TGA CTT CCA AAA AAC TAA ACC TCA CC	144 144	52	3
17H9 *HRASLS3*	MF MR UF UR	TCG GAA GTA GTA ATT TAG GTT TCG A AAT ACG TAC GCC CAT TAT AAA TAC G TTG GAA GTA GTA ATT TAG GTT TTG A TAC ATA CAC CCA TTA TAA ATA CAA A	106 104	60	2
41D12 *GLS2*	MF MR UF UR	AAG GTT TTG TAG AAG GTT TTG AGT ACC TCA CTA AAA TAA TAC CGA ACG TTT GTA GAA GGT TTT GAG TTG G CCT CAC TAA AAT AAT ACC AAA CAC C	116 110	62	2
20F11 *NTN4*	MF MR UF UR	TTT CGT AAA AGG TAG TAG AGA AGC CTC TCG AAA AATACCC ACG AC TTT ATT TTT GTA AAA GGT AGT AGA GAA GTG CCT CTC AAA AAA TCC CAC AAC	119 125	60	2
21A11 *NTN4*	MF MR UF UR	CGT TTA TTT TTT ATA TTT TTT AGC GA CGA AAC TAA ATT AAA ATA TTC CGT C TGT TTA TTT TTT ATA TTT TTT AGT GA CAA AAC TCC CTT CCC CTC TTTC CAT C	107 107	56	3
21F11 *NTN4*	MF MR UF UR	TTT CGT AAA AGG TAG TAG AGA AGC G CTC TCG AAA AAT CCC ACG AC TTT ATT TTT GTA AAA GGT AGT AGA GAA GTG CCT CTC AAA AAA TCC CAC AAC	119 125	57	2
101G6 *GLS2*	MF MR UF UR	TTA TAT TTA AAG GAA AAG GTA TCG A AAC TCC TAA AAC AAA ACA AAA AAC G TTT ATA TTT AAA GGA AAA GGT ATT GA CTC CTA AAA CAA AAC AAA AAA CAA A	143 142	57	2
3 A 11 *MLLT6*	MF MR UF UR	TTC GGT GGA TAT AGG GAA TTT C GTT TTG GTG GAT ATA GGG AAT TTT AAC ACG AAA ATC GAA AAA ACG CAA ACA CAA AAA TCA AAA AAA CAA A	98 102	59	2
66G6 *BARX1*	MF MR UF UR	GTT CGG GAG AGT TTT TGA TAG TC AAA CAA ATT ACC TAA TAA AAA CGA A TTG GGA GAG TTT TTG ATA GTT GT AAA ACA AAT TAC CTA ATA AAA ACA AA	115 114	58 57	2 2
119A6	MF	TCG TAG CGA TAG GTA TAA AGT TAC	100	55	2

NR2E1	MR UF UR	G AAA AAA ACG ACC AAA TCC GA TGT AGT GAT AGG TAT AAA GTT ATG G AAA AAA AAC AAC CAA ATC CAA A	100	55	2
81B1 *PTTG*	MF MR UF UR	GTT CGG GGT TTA GAT GGT TTC TAA CAC TCA TCA TCA AAC AAT TAC CGT T TGT TTG GGG TTT AGA TGG TTT T ACA CTC ATC AAA CAA TTA CCA TT	85 84	56 57	2 2
114E4 *CNTNAP5*	MF MR UF UR	TAG GTT GTG TAG AGG AGA GAG ATA GC ATT TCC CCC GAA TAA ACG AT TAG GTT GTG TAG AGG AGA GAG ATA GTG CAT TTC CCC CAA ATA AAC AAT	96 97	65 60	3 2
127F12 *DLC1*	MF MR UF UR	TGA TTA TTT TTT GTT TGG GGA TC ACC AAA ACT TCC CTT CAC GA TTG ATT ATT TTT TGT TTG GGG ATT AAA CCA AAA CTT CCC TTC ACA A	83 86	62 62	2 2
5D4 *LMXIA*	MF MR UF UR	ATATAGAGTAAAAAGCGACGTTCGT ACTTTTAAACTTACCCAACCTCGA GATATAGAGTAAAAAGTGATGTTTGT ACTTTTAAACTTACCCAACCTCAAA	112 113	59 52	2 2
64E3 *CR2*	MF MR UF UR	GAT GTA AAG TAG GGG GTT AAA AGC ACA ATA ATC CCT CAA AAC TAA CGA A ATG TAA AGT AGG GGG TTA AAA GTG A ACA ATA ATC CCT CAA AAC TAA CAA A	85 84	65 65	2 2

Primers amplify methylated (M) or unmethylated DNA sequence (U). Forward (F), Reverse (R). Primers were all designed using MethPrimer (Li and Dahiya 2002) (http://www.urogene.org/methprimer/).

2.8 **Agarose gel electrophoresis**

2.8.1 Materials

Agarose	*Melford*
dH_2O	
Ethidium bromide solution	*NBS Biologicals*
100bp DNA Ladder	*Invitrogen*
EDTA	*Fisher*
Electrophoresis unit and power pack	*Pharmacia*
GeneGenius bioimaging system	*Syngene*
Glycerol	*Fisher*
Orange G	*Sigma*
Tris Base	*Melford*

2.8.2 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

Both ingredients were heated in the microwave for 90 seconds until the agarose was completely dissolved. It was then allowed to cool to "hand warm" before adding 2 droplets of ethidium bromide solution per 100 ml of TBE.

Loading Buffer

Glycerol	10ml
dH_2O	30ml
Orange G	0.25g

2.8.3 Method

After the PCR step, the products were separated by size using 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% w/v 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.

2.9 Pyrosequencing of bisulphite modified DNA

2.9.1 Brief outline of the technique

Pyrosequencing of bisulphite modified DNA is another method used for assessing methylation status (Tost and Gut 2007). It is a higher throughput technique and involves "sequencing by synthesis" and detection of hydrolysis of pyrophosphate (PPi) by pyrophosphatase. Design of the primers and optimisation can be more difficult than for MSP, however results are quantitative. The reason optimisation can be more difficult is that numerous things can interfere with the final read out. These include whether the binding buffer and beads are in good condition and more specific to an individual set of primers whether amplification is only seen as a result of a reaction of the sequencing primer with the product – and not the product itself forming hairpin loops – or the sequencing primer annealing to an incorrect sequence. Once optimised though it is a very quick and highly reproducible method which provides quantitative results which can readily be compared across experiments and between different institutions. Using this technique it is possible to assess the methylation status of several neighbouring CpGs individually. Like MSP this technique utilises sodium bisulphite modified DNA. The methylation sites are treated as "cytosine/thymine" or "C/T SNPs" (methylSNP) with an allele frequency spectrum spanning the entire range (0-100%). An internal control can be included which is able to confirm the efficiency of bisulphite conversion within the sample.

In this thesis all pyrosequencing was performed on bisulphite modified DNA and therefore for simplicity the term 'pyrosequencing' is used when referring to 'pyrosequencing of bisulphite modified DNA'. All experiments were performed in duplicate and any percentages quoted represent the mean of all relevant samples in both experiments.

2.9.2 Materials

1x Annealing buffer	*Biotage*
Binding buffer	*Biotage*
Denaturation solution (0.2M NaOH)	*Biotage*
70% ethanol	
Oligonucleotides @ 10µM (detailed below in Table 5)	*INVITROGEN*
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*

2.9.3 Method

DNA was first bisulphite modified and PCR performed, as described previously in Chapter 2.5, in a total volume of 25µl including 1µl of modified DNA template using 35 cycles of PCR (unless otherwise stated). Also as previously described in Chapter 2.5.3, adequate bisulphite modification was confirmed by PCR amplification with Calponin primers.

Pyrosequencing primers were designed using the biotage Pyro-Q-CpG software (www.pyrosequencing.com), by inserting the bisulphite modified sequence and selecting the region of CpGs of interest. Either the forward or reverse pyrosequencing PCR primer was biotinylated to allow immobilisation to streptavidin coated sepharose beads after PCR

amplification. 10µl 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^{o}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 thymine (T) at a CpG site indicates unmethylated DNA and incorporation of a C indicates methylation of that given site. The degree of methylation at individual CpG sites is then analysed using the AQ software. An example of the data generated is illustrated in figure 7 below.

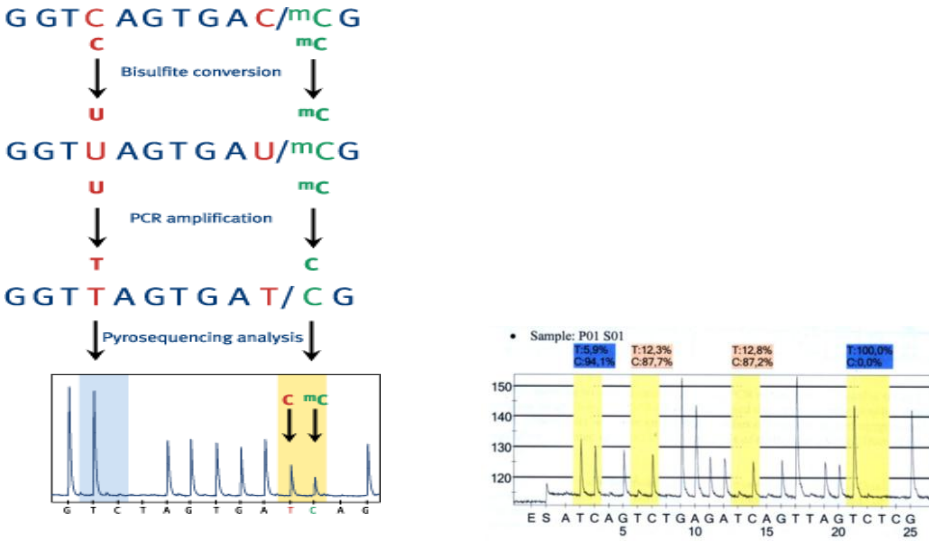


Figure 7 Example of data generated by the pyrosequencing software. C is % methylated, T is % unmethylated at a given CpG site. A adenine, C cytosine, G guanine T thymine M methylated U uracil (http://www.biotage.com/DynPage.aspx?id=22003&search=pyrosequencing)

2.9.4 Pyrosequencing oligonucleotides, cycling conditions and sequence analysed

Pyrosequencing primers were designed using the Biotage PSQ Pyrosequencing software package. Primer details and optimised conditions are shown in table 8 below:

Table 8

Pyrosequencing: primer sequences and optimised conditions.				
Sequence name used/ Gene (if known)	**Primer Sequence Forward (F) / Reverse (R) / Sequencing (S) 5' – 3'**	***Product Size (bp)***	***Annealing Temp (°C)***	***[Mg^{2+}] mM***
5 D 4 *LMX1A*	**FB:** ATG AAT GTG GAG GAT GAG ATA GTT **R:** CCC AAT TTT ACA ATT CTA TTT TCT **S:** CAA TTT TAC AAT TCT ATT TT	323	53	2
119 A 6 *NR2E1*	**F:** TTT GGA GAT ATT ATA GGG GAT TTA **RB:** TCC CTC TAC ATA AAC ATA CAA AA **S:** GGG GTA ATG AAT TT	288	50	2
121 D 9 *CRABP1*	**F:** GAG AAG GTT TTG AGG AGG AGA T **RB:** ACT CTA AAA CCT CAC CCT ACA CTT **S:** GGT TTT GAG GAG GAG AT	59	52	1
24 D 3 *SP5*	**FB:** GGT GGA TTT TTT TTT TAG TAT TTT **R:** CCC AAT TAC AAT CCA AAT ACC C **S:** ACC CCA ACC CTA CCT	207	57	2
101 G6 *GLS2*	**FB:** GAG GAG AGG GGA GAT GAG **R:** CTT ACC CCC ACT CCC ACT A **S:** ACC CCC ACT CCC ACT ATA ATT CT	79	67	3
1 E 7	**F:** GGG ATT GGA AGA GTT GTT TGA **RB:**CAA CCC CAA CAA ACT CAA CTA AA **S:** TGG AAG AGT TGT TTG AGT A	87	64	2
21 G 5 *KIAA1383*	**F:** TTT AGG GGG TAG TTG TAG TAG TAA **RB:** TCC ACC TAC AAC CTA CTA CCC TA **S:** TAC AAC CTA CTA CCC TAC AC	80	60	2
17 G 11 *SIX1*	**F:** TTT AGG ATT TTT GTA GTT GTG GA **R:** CAA AAC TCC AAA CCA ACT CTT AA **S:** TGT GAG TAG TTA TTT TGA GTT AGT	110	57	2

F forward primer, R reverse primer, S sequencing primer, B biotinylated primer.

The sequence to be analysed and the number of CpG sites to be examined for each primer is shown in table 9 below:

Table 9

Pyrosequencing: Analysed sequence and No. of CpG site examined.		
Microarray ID ***Gene***	**Sequence analysed**	**No. CpG sites**
5 D 4 *LMX1A*	CTTTYGATAAAATCTYGAYGTCCTAAACCAYGTTAAAAAYGAAYGTA	6
119 A 6 *NR2E1*	YGGGGATTTTTYGTYGTTGYGTGYGYGGTTTTTTTYGGAAATT	7
121 D 9 *CRABP1*	YGTGGAYGGAYGTAAGTGTAGGGTGAGGTTTTAGAGT	3
24 D 3 *SP5*	CCCCRCTCCTAAAAACTAAATCCCTATATCCRAAAACAACRAAAATTA AAAACCTAACTCCATTTTAAAAAACAAACAACRAAAAAAATCCRTCT ACTAAATCTCCCCTCCRCCAACTACCCACAAATCCCCRAATCTCTCRC AAAAATACTAAAAAAAAAATCCAC	8
101 G6 *GLS2*	CRCTAATTCCCRAATACCCTTCCAAAACAACRCCRCTCATCTCCCCTCT CCTC	4
1 E 7	GTYGGTTGGTTYGGYGGTTAGGTTAGGGYGGGGGYGAGYGTTTAGTT GAGTTTGTTGGGGTTGGA	6
21 G 5 *KIAA1383*	TACCRACTCCTACRAACCCCRCTTACCACCTTACTACTACAACTAC	3
17 G 11 *SIX1*	TYGYGGGGTTYGATYGGAAGGGAYGTTTTTTTTAAGAGTTGGTTTGGA GT	5
111 D1 *OPCML*	GTAYGTATATATATATTTATTYGTTTATAYGYGYGTAGATGTTTTTAGT AYGGGATTTGTTTTTTGTTTYGGGGGATTTAGTGAGTTGGGTTYGAGA TTTGTATTATGTTGTGAGGTA	8

YG is a potential site of methylation on the forward strand otherwise known as “C/T SNP”. CR is a potential CT SNP on the reverse strand.

2.10 Differential methylation hybridisation

2.10.1 Brief outline of the technique

Differential methylation hybridisation (DMH) is a high throughput method of examining the methylation status of several samples in multiple CpG residues. Genomic DNA is digested with MseI (TTAA) and DNA fragments are then ligated to endlinker oligonucleotides and divided into two equal aliquots. One is mock-treated, the other is 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 is 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 are then labelled with cyanine 3 (Cy3) or cyanine 5 (Cy5) and can be hybridised to one of several arrays. This is illustrated in figure 8 below.

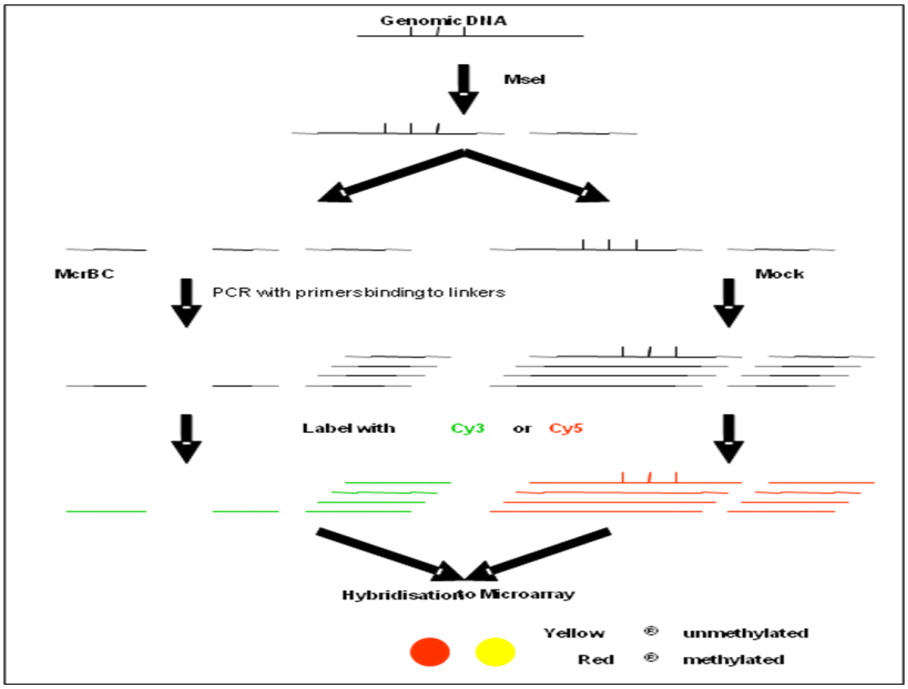


Figure 8 DMH: sample preparation. Genomic DNA is digested with MseI, ligated to endlinkers and divided into equal aliquots. One is mock-treated, the other digested with McrBC. PCR is performed with primers binding to endlinkers. Unmethylated fragments are labelled in both aliquots. Methylated fragments remain intact only in the mock treated aliquot. Amplicons are labelled with Cy3 or Cy5 and hybridised to microarray. If the McrBC sample is labelled green with Cy3 and the mock sample red with Cy5 then if the sample contains methylated CpGs then more red signal will come from the mock sample than green from the digested sample and a red dot will be seen on the array. If sample is unmethylated the mock and McrBC treated samples will amplify equally well and an equal green and red signal will be seen as a yellow spot on the array.

Once samples are spotted to the array they are visualised in the spectrum of red to orange to yellow to green. This is shown below in Figure 9. As described later in the Chapter a computer software package is utilised to quantify the degree of methylation (the intensity of the colour).

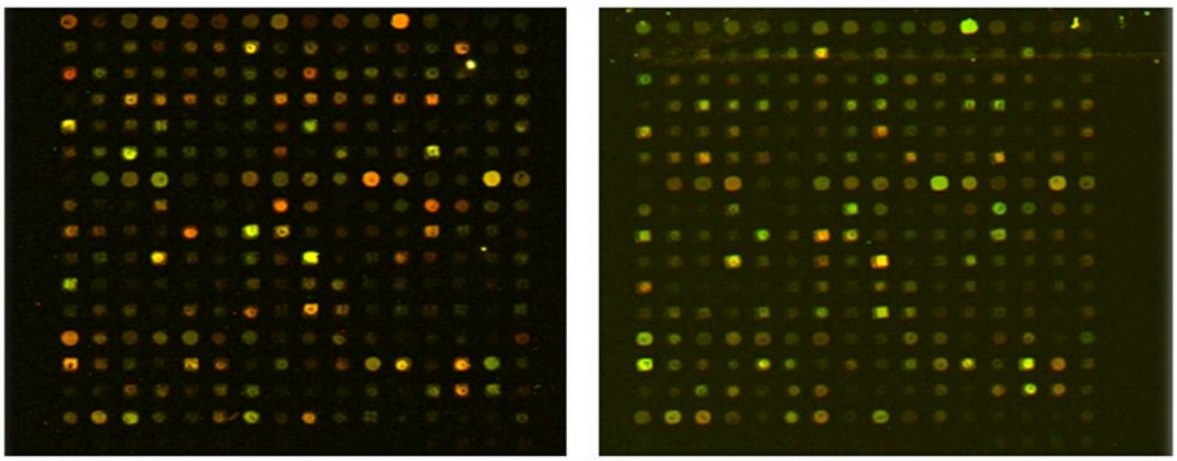


Figure 9. Example of a DMH microarray experiment for the cell line A2780. Left: McrBC digested DNA was labelled green (Cy3), mock-digested DNA was labelled red (Cy5); right: dye swap experiment.

DMH was performed as previously described (Paz, Wei et al. 2003), with some modifications. In the first experiment (performed by Dr Teodoridis) samples were hybridised to the Human CpG 12K Array (Heisler, Torti 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, Charlton et al. 1994). In the second experiment samples were hybridised to the larger Agilent microarray. Arrays were washed with 1xSSC, 0.1% SDS and 0.2xSSC, rinsed with H_2O and scanned with an Axon GenePix 4000A scanner (Molecular devices). GenePix Pro 6.0 was used for image analysis. Dye swap experiments were performed for all cell lines to ensure quality control and reproducibility.

2.10.2 Materials

Axon GenePix 4000A scanner	*Molecular devices*
MseI	*New England Biolabs*
T4 DNA Ligase, 2000U/µl	*New England Biolabs*
Deep Vent exo(-) Thermopolymerase	*New England Biolabs*
McrBC	*New England Biolabs*
BioPrime DNA labeling System	*Invitrogen*
Aminoallyl-dUTP	*SIGMA*
dATP, dCTP, dGTP, dTTP, 100mM each	*Fisher*
Cot-1 DNA	*Invitrogen*
3DNA 2xSDS-based hybridization buffer	*Genisphere*
Nuclear extraction buffer 2 (NEB2)	*New England Biolabs*
Bovine serum albumin	*New England Biolabs*
Annealed H14/H24	*New England Biolabs*
Thermopolymerase buffer	*New England Biolabs*
Guanosine 5'-Triphosphate (GTP)	*New England Biolabs*
H12 and H14 primers	*New England Biolabs*

2.10.3 Method

5 µl of 10x nuclear extraction buffer 2 (NEB2), 5 µl of 10x bovine serum albumin (BSA) (1 mg/ml) were combined in 2.5 ml tubes. 0.8 to 1 µg of DNA was added to this with water (MQ unless otherwise stated) to a final volume of 35 µl for the DNA and water. The mix was vortexed and centrifuged briefly. Finally 5 Ul MseI (10U/ml) was added (MseI kept at -20 °C until needed). 3 drops of mineral oil was layered over the reaction and it was incubated overnight at 37°C. The mastermix is shown in table 10 below:

Table 10

DMH: Mse1 digest	
	Amount in µl per sample
10X nuclear extraction buffer 2 (NEB2)	5
10X bovine serum albumin (BSA) (1 mg/ml)	5
DNA (0.8-1 µg)	According to DNA conc.
H_20	to total 35 µl with DNA
MseI (10U/ml)	5
TOTAL	50

The reaction was then purified using the Qiagen DNA purification kit (see chapter 2.10). Following elution the sample was processed in a speed vac for 2 hours resulting in a dry pellet. 3.5 µl of each of H12 and H24 primers were combined and annealed from 55°C to room temperature. 7 µl of the combined primers was added to the dry pellet which had been reconstituted with 14.5 µl of water. 2.5 µl of 10X T4 Ligase buffer was added. 1 µl of T4 ligase was then added; this had been kept at -20 until needed. The reaction was kept on ice at all times. The reaction was layered over with mineral oil and incubated overnight at 14°C for 14 hours and then kept at 4°C.

Table 11

DMH: Annealing of H12/H24 primers (end linkers).	
	Amount in µl per sample
Annealed H12/H24 primers	7
DNA/H_20	14.5
10XT4 Ligase Buffer	2.5
T4 Ligase (2000U/µl)	1
TOTAL	25

Next the unpurified sample was amplified and ran on a gel. A master mix with 13.7 µl of water, 2 µl of DMSO, 2 µL of 10x thermoPol Buffer, 0.5 µl of H24 primer and 0.4 µl of 10Mm dNTP was prepared on ice. Finally 0.4 µl of deep vent which had been kept at -20°C until needed was added and mixed gently.

Table 12

DMH: Annealing of H24 primer.	
	Amount in µl per sample
H_20	13.7
DMSO	2
10xtthermopol Buffer	2
H24 primer	0.5
10 mM DNTP	0.4
Deep Vent (2U/µL)	0.4
TOTAL	19.0

The reaction was placed on the thermal cycler using the cycling conditions as shown below in table 13:

Table 13

DMH: Cycling conditions for H24 primer annealing.		
	Temperature	*Time in minutes*
1	72°C	5
2	97°C	1
3	72°C	3
4	Go to (2) 24 times	
5	72°	5

A 0.8% agarose gel was prepared (see chapter 2.7). 5 µl of loading buffer was added to the reaction and then 10 µl was loaded onto the gel and run for 30 minutes with a 100 bp ladder. DNA was visualised with UV light using a Syngene GeneGenius Bioimaging System with GeneSnap version 6.03 software. A smear was expected at <500 bp fragment size.

The sample was then purified using the QIA quick columns (see chapter 2.10) and treated in the speed vac for 2 hours, until a dry pellet formed. Next the McrBC digestion was performed. The DNA pellet was reconstituted in 24 µl of water and 12 µl was used for the digest, and 12 µl for a mock treated sample. Two master mixes were made as outlined in the table 14 below.

Table 14

DMH: McrBC and mock digested samples.		
	Digest	*Mock (µl)*
DNA or H_20	12	14
10Xnuclear extraction buffer 2 (NEB2)	2	2
10X Guanosine 5'-Triphosphate (GTP)	2	2
10X bovine serum albumin (BSA)	2	2
McrBC (20 U/µl)	2	NIL
TOTAL	20	20

The reaction was layered over in mineral oil and incubated overnight at 37°C. Next the samples were amplified. A master mix was made as shown in the table below;

Table 15

DMH: Mastermix for PCR amplification of McrBC and mock digested samples.	
	Amount in µl per sample
McrBC digest	20
H_20	230.5
10XThermopolymerase Buffer	30
H24 primer (10 µM)	7.5
DNTP (10 µM)	6
Deep Vent	6
TOTAL	300

The 300 µl reaction was divided equally into 3 PCR tubes (100 µl each) and a PCR reaction was performed as shown in table 16 below.

Table 16

DMH: Cycling conditions for PCR amplification of McrBC/ mock digested samples		
Step	*Temperature*	*Time in minutes*
1	72°C	5
2	97°C	1
3	72°C	3
4	Go to step 2, 20 times	
5	72°C	5

The sample was then purified using the QIAquick column (see chapter 2.10) and the DNA concentration determined using the nanodrop. The DNA concentration was expected to be 2-3 µg in total. Aminoallyl labelling of the amplicon was next performed. 0.3 µg DNA was added to water making a final volume of 33.2 µl. This was then combined with 30 µl of 2.5x random primers solution. The sample was denatured at 95°C for 5 minutes and placed on ice for 2-3 minutes. It was then centrifuged briefly. Next it was combined with the following master mix;

Table 17

DMH: Mastermix for Aminoallyl labelling	
	Amount in µl per sample
10xdNTP (2 Mm A C G, 0.35 Mm T)	7.5
10 mM aa-dUTP	1.8
Klenow (40U/µl)	2.5

This reaction was incubated at 37°C for 5 hours. Next it was purified using the QIAquick columns (see chapter 2.10) and eluted in 2x40 µl of water. The DNA concentration was determined using the nanodrop and the fold increase in concentration was expected to be 13-17 times. The samples were dried using the speed vac for 2 hours. The final part of the reaction, coupling of the dyes to the DNA and hybridisation of the slides, was performed by Dr Teodoridis as previously described (Huang, Perry et al. 1999).

2.11 **DNA purification**

2.11.1 Materials

100% ethanol	Sigma
QIAquick kit and spin columns	Qiagen

2.11.2 Method

As per the manufacturer's instructions, briefly, 5 volumes of buffer PB were added to 1 volume of the PCR reaction and mix. The reaction was then placed in a QIAquick spin column and 2 ml collecting tube and centrifuged for 60 seconds. The flow through was discarded and the column was placed back in the same tube. To wash, 730 µl buffer PE was added to the column and centrifuged for 60 seconds. The flow through was discarded, the column placed back in the tube and then the sample was centrifuged again for a further minute. The column was then placed in a clean 1.5 ml microcentrifuge tube. Unless otherwise stated, to elute DNA 40 µl of water (MQ) was added to the centre of the membrane and centrifuged for 1 minute with this process being repeated once (80 µl total).

2.12 Homogenisation of cell lysates

2.12.1 Materials

QIAshredder columns	Qiagen
Lysate buffer	Qiagen

2.12.2 Method

Following siRNA experiments, ovarian cancer cell lines were spun down to a cell pellet using the Beckman centrifuge at 600 x *g* for 4 minutes. As per the manufacturer's instructions, any liquid was aspirated and the cell pellet was then resuspended in 350 ml of lysate buffer. This was then passed through a QIAshredder column and the lysate was collected in the 2 ml collecting tube, by centrifuging at top speed for 1 minute. The collecting tubes were then fitted with lids and stored at -20°C.

2.13 RNA extraction from cell lines and DNase digest

2.13.1 Materials

DNase free/RNase free water	*Invitrogen*
Ethanol	*Hayman*
QIAshredder spin columns	*Qiagen*
RNeasy mini kit	*Qiagen*
RNase-free DNase set	*Qiagen*

2.13.2 Method

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. DNase-free RNase-free water was used in all steps to minimise the risk of degradation of RNA and complementary DNA (cDNA). Cell lysates were produced as outlined in chapter 2.11. An RNase-free DNase set was used to provide efficient digestion of genomic DNA. The DNase was removed in subsequent wash steps. This was also performed according to the manufacturer's instructions. RNA was eluted once in 30µl of RNase-free H_2O and then stored at -70°C until required.

2.14 cDNA synthesis (Reverse Transcription, RT)

2.14.1 Materials

SuperScript II Reverse transcriptase Kit *Invitrogen*

2.14.2 Method

cDNA was prepared according to the manufacturer's instructions by reverse transcribing the messenger RNA (mRNA) within 1000 ng of total RNA using the Superscript II first strand synthesis system and random primers. A no RTase control was included for each sample. cDNA was then stored at -20^{o}C until required for quantitative reverse transcription PCR (qRTPCR).

2.15 qRTPCR

2.15.1 Brief overview

qRTPCR was carried out using two different methods in this thesis. For the experiments outlined in Chapter 3 investigating the expression of *LMX1A* and *NR2E1* the SYBR green system was used and for the experiments outlined in all other chapters the taqman system was used. qRTPCR can be used to quantitatively evaluate the change in RNA expression

levels of a target gene either by comparing to the expression of a chosen sample within the experiment (relative expression) or by using a standard curve of known concentrations (absolute quantitation). Both methods were used in this thesis. For the absolute quantitation experiments investigating *SP5* expression, a known concentration was produced using a stable vector as described in Chapter 2.16.

In all qRTPCR experiments the expression of the house keeping gene *GAPDH* was analysed in order to allow compensation for variability in the initial concentration and quantity of the tRNA and in the conversion efficiency of the reverse transcription reaction. *GAPDH* was chosen because it had shown consistent expression across several ovarian cell lines by microarray experiment (data not shown).

2.15.2 SYBR Green Quantitative RTPCR (qRTPCR)

2.15.2.1 Materials

DyNAmo HS SYBR green qRTPCR kit	*Finnzymes*
Flat cap strips	*Biorad*
Low 96 well white multiplate PCR plate	*Biorad*
Opticon 2 DNA Engine	*MJ research*
RTPCR oligonucleotides	*Invitrogen*

All other reagents as described in Chapter 2.6

2.15.2.2 Method

qRTPCR 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 3.5. qRTPCR master mixes were made up using the DyNAmo HS SYBR green qRTPCR kit. The 2x master mix provided contained a hot start version of modified Thermus brockiamus DNA polymerase, SYBR green I binding dye, optimised PCR buffer, $MgCl_2$ and dNTPs in a premixed form. Reactions included 1x SYBR green master mix,

150 NG of each forward and reverse primer and 2 µl cDNA made up to 25 µl with dH_20. The optimised conditions are shown in Table 18 below.

Table 18. qRTPCR: oligonucleotide and conditions

Gene	Primer sequence	Genomic position	Product size (BP)	Annealing temp (C)	Mg2+/ mM
GAPDH	F:GTCAAGCTCATTTCCTGGTATG R:GTCTACATGGCAACTGTGAG	Exon 8-9	214	61	2
LMX1A	F:TCATGAACCCTACACGG R:GGGCTCGGCACCATAA	Exon 9-10	143	61	2
N2RE1	F:ATCAACAAGCCGCATTTTAG R:GCCTCCCTGGTTTCCAG	Exon 1-3	160	65	2

Reaction mixes and plates were prepared in the PCR workstation. A negative control containing water instead of template cDNA was included and a standard curve derived from a range of known concentrations of cDNA was set up in triplicate 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 RTPCR reactions, once standardised to *GAPDH*, taken as the concentration of PCR product. Reactions were run on an Opticon 2 DNA Engine according to the cycling conditions in table 19 below:

Table 19. qRTPCR: cycling 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 2x39 times, **8.** Melting curve 70-93°C
LMX1A	**1.** 94°C 15 mins, **2.** 94°C 30 secs, **3**. 63°C 30 secs, **4.** 72°C 30 secs, **5.** 80° C 10 secs, **6.** Plate read, **7.** Go to step 2x42 times, **8.** Melting curve 70-93°C
NR2E1	**1.** 94°C 15 mins, **2.** 94°C 30 secs, **3**. 63°C 30 secs, **4.** 72°C 30 secs, **5.** 80°C 10 secs, **6.** Plate read, **7.** Go to step 2x42 times, **8.** Melting curve 70-93 °C

2.15.3 Taqman Quantitative qRTPCR

2.15.3.1 Materials

Trigene advance (1:100 dilution), *(TM301)*	*Trigene*
Taqman Gene expression mastermix. 5 ml (4369016)	*Applied Biosystems*
DNase/RNase free water (10977-035)	*Invitrogen*
FirstChoice® Human Ovary Total RNA (AM6974)	*Ambion*
MicroAmp fast optical 96 well reaction plates (4346906)	*Applied Biosystems*
Microamp optical adhesive films (4311971)	*Applied Biosystems*
Taqman RTPCR oligonucleotides	*Applied Biosystems*
GAPDH: 4326317E	
SP5: HS01370227-AH	
MAPK: HS01046830	
PX2 thermal cycler	*Thermo Electron Corporation*
StepOnePlus real time PCR machine	*Applied Biosystems*
Thermospecific PICO 17 centrifuge	*Heraeus*

2.15.3.2 Method

Taqman primers were used in the experiments outlined in chapters 4-6. If the CT values of the target gene and *GAPDH* were similar when tested together and individually they were tested in the same reaction; a technique known as multiplexing (The C_T value is a relative measure of the concentration of target in the PCR reaction, see table below). Instead of testing *GAPDH* and the test gene separately in the samples of interest this can be done in one reaction, saving on precious samples and reagents.

In addition to reverse transcribed samples, non reverse transcribed and water samples were included and it was important that no amplification was seen in these. All experiments were performed in triplicate. qRTPCR was performed using the Taqman Gene expression mastermix, probes and StepOne software version 2 (stepone-software.software.informer.com/). The master mix provided contained AmpliTaq Gold® (hotstart taq), buffers and dNTPs. The TaqMan® MGB probe consists of a target specific

oligonucleotide with a reporter dye linked to the 5' end of the probe, a Minor Groove Binder (MGB), and a non fluorescent quencher at the 3' end of the probe (lower background signal allowing more precise quantitation).

For siRNA experiments, 3 master mixes were prepared for each 96 well plate; one for the *GAPDH* standard curve, one for the test gene standard curve and one containing both primers for multiplexing of the water controls and test samples. Each reaction included 10 µl Taqman mastermix, 1 µl of primer (singleplexing) or 1 µl of each primer (multiplexing), 7 µl of water (singleplexing) or 6 µl of water (multiplexing), and 2µl of cDNA, which was prediluted 1/10, with a final volume of 20 µl.

Reaction mixes and plates were prepared in a PCR workstation on ice following washdown with 70% ethanol and trigene solution. For each 96 well plate, three negative controls containing H_2O instead of template cDNA were included along with a standard curve derived from a range of known concentrations of cDNA set up in triplate for each gene. *GAPDH* primers used the VIC reporter and all other primers used the FAM reporter. Reactions were run on an applied biosystems StepOne Real Time PCR system according to the cycling conditions below in Table 20.

Table 20

qRTPCR: PCR cycling conditions using Taqman primers

Step	Temperature	Time
1	50°C	2 mins
2	95°C	10 mins
3	95°C	15 seconds
4	60°C	1 minute
5	Repeat steps 3 and 4, 39 more times	

Taqman primers are customised and validated and although the specific sequence is not available information relating to their alignment can be found on their website for each gene examined (*SP5*).

(https://products.appliedbiosystems.com/ab/en/US/adirect/ab?cmd=ABAssayDetailDisplay&assayID=Hs01370227_mH

Several factors are important when analysing the data generated by qRTPCR and some key definitions which were considered in the results section are listed in table 21 below.

Table 21

qRTPCR: Definitions of terms used when analysing data

Name	Definition
C_T	Intersection between an amplification curve and a threshold line. A relative measure of the concentration of target in the PCR reaction. The threshold is set above the background and within the exponential growth phase of the amplification curve.
Efficiency	If efficiency is maximal (=1) the quantity of PCR product generated at each cycle is optimal, reflected in the amplification plot. If the efficiency decreases the quantity of PCR product generated at each cycle will decrease and the amplification plot will be delayed. The optimal efficiency is between 90 and 110%.
Dynamic range	The number of replicates and logs of template concentration required for accurate results. For these experiments 3 replicates and 5 logs were used.
R^2	A statistical term that defines how good one value is at predicting another. If R^2=1 then you can perfectly predict the value of X (quantity) with the value of Y (C_T)
Precision	The standard deviation (square root of the variance). The greater the SD the lower the ability to distinguish between 2-fold dilutions

Three main steps were taken when analysing the data generated using the Taqman system.

Firstly the amplification plots for the entire plate are reviewed. The amplification plot is the plot of fluorescent signal versus cycle number. The higher the starting copy number of the nucleic acid target the sooner a significant increase in fluorescence is observed. Secondly the standard curve was reviewed. For an experiment to have been successful it is important for replicates to have similar values and for all values to fit well onto one line; defined by the R^2 value. Finally, the baseline and threshold values, used to determine the threshold cycles (C_T) for the amplification curves were set automatically using the Sequence detection system (SDS). For the results of an experiment to be deemed valid it

was important to have a high R_2 value, an efficiency value between 80-110% and amplification of both the reference and test gene ideally within 25 cycles of PCR. Finally amplification should be seen in the reverse transcribed samples only with no amplification in the non reverse transcribed samples or water blanks.

2.16 DNA cloning using miniprep (*SP5, GAPDH, ß-ACTIN* for qRTPCR)

2.16.1 Recipes

Lysogeny (LB) broth

800ml H_2O

10g Bacto-tryptone.

5g yeast extract.

10g NaCl.

pH to 7.5 with NaOH.

Adjust volume to 1L with dH_2O

Sterilize by autoclaving

2.16.2 Materials

TOPO TA cloning® kit for sequencing	*Invitrogen*
(reagents, pCR®4-TOPO® vector, and One shot® TOP10 chemically competent *E. coli*)	
LB Broth	*Imperial College london*
S.O.C. medium	*Qiagen*
Ampicillin	*Qiagen*
LB agar plates with ampicillin	*Imperial College London*
QIAquick® gel extraction kit	*Qiagen*
QIAprep® miniprep kit	*Qiagen*
Cooling Centrifuge	*Fisher*
Shaking incubator	*New Brunswick Scientific*
GeneGenius Bioimaging System	*Syngene*
Nanodrop ND-1000 spectrophotometer	*Nanodrop*

Taq polymerase results in a single ‘A’ overhang on the 3 prime end of all PCR products. The vector supplied with this kit also has a 3 prime ‘T’ overhang which allows the PCR to be ligated efficiently into the vector.

2.16.3 Method

DNA cloning was performed using the Invitrogen TOPO TA Cloning Kit for sequencing. qRTPCR was performed as described in Chapter 2.14. 20 µl of the PCR product and 5 µl of loading buffer were loaded and then electrophorised on a 1% agarose gel for 30 minutes as described in Chapter 2.7. The gel was then placed in the gel imaging machine and using the UV function the product was visualised and cut out using a sterile scalpel. This was then dissolved using the qiagen qiaquick gel extraction kit as per the manufacturer’s instructions. 4 µl of the digested gel were combined with 1 µl of salt and these were mixed by pipetting up and down. Following this 1 µl of the TOPO vector was added and combined by swirling gently (it is important not to pipette it up and down). The reaction was left for 5 minutes and then placed on ice for 30 minutes.

During this time the chemically competent cells were removed from the minus 80 °C storage and thawed on ice. Superoptimal broth (SOC) media was also removed from the fridge and warmed to room temperature. A heating block was prewarmed to 42 °C. After the 30 minute incubation on ice 2 µl of the reaction was gently placed into the vial of chemically competent cells (which were kept on ice at all times). This reaction was left for a further 30 minutes and then placed at 42 °C for 30 seconds. Following this the vials were immediately placed back on ice and left for 2 minutes. Next 250 µl of SOC media was added to each and they were placed at 37 °C with shaking for 1 hour. After this they were spread onto a pre-made ampicillin/ agar plate and left in the incubator overnight.

Only those colonies which had taken up the TOPO vector should grow in the ampicillin environment. Colonies were then picked using a pipette tip and placed in a falcon tube which contained 3.5 ml LB broth and 3.5 µl of ampicillin. They were then left in a shaking incubator at 37 °C overnight. The following morning a miniprep was performed as per the manufacturer's instructions using the qiagen QIAprep kit.

Next 2.5 µl of this was used to perform an EcoR1 digest. 2 µl of 10x EcoR1 buffer, 14 µl of water and 2.5 µl of miniprep were mixed by pipetting up and down. 1.5 µl of EcoR1 enzyme was then added and mixed gently. The reaction was then placed on a heating block at 37 °C for 90 minutes. Afterwards 5 µl was combined with 1 µl of orange G loading buffer and ran on a 1% agarose gel for 35 minutes. If the reaction was not to be run on a gel immediately then it was placed on ice for 10 minutes to stop the ecoR1 reaction (and then stored at minus 20 °C). If the cloning reaction had been successful then for *SP5* a PCR product was seen at 190 bp.

2.17 DNA Cloning using maxiprep (cloning of *SP5*, *KIAA1383* and empty vector for over expression experiments)

2.17.1 Materials

Plasmid DNA cloned in vector as outlined in chapters 2.22 *(KIAA1383*) and 2.23 (*SP5*)

37 °C shaking incubator	*New Brunswick Scientific*
Centrifuge	*Fisher*
250 ml conical flask	*Corning*
Hispeed Plasmid Purifation Maxi Kit	*Qiaaen*

All other reagents as listed in chapter 2.16.1

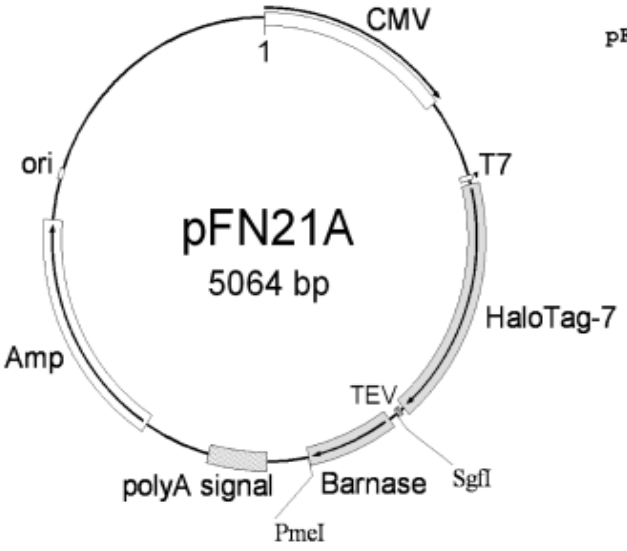
2.17.2 Method

SP5 and *KIAA1383* were provided already cloned into their respective vector systems. The empty vector for both systems was also cloned. The work bench was cleaned down using trigene and all work was carried out close to a flame (from Bunsen burner). Similarly to as described above, both genes were separately transformed into chemosensitive bacteria and colonies grown on agar plates containing ampicillin. Plates were left overnight in a 37 °C incubator. 16 hours later a single colony was selected and placed into 5 ml of Lysogeny (LB) broth containing 5 µl of ampicillin. This starter amplification was placed on the shaking incubator at 37 °C for 6 hours. Next 400 µl of the reaction was taken and placed into a 500 ml conical flask containing 200 ml of LB media containing 200 µl of ampicillin. The conical flask was sealed using tinfoil and then placed on the shaking incubator at 37 °C for 16 hours. It is important that the flask holds at least twice the volume of the reaction. After 16 hours the contents was transferred to a 250 ml bottle with lid and spun at 3200 xg for 15 minutes at 4°C. Following centrifugion the supernatant was removed, sterilised in trigene and discarded.

The cell pellet was then used as the basis for the hispeed maxi prep which was carried out according to manufacturer's instructions (with the exception of the DNA precipitation step which was carried out for 30 minutes rather than 5 minutes and the first three buffers which were used at double the volume). DNA was eluted in 1000 µl TE buffer and passed through the qiaprecipitator. This same elute was then passed through the qiaprecipitaor a second time in order to increased the concentration of DNA obtained.

The DNA was then analysed on the nanodropper and the conc. and 260/280 and 230/280 ratios noted. DNA of adequate quality was expected to have values of 1.8 and 1.9 respectively. If the ratio was lower then protein or solvent contamination respectively were assumed and the maxiprep was repeated. DNA was stored at minus 20°C. Any cloned DNA was sent for sequencing.

Glycerol stocks were made by taking 100 µl of the second incubation liquid and combining with 100 µl of glycerol/LB mix (20 µl glycerol and 80 µl LB media). Stocks were stored at minus 80°C. The vector map for *KIAA1383* is shown below in Figure 10 (below) and the vector map for *SP5* is shown later in chapter 6.8:




```
pFN21A   5064 bp
       1-742: CMV promoter
    1033-1052: T7 RNA polymerase promoter (-17 to +3)
    1067-1957: HaloTag-7 CDS
    1970-1990: TEV protease recognition sequence
    1997-2004: Sgf I recognition site
    2028-2363: Barnase CDS
    2365-2372: Pme I recognition site
    2524-2745: SV40 late polyadenylation signal
    3006-3866: beta-lactamase (Amp) CDS
    4021-4057: ColE1-derived plasmid replication origin
```

Figure 10. Vector map for pFN21A vector which over expressed *KIAA1383* (www.kasuza.com).

2.18 **Cell culture**

All tissue culture media was stored at 4^{o}C

2.18.1 Materials

Cryotubes	*Nunc*
Dimethyl sulfoxide (DMSO)	*Fisher*
Fetal Bovine Serum (FBS)	*Autogen Bioclear*
L-Glutamine 200mM	*Gibco*
Petri dishes (5cm and 10cm)	*Sterilin*
Penicillin-Streptomycin (15140-122)	*Invitrogen*
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*
6, 24 and 96 well tissue culture plates	*Iwaki*
TrypLE express (12604).	*Gibco*
70 umI sieve (734-0003).	*VWR*

2.18.2 Recipes

PBS (Phosphate Buffered Saline)

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

RPMI plus +/- 2x penicillin/ streptomycin

RPMI 1640	500ml
L-glutamine (2mM, final)	5ml
Penicillin/streptomycin	2ml
(10 000 u penicillin+10 000 μg streptomycin/ ml)	
FBS (10% final)	50ml

2.18.3 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^{o}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.

2.18.4 Ovarian cancer cell lines

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). Ten isogenically matched cisplatin-resistant cell lines were used; A2780cp70 (Behrens, Hamilton et al. 1987) and MCP1-9 (referred to as the multi-step clones), (Brown, Hirst et al. 1997). These were derived by multiple exposures to cytotoxic levels of cisplatin and showed a 2-5 fold resistance to cisplatin in clonogenic assays. Six

additional cisplatin-resistant cell lines, derived from exposing A2780 cells to a single dose of cisplatin (McLaughlin, Stephens et al. 1991), were used which included C1cis6, C2cis6, C2E3, C3Cis6, C5E4 and C5E4(15). These are referred to as the single step clones. The final pairs were OVIP and OVIP DDP and CH1 and CH1CISR (McLaughlin, Stephens et al. 1991).

In addition sensitive and resistant ovarian cancer cell line pairs derived from patients were used. PEO1 (a patient with poorly differentiated serous adenocarcinoma after cisplatin, 5FU and chlorambucil chemotherapy) and PEO1CDDP (laboratory generated resistant cell line following further cisplatin treatment), PEO4 (collected after clinical relapse) and PEO6 (collected prior to death); PEO14 (a patient with well differentiated serous adenocarcinoma, collected prior to treatment) and PEO23 (following relapse after cisplatin and chlorambucil); PEA 1 (a patient with poorly differentiated adenocarcinoma collected prior to treatment) and PEA2 (at relapse following cisplatin and prednimustine) (Langdon, Lawrie et al. 1988). All of these cell lines are shown in Figure 11 below.

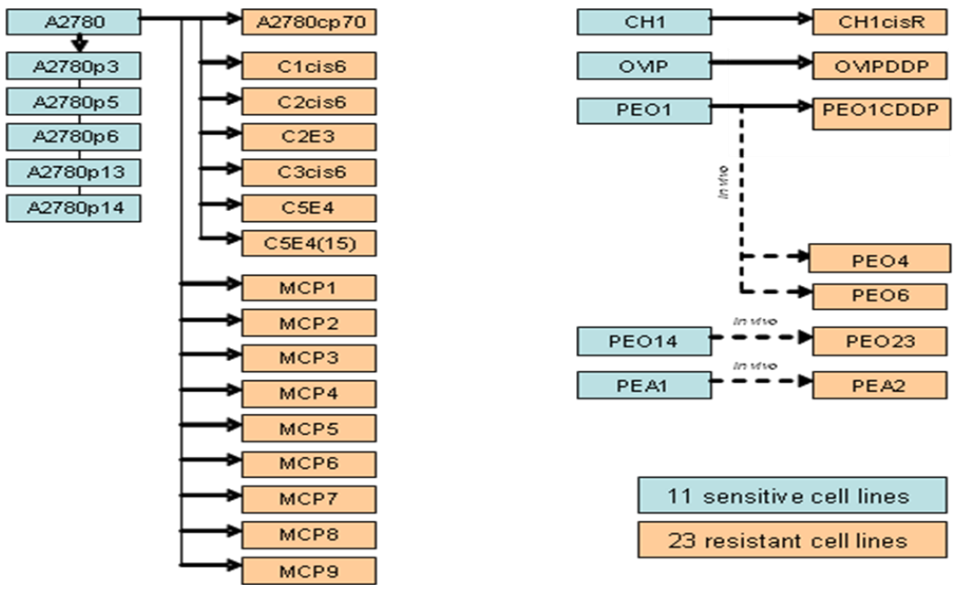


Figure 11. 34 epithelial ovarian cancer cell lines panel. Blue sensitive cell lines, peach resistant cell lines. Solid line *in vitro* generated resistant clones, Broken line *in vivo* generated resistant clones.

Cell stocks were formed by freezing 10^6 cells in 1ml FCS 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.

2.19 **siRNA**

2.19.1 Materials

Hiperfect transfection reagent	*Qiagen*
SP5 siRNA (127988 F08, 127988 F09)	*Invitrogen*
MAPK siRNA (1022564)	*Qiagen*
siGENOME non-targeting siRNA pool (d-0012-6-13-05)	*Dharmacon*
All stars® siRNA (1027280)	*Qiagen*
Cyclophilin B	*Dharmacon*

All other reagents as outlined in Chapter 2.16

2.19.2 Recipes

Serum free media

RPMI 1640	100 ml
L Glutamine	1 ml

2.19.3 Method

Small interfering RNA (siRNA) are double stranded RNA molecules, usually between 20 and 25 nucleotides long, which, among other functions, are involved in the RNA interference pathway. They can therefore be used to modulate gene expression. They have 2-nt 3' overhangs on either end and each strand has a 5' phosphate and a 3' hydroxyl group. If the gene sequence is known it is possible to design an siRNA exogenously that may interfere with the expression of that gene and it is therefore an experimental approach that can allow analysis of the phenotypic effects of suppression of a specific gene's expression. The siRNA experiments described result in a transient reduction of gene

expression which can then be confirmed and quantified at the mRNA level by qRTPCR (or the protein level by western blot analysis) (Dennis-Sykes, Miller et al. 1985).

In order to check that the siRNA has been successfully integrated into the cell, but without off target effects, it is important to run adequate positive controls. siRNAs targeting ubiquitous and highly expressed housekeeping genes are commonly used controls; in this case MAPK was used which has been proven to cause high knockdown of its target gene. MAPK knockdown was confirmed by qRTPCR. As it is not possible to exclude off-target effects as a result of knocking down a gene of interest it is important to measure general off-target effects by using a scrambled control that should not interfere with any gene expression. All Stars® scrambled siRNA (Qiagen) was used in all experiments as the negative control and knockdown again assessed by qRTPCR. All Stars® is known to have no homology to any mammalian gene. In addition, another positive (cyclophilin) and negative (siGENOME non-targeting siRNA) control were tested and these showed the comparable results.

Firstly, qRTPCR experiments were performed to confirm which cell lines that the gene of interest was expressed in. These cells were then transfected with 3 different siRNA's for the same gene at two different concentrations. This was to find the optimal siRNA and concentration for knockdown. These initial experiments were performed using the concentrations of transfection reagent suggested by the manufacturer and all optimal siRNA doses are referred to in the text and tables below. For each experiment a minimum of 5 conditions were analysed - an untreated sample where cells were passaged and seeded in media only; a mock treated sample where the sample was treated with the transfection reagent but no siRNA; and a positive control, negative control and the test siRNA. The positive control was then examined by qRTPCR following RNA extraction and conversion to cDNA (see chapters 2.13-2.15) in order to confirm adequate knockdown. For MAPK a decrease in gene expression of at least 80% was expected to confirm adequate transfection.

If transfection was found to be adequate the knockdown of the test gene was next examined, again as outlined in chapters 2.12 - 2.14.

Cells were grown up as outlined in chapter 2.17 in 75 ml flasks. It is important for cells to be approximately 60% confluent for efficient transfection in siRNA experiments. Once this was the case the experiment was started. On day 1, media was aspirated off and the cells were washed in 10 ml of PBS. This was then aspirated off and 6 ml of TrypLE^{TM} was added. Flasks were placed flat back in the incubator for 10 minutes at standard conditions (37^{o}C 5%CO_2 in air). Trypsanisation of cells was confirmed by microscopy and then the cells and trypsin were passed through a 70 um sieve into a 30ml Falcon tube to remove clumps of cells. 10 mls of RPMI media was washed around the flask and then this was also passed through the sieve. The falcon tube was then centrifuged in a Beckman centrifuge at 600 xg for 4 minutes. Any liquid was aspirated and the cell pellet was then resuspended in 10 ml of RPMI plus media containing 2x penicillin and streptomycin. Ten µl of cells were mixed with 10 µL of trypan blue 0.4% and then counted twice using a Neubauer haemocytometer.

These initial experiments were performed in 24 well plates and $20x10^4$ cells were seeded per well in a volume of 500 µl. Colleagues had previously found this to be the optimal plating density for these cell lines. The plate was then either placed in a humidified incubator at 37^{o}C 5% CO_2 in air for 4 hours for the cells to settle and attach or immediately transfected (within 30 minutes of plating).

Next the siRNA's were defrosted on ice and a mastermix was made up for each test condition. Serum free media was added firstly and then siRNA's where appropriate. This mixture was then vortexed. Hiperfect transfection reagent was then added, the mixture vortexed and then left for 10 minutes at room temperature. Finally 100µl of the mastermix

was added drop wise onto each well attempting to evenly disperse it amongst the well. Mastermixes are outlined in table 22 below:

Table 22. siRNA: Mastermixes for initial experiments (24 well plates).

Sample	SFM* (µl)	2 µM siRNA (µl)	Hiperfect (µl)
Untreated	100	0	0
Mock	100	0	3.25
MAPK +/-other positive	100	3	3.25
All stars +/-other negative	100	3	3.25
SP5/ test gene	100	1.5	3.25

SFM* serum free media, see recipes

Cells were then placed in the incubator at standard CO_2 and temp. On days 2 and 3 cells were observed my light microscopy and any obvious effects noted e.g. suspected cell death. On day 4 in exactly the same way as on day 1 cells were trypsanised, counted and spun down into a cell pellet. The cell pellet was then resuspended in 350 µl of lysate buffer, homogenised using the Qiashredder columns and stored at -20^{o}C prior to RNA extraction, cDNA conversion and qRTPCR as described in chapters 2.13-2.15.

2.20 **Induction of apoptosis as measured by Caspase-Glo®**

2.20.1 Materials

Caspase-Glo® 3/7	*Promega*
White ‘culturPlate 96’ plates	*PerkinElmer*
Optima luminometer	*Lumistar*
Rocking table	*Luckham*

2.20.2 Method

Caspase-Glo® results in cell lysis followed by caspase cleavage of the substrate releasing free aminoluciferin which is consumed by luciferase generating a luminescent signal. The signal is proportional to caspase activity. At each timepoint (24, 48 and 72 hours) 100 µl of caspase was added directly to cells. It was then placed on a shaking plate at room temp with the plate protected from light using tin foil. This reaction was left for 2 hours. Following this the lid of the plate was removed and analysis performed in an Optima lumistar luminometer using the associated software. The caspase solution was stored at minus 20 °C when not in use and protected from light using tinfoil.

2.21 **MTT**

The (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used in three sets of experiments in this thesis; Firstly when *SP5* was knocked down in order to assess the effect on chemosensitvity, secondly in the caspase experiments as a means of normalising apoptosis for the number of surviving cells and thirdly in order to assess whether over expression of *SP5* had an effect on chemosensitivity.

The MTT assay is an alternative to a clonogenic assay, which measures the ability of cells to form colonies and proliferate following a period of drug exposure. The MTT assay has the advantage of being less time consuming and allows a number of assays to be performed simultaneously.

PEO14 and PEO23 cell lines were plated in 96 well plates, in triplicate for each condition. 24 hours later they were exposed to a range of cisplatin doses when in the exponential phase of growth. After 24 hours, cisplatin was removed and cells were allowed to proliferate for 48 hours. The percentage of surviving cells was then determined indirectly by MTT reduction. MTT is a water soluble tetrazolium dye that is reduced by live but not

dead cells to form a water insoluble purple formazan product. This was dissolved in a suitable solvent (MTT Stop solution) and the amount of product determined spectrophotometrically.

2.21.1 Materials

Multi channel pipette	*Thermo Scientific*
Troughs	*Beckman Coulter*
Dimethyl sulfoxide (DMSO)	*Sigma*
3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl-Tetrazolium bromide (MTT)	*Sigma*
Dispenser microfill	*Fisher*
Plate reader	*Biotek*
Microplate photometer	*Labsystems*
Sorensens glycine buffer	*Lab systems*
Routine tissue culture materials	*See section 2.16.2*
MTT stop solution	10% SDS with 0.01% conc. HCL

2.21.2 Method

In the experiments outlined in Chapters 6.5 and 6.8 MTT experiments all included 4 wells treated with media only and then a range of concentrations of cytotoxic drug for each siRNA condition, in quadruplicate. Flasks were incubated at standard conditions and inspected days 2-3 and any obvious effects e.g. increased cell death noted. On day 4 cells were again washed with 10 ml of PBS, trypsinised using 6 ml of TripLE Express and counted having been passed through a sieve as described previously. They were then seeded at a density of $1x10^4$ cells in 160 µl of 'RPMI plus' media per well in a 96 well plate for the first experiments and $0.3x10^4$ for the experiments combined with the caspase assay. They were incubated at standard conditions for 24 hours. A serial 5 fold dilution of cytotoxic drug was prepared in PBS (for siRNA expt) and 6 fold dilution (for over expression Expt) and 20 µl added to the cells. A further 20 µl of media was then added to

each well except for control wells that received 40 µl of media instead .All wells had a final volume of 200 µl. A multichannel pipette was used to dispense the drug starting at the lowest drug concentration.

On day 5, the media was replaced with 200 µl of fresh media so that the drug was removed. Cells were incubated at standard conditions until day 7 when the MTT assay itself was performed. For this the MTT solution was warmed for 1 hour at 37°C. MTT was dissolved in PBS at a concentration of 2 mg/ml. 50 µl of MTT was then added to each well using the auto pipette. The plates were incubated for 1 hour at 37^{o}C. The MTT was then flung off and the plates blotted upside down with paper. 200 µl of DMSO was added to each well and the plates were left on an agitator for 15 minutes. The plate reader was switched on and a 540 nm filter inserted. The plates were read using the ASCENT programme. This provides results in a format that can be transferred to excel.

In the experiment reported in Chapter 6.6 the MTT was used simply to normalise the caspase results and therefore the use of cisplatin was omitted. Cell lysates were prepared as described in chapter 2.11 for each time point.

Caspase plates were read on the Lomita optima illuminometer plate using the corresponding software and data exported into excel. MTT was read on the spectraMaxi190 at 570 nm using Surtax Pro software and data again exported into excel. The average was taken for each triplicate and then the caspase results divided by the MTT results. This provided a control for altered proliferation in different conditions. RNA was later extracted from cell lysates and reverse transcription performed as described in Chapters 2.12 - 2.14.

2.22 Outline of siRNA knockdown experiment used for caspase 3/7 analysis normalised to MTT

2.22.1 Materials

All reagents as outlined in Chapter 2.18, 2.19 and 2.20

2.22.2 Method

siRNA was performed directly onto 96 well plates in a similar manner to that described in chapter 2.18 with some modifications. On day one $0.3x10^7$ cells in 80 µl of RPMI plus media (containing 2x penicillin and streptomycin) were transfected in triplicate onto white 96 well plates for later caspase analysis (Chapter 2.19). Simultaneously $0.3x10^7$ cells in 80 µl media were seeded on clear 96 wells in quadruplicate for later RNA extraction and qRTPCR. A third clear plate had the same cell numbers plated in triplicate for a later MTT, as described in Chapter 2.20. The same five conditions were used as described in the previous chapter; namely untreated, mock treated, all stars scrambled control, MAPK control and *SP5* nm siRNA. Plates were prepared for analysis at 24, 48 and 72 hours. The siRNA transfection was performed approximately 4 hours after the cells had been seeded. A mastermix containing serum free media, hiperfect +/- siRNA for each condition was prepared in 1.5 ml tubes and vortexed and left at room temp for 10 minutes. Allstars and *SP5* were used at 5 nm conc. and MAPK at 10 nm conc. Untreated wells contained 20 µl of serum free media only, mock treated cells contained 19.8 µl of SFM and 0.2 µl of hiperfect. Allstars and *SP5* contained 19.5 µ, 0.2 µl hiperfect and 0.3 µl siRNA. MAPK contained 19.2 µl SFM, 0.2 µl hiperfect and 0.6 µl siRNA. They were then left in the

incubator at 37 degrees °C and 5% CO_2. Separate plates for caspase, MTT and cell lysates were then removed at each time interval (24, 48 and 72 hours) and analysed as described in Chapters 2.19, 2, 20 and 2.11 respectively.

2.23 Flow cytometry and cell cycle analysis following transient over expression of *KIAA1383* +/- cisplatin in PEA2.

2.23.1 Materials

pFN21A vector with halotag	*Promega*
- FHC00819	
- FHC02806	
Effectene transfection kit (301425)	*Qiagen*
DiACFAM halotag ligand	*Promega*
Propidium iodide (P4170)	*Sigma*
RNase A (R4875)	*Sigma*
Triton X100 (X100 500 ml)	*Sigma*
Polystyrene FACS tubes (352052)	*Becton Dickinson, Oxford, UK (BD)*
FACScalibur	*Becton Dickinson, Oxford, UK*
Cell quest analysis software	*Becton Dickinson, Oxford, UK*
FloJo 7.6.1 cell cycle analysis software	*FloJo*

Other reagents and kits as described in chapter 2.17 (maxi prep) and 2.18 (Cell culture)

2.23.2 Methods

2.23.2.1 Transient transfection

The effect of over expressing *KIAA1383* in PEA2 was assessed by cell cycle analysis. *KIAA1383* were supplied within the pFN21A vector system which contains a halotag. On day one, $2x10^5$ cells were seeded in 1600 µl media containing 2X penicillin and

streptomycin onto two 6 well plates. Plate one was later transfected with empty vector +/- cisplatin in triplicate and plate two with overexpressing vector +/- cisplatin, in triplicate.

On day 2 if cells were 40-80% confluent transfection was performed. This was performed as described by the manufacturers. Briefly, 0.4 µg DNA had previously been eluted in TE buffer (chapter 2.17). This was combined with EC buffer to a final volume of 1400 µl. 3.2 µl was added and the mixture vortexed. It was then incubated at room temperate for 5 minutes and centrifuged for 15 seconds. 10 µl of effectene reagent was added to the DNA enhancer mix and vortexed for 10 seconds. This was incubated at room temperate for 5-10 minutes. While complex formation was occurring the media was aspirated off the cells and a PBS wash was performed. 1000 µl of fresh media was then added to each well. 600 µl of media was then added to the complexes and mixed by pipetting up and down twice. This was then added immediately to wells dropwise. The dish was swirled gently to aid even distribution. The plates were placed in a 37 °C 5% CO_2 incubator for 48 hours.

On day 4 for the cisplatin treated cells 25 µM cisplatin in 2000 µl media was added and for the others a simple media change performed. Plates were again incubated at 37 °C degrees and 5% CO_2.

2.23.2.2 Addition of fluorescent ligand.

On day 5 the fluorescent halotag was added as per the manufacturer's instructions. Firstly the media was changed in all wells in order to remove cisplatin. The fluorescent tag was diluted 1/1000 in fresh warm media and 650 µl pipetted into each well. This was incubated for 15 minutes at 37 °C and 5% CO_2. The ligand was then gently replaced with 1000 µl warm fresh media. This was repeated twice further with PBS finishing with 1000 µl of warm medium. Cells were incubated in an incubator for 30 minutes to wash out unbound ligand. The cells were washed with PBS and the medium replaced for a final time with 2000 µl fresh warm medium.

2.23.2.3 Propidium Iodide fixing and staining

Cells were next fixed, permeabilised and stained with propidium iodide containing RNase A. Firstly cells were washed with PBS, trypsinised and centrifuged at 400 g. The supernatant was removed and they were resuspended in 1 ml of PBS in a 1.5 ml eppendorf tube. They were further centrifuged at 2500 rpm for 5 minutes. They were next resuspended in 1 ml of cold 70% ethanol which was added dropwise while vortexing. This reaction was then incubated at minus 20 for 30 minutes. The reaction was then centrifuged for 5 minutes at 400g. The supernatant was removed; 10 µl of each reaction was place in a separate eppendorf. All tubes were then resuspended in 1 ml PBS. 500 µl of a mastermix containing 0.2% Triton X, 50 µg/ ml propidium iodide and 100 µg/ml RNase A was then added and incubated for the propidium iodide stained cells and 500 µl PBS added to the corresponding non stained control samples (which were made up of 10 µl of the initial reaction resuspended in PBS). Eppendorfs were protected from sunlight using foil. These reactions were incubated at 37 °C for 30 minutes. After this cells were spun at 2500 RPM for 5 minutes and the supernatant removed. Each vial of cells was resuspended in PBS and transferred to a polystyrene FACS tube just prior to analysis. Each sample was then analysed using cell quest analysis software. 20 000 events were counted. Cells were gated according to forward and side scatter and then FLT2 area and width. Cell cycle analysis was performed on the remaining cells using FlowJo 7.6.1. DiACFAM crosses the extracellular membrane and stains green and Propidium Iodide stains DNA red. The controls used in the initial set up of the FACS machine are listed below:

1. PEA2, no plasmid, fluorescent tag, no PI
2. PEA2, no plasmid, fluorescent tag, PI
3. PEA2, plasmid, no fluorescent tag, no PI
4. PEA2 , plasmid, no fluorescent tag, PI

2.24 Stable over expression of *SP5* in A2780 cp70 with subsequent MTT experiment using cisplatin at varying doses

2.24.1 Materials

pCMV6-AC-GFP vector with *SP5* *(and empty vector only control)*	*Origene*
Effectene transfection kit (301425)	*Qiagen*
HiSpeed Plasmid maxi prep kit (12662)	*Qiagen*
RPMI 1640 + L-glutamine (21875-091)	*Invitrogen*
G418 sulphate (P25-011)	*PAA*
Tryple Express (12604013)	*Invitrogen*
PBS	*In-House*
6 well plates (353046)	*Falcon*
24 well plate (142475)	*Nunc*
T25 vent cap (430639)	*Corning*
T75s vent cap (430641)	*Corning*
96 well plates (Costar 3596)	*Corning*
Stericup GP filter unit 250ml (SCGPU02RE)	*Millipore*
MTT 2mg/ml	*Sigma*
Countess cell counter	*Invitrogen*
Countess chamber slides	*Invitrogen*
DMSO analytical grade	*Fisher*
Synergy 2 plate reader	*Biotek*
Gen5 v1.05 software	*Biotek*
Scout Pro balance	*Ohaus*

Other reagents and kits as described in chapter 2.13-2.14, 2.16 (RNA extraction, cDNA synthesis and qRTPCR), 2.18 (Cell culture), 2.21 (MTT).

2.24.2 Method

A2780 cp70 cells were plated at 3 different densities in duplicate 6-well plates; 0.5 x 10^5, 1 x 10^5, 2 x 10^5. These were then incubated overnight (~22hrs) prior to transfection. As described in Chapter 2.22.2.1, 0.4µg of DNA was used per well of plate, *SP5* and vector only transfections in were performed in parallel at each cell density. Briefly, the volume of DNA containing 0.4µg was made up to 100µl using enhancer (EC) buffer and then 3.2µl of enhancer solution was added before vortexing for 1 sec and incubating for 5 minutes at room temp. 10µl of effectene reagent was then added and mixed by pipetting 5 times and further incubated for 10 minutes.

The medium on the cells was then removed and replaced with 1600µl fresh medium. 600µl medium (normal medium for cell culture was used for this i.e. including serum) was then added to the transfection mix and mixed by pipetting twice. This solution was then immediately added, dropwise, to the appropriate well.

Twenty four hours post transfection cells were between 40-60% confluent. Each well of plate was passaged into a 100mm dish. Cells were transferred from 9.5cm^2 growth area to 55cm^2 area giving an approximate split ratio of 1/6 (between 1/5 and 1/10 is recommended). From this point onwards cells were maintained in medium containing 0.5mg/ml G418 sulphate to select for cells that had taken up the plasmids containing the resistance gene. The medium was replaced every 2 to 3 days to maintain a constant concentration of antibiotic.

Four to five days post passage some colonies were visible. 6 days after passage, cells from the highest density were too confluent to select colonies. Therefore cells were harvested for RNA to check expression on *SP5 (*see Chapter 2.14*)*. Results were encouraging

showing that *SP5* was being expressed at a high level in the expected clones and no *SP5* was detected in the vector only control.

Fourteen days after passage many individual colonies were visible in the remaining duplicate cultures. For each plate which was *SP5* transfected, five individual colonies were removed (using 1µl of trypsin and pipette tip) and placed into a single well of 24-well plate. After 5 colonies were removed from each plate (to give 10 in total labelled A-J) and the remaining cells from each plate were pooled to give pools A and B and passaged into T75 flasks. The 'vector only' cultures had fewer visible colonies so were combined to create a single vector only pool (passaged into a T75 flask). Cells were then bulked up as quickly as possible so that cells could be harvested for RNA and cyropreserved for future use (see Chapter 2.12).

2.25 Statistical methods of analysis

2.25.1 Student's t-test

The t-test assesses whether the means of two groups are significantly different from each other. This analysis is appropriate whenever you want to compare the means of two groups. In this thesis a 2 tailed t-test was used assuming that the two samples could have unequal variance.

2.25.2 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.

2.25.3 Logistic regression

Logistic regression (Mayers 1963) is used to predict the probability of occurrence of an event by fitting data to a logistic curve. It is a generalized linear model used for binomial regression. It can make use of several predictor variables that may be either numerical or categorical. In this thesis it was used for assessing the correlation between methylation of our candidate genes (used as a continous variable) with response to first line platinum-based chemotherapy in two cohorts of ovarian cancer patients. The significance level was set at $p<0.05$.

2.25.4 Cox proportional hazards model

Proportional hazards models are a class of survival models in statistics. Survival models relate the time that passes before some event occurs to one or more covariates that may be associated with that quantity. Survival models consist of two parts: the underlying hazard function, describing how the hazard (risk) changes over time at baseline levels of covariates; and the effect parameters, describing how the hazard varies in response to explanatory covariates. The proportional hazards condition (Breslow 1975) states that covariates are multiplicatively related to the hazard. Sir David Cox observed that if the proportional hazards assumption holds then it is possible to estimate the effect parameter(s) without any consideration of the hazard function. This approach to survival data is called application of the Cox proportional hazards model (Cox 1972), sometimes abbreviated to Cox model or to proportional hazards model. In this thesis it was used when assessing the effect of methylation of a candidate gene on survival, where methylation was used as a continuous variable (without using any arbitrary cut-off). The assumption of the proportional hazards was examined by fitting general linear regression model of weighted residuals of the Cox model, and test the non-zero slope of the model as previously described (Grambsch 1994). The significant level was set at $p<0.05$ (two-sided).

2.25.5 Kaplan-Meier

The Kaplan–Meier estimator is used for estimating the survival function from life-time data (Kaplan 1958). In this thesis it was used to demonstrate if the survival of two groups of patients, with high/low methylation of a candidate gene determined by median of methylation across all the patients, is significantly different.

A plot of the Kaplan–Meier estimate of the survival function is a series of horizontal steps of declining magnitude which represent the true survival function for that population. This method can take into account some types of censored data, for example, if a patient withdraws from a study, i.e. is lost from follow-up before the final outcome e.g. death is observed. On the plot, small vertical tick-marks indicate losses, where a patient's survival time has been right-censored.

In this thesis it was used for two main reasons: firstly it provides a visual representation of the data and secondly it was used to analyse the combined data set, as a means of confirming any association or lack of association with methylation of a candidate gene. Compared to the Cox regression model (Cox 1972) it has the disadvantage of needing to use an arbitrary cut-off rather than a continuous variable, to divide the groups - in this case high or low methylation, based on the median.

2.25.6 PAM

Prediction Analysis Microarrays (PAM) is a statistical technique used to identify groups of genes that discriminate best between classes. In this technique the class gene centroid is shrunken towards the overall centroid by a threshold amount, determined by cross validation, following standardisation of each gene by its within class standard deviation

(Tibshirani and Efron 2002). This technique was originally designed to interrogate gene expression microarray data and has also been used to interpret DMH data.

2.25.6.1 Pre-processing of DMH data

Since the Agilent custom-designed microarray was originally designed for detecting copy number alterations (CNA), Agilent Feature Extraction software does not provide quality control (QC) reports tailored for DNA methylation analysis. Therefore, 4 features shown in Table 23 as indicators of DMH data quality, rather than using the QC report which is generated for comparative genomic hybridisation (CGH) microarrays by Agilent Feature Extraction Software. These 4 features indicate the outliers of non-uniformity and population at background and foreground in green and red channels, respectively. The highest consistency (R^2=0.95) between duplicates was achieved using these 4 features to exclude probes not fulfilling required data quality. Probes with signal intensities over 65000 (signal saturation) were excluded from the analysis due to the signal saturation. The probes with low intensities were also not taken into consideration.

Table 23: PAM: Features for quality control in DMH assay

Features (Green)	Features (Red)	Types	Description*
gIsFeatNonUnifOL	rIsFeatNonUnifOL	Boolean	Boolean flag indicating if a features is a NonUniformity Outlier or not. A feature is non-uniform if the pixel noise of feature exceeds a threshold established for a 'uniform' feature.
gIsBGNonUniOL	rIsBGNonUniOL	Boolean	The same concept as above but for background
gIsFeatPopnOL	rIsFeatPopnOL	Boolean	Bolean flag indicating if a feature is Population Outlier or not. Probes with replicate features on a microarray are examined using population statistics. A feature is a population outlier if its signal is less than a lower threshold or exceeds an upper threshold (1.42×IQR).
gIsBGPopnOL	rIsBGPopnOL	Boolean	The same concept as above but for background

* Reference Guide of Agilent Feature Extraction Software (www.genomics.**agilent**.com/files/.../G4460-90026_FE_**Reference**.pdf)

2.25.7 SAM

Significance analysis of microarrays (SAM) is used to identify individual sequences which best discriminate between classes. SAM uses an extension of the t-statistic to assign each gene a score based on the change in hybridisation relative to the standard deviation of repeated measurements (Tusher, Tibshirani et al. 2001). SAM then uses permutations to estimate the false discovery rate (FDR) for significant genes which have a score over a certain threshold. Following the initial DMH experiment a cut off is arbitrarily chosen, between the ratios of raw signal intensities (undigested/ digested), to identify methylated loci.

This technique has also been used frequently to analyse gene expression microarray experiments and subsequently to analyse DMH data. The data is normalised assuming a normal distribution and symmetry (that only some sequences will show a change in methylation and that as many sequences will gain methylation as will lose it between groups).

2.25.8 MLDA

Methylation linear discrimination analysis is a statistical technique recently developed for analysing CpG island microarray hybridisation data (Dai, Teodoridis et al. 2008). It uses a linear regression model to identify loci which are differentially methylated e.g. between tumour pairs or isogenically matched sensitive and resistant cell lines. Three linear models are constructed. The first one is constructed from the log-transformed signal intensities of unmethylated features (mitochondrial sequences). This is used as a reference for unmethylation. Next an intermediate model is constructed using the point corresponding to the 97.5-quantiles residual below the first linear regression line. The third model is used

as a reference for methylation and is generated using features with a standardised residual of less than 2 from in the intermediate model.

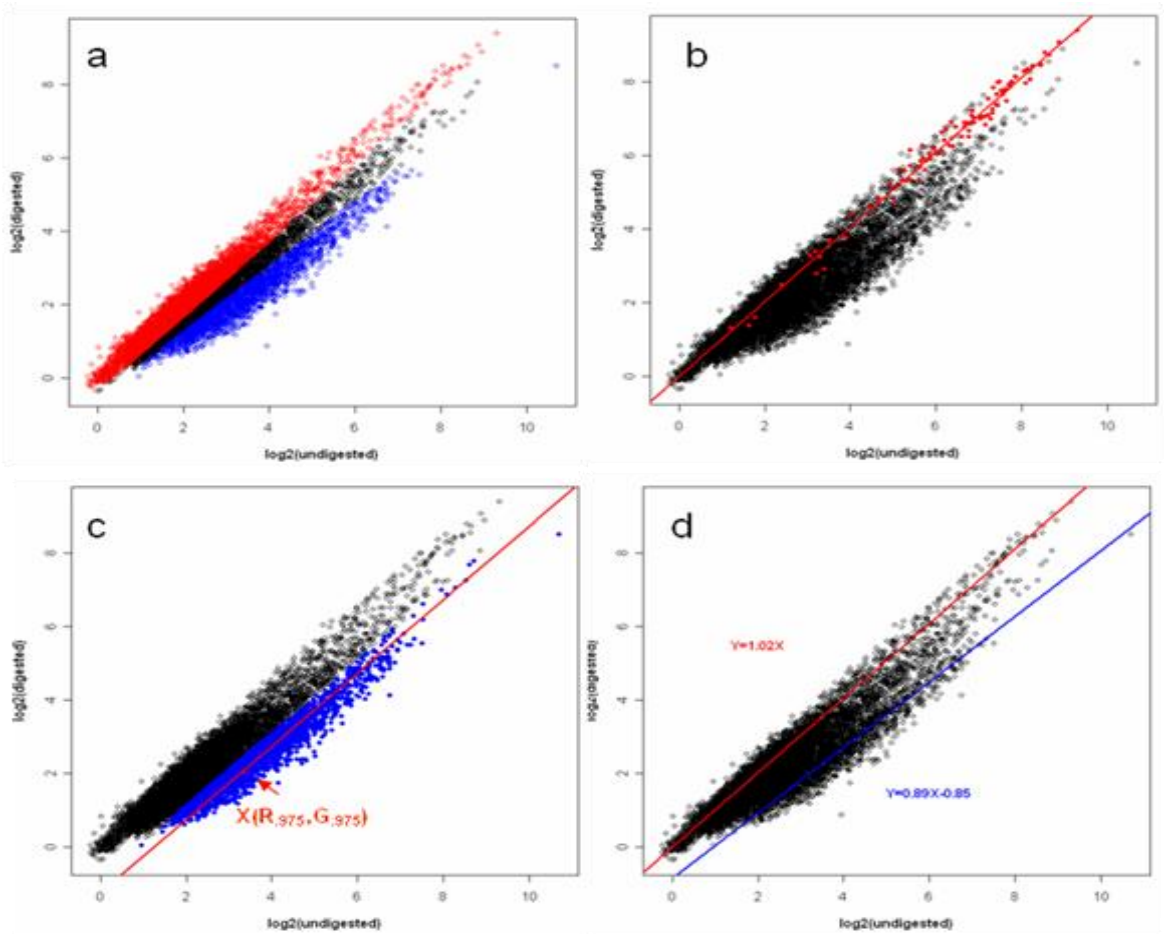


Figure 12. MLDA: An illustration of unmethylated and methylated model construction in MLDA in the A2780 cell line. a: Three patterns can be observed on the scatter plot of log-transformed Cy3 (undigested) against log-transformed Cy5 (digested) intensities. b: The unmethylated model constructed using 94 mitochondrial sequences as a unmethylation reference. c: The intermediate model constructed through the 97.5 quantile residual. The point X is the 97.5 quantile residual. The microarray probes coloured in blue (standardised residual to the intermediate model is less than 2) are selected to construct the methylated model. d: Methylated (in blue) and unmethylated (in red) models in A2780 cell line.

The log likelihood ratio of a locus being methylated is proportional to the difference between the squared standardised residual from the methylated line and that from the unmethylated line. Features consistently identified as methylated are assigned a score of 1 and those unmethylated a score of -1; the rest are assigned a weighted score corresponding to their location of the plot of log likelihood ratios. The averaged score for each locus is calculated, in for example the sensitive and resistant cell line, and plotted against each

other. The weighted scoring system used to allocate a sensitive and resistant score is illustrated in Figure 13 below.

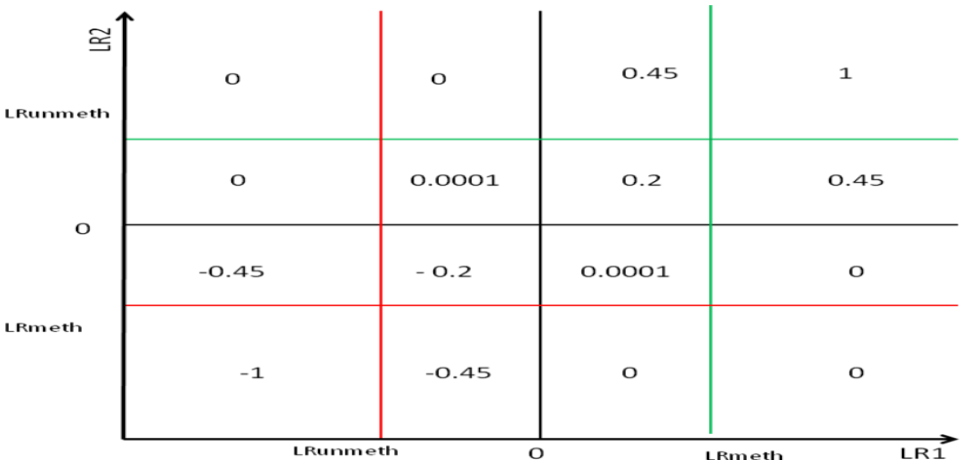


Figure 13. MLDA: Weighted scoring system. The microarray probes consistently identified as methylated candidates on dye-swap arrays were scored 1; unmethylated microarray probes were scored -1. The rest of the microarray probes were assigned a weighted score based on their location on the plot. LRmeth: log likelihood ratio cut-off for methylated loci; LRunmeth:log likelihood ratio cut-off for unmethylated loci. LR: log likelihood ratio-swapped dyes. (Dai, Teodoridis et al. 2008).

A robust regression model is then fitted to these data and assumed to follow a normal distribution so that outliers can be used to identify the differentially methylated loci between the sample classes. This is shown in Figure 14a & b below:

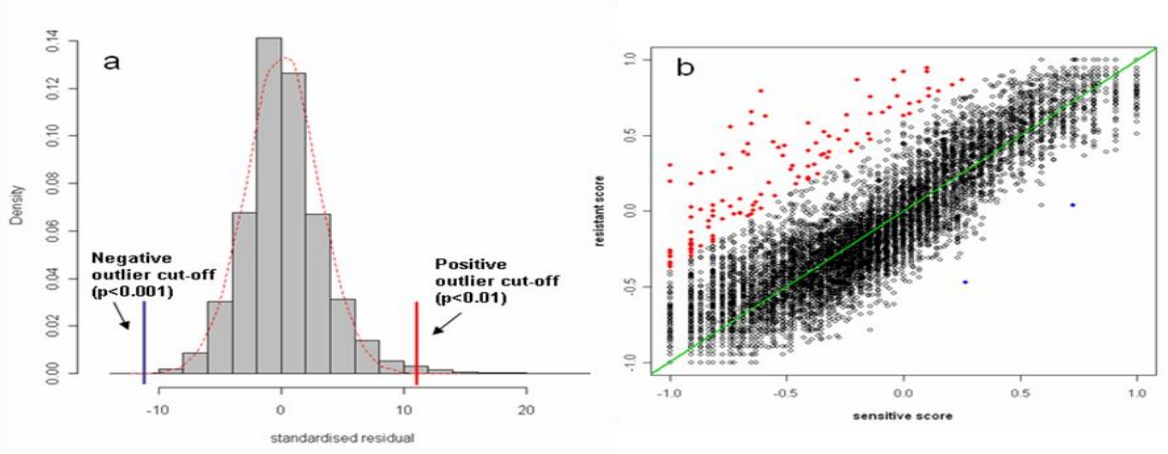


Figure 14. MLDA: Outliers identifications: a: Distribution of the observed (histogram) standardised residuals and the theoretical distribution based on the fitted model (dashed smooth line in red). The red and blue solid line are the positive and negative cut-offs, respectively. b: Scatter plot of sensitive scores against resistant scores in A2780 series cell lines. The hypermethylated loci are coloured in red and hypomethylated loci are in blue. The robust regression model is Y = 0.9956X + 0.0019. Please refer to Figure 6 of Dai et al. (Dai, Teodoridis et al. 2008)

MLDA is more comprehensively explained and illustrated in Dai et al (Dai, Teodoridis et al. 2008).

3 Characterisation of loci showing differential methylation in cisplatin resistant lines identified by PAM.

3.1 Background and aims.

The aim of the experiments described in this Chapter were to identify and validate novel DNA methylation markers for acquired drug resistance in ovarian cell lines and evaluate their relevance to acquired resistance in patient samples. In a collaboration between Dr. Tim Huang (Ohio State University, Columbus, Ohio) and Dr. Jens Teodoridis (when at the University of Glasgow), DMH was performed with hybridisation onto a 12K CGI microarray (university Health Network, Toronto, Canada) (Heisler, Torti et al. 2005). The aim was to use DMH to detect genome wide changes in CpG island (CGI) methylation in DNA from 34 well characterised, matched ovarian cancer cell line models (see chapter 2.17.4) and identify DNA sequences whose methylation status discriminated between cisplatin-sensitive and cisplatin-resistant ovarian surface epithelial cancer cell lines.

DMH and initial data analysis was performed by Dr Teodoridis and Ms Wei Dai and they performed all normalisation of the data and compiled lists used to identify potential candidate genes. This is now briefly summarised.

Once the hybridisation had been performed signal intensities were corrected for background intensity. Weak (equal or less than the average plus two standard deviations of background signals) and frequently missing signals from each sample data set were removed and then PAM (Tibshirani and Efron 2002) was applied to the microarray data using PAM 2.0 for Excel. For further details see PAM in methods section (Chapter 2.25.4). In PAM results are obtained by gradually removing sequences which are creating noise by

increasing a threshold which is estimated by cross validation (Tibshirani and Efron 2002) (also see chapter 2.24.4).

In this experiment the following filters were used in order to achieve a manageable number of sequences; class probabilities for resistant (0.66) and resistant (0.34). For a more detailed description of these please refer to Tibshirani and Efron 2002. By changing these filters smaller or much larger lists could be generated but it was considered that these criteria, which detected 41 sequences as differentially methylated, would provide a manageable number of loci from which to identify candidates for further individual characterisation.

Of the 41 sequences identified, 13 had a CGI which overlapped the first exon/ promoter. A CGI was defined as a stretch of DNA of at least 200bp long with at least 50% GC content (http://data.microarrays.ca/cpg/faq.htm). The 13 sequences mapped to 12 genes. This is shown in table 24 below. All 41 sequences are shown and then black type is used to highlight those which were 5' and contained a CGI. Grey highlighting function used to further show the loci that were selected for further individual characterisation.

Table 24. CGI and corresponding genes identified from DMH and PAM

	Microarray Identifier	Chromosomal location	CpG Island	5'	Gene
1	66_G_6	9q22.32	Yes	No	-
2	46_A_12	8p11.21	No	No	-
3	80_H_5	NBA			
4	**119_A_6**	**6q21**	**Yes**	**Yes**	***NR2E1***
5	47_A_12	12q14.1	No	No	-
6	**81_B_1**	**5q33.3**	**Yes**	**Yes**	***PTTG1***
7	17_G_11	14q23.1	Yes	No	*SIX1*
8	**114_E_4**	**2q14.3**	**Yes**	**Yes**	***CNTNAP5***
9	**127_F_12**	**8p22**	**Yes**	**Yes**	***DLC1***
10	42_D_9	NBA			
11	**5_D_4**	**1q23.3**	**Yes**	**Yes**	***LMX1A***
12	20_A_1	9q32	No	No	*ZFP37*
13	41_D_9	12q13.12	Yes	Yes	*WNT1*
14	47_D_8	4p11	No	No	-
15	109_A_6	11q12.3	Yes	No	*AHNAK*
16	**64_E_3**	**19q32.2**	**Yes**	**Yes**	***CR2***
17	113_E_10	18q11.2	No	No	-
18	**6_D_4**	**1q23.3**	**Yes**	**Yes**	***LMX1A***
19	17_H_9	11q13.1	Yes	Yes	*HRASLS3*
20	35_A_11	12q24.13	Yes	No	-
21	23_A_5	> one CL			
22	49_E_1	1q12	No	No	-
23	109_B_5	NBA			
24	39_E_1	16p13.3	No	No	*C1QTNF8*
25	3_A_11	17q12	Yes	Yes	*MLLT6*
26	70_B_3	5q23.2	No	No	
27	40_E_1	16p13.3	No	No	*C1QTNF8*
28	50_D_5	3q22.1	Yes	No	*AMOTL2*
29	14_E_8	NBA			
30	27_F_7	NBA			
31	6_G_10	21q22.3	No	No	*RIPK4*
32	17_H_6	6q23.3	Yes	Yes	*TNFAIP3*
33	50_F_1	NBA			
34	69_B_5	20p12.2	Yes	Yes	*JAG1*
35	104_G_6	1q21.3	Yes	Yes	*THEM4*
36	36_H_5	19q13.42	No	No	
37	100_G_11	NBA			
38	7_D_1	20q13.12	No	No	
39	121_H_10	2q21.2	No	Yes	
40	47_E_5	NBA			
41	100_G_12	NBA			

NBA no blat alignment, > one CL more than one chromosomal location 1http://data.microarrays.ca/cpg/searchsingleclones.htm. Black type used to highlight sequences which were 5' and mapped to a CGI. Bold and grey highlight used to show sequences which were selected for further validation.

3.2 Methylation of candididate loci in epithelial ovarian cancer cells lines by MSP and pyrosequencing.

The aim of this experiment was to confirm in the laboratory that the candidate loci arising from the PAM analysis did indeed show increased methylation in the resistant ovarian cancer cell lines. Initially MSP was employed as a rapid method of characterising multiple loci. As described in Chapter 2.9 MSP is a highly sensitive method for detecting methylation (Herman, Graff et al. 1996) and using serial dilutions of the positive and negative controls semi quantitative results can be obtained. For MSP and pyrosequencing all DNA was bisulphite modified as described in chapter 2.7 and a PCR was performed using Calponin primers to check for complete modification of DNA (Rand, Qu et al. 2002).

For MSP semi-quantitative results were obtained using 1/10 and 1/20 dilutions of IVM: N and only including samples with a band of at least the same intensity as the 1/10 dilution. IVM is *in vitro* methylated DNA and N is normal whole male genomic DNA and represents the background level of methylation normal tissues. Care was taken that no band was seen in the negative control or water samples and that all samples had been completely modified prior to starting. No more than 35 cycles of PCR were used to minimise the chance of amplifying contaminant DNA. Each experiment was performed 3 times and the average of the experiments was used. In all figures a 100 bp ladder is shown. The same 34 ovarian cancer cell lines which had been used to prepare the DMH were used to investigate the methylation state of candidate loci in the laboratory (see Figure in chapter 2.17.4).

Any loci which showed increased methylation by MSP were next examined by pyrosequencing (of bisulphite modified DNA). For pyrosequencing experiments IVM and

N was again used as positive and negative controls respectively. In all pyrosequencing experiments the percentage of CpG methylation in human male genomic DNA (negative control) was used to normalise each result to the background level of methylation seen and eliminated any "noise".

Results were very reproducible between different experiments and bisulphite modifications and this is illustrated in Figure 15 below, where the values obtained for N in 6 independent experiments are shown. Here, using *SIX1* gene methylation as an example, 3 independent bisulphite modification of N are shown. (Experiments 1, 2 and 3 were performed using one sample; experiment 4 using a second and experiments 5 and 6 using a third).

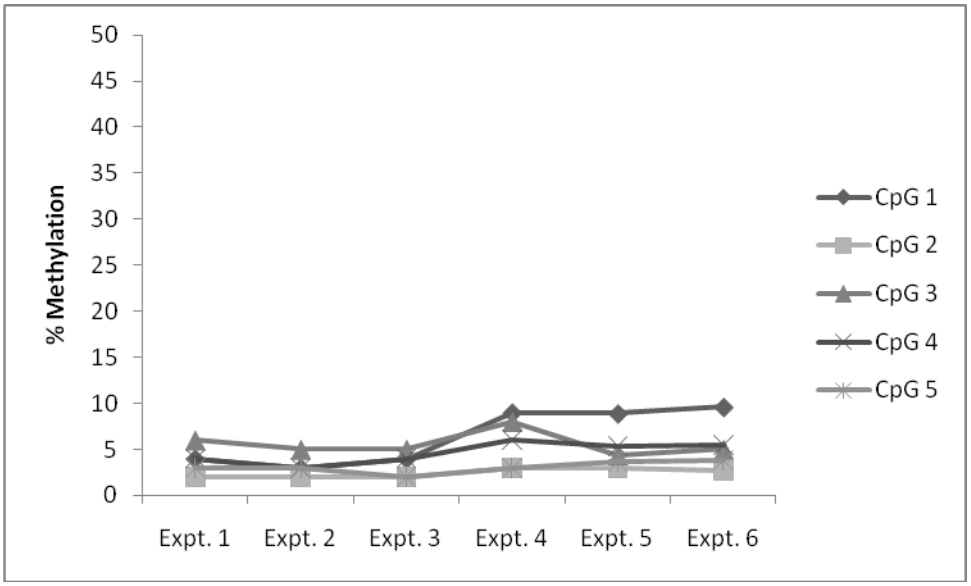


Figure 15. Variation in methylation of 5 adjacent CpGs, in *SIX1* gene, in 6 experiments using human male genomic DNA (negative control) by pyrosequencing demonstrating reproducibility between experiments.

3.2.1 Examination of candidate loci in cell lines by MSP

Seven sequences mapping to six genes (*LMX1A, NR2E1, CR2, CNTNAP, DLC1, PTTG*) were chosen at random from this list to be further characterised. Five of the genes were expected to show an increase in methylation in the resistant cell lines and one gene (*PTTG*) was expected to show the opposite. Methylated and unmethylated primers were designed to examine the methylation status of these genes in the bisulphite modified A2780 sensitive and resistant cell lines (see Figure Chapter 2.17.4). *PTTG* had a negative score by PAM and was therefore expected to show less methylation in the resistant cell lines and was therefore added to the five loci that were chosen to be characterised as a check of the data. We would expect it to show a decrease in methylation in the resistant cell lines although it actually showed no differential methylation. The locus corresponding to *CR2* did not show an increase in methylation in the resistant cell lines by MSP and it was not possible to optimise primers to investigate *CNTNAP*.

NR2E1, *LMX1A* and *DLC1* showed increased methylation in the resistant ovarian cancer cell lines by MSP and were therefore also examined by pyrosequencing of the bisulphite modified DNA. The results for these candidate loci are now discussed in more detail.

3.2.2 Methylation of DLC1 by MSP and pyrosequencing

Methylation of *DLC1* was first examined by MSP. A degree of methylation was observed in the A2780 sensitive cell lines with a further increase in methylation in the A2780 multistep resistant cell lines (especially MCP1, 3, 4, 6, 7 and 9). No methylation was seen in PEA1 and 2 or indeed any of the other non-A2780 based cell line pairs. The results are shown for the methylated *DLC1* primer in Figure 16 below:

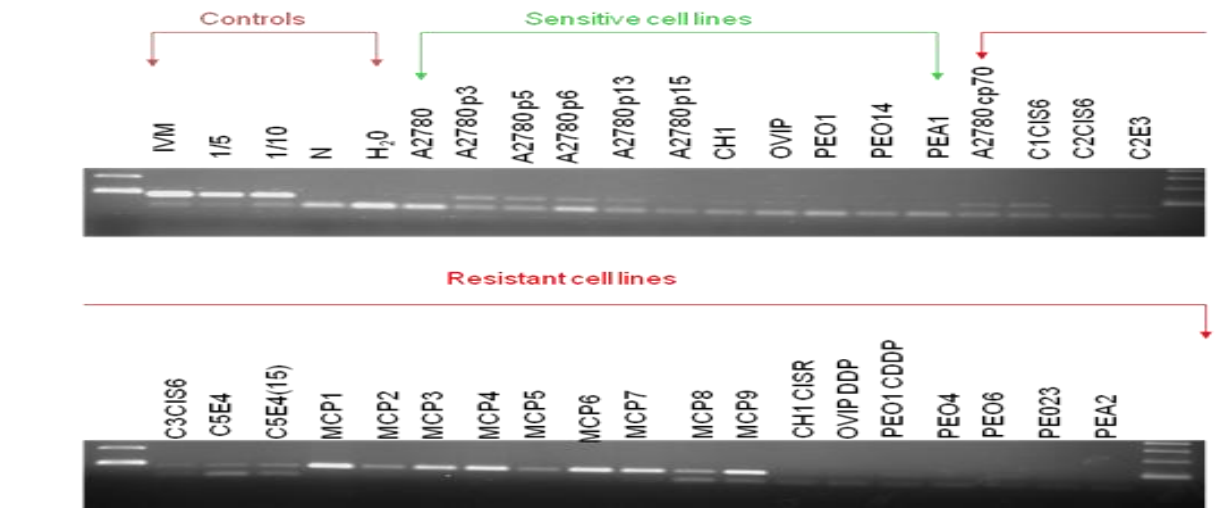


Figure 16. Methylation of *DLC1* by MSP. 100 BP ladder extreme right and left of gel. IVM invitromethylated DNA (positive control), 1/5 dilution of IVM :N, 1/10 dilution of IVM/N, N is whole male genomic DNA, H2O water control. Upper band is PCR product, lower band is primer dimer. Sensitive cell lines in green, resistant cell lines in red (see Chapter 2.6.4 for primer details). Summary of sensitive and resistant cell lines shown in chapter 2.17.4) p15 should read p14.

By pyrosequencing, the background methylation was found to be 4.6% and a statistically significant difference was seen in the level of CpG methylation in the sensitive A2780 cell lines (42.7%) and in the resistant cell lines (A2780cp70 and MCP1-9) (60.2%) (student t test $9.14x10^{-9}$). As noted by MSP minimal methylation was seen in the other ovarian cancer cell line pairs. The results are shown in Figure 17 below:

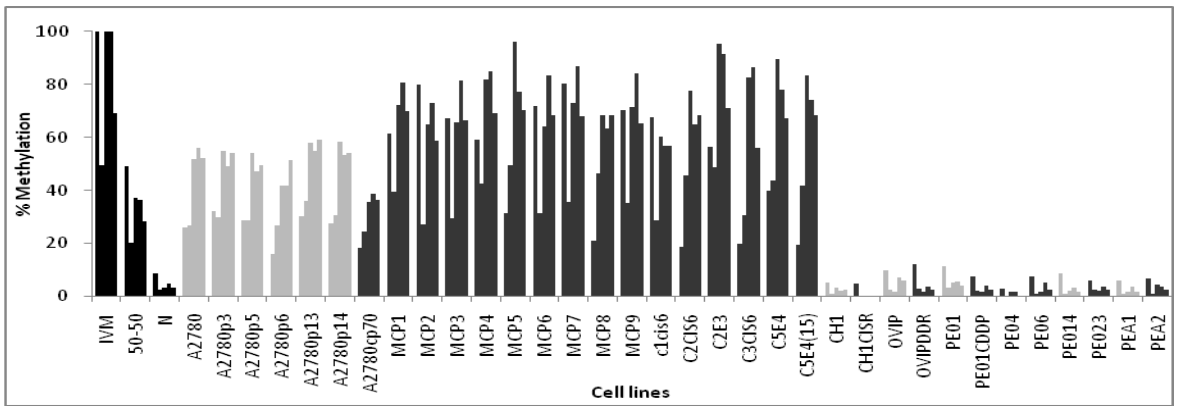


Figure 17. Methylation of *DLC1* in the 34 cell line panel by pyrosequencing. Primers designed across 5 adjacent CpG residues and each bar represents an individual CpG. Sensitive cell lines shown in light grey with resistant cell lines immediately to the right in dark grey. Controls in black are IVM (invitromethylated DNA positive control), a 50:50 mix of IVM and N, and N (indication of background methylation in normal tissues) (see chapter 2.8.4 for primer details).

The pyrosequencing results therefore showed the same trends as the MSP but with the added quantitative information.

3.2.3 Methylation of NR2E1 by MSP and pyrosequencing

The CGI at *NR2E1* showed a clear differentiation between the sensitive and resistant A2780 based cell lines by MSP with all of the sensitive cell lines showing virtually no methylation. Some methylation was observed in OVIP (sensitive) and this was increased in OVIP DDP (resistant). A small amount of methylation was seen in CH1 (sensitive) which again was marginally increased in CH1CISR (resistant). PEO1 (sensitive) showed some methylation which again was more in the resistant cell lines (PEO1 CDDP, PEO4, PEO6). These results are illustrated below in Figure 18.

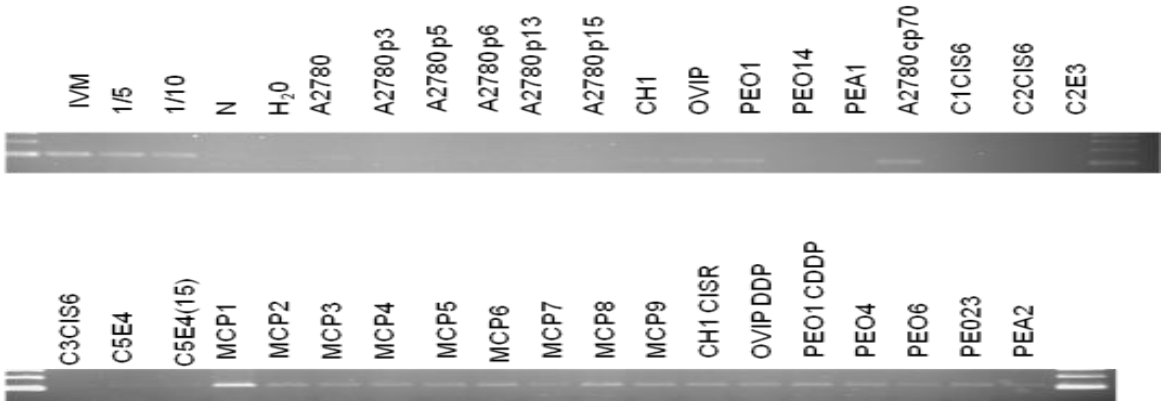


Figure 18. Methylation of *NR2E1* in the 34 cell line panel by MSP. 100 BP ladder extreme right and left of gel. IVM invitromethylated DNA (positive control), 1/5 dilution of IVM :N, 1/10 dilution of IVM/N, N is whole male genomic DNA, H_2O water control. Sensitive cell lines all shown first then resistant cell lines starting at A2780 CP70. Summary of sensitive and resistant cell lines chapter 2.17.4. For primer details see Chapter 2.6.4.

These results were supported by pyrosequencing, where the level of CpG methylation in whole male genomic DNA was 1.0% with 1.4% methylation in the sensitive A2780 cell lines and 50.9% methylation in the resistant A2780 based cell lines (A2780 cp70 and MCP 1-9). The differential methylation was shown to be statistically significant for the A2780 based comparison (student t test $p=1.82x10^{-29}$). Similarly to the MSP, a small increase in methylation was seen between OVIP (sensitive) and OVIP DDP (resistant) and this difference was statistically significant (student t test p=0.03). The pyrosequencing results are shown in Figure 19 below:

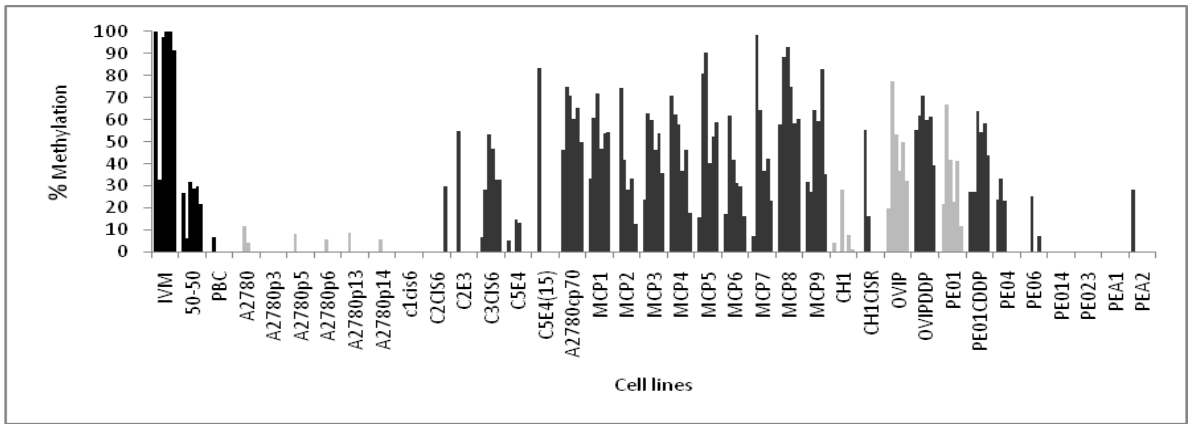


Figure 19. Methylation of *NR2E1* in the 34 cell line panel, by pyrosequencing. Primers designed across 6 adjacent CpG residues. Sensitive cell lines shown in light grey. Corresponding resistant cell lines shown to the right in dark grey. Controls in black. Primer details as outlined in Chapter 2.8.4

MSP and pyrosequencing were shown to demonstrate the same trend of results with the pyrosequencing providing additional quantitative information. Both methods demonstrated the clearest increase in methylation seen in the A2780 derived cell lines but a small increase between CH1 and CH1CISR and PEA1 and PEA2. As previously a smaller degree of methylation was seen the single step A2780 resistant clones (C1CISR to C5E415) compared to the multistep clones (MCPs). This would was as predicted as the multistep clones were treated with repeated cisplatin treatment and changes associated with resistance should therefore be more pronounced.

3.2.4 Methylation of LMX1A by MSP and pyrosequencing

Next the CGI within *LMX1A* was examined by MSP. As shown in figure 20 some methylation was seen in the A2780 sensitive cell lines although this was deemed to be more consistently detected in the platinum resistant derivatives. A higher level of methylation was seen in CH1 (sensitive) than its corresponding resistant pair CH1CISR, and the same level of methylation was seen for OVIP (sensitive) and OVIP DDP (resistant), and PEO1 (sensitive) and PEO1CDDP (resistant). An increase in methylation was seen from PEO1 (sensitive) to PEO4 (resistant) but not the other *in vivo* derived cell line in this pair; PEO6. A decrease in methylation was seen from PEA1 (sensitive) to PEA2 (resistant).

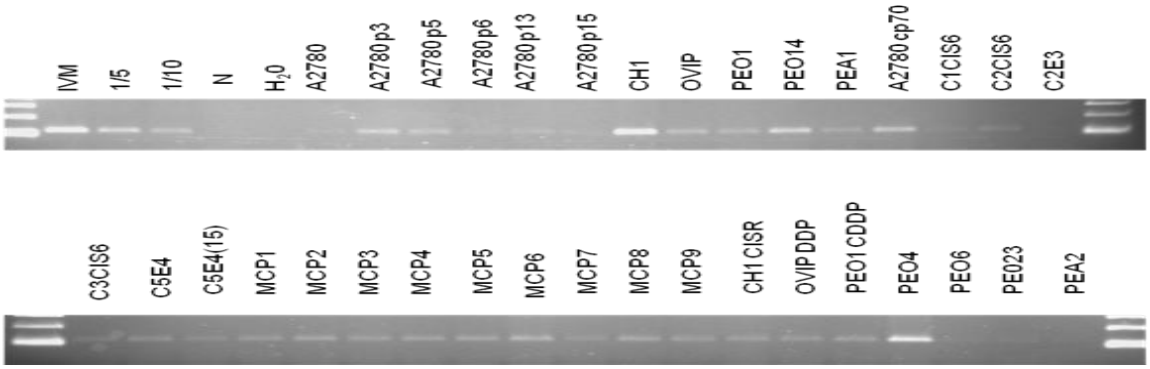


Figure 20. Methylation of *LMX1A* by MSP. 100 BP ladder extreme right and left of gel. IVM invitromethylated DNA (positive control), 1/5 dilution of IVM :N, 1/10 dilution of IVM/N, N is whole male genomic DNA, H2O water control. Sensitive cell lines all shown first then resistant cell lines starting at A2780 CP70. Summary of sensitive and resistant cell lines chapter 2.17.4. For primer details see Chapter 2.6.4

By pyrosequencing the background level of methylation was much higher than had been seen for the previous two genes at 17.3%. The differential methylation seen between the sensitive and resistant A2780 based cell lines was reproduced by pyrosequencing with a statistically significant increase in methylation seen in the resistant lines (21.7% vs. 45.3%), (student t test $p=1.36x10^{-10}$). The increase seen between PEO1 and PEO4 which had been observed by MSP was not confirmed by pyrosequencing and none of the non-

A2780 based comparisons were statistically significant. The results are shown in Figure 21 below.

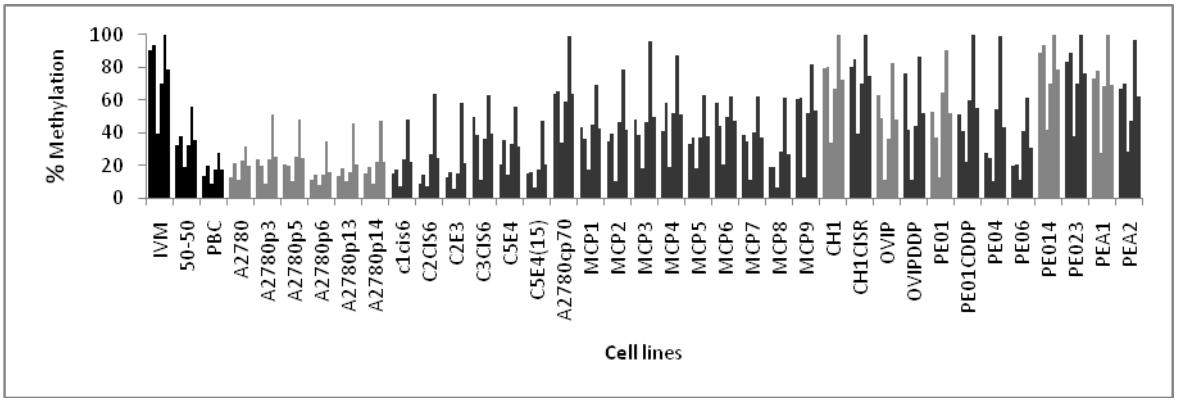


Figure 21. Methylation of *LMX1A* in 34 cell line panel by pyrosequencing. Primers designed across 6 adjacent CpG residues. Sensitive cell lines shown in light grey. Corresponding resistant cell lines shown to the right in dark grey. Controls in black. See chapter 2.8.4 for primer details.

These loci were identified by comparing 34 cell lines by PAM. The A2780 sensitive and resistant cell lines dominated this comparison and it could therefore be predicted that differential methylation would be most pronounced there. For *DLC1* the differential methylation was essentially confined to the A2780 sensitive vs. MCP comparison. *NR2E1* showed increased methylation in this comparison but also in some of the other cell line pairs such as CH1/ CH1CISR and PEA1/ PEA2.

DLC1, NR2E1 and *LMXIA* were next examined in primary EOC tumours in order to assess the frequency and heterogeneity of methylation in primary tumours. If methylation of these genes was important in the *in vivo* setting then one would predict that methylation would be seen in a proportion of the primary tumours.

3.3 Examination of candidate loci in primary ovarian cancer tumours

The aim of this experiment was to take the loci identified from the first DMH experiment in cell lines and assess whether significant methylation was observed in patient samples. It is extremely difficult to obtain samples from patients at the time of resistant disease and therefore tumours from patients at diagnosis were used. A large number of these primary tumours were available as described in Chapter 2.4.3 and 2.4.4. We selected patients with advanced stage 3 and 4 disease and excluded clear cell and mucinous pathology. As survival data was available for many of these patients any correlation with survival could also be sought, in order to see if any of these loci could be utilised as a prognostic biomarker. There is a limitation to this approach as it is possible that a gene important in acquired drug resistance might only show increased methylation in the resistant or post treatment sample – and not in the primary tumour.

DNA from all of the tumours were bisulphite modified as previously described in Chapter 2.5 and a calponin PCR used to check for adequate modification of all samples (Chapter 2.5.3). Since the patient samples are highly precious and it was hypothesised that if no significant methylation was seen in the first twenty samples it would be unlikely to be detected in the second set, and so the test set was subdivided into two. Initially a smaller test panel of 22-23 tumours were examined and if any samples showed ≥50% methylation by pyrosequencing this was confirmed in a larger set of 54 tumours (test set (ii)). Test set (I) and (ii) were then analysed to see if there was any correlation with PFS, OS and/ or response in a univariate analysis. If a correlation with survival was found then the results were confirmed in an independent validation set of 67 tumours. If the univariate association between the gene and response was confirmed then a multivariate analysis integrating stage and grade was performed on the combined data sets.

3.3.1 CpG methylation of DLC1 by pyrosequencing

DLC1 had shown increased methylation in the resistant cell lines and it was first examined in the smaller test set of primary tumours (known as test set (I)). As shown in Figure 22 below no samples showed methylation of ≥50% (the level of methylation likely to be required to result in gene silencing) - and in fact none showed methylation of greater than 20%.

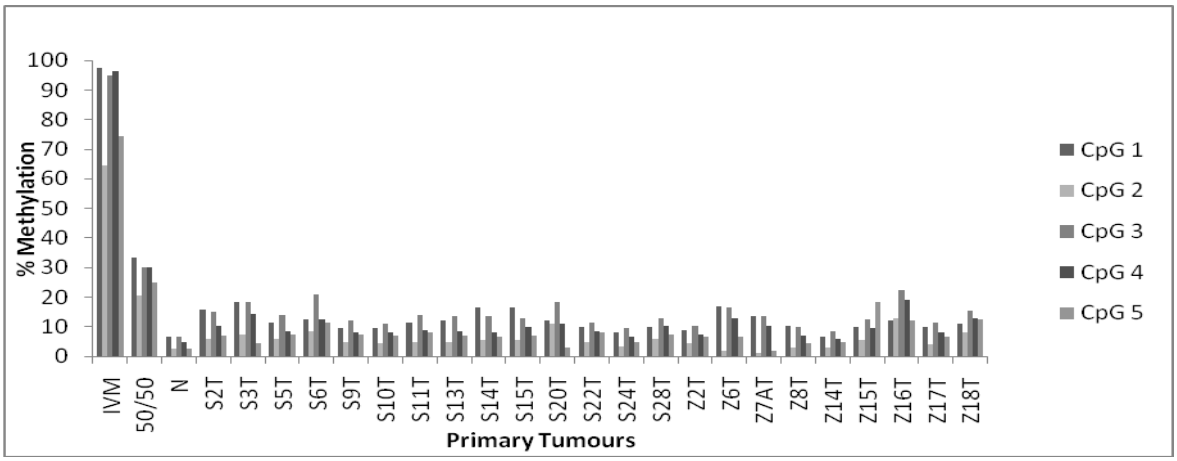


Figure 22. Methylation of *DLC1* in panel (I) of 23 primary tumours by pyrosequencing. IVM invitromethylated DNA (positive control), 50:50 a 50% mix of IVM and N, N whole male genomic DNA (background control). Pyrosequencing primers designed to analyse methylation status of 5 adjacent CpG residues (each shown in a different colour).

These results suggest that methylation of *DLC1* in primary EOC specimens is infrequent and hence a rare event in primary ovarian cancer. Further examination of a larger panel of primary tumours was therefore not pursued as its low level of methylation in primary tumours might reduce its potential usefulness as a prognostic biomarker. However, methylation of *DLC1* could be selected for during chemotherapy and only chemonaive tumours were examined and therefore methylation of *DLC1* was also examined in matched pairs of pre and post residual disease tumours (see chapter 3.4).

3.3.2 Methylation of LMX1A (5D4) and NR2E1 (119A6) in primary tumours by MSP

Methylation of the two CGIs associated with *LMX1A* (5D4) and *NR2E1* (119A6) had previously been examined in a similar panel of DNA from primary tumours, using MSP, by Dr Catriona Hardie, and these results are shown below.

The methylation status of these two sequences were analysed using MSP in DNA from 199 primary epithelial ovarian tumours which included 16 early stage (I/II) and 183 late stage (III/IV). The tumours analysed comprised 125 samples from the prospective DNA methylation study, and 74 samples from the retrospective study, as described in Chapter 2.4. *NR2E1* and *LMX1A* were methylated in 12.6% (25/199) and 61.8% (123/199) of the ovarian tumour samples respectively as shown below in Table 24. Methylation frequencies for *NR2E1* and *LMX1A* were higher for early versus late stage disease with *NR2E1* being methylated in 18.8% (3/16) versus 12% (22/183) and *LMX1A* being methylated in 75% (12/16) versus 60% (111/183) respectively. No correlation with PFS, OS or response to chemotherapy for either gene was observed.

Table 25 Methylation frequencies of identified sequences in epithelial ovarian tumours. Methylation frequency % (number methylated/total number of samples).

	Methylation frequency of identified sequences	
Samples (N)	***NR2E1 (119A6)***	***LMX1A (5D4)***
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)
Overall ovarian tumours (199)	12.6 (25/199)	61.8 (123/199)

3.4 Examination of candidate loci in matched tumour pairs from patients at diagnosis and time of surgery for residual disease

It is possible that a locus that could be a useful biomarker for acquired drug resistance might not show methylation in primary tumours. Often this 'methylation mark' is thought to be selected for during the course of chemotherapy and therefore unless one investigates tumours from the time of residual disease or relapse a potentially useful candidate locus could be missed. However matched pre- and post- chemotherapy samples are a much more scarce resource and this limits their use. The expectation when using samples taken at the time of surgery for residual disease is that any cancer left behind following chemotherapy must have been resistant to the treatment that was administered. It should be noted that detailed clinical data was not available for the matched pairs and it is therefore not possible to distinguish whether these represented platinum sensitive or resistant relapse.

The aim of the next experiment was to address this question by taking the loci that had shown increased methylation in the *in vitro* generated cisplatin resistant cell lines and investigating whether an increase in methylation was also seen between samples from patients at diagnosis and when they had surgery for residual disease following a course of adjuvant chemotherapy; the assumption being that disease still present following chemotherapy is by definition more resistant. As outlined, in Chapter 2.4.1, 10 of the 12 pairs of ovarian surface epithelial tumours were analysed (chapter 2.4.1). These patients had had a biopsy taken at the time of primary surgery and then a second one taken following a course of adjuvant chemotherapy.

3.4.1 CpG methylation of DLC1 by pyrosequencing

The percentage of background methylation was 3.5% and the average methylation in the pre-treatment samples was 4.97% (range 3.5-6%) and post- treatment samples was 4.85% (range 3.8-9.1). *DLC1* was therefore not shown to be significantly methylated in any of the pre-treatment 'sensitive' or post-treatment 'resistant' pairs. This is shown in Figure 24 below:

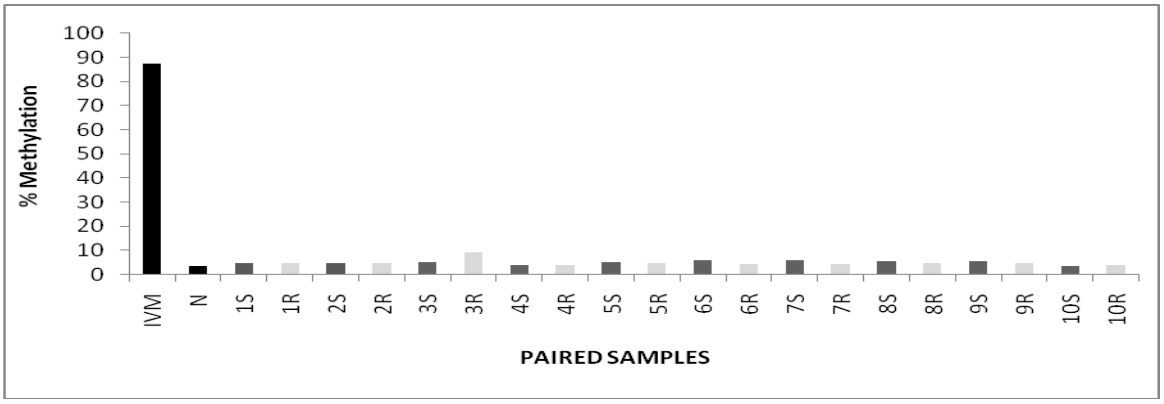


Figure 24. Methylation of *DLC1* in matched pairs by pyrosequencing. Controls in black; IVM invitromethylated DNA, N normal male genomic DNA; Dark grey is pre treatment samples, Light grey is sample following chemotherapy at time of debulking surgery.

3.4.2 NR2E1 and LMX1A by pyrosequencing

NR2E1 and *LMX1A* methylation were also examined by pyrosequencing. For *NR2E1* (Figure 24) and *LMX1A* (Figure 25), there was a background level of 3% and 10.3% respectively (as before, the percentage methylation in normal male genomic DNA). *NR2E1* 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 24 below. *LMX1A* 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 25. These results were not affected by the subsequent genotyping studies which excluded pairs 7 and 12 (which had been shown not to be pairs from the same patients). Histological analysis showed that the percentage of

tumour cells in all paired samples were almost equal which would indicate that the obvious increase in methylation seen in *NR2E1* post-chemotherapy was not due to a quantitative increase in tumour cells following treatment or simply enrichment of tumour cells after chemotherapy.

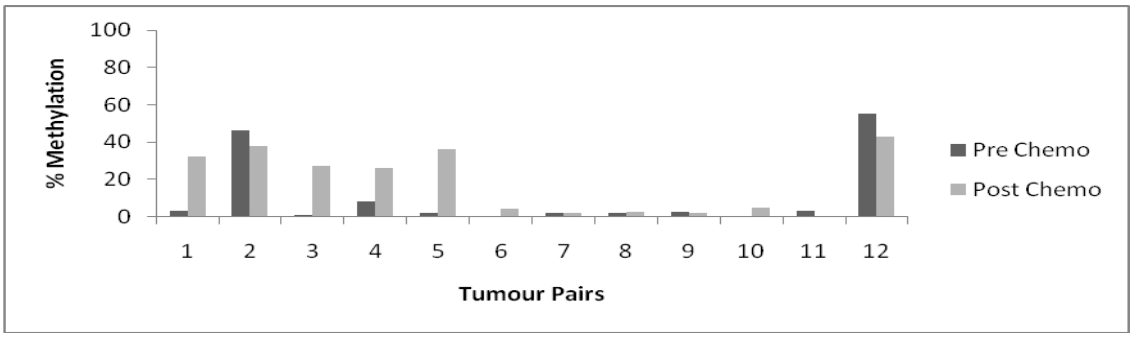


Figure 25. Pyrosequencing analysis of *NR2E1* in 12 paired pre-chemotherapy samples (dark grey) and residual disease post-chemotherapy samples (light grey). Average CpG methylation over 7 sites for each matched pair. Background methylation in DNA from whole male blood of 3%. Pairs 7 and 12 later removed after genotyping studies.

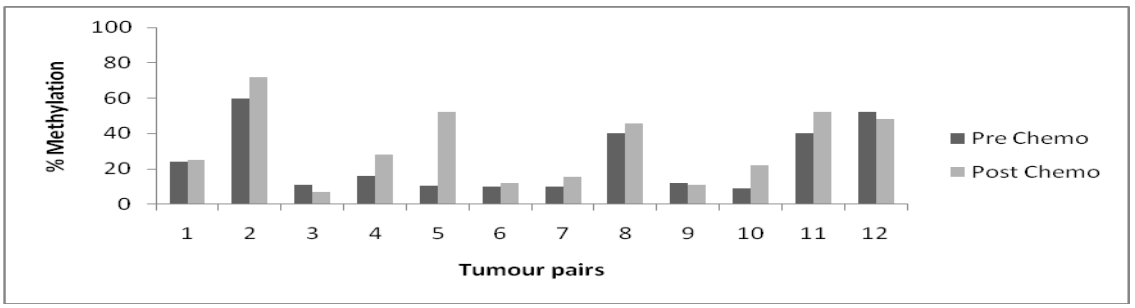


Figure 25. Pyrosequencing analysis of *LMX1A* in 12 paired pre-chemotherapy (dark grey) and residual disease following chemotherapy (light grey). Average CpG methylation over 5 sites for each matched pair. Background methylation in DNA from whole male blood of 10.3%. Pairs 7 and 12 later removed after genotyping studies.

In summary, these findings suggest that *NR2E1* could be a potential biomarker of acquired drug resistance in ovarian cancer, given the increase in methylation in some of the matched tumour pairs as well as the cell lines, however this would require prospective validation. *LMX1A* is less likely to be a useful clinical marker given the higher background level of methylation in the sensitive cell lines; however the high level of methylation seen in the primary tumours is very interesting and it was felt both loci warranted further investigation. The first step was to investigate whether the change in methylation at the

promoter of these genes correlated with a decrease in mRNA expression and this work was carried out in collaboration with Dr Hardie and Dr Teodoridis.

3.5 Correlation between *LMX1A* and *NR2E1* promoter methylation and mRNA expression

The mRNA expression levels of *LMX1A* and *NR2E1* were quantified in A2780 and A2780p6, vs. MCP1, MCP6 and MCP9 using qRTPCR (see Chapter *2.14*). In order to assess whether the change in expression was associated with a change in methylation in cell lines were treated with decitabine and it was hypothesized that this would result in re expression of the silenced genes. This was indeed the case although again the changes seen in *LMX1A* were less marked. This fits with the methylation pattern that was seen where *LMX1A* was more methylated in the sensitive cell lines than *NR2E1*. The results are shown in Figure 26 below:

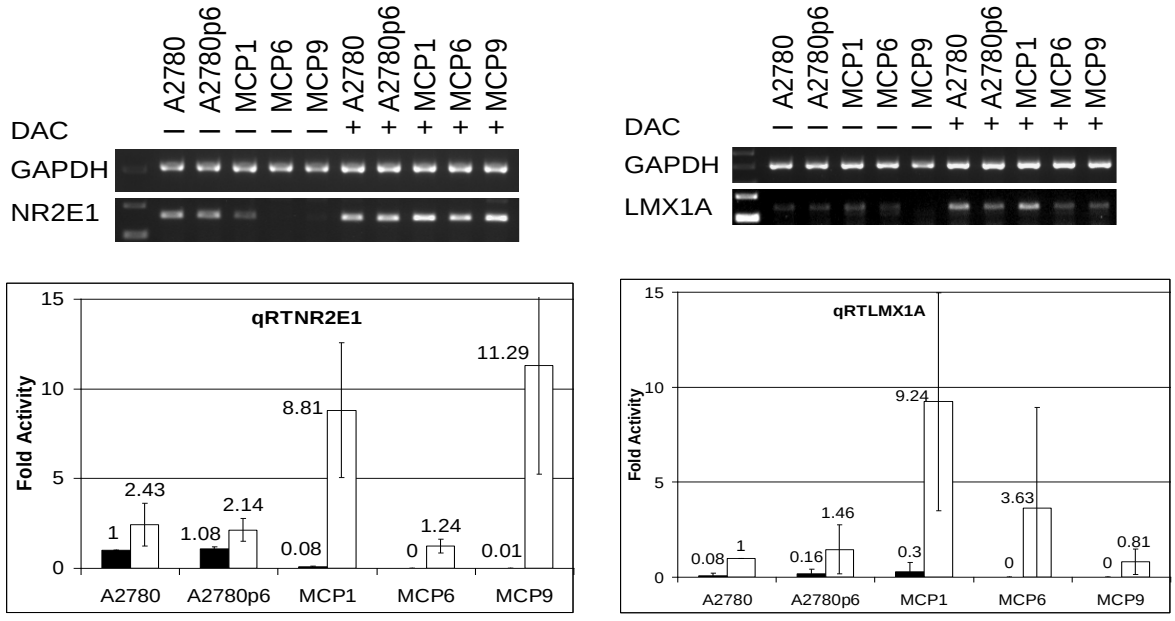


Figure 26. qRTPCR values for *NR2E1* and *LMX1A*. *GAPDH* qRTPCR was used for normalisation and values are from 3 independent experiments. Values represent the mean of three replicates in triplicate experiment ± 1 standard deviation. Filled bars: untreated cells, open bars: DAC-treated cells. (DAC = Decitabine) (please note Fold activity in Y axis should read relative expression but this figure was compiled by Dr Hardie and raw data is no longer available).

3.6 **Discussion**

Two genes from this analysis, *LMX1A* and *NR2E1*, appear to be interesting candidates for future research in ovarian cancer. Neither has been reported to have a role in ovarian cancer previously. *LMX1A* is located on chromosome 1q22-q23. It acts as a transcriptional activator and is required for development of the roof plate and subsequently for specification of dorsal cell fates in the CNS and developing vertebrae. It is highly preserved amongst species, from humans to zebrafish (www.genecards.org/cgi-bin/carddisp.pl?gene=*LMX1A*).

Liu and colleagues observed a potential link between *LMX1A* methylation and cancer last year, reporting its role as a metastasis suppressor in cervical cancer (Liu, Chao et al. 2009). They had previously identified that it was methylated in 89.9% of squamous cell carcinomas of the cervix compared to 6.7% in normal cervix and they therefore planned a study of the functional implications of this methylation. The authors found that over expressing *LMX1A* significantly reduced colony formation in two cell lines and that this also corresponded with a less invasive phenotype using a matrigel invasion assay. They were able to reverse both these phenotypes using *LMX1A* shRNA. In addition transcription factors known to be associated with EMT were assessed by RTPCR following over expression and knockdown. Both *LMX1A* transfectants exhibited up-regulation of the epithelial marker CDH1 and down regulation of the mesenchymal markers CDH2 and vimentin. RNA interference of *LMX1A* transfectants reversed the expression of CDH2 in HeLa cells and vimentin in CaSki cells. RNA interference of *LMX1A* significantly increased the expression of transcription factors known to be involved in EMT – *SNAIL*, *SLUG* and *TWIST*. It was therefore concluded that *LMX1A* mediated cancer invasion through EMT related events (Liu, Chao et al. 2009).

In addition the authors had found that *LMX1A* inhibited tumour growth and metastases in a xenograft model and that although *LMX1A* was epigenetically silenced in invasive cancers it was expressed in precancerous cervical lesions. These findings would fit with our finding that *LMX1A* is highly methylated in primary tumours and more methylated in post-chemotherapy samples and resistant cell lines and that the increase in methylation correlates with a decrease in expression at the mRNA level. It is clearly important therefore to investigate the functional role of methylation in ovarian cancer and the techniques used by Liu and colleagues could be applied to an ovarian cancer model system.

In the chick developing spinal cord *LMX1A* has been shown to induce expression of *WNT1* (Chizhikov and Millen 2004). The WNT pathway has been implicated in numerous tumour types including brain (Ellison, Dalton et al.; Lindsey, Hill et al.; Clifford, Lusher et al. 2006), colon (You, Bryant et al. 2007; Hope, Planutis et al. 2008; Najdi, Syed et al. 2009; Scholtka, Schneider et al. 2009), and endometrial cancers (Ellis and Ghaem-Maghami 2011) and recently our group has uncovered methylation of multiple promoter CGIs of *WNT* pathway genes is associated with PFS of ovarian cancer patients (Dai, Teodoridis et al. 2010). Another recent paper has identified *LMX1A* as having a role in the differentiation of human embryonic stem cells into midbrain dopamine neurons in culture and after transplantation into a Parkinson's disease model (Cai, Donaldson et al. 2009) and this highlights another potentially very interesting area of future research.

Given that *LMX1A* is known to be involved in developmental processes and that this has been shown to be epigenetically regulated this raises the question as to whether epigenetic regulation of *LMX1A* could be important in ovarian cancer initiating cells. Using differential Hoechst dye uptake, colleagues in our laboratory have recently been able to identify a side population within ascites that is thought to correspond with the tumour initiating population (Rizzo, Hersey et al. 2011). It would therefore be possible to assess

the methylation and expression of *LMX1A* in these side populations compared to the normal cell population.

NR2E1 is another transcription factor with a role in various CNS development processes. Again it is highly conserved amongst species; *tlx* is the mouse homologue (www.genecards.org/cgi-bin/carddisp.pl?gene=*NR2E1*). Liu and colleagues have shown that *tlx* induces long term neural stem cell expansion and brain tumour initiation (Liu, Wang et al. 2010). They demonstrate that *tlx*, which in the adult is expressed exclusively in the astrocyte-like B cells of the subventricular zone, acts as a key regulator of neural stem cell expansion and brain tumour initiation from these neural stem cells. They found that over expression of *tlx* antagonises age-dependent depletion of NSCs and results in increased production of new neurone in the ageing brain. These cells then lead to the development of glioma like lesions and gliomas and this process was accelerated with p53 loss. The *tlx* induced NSC expansion was associated with increased angiogenesis and migration and this is interesting as one of the most promising new therapy to have merged for gliomas in recent years is AZD2171 (cediranib) which is a pan VEGF inhibitor. This agent showed a very impressive partial response rate of 56% in the recurrent/ resistant disease setting, in a recent Phase II study reported at ASCO (Batchelor, Duda et al. 2010). In addition Liu and colleagues showed that the area of over expressed *tlx* corresponded with a subpopulation overexpressing Nestin, the known neural stem cell marker.

tlx, is an upstream regulator of *PAX2* (Yu, Chiang et al. 2000) and suppresses *PAX2* expression in mice - Downregulation of *PAX2* can enhance cisplatin sensitivity (Hueber, Waters 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, Dziarmaga et al. 2006), although any involvement in drug resistance mechanisms remains to be investigated.

As discussed in the introduction drug resistance could be a result of enrichment of resistant clones selected for during the course of chemotherapy – or because of the persistence of a tumour initiating/ sustaining population. The loci identified in this chapter could be implicated in either of these processes and the work so far does not address this question. *NR2E1* induces cisplatin-induced apoptosis (Dziarmaga, Hueber et al. 2006; Hueber, Waters et al. 2006), and cisplatin-based chemotherapy may select pre-existing subpopulations with epigenetically silenced *NR2E1*, whilst on the other hand *LMX1A* and *NR2E1* have both been implicated in neural stem cell and potentially cancer initiating processes as described. In ovarian cancer important future work would include characterising the phenotypic effects of over and under expressing this gene and also investigating whether it is associated with known stem cell markers as was seen in the mouse model.

DLC1 is a gene which encodes a GTPase-activating protein (GAP) that is a member of the rhoGAP family of proteins which play a role in the regulation of small GTP-binding proteins. GAP family proteins participate in signalling pathways that regulate cell processes involved in cytoskeletal changes (Ullmannova-Benson, Guan et al. 2009). It is known to function as a tumour suppressor gene in multiple tumour types including prostate, hepatocellular, Hodgkins lymphoma, nasopharangeal, oesophageal, cervical and gallbladder cancers (Yuan, Durkin et al. 2003) (Garcia, Manterola et al. 2009) (Guan, Zhou et al. 2006) (Peng, Ren et al. 2006) (Wong, Lee et al. 2003) and a link between epigenetic regulation via DNA methylation and gene expression has been reported.

However methylation of *DLC1* has not been reported previously in ovarian cancer. This gene has multiple transcript variants due to alternative promoters and alternative splicing and it is possible that this is the reason that I was not able to distinguish an increase in methylation in resistant cell lines, primary tumours or post chemotherapy samples (Wilson, McGlinn et al. 2000). Gene ontology analysis shows that this gene is involved in neural

tube closure, along with its role in cytoskeleton organisation. This raises the question as to whether like *LMX1A* and *NR2E1* it could have a role in neural stem cell differentiation.

3.7 **Conclusion**

The aim the experiments outlined in this chapter was to further characterise loci identified as differentially methylated between sensitive and resistant A2780 human ovarian cell lines, by DMH using a 12K CGI microarray, and attempt to identify candidate biomarkers of acquired drug resistance or identify key genes or pathways involved in ovarian cancer development.

Methylation of *NR2E1* and *LMX1A* was increased in resistant cell lines and matched tumour pairs and therefore could represent loci that are selected for during the course of chemotherapy. This would however require prospective validation within a clinical trial setting and the number of matched tumour pairs was both limited and not annotated in terms of survival– it is not known whether the matched samples represent platinum sensitive or resistant disease and this is clearly important. In addition as discussed the biological subtypes of ovarian cancer clearly have now been identified as having a key role in the response to chemotherapy (Lalwani, Prasad et al. 2011), However at the time of analysis the subtypes of EOC used in these samples was not determined.

As only 3 from an expected 6 genes were identified as potential novel markers of acquired drug resistance in epithelial ovarian cancer, from an original list of 41 sequences, this was deemed to be a relatively low yield. There could have been various reasons for this. Since the original DMH experiment (Heisler, Torti et al. 2005) larger and better annotated arrays are now available. The human genome is thought to consist of approximately 45000 CGIs and this library included only 12000 of these (Cross, Charlton et al. 1994). The annotation of this library frequently changed so it is possible that genes were missed that should have been investigated or vice versa. Cross et al demonstrate in the initial validation of DMH

which uses an MSe1 digest in combination with an MBD binding column that 77% were likely to be CGI. Ten percent represented rDNA and 10% were bulk DNA (Cross, Charlton et al. 1994). In the table at the beginning of this chapter it is shown that 13 of 41 sequences fulfilled our criteria of being a CGI and having a 5' location and this would argue that there are significantly more false positives in the library than proposed.

In addition the statistical method used to extract candidate loci from the DMH data was originally developed for the interpretation of gene expression microarray experiments and it is possible that vital information is lost by not taking into account the unique biological differences between changes in methylation and changes in expression. In expression experiments a small number of losses and gains in expression are seen whereas when analysing methylation a larger number of changes are seen and these tend to be asymmetrical – with more loci showing an increase in methylation in the resistant cell lines than a decrease. The normalisation that is necessary in RNA microarray experiments could be detrimental when analysing methylation data and valuable information could be lost.

The 34 cell lines panel is weighted towards the A2780 cell line series which is *in vitro* generated and it is possible that sequences and hence genes identified from this panel could have less biological relevance. Of note for all genes in this chapter although a clear increase in methylation was seen in the A2780 sensitive and resistant cell lines it was minimal in the other cell line pairs raising the question as to whether this is an A2780 effect only, and therefore of less biological relevance.

MSP was initially used to screen the 6 genes that were selected for further characterisation. For *PTTG* a decrease in methylation was expected but instead no methylation in any cell line was seen. For *CNTNAP* it was not possible to optimise primers and for *CR2* no increase in methylation was seen in the resistant cell lines. MSP is non-quantitative and optimisation and analysis of the gels relatively subjective. Since the start of this thesis pyrosequencing of bisulphite modified DNA has effectively replaced this technique.

With both MSP and pyrosequencing it is possible to gain a false negative result because the primers were not designed to amplify exactly the same area where the maximal difference in methylation was identified by PAM and similarly both techniques only investigate a few CpG residues so it is possible to miss increased methylation by a relatively small change in primer location. Using MSP false positive results can be obtained from incompletely methylated DNA (although a calponin PCR had been preformed to limit the effect of this).

After confirming an increase in methylation in the ovarian cisplatin resistant cell lines I investigated for methylation in primary tumours. The assumption was that if methylation was playing a role in these tumours that a heterogeneous pattern of methylation would be seen in the tumours – with the anticipation that this would then be increased in resistant tumours had these been available. However *DLC1* showed no methylation in primary tumours and was excluded from further analysis. There is a risk with this as if this gene was a marker of acquired resistance it would not necessarily need to show this heterogeneous pattern of methylation at presentation that we predicted.

In Chapter 7 the methylation status of these loci are re-examined in primary tumours on the OGT customised array. This gave an additional chance to investigate the loci for which MSP or pyrosequencing primers were not optimised or where differential methylation had not been observed (*CNTNAP* and *CR2*).

The tumour pairs that were available were obtained from patient's pre- and post-chemotherapy at the time of surgery for residual disease. If the patients had had a sufficient response to be considered eligible for such an approach it is possible that the disease that was left behind was not drug resistant and therefore these tumour pairs would not be suitable for detecting changes in methylation associated with resistant disease. No clinical details were available for these samples to indicate response to treatment. At the time of subsequent chapters the relapsed disease pairs were available and it would be

interesting to go back and pyrosequence these (assuming they represent chemoresistant relapse).

The correlation between methylation and expression was next examined. This was done in a limited number of cell lines and not performed in tumours as RNA was not available. For these candidates to be of true biological relevance it needs to be demonstrated that increased methylation at the promoter consistently correlates with decreased mRNA gene expression and that this can be reversed with a demethylating agent – both in cell lines and in tumours. In addition it would be important to assess the effect of methylation on protein expression using for example western blot analysis. Another important series of experiments would assess the functional impact of increased or decreased expression of the genes. As *NR2E1* and *LMX1A* have both been associated with embryonic stem cells and perhaps tumour sustaining cells it would be very interesting to investigate this in the ovarian cancer setting.

In the following chapters an attempt was made to address some of these questions.

4 Characterisation of loci showing differential methylation in cisplatin resistant lines identified by methylation linear discrimination analysis (MLDA).

4.1 Background and aims

The aim of the experiments in this chapter was to assess the ability of a novel statistical package, MLDA (Dai, Teodoridis et al. 2008), developed in our laboratory, to identify differentially methylated loci from the previously described 12k array. The 16 A2780-based sensitive and resistant cell lines (chapter 2.18.4) were used and candidates investigated by MSP +/- pyrosequencing. It was hoped that these candidates could also represent potential markers of acquired cisplatin resistance.

As outlined in Chapter 2.24.4 and 2.24.5, PAM and SAM were originally developed to interrogate microarray expression data rather than methylation data. They rely on some important assumptions in order to normalise the data. Firstly, that only a small percentage of genes will change expression, or in this case methylation status, and secondly that these changes will show symmetry, i.e. as many will show an increase in methylation as a decrease when comparing, for example, sensitive and resistant ovarian cancer cell lines.

However this is not usually the case when analysing methylation data where many loci are predicted to change their status and this change is likely to be asymmetrical because an ‘enrichment’ is seen with more sequences gaining methylation in resistant cell lines than losing it.

It was predicted that MLDA could therefore have advantages in detecting loci that optimally discriminate these isogenically matched ovarian cancer cell lines, without using

arbitrarily chosen cut offs or losing vital information as a result of over normalisation of the data.

As this was the first time sequences generated using MLDA had been examined in the laboratory we wished to do this in the most unbiased manner possible. Using the MLDA score, PAM and SAM an extensive list of candidate sequences was generated when comparing the more limited panel of 16 sensitive and resistant epithelial surface ovarian cancer cell lines, referred to as the A2780 cell lines in future text. These included A2780, A2780 p3, A2780 p5, A2780 p6, A2780 p13 and A2780 p14 (6 sensitive) and A2780 CP70 and MCP 1-9 (10 resistant). Nine sequences were chosen entirely at random, which it was hoped reflected samples that ranked high and low by MLDA/PAM/SAM, from the generated list and firstly examined in the same 16 ovarian cancer cell lines, by MSP. The MLDA ranking for each sequence is shown in table 26 below:

Table 26 Candidate loci from MLDA analysis of 16 A2780 cell lines.

Loci	Gene	Chromosome	Sensitive Score*	Resistant Score**	MLDA Ranking***
3 A 11	*MLLT6*	17	-0.86	0.01	**27**
17 H 9	*HRASLS3*	11	0.10	0.92	**34**
20 F 11	*NTN4*	12	-0.81	0.03	**41**
21 A 11	*NTN4*	12	-0.86	0.25	**9**
24 D 3	*SP5*	12	0.25	0.71	**75**
38 D 7	*AGBL2*	11	-0.91	0.18	**11**
41 D 12	*GLS2*	12	-0.81	0.00	**36**
101 G 6	*GLS2*	12	-0.91	0.025	**21**
121 D 9	*CRABP1*	15	-0.65	0.65	**2**

Sensitive score 1* is the average MLDA score in the sensitive cell lines , Resistant score 2** is the average MLDA score in the resistant cell lines. MLDA ranking*** is the rank of standardised residuals to the robust regression line constructed by the averaged sensitive scores against averaged resistance scores. Further information is available in table 2, page 9, Dai et al (Dai, Teodoridis et al. 2008).

4.2 Examination of candidate loci in ovarian cancer cell lines by MSP.

The aim of these experiments was to confirm in the laboratory that the candidate loci predicted by the MLDA analysis showed increased methylation in the same resistant ovarian cancer cell lines. By MSP, 8 of 9 sequences showed increased methylation in resistant cell lines (all except 3A11/ *MLLT6*) (Figure 27 below). The 8 sequences mapped to 6 known genes; 121D9 (*CRABP1*), 24D3 (*SP5*), 38D7 (*AGBL2*), 17H9 (*HRASLS3*), 41D12 and 101G6 (*GLS2*), 21F11 and 21A11 (*NTN4*).

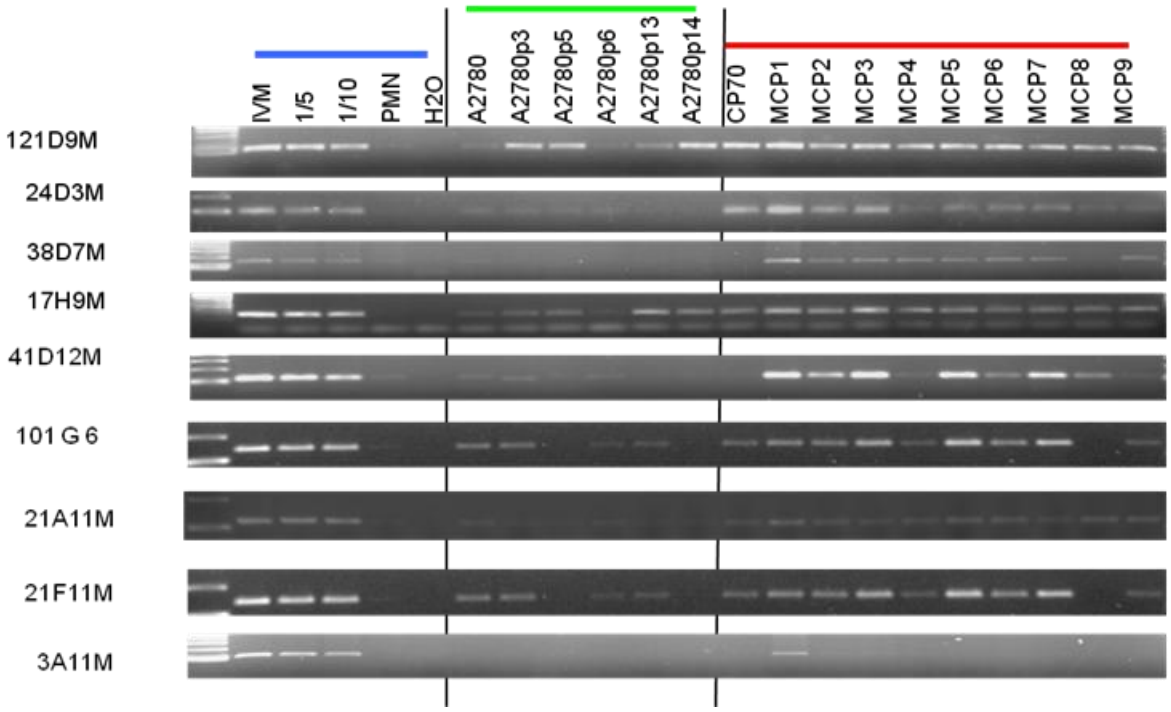


Figure 27. Methylation of candidate loci from A2780 analysis by MSP. Blue line above controls, Green line above sensitive cell lines, Red line above resistant cell lines. IVM *in vitro* methylated/positive control, PMN peripheral mononuclear cells/ normal whole male genomic DNA. 1/5 and 1/10 serial dilutions of positive in negative control.100 BP ladder to the left. Primer details are shown in chapter 2.6.3. Loci names are shown on the left of each gel.

The eight sequences which had confirmed increased methylation in the resistant cell lines were examined in a panel of 20 bisulphite modified primary tumours by MSP to identify loci whose methylation would be of potential clinical relevance and worthy of further detailed investigation. ('retrospective samples', Chapter 2.4.3). Loci mapping to three

genes; 24D3 (*SP5*), 101G6 (*GLS2*) and 121D9 (*CRABP1*), all showed increased methylation in >20% of samples. As the subsequent pyrosequencing results provide more quantitative data the MSP results are not shown. The three loci were next pyrosequenced in a larger 28 cell lines panel (the 34 cell line panel, without the single step clones, C1cis6, C2cis6, C2E3, C3Cis6, C5E4 and C5E4(15) , (see Chapter 2.17.4) prior to pyrosequencing in the full panel of primary tumours.

4.2.1 CpG Methylation of GLS2 (101G6) in ovarian cancer cell lines by pyrosequencing

Although only the A2780 based sensitive and resistant cell lines were used for MSP it was decided to use an expanded panel of cell lines which included the *in vivo* pairs for pyrosequencing (see chapter 2.17.4). It was anticipated that an increase in methylation would be seen in the A2780 resistant cell lines as these had been the cell lines used in the original MLDA analysis but whether an increase in methylation would be seen in other independent cell lines was unknown. If an increase in methylation in these independent cell lines was observed it was hoped that this would reflect a more biological meaningful locus – in terms of the likeliness that it played a role in acquired drug resistance.

All pyrosequencing experiments were performed in duplicate and percentages represent the mean of both experiments for the relevant experiments. An increase in mean methylation between the A2780 sensitive and resistant cell lines was confirmed (18.5% to 46.4%) (student t-test $p=1.48x10^{-9}$) however these changes were not observed in the non-A2780 cell lines and of note in the PEA1&2 pairing a statistically significant decrease in the mean methlyation in the resistant cell line was observed (75.6 to 11%) (student t-test p=0.0014). Results are shown in Figure 28 below:

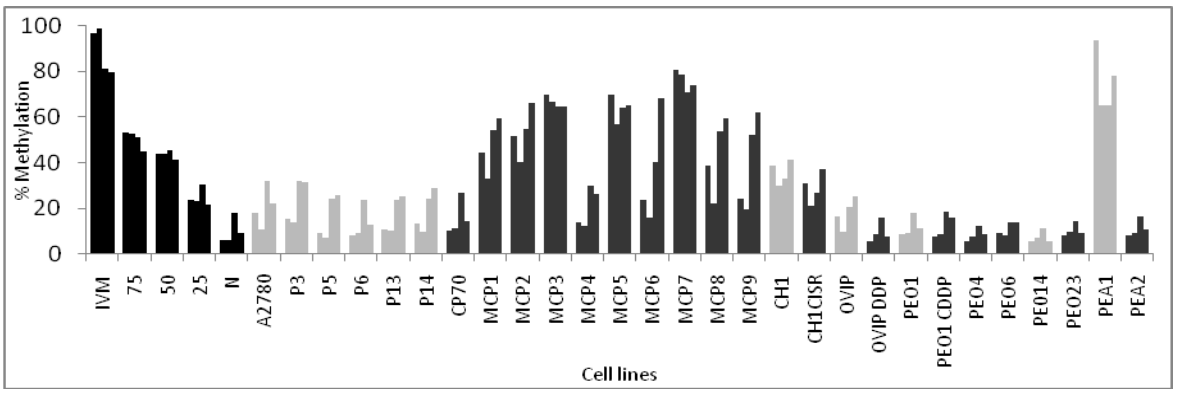


Figure 28. Methylation of *GLS2* in panel of 28 ovarian cancer cell lines by pyrosequencing. Controls in black: IVM (*In Vitro* Methylated DNA, positive control), serial dilutions of IVM/N, N (whole male genomic DNA, background control) shown. Sensitive cell lines in light grey and corresponding resistant cell lines shown in dark grey. Bars represent 4 adjacent CpG residues.

4.2.2 CpG Methylation of CRABP1 (121D9) in ovarian cancer cell lines by pyrosequencing

In *CRABP1* a highly significant difference was seen in the mean methylation between the sensitive and resistant A2780 based cell lines, from 12.7 to 57.7% (student t-test $p=1.77x10^{-22}$), with a background mean methylation level of 6.6%. The differences between CH1 and CH1CISR, and PEO1 and its corresponding resistant cell lines were not statistically significant but the decrease in mean methylation between PEO14 and PEO23 and PEA1 and PEA2 were found to be significant (student t-test p= 0.0004 and p=0.000127 respectively). This is shown in Figure 29 below:

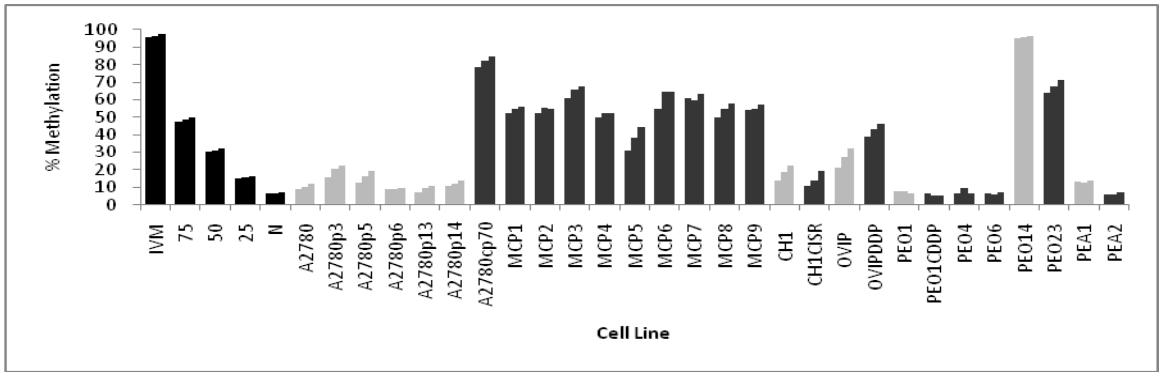


Figure 29. Methylation of *CRABP1* in panel of 28 ovarian cancer cell lines by pyrosequencing. Controls in black: IVM (*In Vitro* Methylated DNA, Positive control), serial dilutions of IVM/N, N (whole male genomic DNA, background control) shown. Sensitive cell lines in light grey and corresponding resistant cell lines shown in dark grey. Bars represent 4 adjacent CpG residues.

4.2.3 CpG Methylation of SP5 (24D3) in ovarian cancer cell lines by pyrosequencing

The percentage mean methylation of IVM was 94.9% which is what would be expected but the background level of methylation was found to be much higher than usual at 20.7%. Although a statistically significant difference in methylation was seen between the A2780 sensitive and resistant cell lines, with 42.5% and 91.6% methylation respectively (student t-test $p=1.6x10^{-14}$), for most other cell lines very high levels of methylation were seen in sensitive and resistant cell lines (with the exception of CH1CISR which showed methylation of 60.4%. The lowest value found for the rest of the cell lines was >90%) (student t-test p=0.003). These very high levels of methylation were more marked than had been seen by MSP. This is illustrated in Figure 30 below:

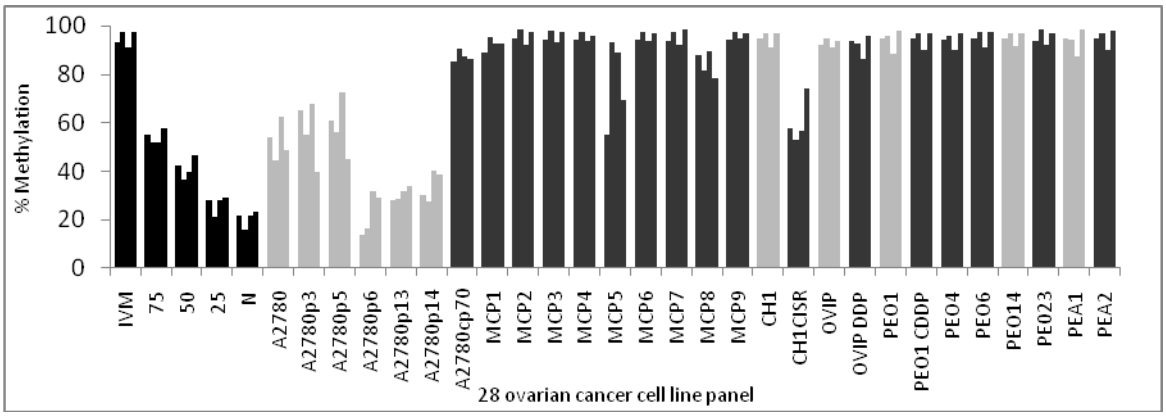


Figure 30. Methylation of *SP5* in 28 ovarian cancer cell lines by pyrosequencing. Controls in black: IVM (*In Vitro* Methylated DNA, Positive control), serial dilutions of IVM/N, N (whole male genomic DNA, background control) shown. Sensitive cell lines in light grey and corresponding resistant cell lines shown in dark grey. Bars represent 4 adjacent CpG residues.

Although this meant that methylation of this locus was less likely to be useful as a marker for acquired drug resistance it was still felt that it was still potentially a very interesting target in epithelial ovarian cancer and that it could highlight an important pathway in ovarian cancer pathogenesis. Along with *GLS2* (101G6) and *CRABP1* (121D9), *SP5* (24D3) CpG island methylation was therefore next examined in primary tumours.

4.3 Examination of candidate loci in primary tumours

4.3.1 CpG methylation of GLS2 (101G6)

Methylation of the loci 101G6 within *GLS2* was next assessed in the test set of 29 tumours. Only one sample showed methylation significantly greater than that seen in N (which as previously was used to represent the level of background methylation) with a 'p' value of 0.03. The mean methylation across all samples was 10.9 vs. 10.5% in normal. As only one sample showed significantly increased methylation this locus was not investigated in the larger test set. It had been decided that if no samples in the test set showed methylation of ≥50% in an individual sample that methylation of this locus in ovarian cancer must be rare. We were able to use a pre-defined cut-off in individual samples because pyrosequencing provides quantitative data, compared to the MSP data which were only semi quantitative. An alternative way to choose a cut-off for this data would have been to chart the methylation of the gene in interest and decide on a cut-off based on the distribution of the curve. The results are shown in Figure 31 below. As stated previously it was possible that increased methylation may not be seen in the primary tumour but still play a key role at the time of relapse when methylation could be acquired or enriched for. Although this locus was not further characterised at this stage, it was included in the OGT arrays (see chapter 7).

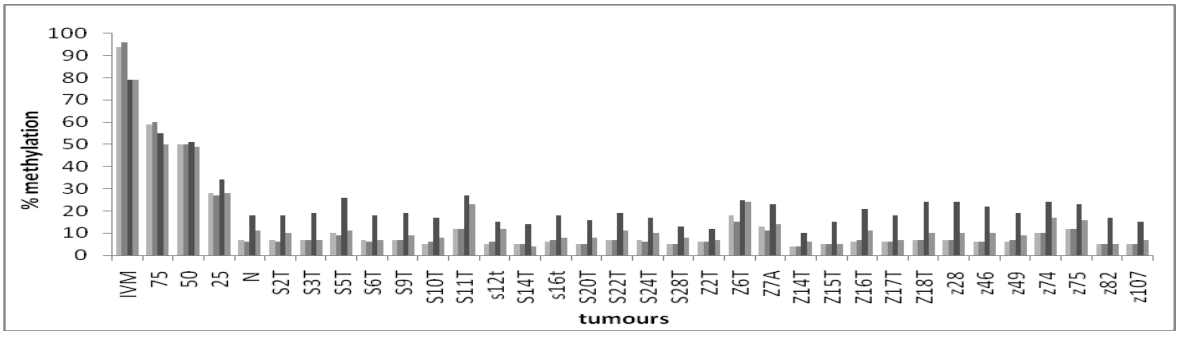


Figure 31. Methylation of *GLS2* in test set (I) of 29 primary EOC by pyrosequencing. IVM *in vitro* methylated DNA, N normal male genomic DNA, 75,50 and 25 represent serial dilutions of positive and negative control. Each of four bars is an adjacent CpG residues.

4.3.2 CpG Methylation of CRABP1 (121D9)

121D9 which mapped to the gene *CRABP1* also did not show increased methylation in the panel of primary tumours when examined by pyrosequencing (see figure 32 below) and, as was the case for *GLS2*, was not investigated any further at this stage as it was felt it was unlikely to be a useful biomarker if so little methylation was seen in the test set of primary tumours. It was however re-included when methylation in the primary tumours was investigated in the OGT customised arrays (see chapter 7).

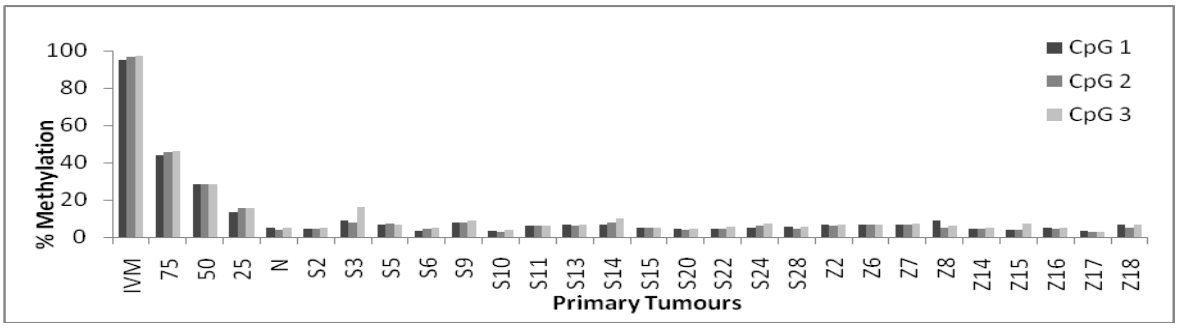


Figure 32. Methylation of *CRABP1* in test set (I) of 23 primary EOC tumours by pyrosequencing. IVM *in vitro* methylated DNA, N normal male genomic DNA, 75,50 and 25 represent serial dilutions of positive and negative control.

4.3.3 CpG Methylation of SP5 (24D3)

In contrast 24D3, which mapped to *SP5*, showed a very high level of methylation in the majority of tumour samples and also a higher than average level of methylation in the negative control (22.3%). As previously twenty bisulphite modified patient samples were first examined and if methylation was seen in these a further 54 samples were investigated making up the test set of 74 samples. If methylation was seen in the test set a further 66 independent tumours were examined. The average methylation in the tumours was 74.2% with only one sample showing methylation of less than 50% (sample No.15, 45.9%). Methylation for all the tumours in the initial test panel is illustrated in figure 33 below.

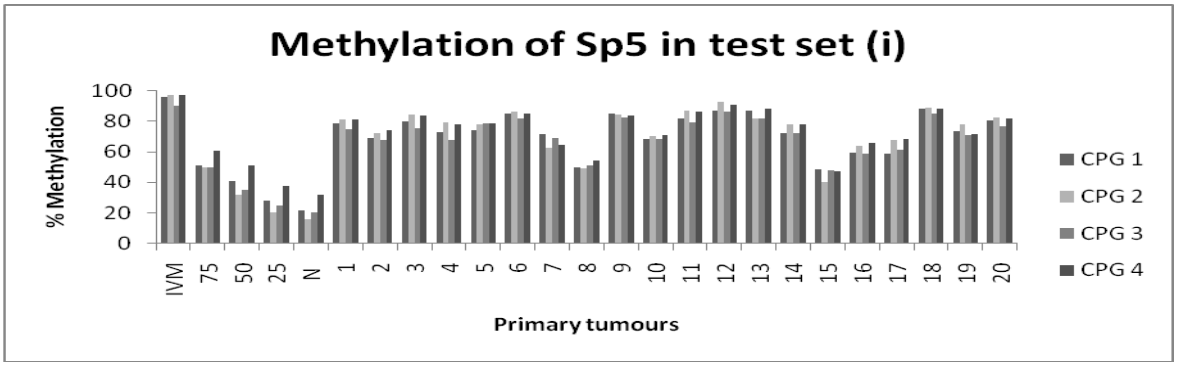


Figure 33. Methylation of *SP5* in test set (I) of 20 primary EOC tumours by pyrosequencing. IVM invitromethylated DNA, N normal male genomic DNA, 75,50 and 25 represent serial dilutions of positive and negative control.

As *SP5* showed high levels of methylation in the initial test panel it was next examined in an additional panel of 54 primary tumours. The mean methylation was 74% and 6 tumours had a mean methylation level of ≤50% (1.1%). Again the level of mean methylation in the negative control was high (22.3%). (Figure 34a overleaf). Here the mean methylation seen in 4 adjacent CpGs is shown and tumours with lower than 50% methylation highlighted in black.

These very high levels of methylation were confirmed in an independent validation set of primary tumours from patients in both the retrospective and prospective data sets (see chapter 2.4.3 and 2.4.4). The pyrosequencing results for a further 66 samples are shown in Figure 34b below. Here the background level of methylation was 17.63%, the mean methylation across all tumours 68.45%, and 1.2% (8) of tumours had a methylation level of ≤50%.

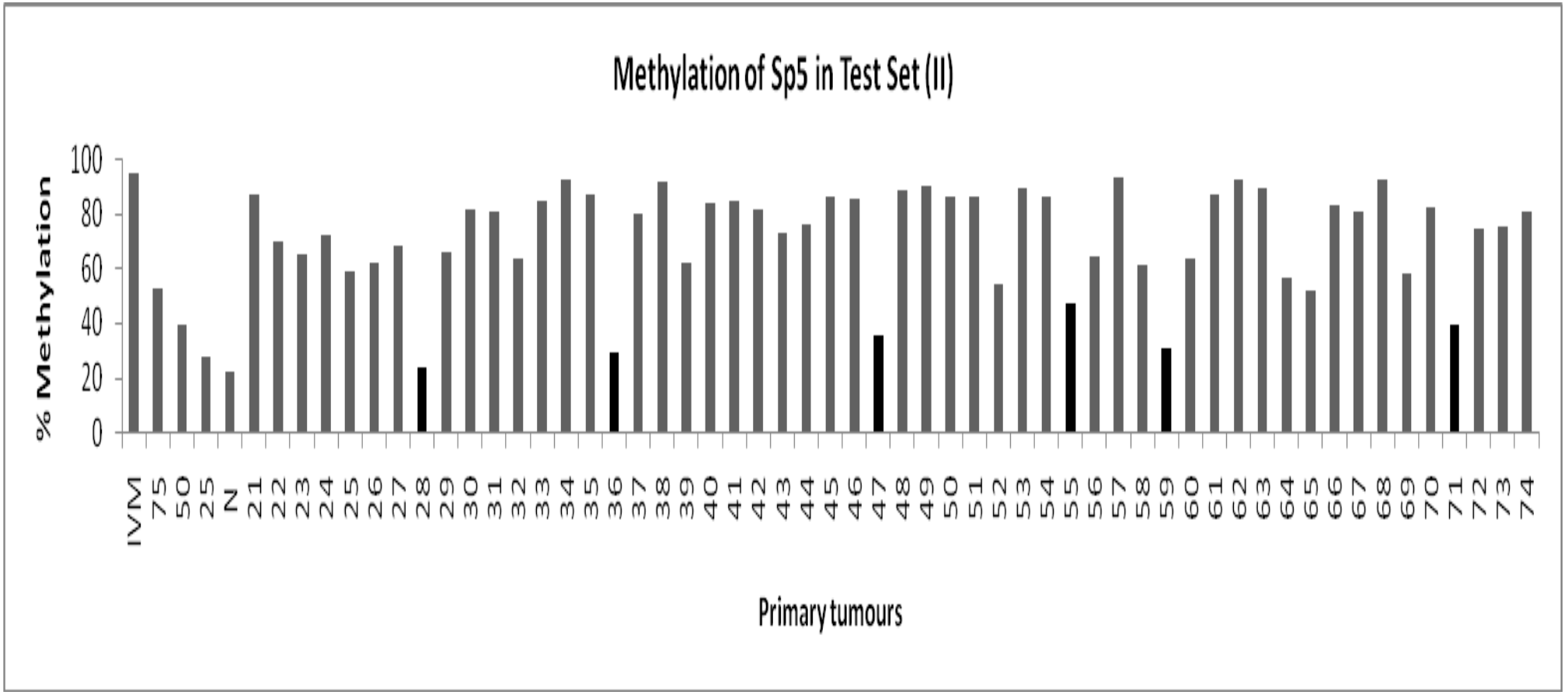


Figure 34a. Methylation of *SP5* in test set (ii) of 54 primary EOC tumours by pyrosequencing. Bars represent four adjacent CpGs. Grey bars: controls and tumours with methylation >≥50%. Black bars: tumours with <50% methylation. IVM invitromethylated DNA, N normal male genomic DNA, 75,50 and 25 represent serial dilutions of positive and negative control.

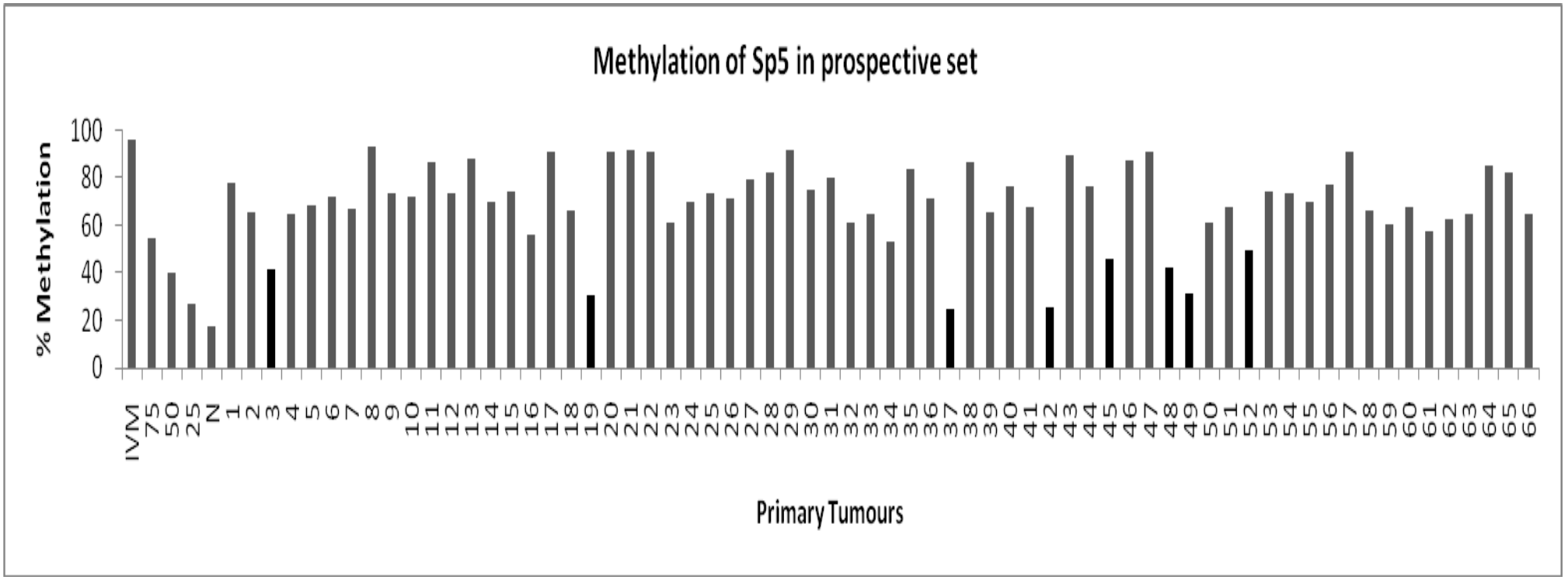


Figure 34b. Methylation of *SP5* in the validation set of 66 primary EOC tumours by pyrosequencing. Bars represent four adjacent CpGs. Grey bars: controls and tumours with methylation >≥50%. Black bars: tumours with <50% methylation. IVM *in vitro* methylated DNA, N normal male genomic DNA, 75,50 and 25 represent serial dilutions of positive and negative control.

4.3.4 Effect of methylation of SP5 (24D3) on patient survival and response to chemotherapy

The aim of the experiments described in this next subchapter was to investigate whether the level of methylation of *SP5* correlated with response to chemotherapy or survival for patients. The test and validation sets were analysed separately for progression free survival (PFS) and overall survival (OS), by Cox regression analysis and subsequently both data sets were combined in a Cox regression multivariate analysis with stage, grade, age and histological subtype. Known clear cell and mucinous tumours were excluded. Response to chemotherapy was assessed using logistic regression in the combined data set. All statistical analysis was performed using SPSS with the help of Wei Dai.

4.3.4.1 Response to chemotherapy (logistic regression)

It was possible to assess 85 out of 140 patients for response to chemotherapy, taking the test and validation sets together. Methylation was used as a continuous variable and no relationship was observed. Various analyses were preformed comparing different radiological responses but in all cases the results were comparable. This is shown in table 27 below.

Table 27 Relationship between *SP5* methylation and response to chemotherapy according to different groupings of patients by radiological response.

	Methylation level *(SP5)* %		Logistic regression model		
	Responders	Non-responders			
	(Mean±SD)	(Mean±SD)	OR	95% CI	P
CR+PR+SD (n=73) vs. PD (n=12)	70.1±16.5	76.6±9.4	1.03	(0.98, 1.08)	0.244
CR+PR (n=59) vs. PD (n=12)	72.7±16.9	76.6±9.4	1.02	(0.97, 1.06)	0.444
CR+PR (n=59) vs. SD+PD (n=26)	72.7±16.9	69.1±13.0	0.99	(0.96, 1.02)	0.331

OR: odd ratio (OR>1 the patients with increased methylation are more likely to have poor response to first-line chemotherapy);
95% CI: 95% confidence interval; p value: Wald test

4.3.4.2 Methylation of *SP5* and prognosis (Cox regression and Kaplan-Meier analysis)

In addition there was no correlation between *SP5* methylation and PFS or OS. Complete data was available for 65 of 74 patients in the test set and 64 of 66 in the validation set in order to investigate the effect of *SP5* methylation on PFS. By univariate Cox model analysis, *SP5* methylation showed a trend towards higher methylation correlating with PFS in the test set, however this was not statistically significant (two-sided score test p=0.064, HR 0.987, 95% CI 0.973-1.001) and no relationship was seen between *SP5* methylation and OS (two-sided score test p=0.126, HR 1.015, 95% CI 0.996-1.034). The lack of statistically significant correlation between *SP5* and PFS or OS was confirmed in the validation set (two-sided score test PFS p=0.884, HR 1.001, 95% CI 0.987-1.015), (OS two-sided score test p=0.619, HR 0.996, 95% CI 0.981-1.012). The results are shown in table 28 below:

Table 28 Summary of statistics for *SP5* methylation by univariate analysis

	'P' value	HR	95% confidence interval
PFS test set (N=65)	0.064	0.987	0.973-1.004
PFS validation set (N=64)	0.884	1.001	0.987-1.015
OS test set (N=65)	0.126	1.015	0.996-1.034
OS validation set (N=64)	0.619	0.996	0.981-1.012

*P value is two-sided score test of the univariate Cox model. HR: hazard ratio. HR>1 indicates the increased risk of disease progression/death associated with the increase of methylation. HR<1 indicates the decreased risk of disease progression/death associated with the increase of methylation.

The prognostic value of methylation at *SP5* was then adjusted by stage, grade, age and histological subtype in the multivariate Cox model. Each of the variables in the multivariate analysis was tested individually by Wald test for its impact on PFS and OS. Complete data was available for 102 of the 140 patients for PFS and 104 for OS. As predicted *SP5* methylation did not have a significant impact within a multivariate setting (PFS p=0.177, 95% CI 0.979-1.004), (OS p=0.320, 95% CI 0.993-1.023). Each of the variables in the multivariate analysis was tested individually for its impact on PFS and OS.

For PFS, patients with endometriod subtype had a better PFS (p=0.013, HR 0.364, 95% CI 0.164-0.811). Patients with stage 3 and 4 cancer had a significantly worse PFS (stage 2 p=0.699, HR 0.715, 95% CI 0.131-3.909), (stage 3 p=0.002, HR 4.826, 95% CI 1.757-13.255) and (stage 4 p=0.001, HR 5.965, 95% CI 2.046-17.393). Grade and age did not have a significant impact. For overall survival stage was the only variable that had a statistically significant impact on survival (p=0.01, Wald test). As a further check on the data and in order to produce the results in visual form Kaplan-Meier analysis was performed. In case the study had been underpowered to detect a difference in methylation, due to sample size, the test and validation sets together (n=140), using median methylation as the cut off, and this confirmed no significant relationship between *SP5* methylation and PFS (log rank test of survival curves in both groups p=0.098) (Figure 35) or OS (log rank test of survival in both groups p=0.963) (Figure 36).

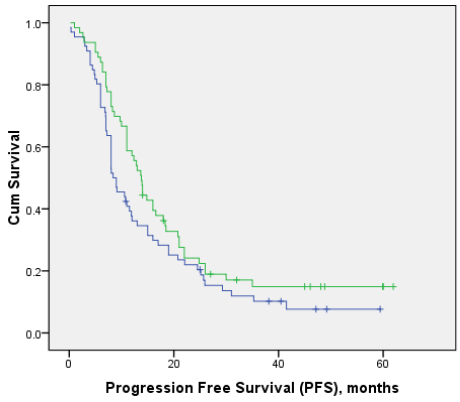


Figure 35. Kaplan-Meier graph showing the effect of *SP5* methylation on PFS in primary EOC (combined test and validation set). 'P'=0.098 (log rank test, median was used as the cut-off). Blue low methylation, green high methylation. Survival in months. Cum Survival cumulative survival. N=102.

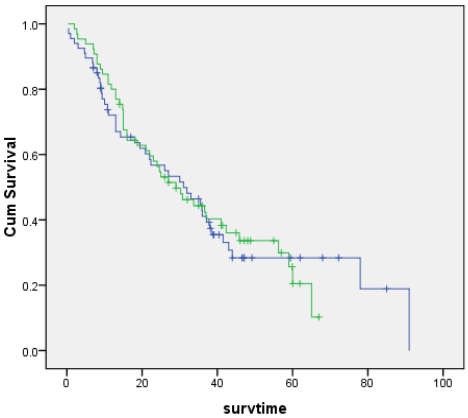


Figure 36. Kaplan-Meier graph showing the effect of *SP5* methylation on OS in primary EOC (combined test and validation set). 'P'=0.963 (log rank test, median was used as the cut-off). Blue low methylation, green high methylation. survtime Overall Survival time in months. Cum Survival cumulative survival. N=104.

4.4 Examination of *SP5* (24D3) in Matched pairs

In order to investigate whether the changes seen *in vitro* had significance for patients in terms of acquired drug resistance matched pairs of tumour pre- and post-chemotherapy were obtained. If methylation is selected for during the course of treatment then in pairs where minimal methylation was seen in the pre- treatment sample an increase in methylation in the subsequent sample could herald a clinically useful methylation marker. Two sets of samples were available; from patient's pre- treatment and at the time of surgery for residual disease and from patient's pre- chemotherapy and at the time of relapse. In principle the relapsed patients should be the most representative of acquired drug resistance although it was hoped that for patients with residual disease following chemotherapy that this is by definition also at least partly resistant to chemotherapy.

4.4.1 Residual disease

8 pairs of samples from patients pre-chemotherapy and at the time of surgery for residual disease were available. A description of these is available in Chapter 2.4.1. No significant difference was seen between pre- and post- treatment samples ($p=0.61$, two-sided student t-test comparing all pre- vs. all post- samples). This is with the caveat that these patients had not relapsed and did not therefore necessarily have resistant disease. In addition ovarian cancer is known to be a heterogeneous disease and it is possible that the DNA extracted was from a part of the tumour that was less aggressive. Figure 37 below show the results for each individual pair.

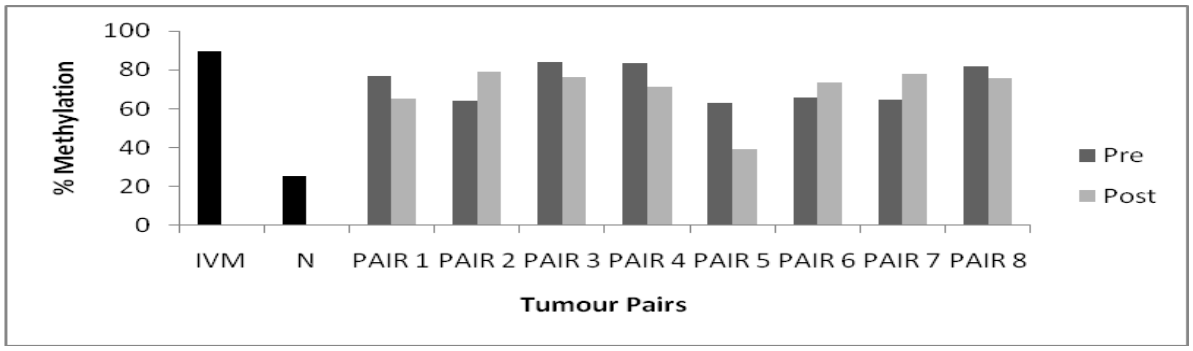


Figure 37: Methylation of *SP5* in residual disease pairs by pyrosequencing. Controls in black: IVM *in vitro* methylated DNA, N normal male genomic DNA. Each bar represents the average of four adjacent CpG's. Dark grey pre- chemotherapy, Light post- chemotherapy.

4.4.2 Relapsed disease

These results were confirmed by examining *SP5* methylation in the relapsed pairs were again no significant difference was seen between pre- and post- relapse (two-sided student t-test p=0.93) although as stated in the methods section (2.5.2) and the discussion in the previous chapter it is not known whether the relpased disease samples represent sensitive or resistant relapse. The results are shown in Figure 38 below.

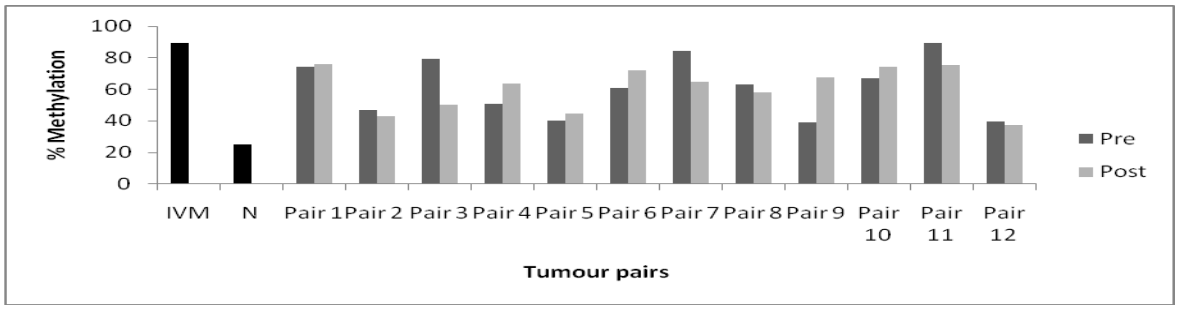


Figure 38: Methylation of *SP5* in relapsed disease pairs by pyrosequencing. Controls in black: IVM *in vitro* methylated DNA, N normal male genomic DNA. Each bar represents the average of the 8 samples in 4 adjacent CpG's. Dark grey pre-chemotherapy, Light grey post-chemotherapy (n=2).

It is perhaps not surprising that *SP5* did not show differential methylation between pre- and post- treatment samples given the high level of methylation demonstrated in primary tumours and cell lines and given the highly statistical significant correlation between *SP5* methylation and response to first line chemotherapy we were keen to investigate this locus further. We wished to clarify whether *SP5* methylation was tissue specific and for the

tumours that appeared to lose methylation whether this occurred as an early or late phenomenon.

4.5 Examination of *SP5* (24D3) in other tissues

Usually when investigating candidate loci in a panel of primary tumours low levels of methylation or a mixed pattern of methylation are seen. In this case a high level of methylation was seen in the majority of samples, with a small proportion of samples showing much lower methylation. Therefore it was hypothesised that the tumours with a low level of methylation could be losing methylation rather than the other samples gaining it. In an attempt to address this question the methylation of eight PBMCs, 4 nOSE, 4 normal adjacent, 4 samples which had shown low methylation, 4 samples of normal adjacent to the samples that had shown low methylation and the entire collection of primary tumours was compared and the results are shown in Figure 39 below;

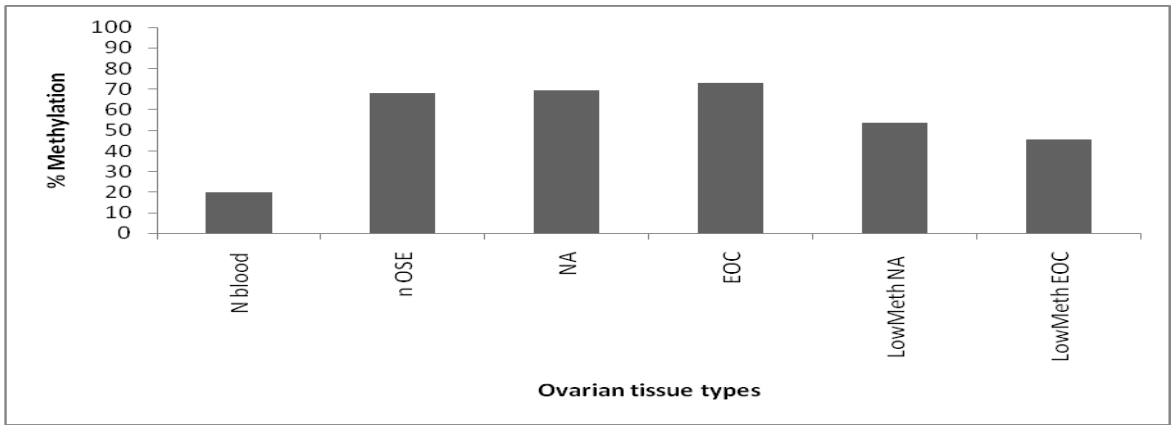


Figure 39. Methylation of *SP5* in various ovarian tissue by pyrosequencing. N blood whole male blood peripheral blood mononuclear cell, n OSE normal ovarian surface epithelium, NA normal adjacent, EOC epithelial ovarian cancer, LowMeth NA normal adjacent next to samples that had shown low methylation, LowMethEOC tumour samples that had shown less than 50% methylation. Each bar represent the average across all CpGs for samples of the same type. (N=2).

The highest percentage methylation was seen when the whole collection of EOC's were taken together. A significant difference was observed in the methylation between this

group and both the tissue adjacent to the tumours which had shown low methylation (student t-test $p=4.8x10^{-11}$) and the tumours themselves which had shown low methylation (student t-test $p=8.8x10^{-15}$). In addition the decrease in methylation between the tissue which was adjacent to the tumours that had shown low methylation and the low methylation tumours themselves was significant (student t-test p=0.03). This could suggest that methylation maybe first lost in the adjacent normal with a further loss of methylation in a distinct group of tumours, although the numbers investigated are clearly small. This pattern could indicate the role of methylation in maintaining tissue specific methylation patterns.

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4.6 **Discussion**

Eight potentially interesting candidates were identified from this experiment and the work of others with relation to these will now briefly be summarised.

CRABP1 is cellular retinoic acid binding protein. This gene encodes a specific binding protein for a vitamin A family member and is thought to play an important role in retinoic acid mediated differentiation and proliferation. It is located on chromosome 15 and is highly conserved among species (www.genecards.org/cgi-bin/carddisp.pl?gene=CRABP1). SAGE databases demonstrate it is expressed in the brain, retina and spinal cord and also in skin, breast and ovarian cancers although to a lesser extent (www.genecards.org/cgi-bin/carddisp.pl?gene=CRABP1). It has been shown to be a frequently methylated tumour suppressor gene in oesophageal cancer, colorectal cancer, ovarian cancer and thyroid cancer (Huang, de la Chapelle et al. 2003; Lind, Kleivi et al. 2006; Ogino, Brahmandam et al. 2006; Tanaka, Imoto et al. 2007; Wu, Lothe et al. 2007).

AGBL2 is ATP/GTP binding protein-like 2. It is located on chromosome 11 and is thought to play a role in the processing of tubulin (which could be of relevance given paclitaxel is one of the mainstays of ovarian cancer chemotherapy). It is expressed in a wide range of normal and cancer tissues according to the Genenote data base and expression is seen in a wide range of normal tissues using the eNorthern and SAGE databases (www.genecards.org/cgi-bin/carddisp.pl?gene=AGBL2). Characterisation of this gene, in normal tissue or cancer, or whether it is epigenetically regulated has not previously been reported.

HRASLS3, HRAS-like suppressor 3 is a tumour suppressor gene that may be involved in interferon-dependent cell death. It is located in chromosome 11 and shows conservation from the mouse to humans. It is expressed in a variety of normal tissues and cancers according to the Genenote, eNorthern and SAGE databases (www.genecards.org/cgi-bin/carddisp.pl?gene=HRASLS3). Two authors have proposed its role as a tumour suppressor in ovarian cancer although whether gene transcription is epigenetically regulated has not been examined (Sers, Husmann et al. 2002; Nazarenko, Schafer et al. 2007).

GLS2 encodes a protein which is important in the regulation of glutamine metabolism. It is located on chromosome 12 and highly conserved among species. It is expressed in a wide variety of tissues according to the Genenote database and a wide range of cancers according to the eNorthern data set (www.genecards.org/cgi-bin/carddisp.pl?gene=GLS2). There are a small number of publications on this gene. It is thought to be a target of p53 (Hu, Zhang et al. 2010) and when glioma cells were transfected using cDNA, reduced survival, migration and proliferation was observed (Szeliga, Sidoryk et al. 2005). It could therefore be hypothesised that it is a tumour suppressor gene that might be epigenetically regulated.

NTN4 belongs to a family of proteins related to laminins and is thought to play an important role in neural, kidney and vascular development. It is located on chromosome 12 and conserved from mouse to humans. Expression has been noted in a wide variety of tissues and also in liver, pancreatic, breast, thyroid and ovarian cancer cell lines, according to the eNorthern and SAGE databases (www.genecards.org/cgi-bin/carddisp.pl?gene=NTN4). It has not been reported, to date, to have a role in ovarian cancer, or to be epigenetically regulated although recently a paper was published by Nacht et al describing its role in inhibiting angiogenesis (Nacht, St Martin et al. 2009).

SP5 is a transcriptional activator, located on chromosome 2, which has a role in the coordination of changes in transcription required to generate the developmental pattern in the developing embryo. Using the SAGE database it is shown to be expressed in the brain colon, pancreas, prostate and placenta but expression has not been demonstrated in any cancers. It is highly conserved among species and has two CGIs (www.genecards.org/cgi-bin/carddisp.pl?gene=*SP5*). The primers in this thesis amplified an area in the larger CGI which is located within the promoter.

SP5 is known to be a transcription factor which antagonises *SP1* (Harrison, Houzelstein et al. 2000) and is a downstream target of Wnt signalling (Takahashi, Nakamura et al. 2005; Weidinger, Thorpe et al. 2005; Chen, Guo et al. 2006; Fujimura, Vacik et al. 2007). As Wnt is known to be dysregulated in EOC and has also been implicated in the pathogenesis of tumour initiating or sustaining cells this makes loss of methylation in tumours which regain expression of *SP5* an important novel observation. We have recently shown that methylation of key genes in the Wnt pathway has an impact on PFS on ovarian cancer (Dai, Teodoridis et al. 2010) and this adds further weight to the notion that methylation plays an important role in ovarian cancer drug resistance.

SP5 has been shown to be dynamically expressed during CNS development (Harrison, Houzelstein et al. 2000; Treichel, Becker et al. 2001; Weidinger, Thorpe et al. 2005) but it was only recently noted to show increased expression in colorectal, gastric and hepatocellular cancers with a negative impact (Chen, Guo et al. 2006). Chen et al, using an inducible gene expression system combined with microarray analysis found that over expression of *SP5* in MCF7 cells resulted in significant growth promotion supporting our results (Chen, Guo et al. 2006). This fits with the high levels of methylation seen in the cell lines and majority of primary tumours. The authors also identified downstream targets of *SP5* in the microarray experiment; many of these genes have been implicated in ovarian cancer and drug resistance and some have been shown to have epigenetic modulation of their gene expression – they include *p21, TGFB1, MDM2, ABCG2* and *ABCC3* (see Chapter 1.3).

4.7 **Conclusion**

In these experiments the aim was to validate MLDA as a statistical technique capable of identifying sequences that gain methylation in resistant cell lines by DMH (Dai, Teodoridis et al. 2008). The hypothesis being that such loci could represent candidate biomarkers of acquired platinum resistance. It should be noted though that for this reason it was not the top ranking sequences that were analysed but instead random sequences, which ranked from 2 to 75. Eight of nine sequences showed increased methylation in the A2780-derived resistant cell lines and we can therefore concluded that MLDA is at least as good at identifying candidate loci as PAM or SAM.

Methylation of *SP5* was not associated with response to chemotherapy or PFS or OS. Given that only a small number of tumours showed a decrease in methylation it is possible

that both the response and survival analysis were underpowered. However analysis of the matched pairs also showed no significant differences.

However it remains possible that a decrease in methylation at *SP5* could correlate with an increase in expression and have functional significance – given the other publications relating to this gene. If this is the case then the DMH experiment may have highlighted an important gene (*SP5*) or pathway (*Wnt*) in ovarian cancer.

In conclusion, in this chapter we demonstrated that MLDA (Dai, Teodoridis et al. 2008), a novel statistical technique developed in our laboratory, was able to identify loci which showed differential methylation between sensitive and resistant A2780 based cell lines. Given that loci were chosen at random to be characterised and ranked up to 75th (by MLDA ranking) it was encouraging that all but one locus validated in the same cell line panel.

For *SP5* we hypothesised we would see an increase in methylation in the resistant cell lines and a heterogeneous pattern of methylation in the primary tumours and although *SP5* did not show this pattern and instead showed a very high level of methylation in nearly all cell lines and primary tumours, it was thought that this in itself was a interesting observation.

In addition it appeared that rather than sequences gaining methylation that it may be a small cohort of samples that were losing methylation (which could be causing an increase in gene expression) - and a negative phenotypic effect from over expression of this gene would fit with the published work of others, as outlined above. As a result we decided that further more functional experiments were warranted and these are described in Chapter 6. Other candidates were excluded from further analysis due to the low levels of methylation seen in primary tumours, but as was discussed in the last chapter this may be flawed logic.

There was concern though that the changes that are seen in the A2780 resistant series were not seen in the *in vivo* generated resistant cell lines and even sometimes showed the opposite, for example in the case of *CRABP1* where a decrease in methylation was seen between PEO14 and PEO23. Further refinement in the approach could be achieved by identifying candidate loci from methylation changes seen between *in vivo* generated sensitive and resistant cell lines (PEA1 and PEA2, PEO14 and PEO23) as opposed to *in vitro* generated cell lines (A2780 sensitive lines, A2780 cp70 and MCP 1-9), by DMH. This question was addressed by the experiments described in the next chapter.

5 Characterisation of loci showing differential methylation between patient derived cell line pairs by MLDA

The experiments described in this chapter aimed to investigate whether using sensitive and resistant cell lines, where the resistance was generated within patients during chemotherapy, identified more clinically relevant methylation biomarkers of acquired drug resistance in ovarian cancer. In order to address this, PEA1 (sensitive) vs. PEA2 (resistant) and PEO14 (sensitive) vs. PE023 (resistant) were used instead of the A2780 panel. These cell lines were generated from patients with epithelial ovarian cancer who had been treated with platinum and subsequently developed resistance, and are referred to in the text as the *in vivo* cell lines (Langdon, Lawrie et al. 1988).

Loci which showed increased methylation in the resistant *in vivo* ovarian cancer cell lines were identified using MLDA. Figure 40a below shows how candidate loci are identified. Those of interest are seen as outliers on this line. The six loci we were particularly interested in were those that gained maximal methylation in the resistant pair and these are shown in figure 40b within the red box.

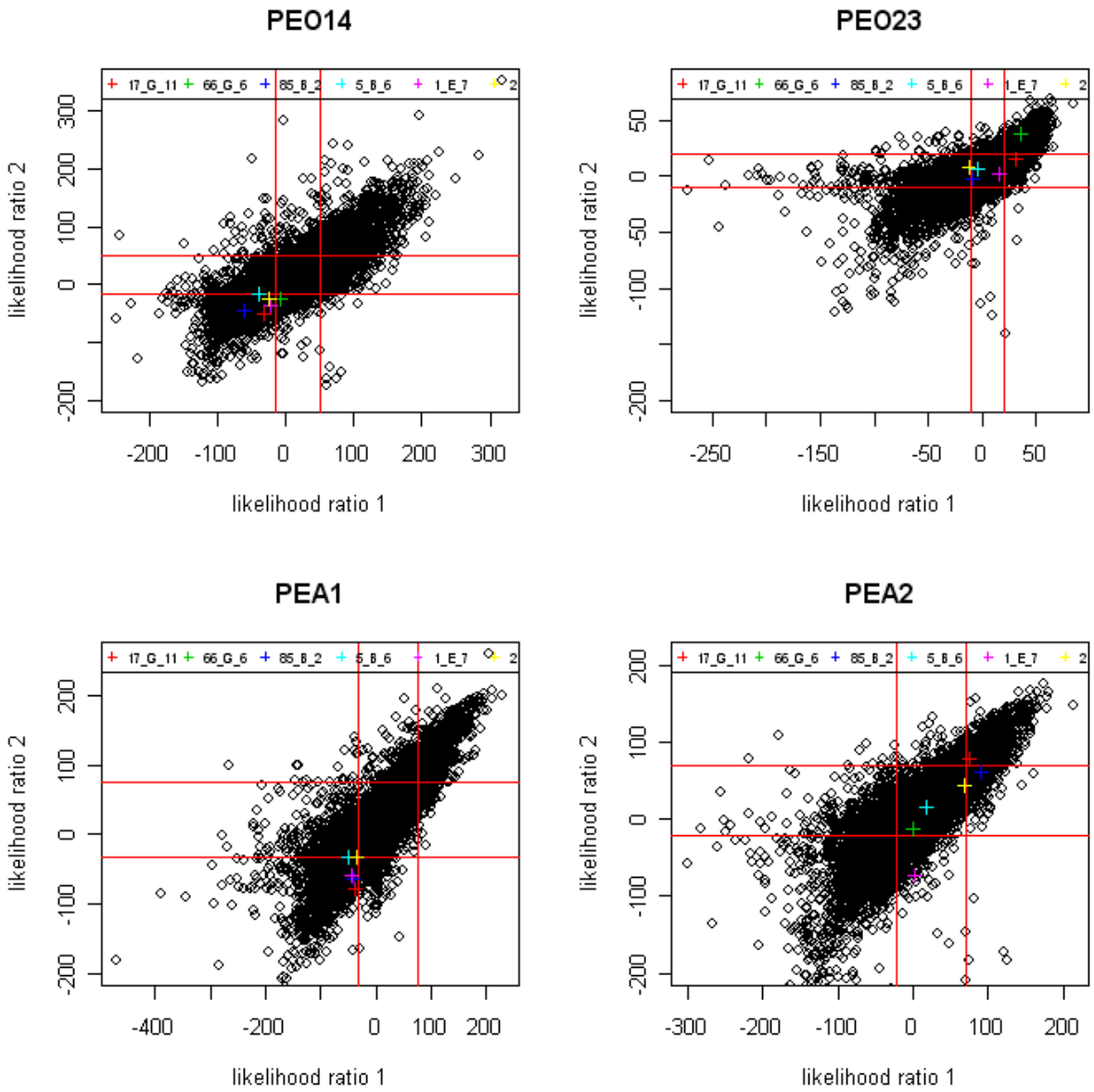


Figure 40a. MLDA: Sensitive and resistant scores for 6 loci which gain methylation in resistant cell lines. The top two diagrams show the score in the sensitive cell line PEO14 and the resistant cell line PEO23. The bottom two diagrams show the same for the PEA1 and PEA2 pairing. Coloured crosses highlight the position of the 6 loci within the whole data set and they can be seen to move from an area associated with less methylation to one of more methylation, between the sensitive and resistant cell lines (for a more complete explanation of how these figures are derived please refer to Dai et al, Figure 3 and Figure 7). (Dai, Teodoridis et al. 2008).

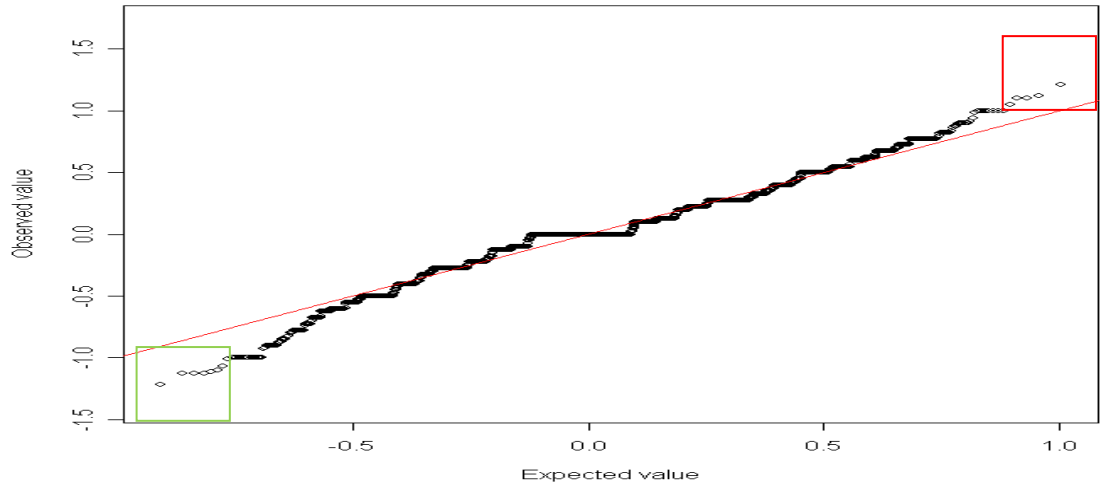


Figure 40b. Identification of hypo- and hyper- methylated outliers by MLDA. Loci which lose methylation in the resistant cell lines are shown as non filled circles within green box. Loci which gain methylation in resistant cell lines are shown as non filled circles within the red box.

Of the 6 sequences which showed increased methylation in the two resistant cell lines by MLDA score, one locus did not contain a CGI and another had more than more BLAT (Blast like alignment tool) alignment and we therefore selected 4 sequences to characterise in more detail in this chapter. These are shown in table 29 below. We did not choose to characterise the sequences that showed a decrease in methylation in the resistant cell lines. This was a pragmatic decision given that the characterisation of each locus is time consuming. We hypothesised that a gain in methylation in the resistant cell lines would be more functionally relevant and have more potential as a biomarker than a loss in methylation.

Table 29 List of sequences gaining methylation in PEA2 and PEO23 (resistant) cell lines.

MLDA Rank	Microarray Identifier[1]	Gene Symbol	CGI[2]	PEO14	PEO23	PEA1	PEA2	Residual score 1*	Residual score 2*
1	85B2	*LOC113230*	Y	-1	-0.2	-1	0.45	0.8	1.45
2	21G5	*KIAA1383*	Y	-1	0	-1	0.2	1	1.2
3	17G11	*SIX1*	Y	-1	0.45	-1	1	1.45	2
4	66G6	-	Y	-0.45	1	-1	0	1.45	1.0001

[1]http://data.microarrays.ca/cpg/searchsingleclones.htm. [2]CGI Gardener Garden Y yes, N no. *The residual score is the difference of MLDA score between pair of cell lines (1: PEO14 vs. PEO23; 2: PEA1 vs. PEA2), and positive residual score indicates increased methylation in the resistant cell line. MLDA score (see MLDA paper Figure 3) (Dai, Teodoridis et al. 2008) representing how consistently the locus was methylated (positive score) or unmethylated (negative score) in duplicates.

5.1 Examination of candidate loci in cell lines

The next aim was to confirm by pyrosequencing the methylation status of candidate loci identified from the MLDA analysis. The 28 cell lines, as described in chapter 3, were used (the full 34 cell lines panel minus the single step clones). It was possible to optimise pyrosequencing primers for 3 of the 4 sequences which contained a CGI and the other, 66G6, was examined by MSP. The results are discussed below.

5.1.1 CpG methylation of LOC113230 (85B2) in 28 cell lines by pyrosequencing

The results for *LOC113230* (85B2) are shown in Figure 41 below (two-sided student t-test p=0.46 when comparing all senstive vs all resistant).

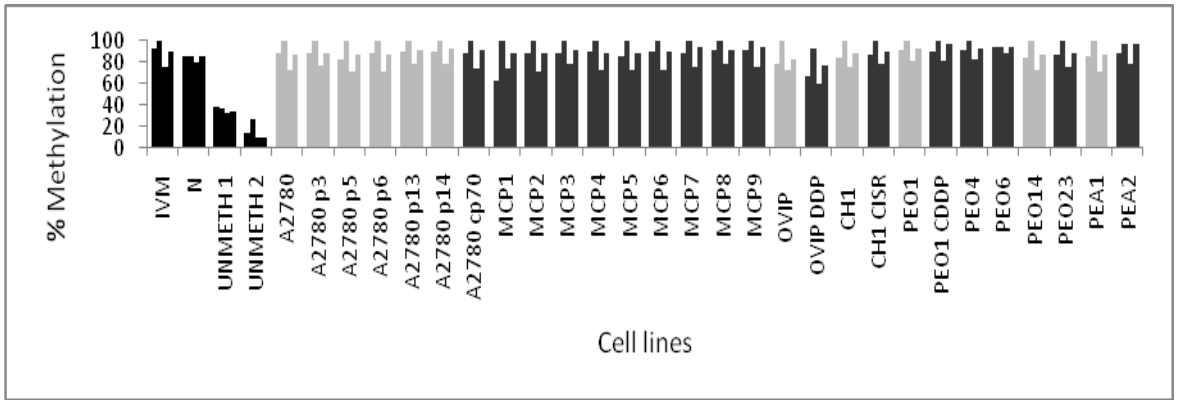


Figure 41. Methylation of *LOC113230* in the 28 cell line panel by pyrosequencing. Controls in black: IVM *in vitro* methylated DNA, Positive control), serial dilutions of IVM/N, N (whole male genomic DNA, background control) shown. Unmeth 1 and 2 whole genome amplified unmethylated DNA. Sensitive cell lines in light grey and corresponding resistant cell lines shown in dark grey. Bars represent 6 adjacent CpG residues.

From this experiment *LOC113230* (85B2) would appear to be a false positive result. Although it appeared that there was differential methylation between the sensitive and resistant cell lines on the DMH experiment this was not seen by pyrosequencing. On this occasion because very high levels of methylation were seen in PBMC's an alternative negative control was used and this is labelled UNMETH 1 and 2 (whole genome amplified unmethylated DNA). The limitation of this approach is that pyrosequencing only examines a handful of CpG sites however attempts to optimise alternative primer sets were not successful for this locus.

It was decided not to attempt to further characterise this locus at this time but instead to address the methylation status in the independent arrays, as outlined in chapter 7.

5.1.2 CpG Methylation of SIX1 (17G11) in 28 cell lines by pyrosequencing

SIX1 showed a distinct increase in methylation in 4 out of 6 cell line pairs, most remarkably between the A2780 sensitive cell lines and the cisplatin resistant MCP lines with an increase in mean methylation from 44.56% to 72.35% (A2780 sensitive vs. A2780 cp70 and MCP1-9, student t-test p=0.0004), the PEO1 sensitive cell line (25.5%) and the resistant corresponding cell lines PEO4, PEO6 and PEO1CDDP (38.63%) (student t-test p=0.021) and between the PEA1 and 2 pairing where an increase of 2.5% to 83.8% was seen (student t-test p=0.0008). The background level of mean methylation for these experiments was 12.5%. Whereas most of the loci investigated in the previous chapters had shown increased methylation in the A2780 based cell lines but not in the others this loci showed differential methylation in both the A2780 and *in vivo* derived pairs and this result was seen as highly encouraging. The pyrosequencing results are shown in figure 42 below:

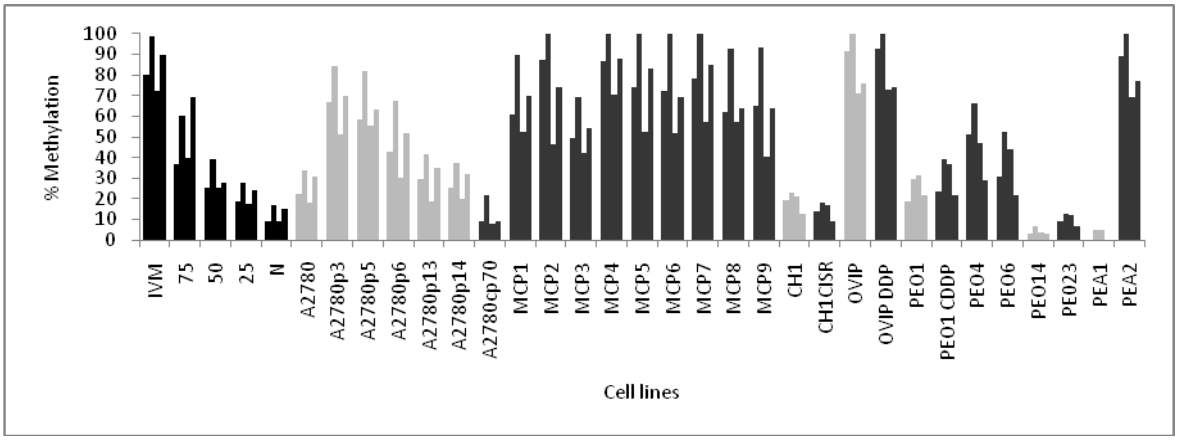


Figure 42. Methylation of *SIX1* in the 28 cell line panel by pyrosequencing. Controls in black: IVM *(in vitro* methylated DNA, positive control), serial dilutions of IVM/N, N (whole male genomic DNA, background control) shown. Sensitive cell lines in light grey and corresponding resistant cell lines shown in dark grey. Bars represent 4 adjacent CpG residues.

5.1.3 CpG Methylation of KIAA1383 (21G5) in 28 cell lines by pyrosequencing

KIAA1383 also showed an increase in mean methylation in several of the resistant cell line pairings (3 of 6 pairings). In the A2780 cell line groupings there was an increase in mean methylation from 50.1% to 66.8% (student t-test p=0.000461). In the PEO14 and PEO23 pairing there was an increase in mean methylation from 5.5% to 20.33% (student t-test p=0.000878) and in the PEA1 and PEA2 pair there was an increase from 4.83% to 30.33% (student t-test p=0.005582). This was with a background level of methylation of 4.99%. The results are shown in Figure 43 below:

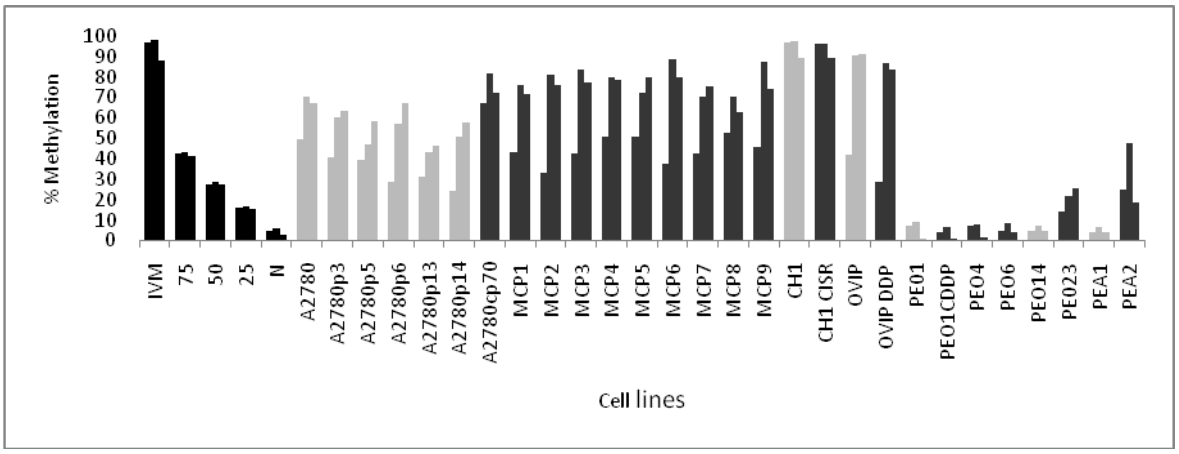


Figure 43. Methylation of *KIAA1383* in the 28 cell line panel by pyrosequencing. Controls in black: IVM *in vitro* methylated DNA, positive control), serial dilutions of IVM/N, N (whole male genomic DNA, background control) shown. Sensitive cell lines in light grey and corresponding resistant cell lines shown in dark grey. Bars represent 3 adjacent CpG residues.

5.1.4 CpG Methylation of 66G6 in 22 A2780 based cell lines by MSP

One further sequence, 66G6, could not be optimised for pyrosequencing and was examined by MSP in the A2780 cell lines where it showed increased methylation in the resistant cell lines had been confirmed here also. These results are shown in figure 44 below.

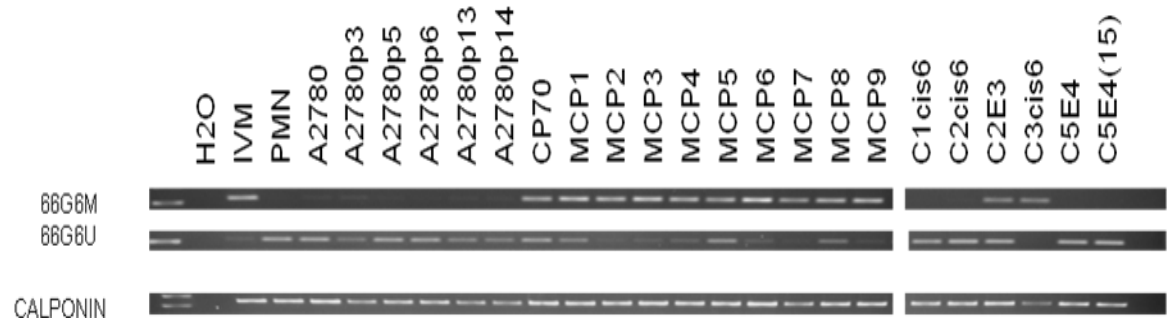


Figure 44. Methylation of 66G6 in 22 A2780 derived sensitive and resistant cell lines by MSP. Validation using MSP of 66G6 in the cell lines analysed by DMH as well as 6 additional cisplatin resistant cell lines, C1Cis6-C5E4(15). (M), methylated primer set and (U), unmethylated primer set. H_2O, reaction without template DNA; IVM, *in vitro* methylated DNA; PMN, DNA from whole male blood. The in vivo cell lines were not available at this time.

5.2 **Matched pairs**

KIAA1383 and *SIX* were next examined in DNA from matched paired samples from patients with ovarian cancer (66G6 was not taken any further as I could not optimise pyrosequencing primers and 85B2 was not examined further because of the very high level of methylation seen in all cell lines).

5.2.1 Pre chemotherapy and residual disease

As described in Chapter 2.4.1, ten of the twelve pairs of samples from patients at diagnosis and at the time of surgery for residual disease, following chemotherapy, were available.

SIX1 showed a background methylation of 6.6%. Although 7 of 10 samples showed some increase in methylation in the relapse samples, for only two samples was the percentage methylation in the pre treatment sample ≤ the background level of methylation and when all pre treatment samples were compared to resistant no significant difference in methylation was seen (student t-test p=0.855). This is illustrated in Figure 45 below.

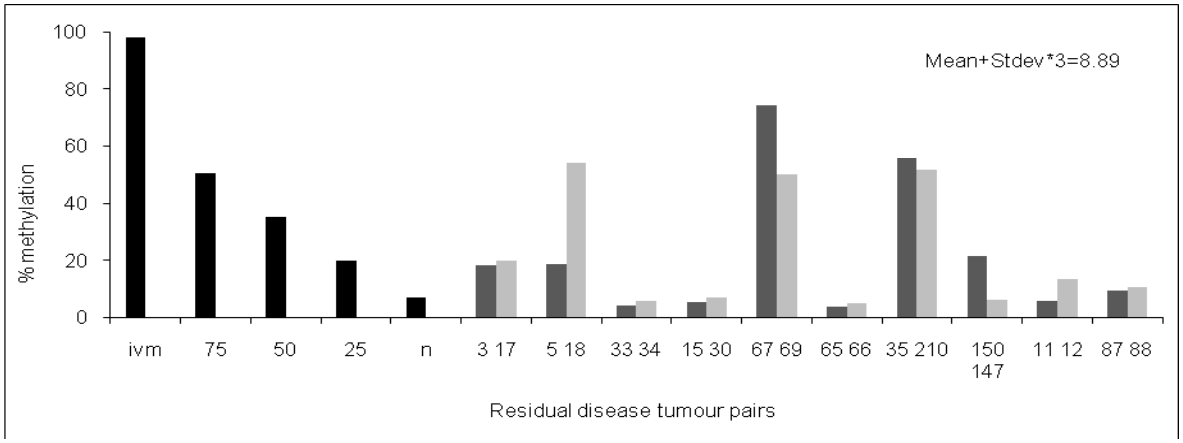


Figure 45. Methylation of *SIX1* in the residual disease pairs by pyrosequencing. Controls in black, IVM *in vitro* methylated DNA, positive control), serial dilutions of IVM/N, N (whole male genomic DNA, background control) shown. Dark grey is pre- chemotherapy sample, Light grey is corresponding post- relapse sample. One bar represents 5 adjacent CpG's.

KIAA1383 was also investigated in these same patient pairs. Background methylation was 8%. Six of 10 samples showed a small increase in methylation in the residual tumour however in only two samples was the pre- residual disease sample ≤ methylated than in the background. For one pair a statistically significant difference was seen between the pre- and post- treatment samples (student t-test p=0.0004). Overall though when comparing all pre- treatment samples to all residual disease samples no significant difference was seen in mean methylation (student t-test p=0.72). This is illustrated in figure 46 below.

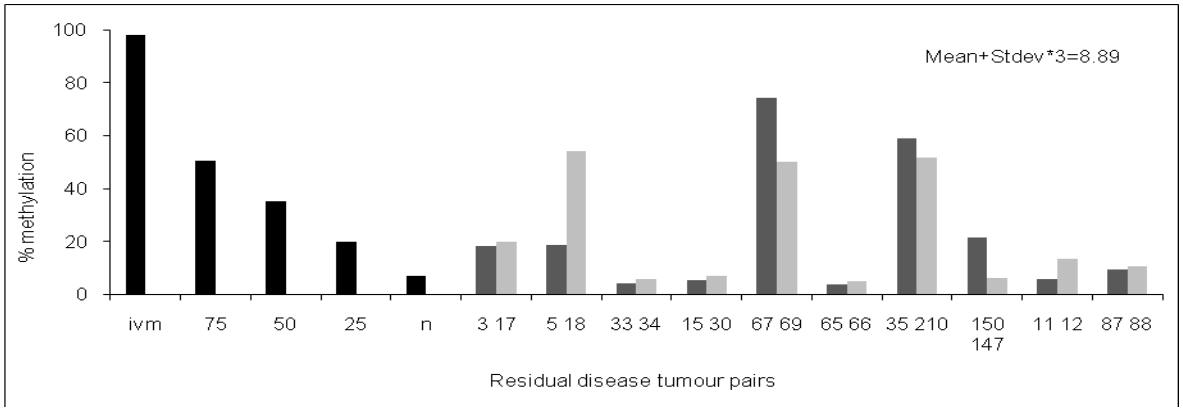


Figure 46. Methylation of *KIAA1383* in the residual disease pairs by pyrosequencing. Controls in black, IVM *in vitro* methylated DNA, positive control), serial dilutions of IVM/N, N (whole male genomic DNA, background control) shown. Dark grey is pre- chemotherapy sample, Light grey is corresponding residual disease sample. One bar represents three adjacent CpGs. Mean+SD3 is mean % methylation plus three standard deviations.

5.2.2 Pre chemotherapy and relapsed disease

For *SIX1*, 1 pair showed a significant increase in methylation in the post treatment sample (student t-test p=0.002) however overall no significant difference was seen between pre- and post- relapse samples (student t-test p=0.29). This is illustrated in figure 47 below.

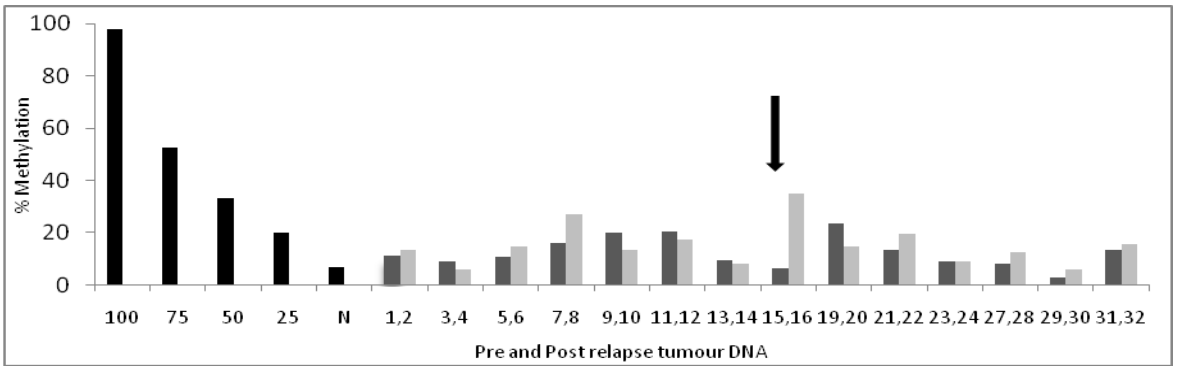


Figure 47. Methylation of *SIX1* in the relapsed disease pairs by pyrosequencing. p=0.29. Black columns show the controls, IVM *in vitro* methylated DNA, positive control), serial dilutions of IVM/N, N (whole male genomic DNA, background control) shown. Dark grey bars pre-treatment, Light grey bars post- relapse. One bar represents the average value for 5 adjacent CpG's. One pair with significant student t-test is highlighted with an arrow, p=0.002.

KIAA1383 was also investigated in the relapsed disease matched pairs. Here an increase in mean methylation was seen in 1 of 4 relevant pairs but again when all pre- vs all relapsed disease pairs were analysed together no significant difference was seen (student t-test p=0.9). This is illustrated in figure 48 below.

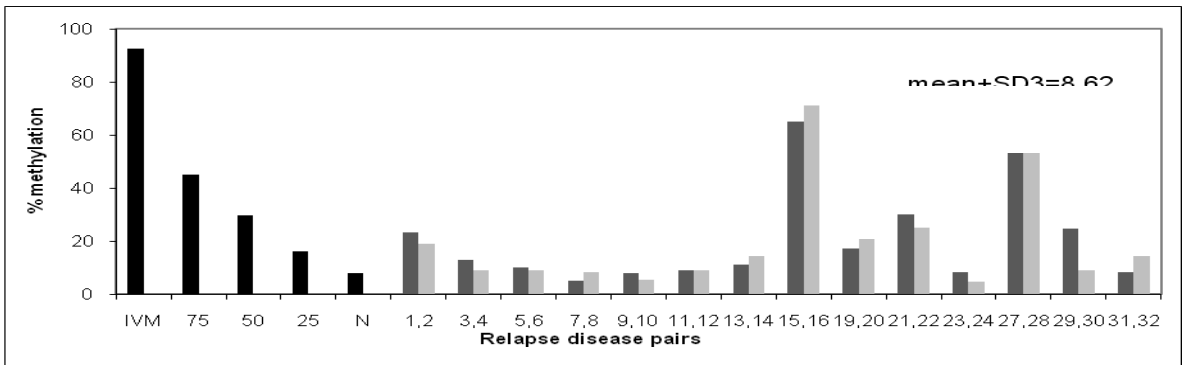


Figure 48. Methylation of *KIAA1383* in the relapsed disease pairs by pyrosequencing. p=0.9 Black columns show the controls, IVM *in vitro* methylated DNA, positive control), serial dilutions of IVM/N, N (whole male genomic DNA, background control) shown. Dark grey bars pre treatment, light grey bars post relapse. One bar represents the average value for 3 adjacent CpG's.

5.3 Examination of candidate loci in primary tumours and correlation with response to chemotherapy and survival

The objective of these experiments was to examine *SIX1* and *KIAA1383* in primary epithelial ovarian cancer tumours and to investigate whether an increase in methylation, such as that had been seen in the resistant cell lines, was again observed. As before these loci were first investigated in the smaller test panel of approximately 20 tumours.

5.3.1 CpG Methylation of SIX1 (17G11) in primary tumours.

Mean methylation in the normal control (N) in the test set was 5.7%. Similar results were seen in the second test set at 6.2%, and the validation set where average methylation of 7.8% was observed. 40% of samples in the first test set had methylation of > 50% methylation. The results for each of the data sets are illustrated in figure 49a-c below and overleaf. As previously for the two larger data sets average methylation across the adjacent CpG's is shown and those samples with >50% methylation are highlighted.

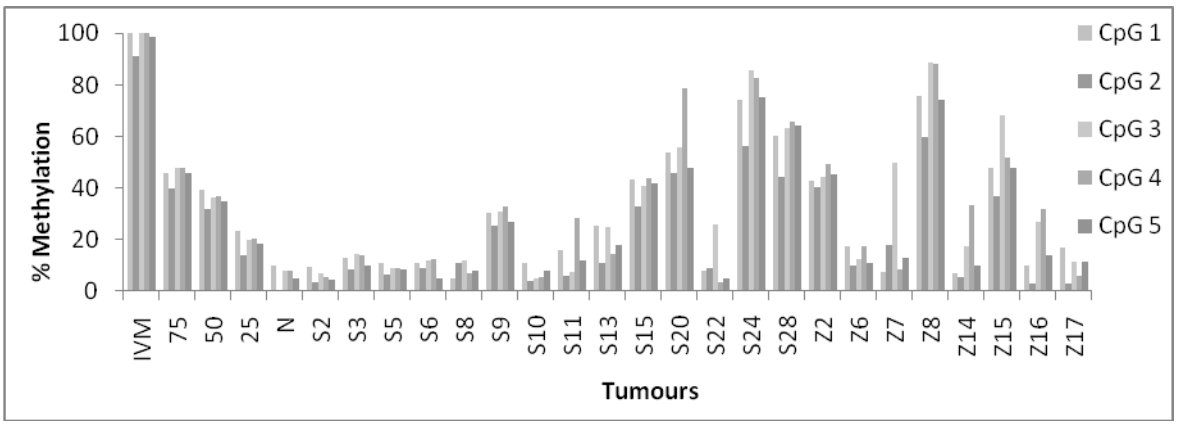


Figure 49a. Methylation of *SIX1* in test set (I) of 22 primary EOC tumours by pyrosequencing. Bars represent 5 adjacent CpG residues. IVM *in vitro* methylated DNA, N normal male genomic DNA, 75,50 and 25 represent serial dilutions of positive and negative control.

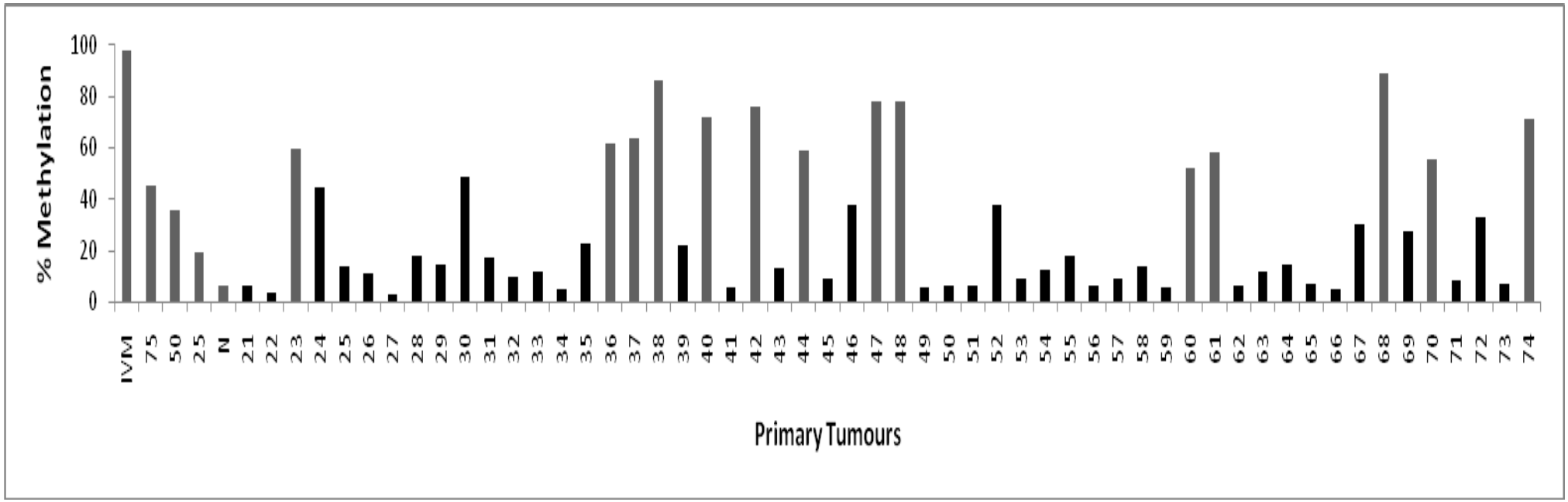


Figure 49b. Methylation of *SIX1* in test set (ii) of 54 primary EOC tumours by pyrosequencing. Bars represent 5 adjacent CpG residues. Grey bars: controls and tumours with methylation >≥50%. Black bars: tumours with <50% methylation. IVM *in vitro* methylated DNA, N normal male genomic DNA, 75,50 and 25 represent serial dilutions of positive and negative control.

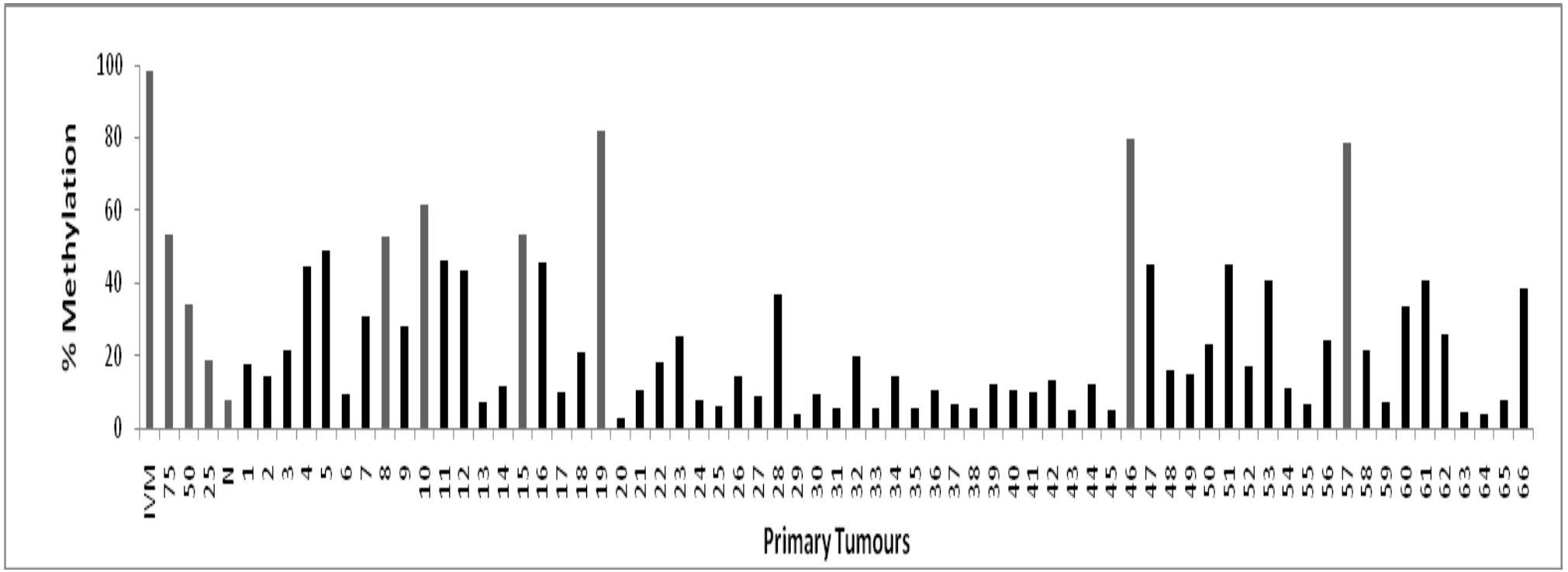


Figure 49c. Methylation of *SIX1* in the validation set of 66 primary EOC tumours by pyrosequencing. Bars represent 5 adjacent CpG residues. Grey bars: controls and tumours with methylation >≥50%. Black bars: tumours with <50% methylation. IVM *in vitro* methylated DNA, N normal male genomic DNA, 75,50 and 25 represent serial dilutions of positive and negative control.

5.3.2 Effect of methylation of SIX1 (17G11) on response to first line chemotherapy

No relationship was seen between methylation of *SIX1* and response to chemotherapy, by logistic regression. It is possible that this is because the analysis was underpowered. Data was only available for 85 patients and whichever way the patient groups were split, in terms of RECIST response, there were only a maximum of 26 in one of the groups. Similarly as before it is possible for a biomarker to be of prognostic value but this not to be attributable to the response to first line treatment and we therefore went ahead with the survival analysis as outlined in the next sub-section. The response results are shown in table 30 below:

Table 30. Relationship between *SIX1* methylation and response (by RECIST).

	Methylation level (*SIX1*) %		Logistic regression model		
	Responders	Non-responders			
	(Mean±SD)	(Mean±SD)	OR	95% CI	P
CR+PR+SD (n=73) vs. PD (n=12)	28.5±25.8	32.1±26.4	1.00	(0.98, 1.03)	0.649
CR+PR (n=59) vs. PD (n=12)	29.2±27.2	32.1±26.4	1.00	(0.98, 1.03)	0.725
CR+PR (n=59) vs. SD+PD (n=26)	29.2±27.2	28.9±22.7	1.00	(0.98, 1.02)	0.936

OR: odd ratio (OR>1 the patients with increased methylation are more likely to have poor response to first-line chemotherapy);
95% CI: 95% confidence interval; p value: Wald test

5.3.3 Effect of methylation of SIX1 (17G11) on patient survival

By univariate Cox regression model no relationship was seen between *SIX1* and PFS in either data set however significant results were seen for OS. The results were conflicting though with an increase in methylation having a negative impact on OS in the test set and the converse true in the validation group. The results are summarised in the table below but effectively mean that analysis of a further group of samples would be necessary to investigate whether there is a positive or negative impact of *SIX1* on survival:

Table 31 Summary of statistics for univariate analysis of *SIX1* methylation and survival.

	p value*	HR	95% confidence Interval
PFS test set (n=65)	0.448	0.997	0.988-1.005
PFS validation set (n=64)	0.083	0.987	0.973-1.002
OS test set (n=65)	**0.043**	**1.01**	**1.000-1.019**
OS validation set (n=64)	**0.037**	**0.983**	**0.966-0.999**

*P value is two-sided score test of the univariate Cox model. HR: hazard ratio. HR>1 indicates the increased risk of disease progression/death associated with the increase of methylation. HR<1 indicates the decreased risk of disease progression/death associated with the increase of methylation. Statistically significant results shown in bold. .

The complete data set was again analysed by Kaplan-Meier, using median methlyation as a cut-off and the results substantiated those of the Cox regression model with *SIX1* not influencing PFS or OS (log rank test of survival in both groups p=0.734 and 0.347 respectively). The PFS in the low methylation group was 11.5 months and in the high methylation group was 11 months. The results are depicted in Figure 50.

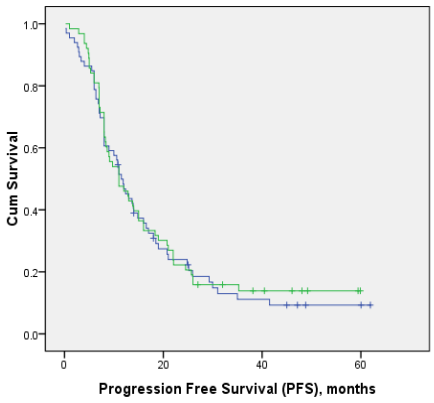


Figure 50. Kaplan-Meier graph showing the effect of *SIX1* methylation on PFS in 140 EOC tumours, in months, p=0.734 (log rank test, median was used as the cut-off)). Blue low methylation, green high methylation.

OS in the low methylation group was 33.7 months and in the high methylation group was 27 months. This was not statistically significant and is a further confirmation of the conflicting univariate analysis – namely that there is unlikely to be a significant

relationship between *SIX1* methylation and overall survival. The Kaplan Meier plots are shown in figure 51 below:

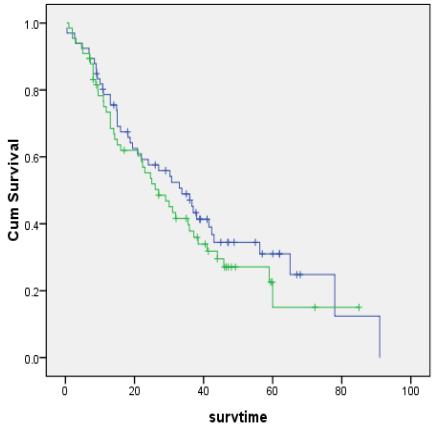


Figure 51. Kaplan-Meier showing the effect of *SIX1* methylation on OS in 140 primary EOC tumours, in months, p= 0.347 (log rank test, median was used as the cut-off)). Blue low methylation, green high methylation.

5.3.4 CpG Methylation of KIAA1383 (21G5) in primary ovarian cancer tumours

For *KIAA1383* the level of background methylation in the test set was 5.5%. Mean methylation of the samples was 31.81% and 20% of samples had methylation of > 50%. It was therefore investigated in the second part of the test set where similar results were seen with methylation in normal of 6% and average methylation in the samples of 32.8% with methylation of >50% in 20% samples. The validation set was therefore analysed in terms of methylation of this candidate. Here methylation in normal was 4.5% and the average methylation in the samples was lower at 22.12% with only 1% of samples showing methylation of 50%. The results are illustrated in figure 52 a-c below.

As before for the initial data set results for individual CpGs are shown whereas for the second two figures average results across 3 adjacent CpGs are shown.

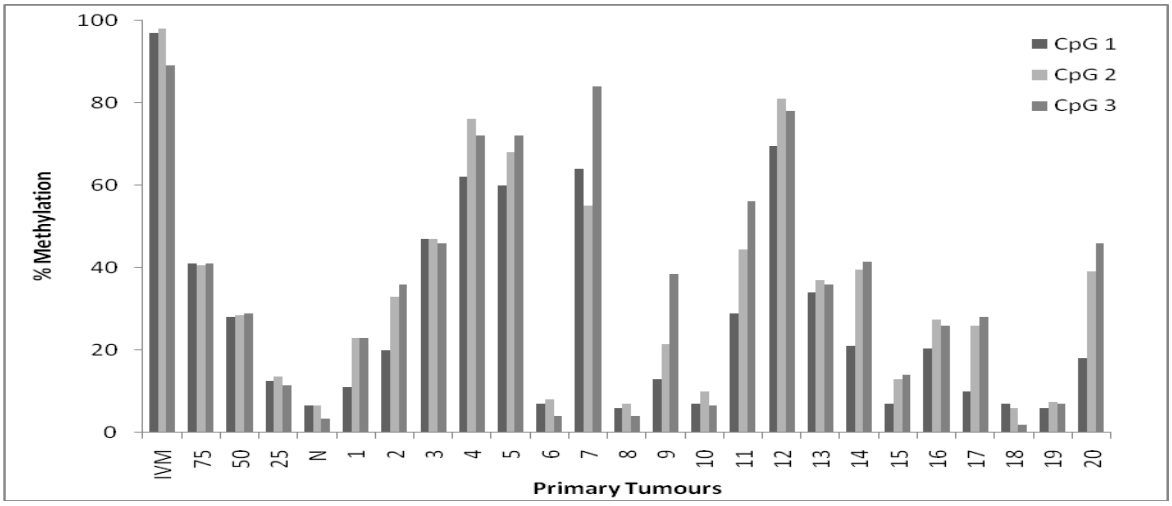


Figure 52a. Methylation of *KIAA1383* in test set (I) of primary EOC tumours by pyrosequencing. Each colour represents an individual CpG. IVM *in vitro* methylated DNA, 75,50 and 25 serial dilutions of IVM with N. N normal whole male genomic DNA.

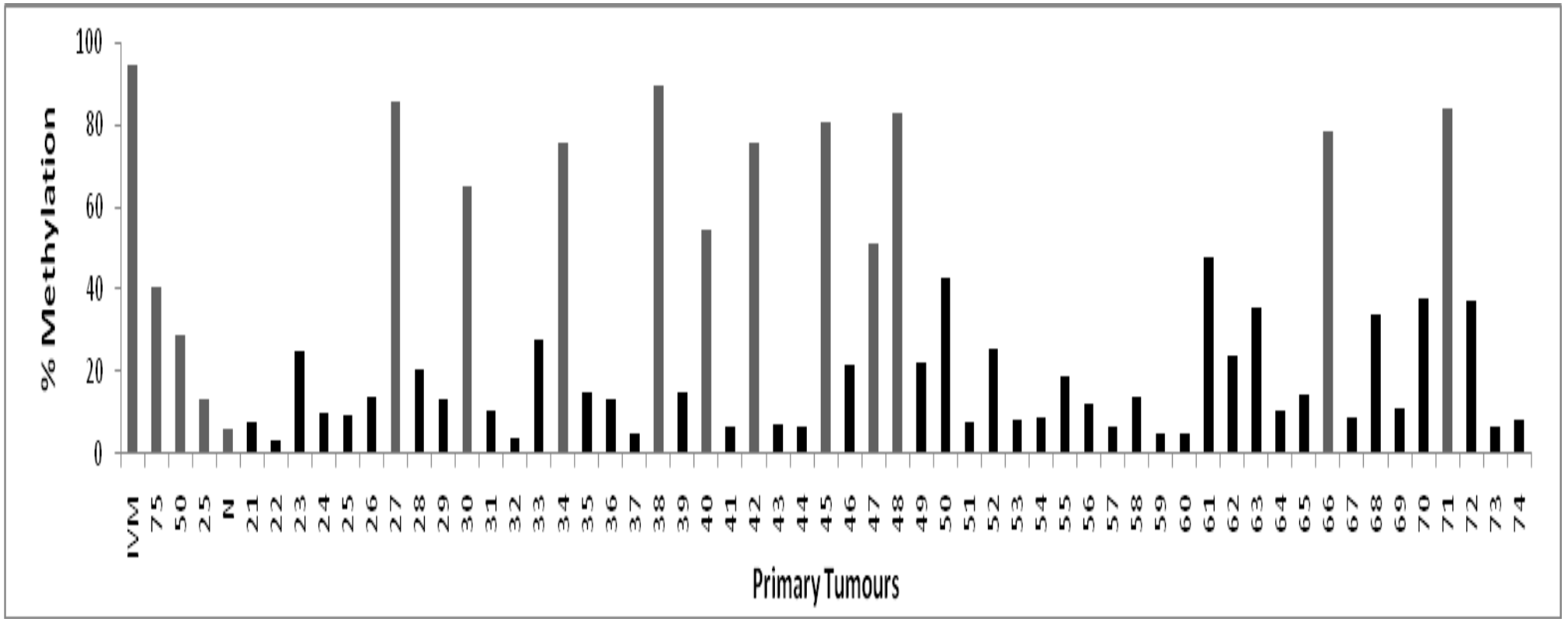


Figure 52b. Methylation of *KIAA1383* in test set (ii) of 54 primary EOC tumours by pyrosequencing. Bars represent 3 adjacent CpG residues. Grey bars: controls and tumours with methylation >≥50%. Black bars: tumours with <50% methylation. IVM *in vitro* methylated DNA, N normal male genomic DNA, 75,50 and 25 represent serial dilutions of positive and negative control.

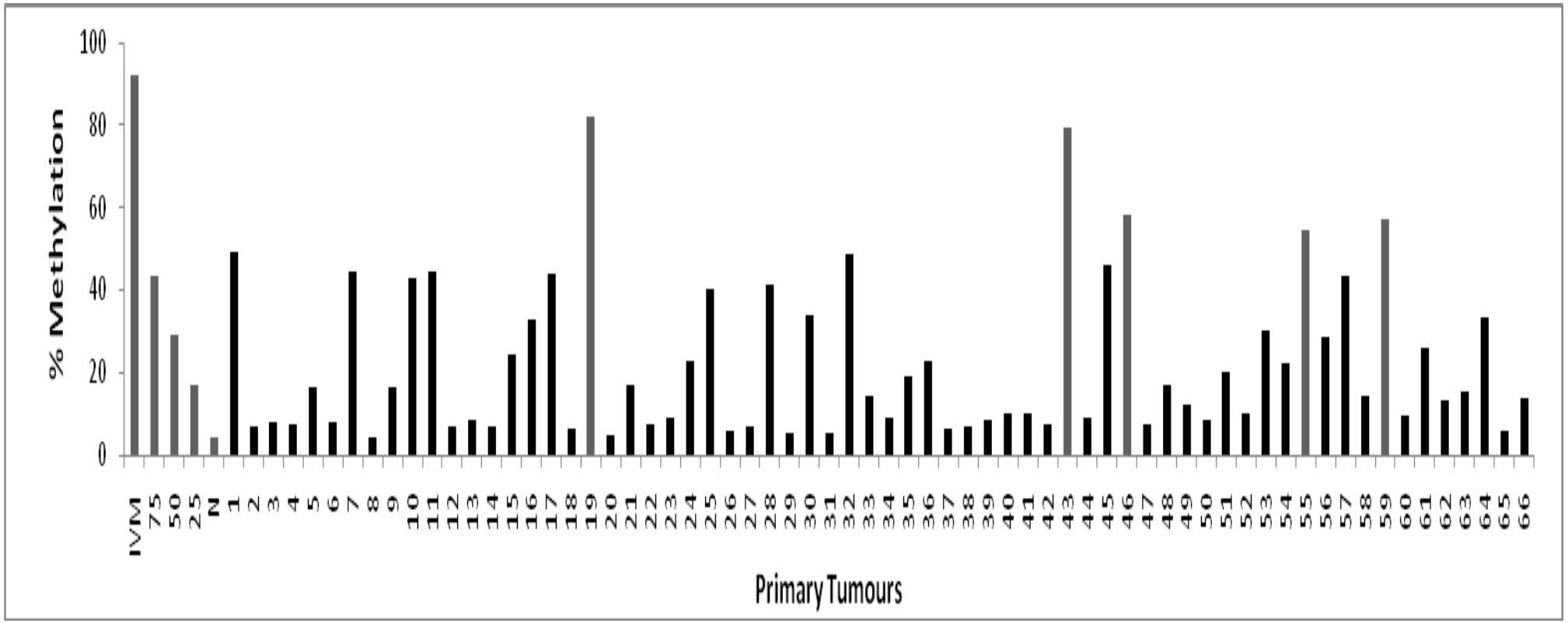


Figure 52c. Methylation of *KIAA1383* in the validation set of 66 primary EOC tumours by pyrosequencing. Bars represent 3 adjacent CpGs. Grey bars: controls and tumours with methylation >≥50%. Black bars: tumours with <50% methylation. IVM *in vitro* methylated DNA, N normal male genomic DNA, 75,50 and 25 represent serial dilutions of positive and negative control.

5.3.5 Effect of methylation of KIAA1383 (21G5) on response to first line chemotherapy

The combined data set was analysed using logistic regression according to the different possible groupings by radiological response; again no statistically significant relationship was seen between methylation of this locus and response to chemotherapy and as explained before this maybe due to underpowering of the analysis. Similarly as before it is possible for a biomarker to be of prognostic value but this not to be attributable to the response to first line treatment and we therefore went ahead with the survival analysis as outlined in the next sub-section. The results for response to treatment are shown in table 32 below:

Table 32. Relationship between *KIAA1383* methylation and response (by RECIST).

	Methylation level (*KIAA1383*) %		Logistic regression model		
	Responders	Non-responders			
	(Mean±SD)	(Mean±SD)	OR	95% CI	p
CR+PR+SD (n=73) vs. PD (n=12)	26.8±23.4	24.9±17.1	1.00	(0.97, 1.03)	0.785
CR+PR (n=59) vs. PD (n=12)	27.2±23.1	24.9±17.1	1.00	(0.97, 1.03)	0.734
CR+PR (n=59) vs. SD+PD (n=26)	27.2±23.1	24.9±21.7	1.00	(0.98, 1.01)	0.652

OR: odd ratio (OR>1 the patients with increased methylation are more likely to have poor response to first-line chemotherapy);
95% CI: 95% confidence interval; p value: Wald test

5.3.6 Effect of methylation of KIAA1383 (21G5) on patient survival

Survival data was available for the majority of the patients. As before, the test and validation sets were analysed for the correlation between methylation and PFS, OS using univariate Cox model, and then adjusted by stage and grade in multivariate Cox model, respectively. The associated between methylation with response was analysed using univariate logistic regression model (see methods Chapter 2.25.3).

In addition to the statistically significant correlation with response to chemotherapy a statistically significant relationship was seen between methylation of *KIAA1383* and PFS

in the test set (two-sided score test p=0.027, HR 0.987, 95% CI 0.975-0.999). This was confirmed in the validation set where a 'p' value of 0.009 was seen (HR 0.980, 95% CI 0.965-0.995). In the test set no relationship was seen between *KIAA1383* methylation and overall survival (two-sided score test p=0.833, HR 0.999, 95% CI 0.986-1.012) however a statistically significant relationship between methylation and OS was seen in the validation set (two-sided score test p=0.011, HR 0.976, 95% CI 0.961-0.995). A summary of the statistics is shown in table 33 below:

Table 33 Summary of statistics for univariate analysis of *KIAA1383* methylation and survival

	P value	HR	95% confidence Interval
PFS test set (n=65)	**0.027**	**0.987**	**0.975-0.999**
PFS validation set (n=64)	**0.009**	**0.980**	**0.965-0.995**
OS test set (n=65)	0.833	0.999	0.986-1.012
OS validation set (n=64)	**0.011**	**0.976**	**0.961-0.995**

*P value is two-sided score test of the univariate Cox model. HR: hazard ratio. HR>1 indicates the increased risk of disease progression/death associated with the increase of methylation. HR<1 indicates the decreased risk of disease progression/death associated with the increase of methylation. Statistically significant results shown in bold. Survival data available for 113 of 140 patients.

As before, histological types, stage, age and grade were all integrated with *KIAA1383* methylation into a multivariate analysis. Histological type (p=0.022) with endometriod tumours reflecting a better PFS (two-sided score test p=0.013, HR 0.364, 95% CI 0.164-0.811), stage (two-sided score test p=<0.0001, with patients with stage 3 and 4 tumours having a worse PFS; Stage 3 two-sided score test p=0.002, HR 4.826, 95% CI 1.757-13.255; Stage 4 two-sided score test p=0.001, HR 5.965, 95% CI 2.046-17.393) and grade (two-sided score test p=0.048) were all shown separately to have a statistically significant impact on PFS but when integrated into the model together only *KIAA1383* methylation had a significant 'p' value (two-sided score test p=0.039, 95% CI 0.979-0.989). Thus, *KIAA1383* methylation is significantly associated with PFS independent of histological type, stage, age and grade.

Next the same factors were examined in terms of OS. Stage was the only factor to have a statistically significant influence on OS (two-sided Wald test p=0.010) with patients with stage 3 and 4 tumours having a poorer OS (stage 3 HR 2.219, stage 4 HR 2.409 although the 'p' values were not significant).

Kaplan-Meier analysis of all 140 tumours confirmed a statistically significant relationship between PFS and *KIAA1383* methylation with patients with less methylation having a worse outcome (p=0.012 log rank test, median as cut-off), low methylation=10 months, high methylation 14 months). This is shown in Figure 53 below:

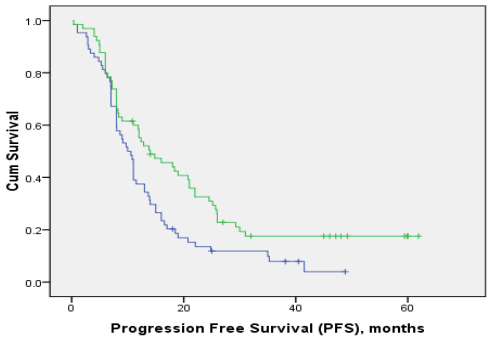


Figure 53. Kaplan-Meier graph of *KIAA1383* methylation and PFS (p=0.012). (log rank test, median was used as the cut-off). Green high methylation, Blue low methylation, Cum survival Cumulative survival.

Although patients with less *KIAA1383* methylation also had a worse outcome in terms of OS (35.9 vs. 22.3 months) this was not statistically significant (p=0.159 log rank test, median as cut-off). This is shown in Figure 54 below:

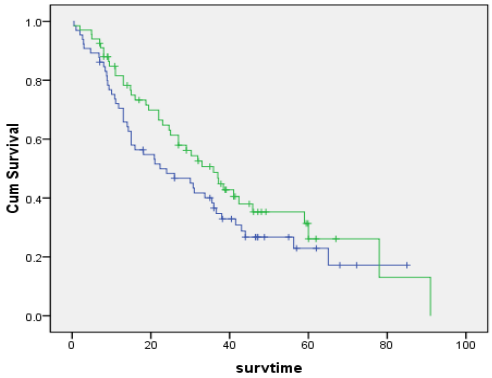


Figure 54. Kaplan-Meier of Methylation of *KIAA1383* and OS (p=0.159)
('p' = log rank test, median was used as the cut-off). Green high methylation, Blue low methylation, Cum survival Cumulative survival.

5.4 Discussion

A smaller list of genes was generated when comparing the two *in vivo* derived cell lines by MLDA.

1E7 maps to *FAM7A2*. This gene is located on chromosome 15. Its biological function is not known and it is not seen to be expressed in Genenote or eNorthern databases (www.genecards.org/cgi-bin/carddisp.pl?GENE=FAM7A2). The results in this thesis demonstrated an increase in methylation between PEA1 and PEA2 but no methylation in primary tumours.

85B2 maps to *LOC113230*. Again there is no published literature on this gene and its function is unknown other than it is located on chromosome 19 and noted to be expressed in some cancer cell lines using the SAGE database (www.genecards.org/cgi-bin/carddisp.pl?gene=*LOC113230*). The CGI at *LOC113230* is highly methylated in sensitive and resistant cell lines.

21G5 maps to *KIAA1383*, a hypothetical protein located on chromosome 1. It is a small gene which just spans one exon. Expression has been noted in some tissues using Genenote and eNorthern databases although of note none of the databases report expression in normal or cancerous ovarian tissue or cell lines (www.genecards.org/cgi-bin/carddisp.pl?gene=*KIAA1383*). There are no publications relating to this hypothetical protein in the literature although in one publication it was noted in a list of genes shown to be expressed in response to the histone methyltransferase inhibitor DZnep (Miranda, Cortez et al. 2009). This drug has recently been shown to suppress histone methylation globally having initially been thought to be selective for the H3K27 and H4K20 marks. This raises the question as to whether histone methylation in addition to DNA methylation is required.

5.5 **Conclusion**

SIX1 and *KIAA1383* were examined in primary tumours as they had shown increased methylation in the resistant cell lines. Encouragingly this increased methylation was not unique to the *in vivo* cell lines they originated from but was also seen in the A2780 cell lines.

In the primary tumours they showed the heterogeneous pattern that we hypothesised we would see if a gene was playing a role in ovarian cancer pathogenesis. However *SIX1* methylation did not correlate with response to chemotherapy or prognosis. Encouragingly though increased methylation of *KIAA1383*, by pyrosequencing, showed a significant correlation with improved PFS and in addition a correlation with improved OS was seen in the validation set.

As was the case for *SP5* though the direction of methylation was in the 'opposite direction' to that which would be predicted by the cell line analysis i.e. an increase in methylation conferred an advantage for the patient. As outlined previously this can be explained biologically by the fact methylation +/- silencing of a gene could have a different impact at the outset of disease compared to later in the disease course.

Given the statistically significant correlations with both response to chemotherapy and PFS it seems likely that *KIAA1383* is a biologically important gene, and a potential prognostic biomarker, however almost nothing is known about it. It is highly conserved among species however other than its amino acid sequence and the low levels of expression seen using microarray experiments it is almost an entirely unknown candidate (http://www.genecards.org/index.php?path=/Search/keyword/*KIAA1383*).

Again here we have hypothesised that a decrease in methylation is resulting in an increase in expression but this needs to be confirmed. Unfortunately I had difficulty examining this locus by qRTPCR. It is unclear whether this is because this gene is not expressed or if it is a problem with the primer design (as the gene only spans one exon). I did however manage to perform an overexpression experiment and the results are discussed in Chapter 6.

Methylation of *SIX1* (17G11) did not correlate with improved response to chemotherapy and consistent results were not demonstrated in terms of prognosis either. It was therefore not selected for further characterisation in Chapter 6, in terms of functional experiments, such as knockdown or over-expression, but it was investigated in the independent arrays described in Chapter 7.

It was not possible to optimise primers to investigate the methylation of 66G6 by pyrosequencing and given the difficulty in quantifying methylation by MSP demonstrated thus far in the thesis a pragmatic decision was taken not to proceed further with investigation of this loci. The difficulty in optimising primers was related to the extreme density of CpGs within the CpG island which made it difficult to position primers. That said it should be noted that it did appear to be an interesting candidate within the A2780 cell lines (the in vivo cell lines were not available at the time) by MSP and perhaps further investigation of its expression by qRTPCR, as a first step, is warranted.

In conclusion, if one excludes 66G6 as it was not characterised in the primary tumours due to assay limitations, this chapter has demonstrated that using the *in vivo* derived cell lines to identify genes resulted in two of three candidates examined showing a correlation with PFS or OS.

MLDA had originally identified six loci mapping to 4 genes containing CGIs and therefore this was felt to be very encouraging, as there was a high correlation between loci

identified and number correlating with survival. Interestingly though it was higher methylation for both candidates that correlated with improved survival for patients and this at first glance is counter intuitive given that the loci were identified by MLDA because they showed increased methylation in the resistant cell lines. This is possible to explain biologically though in that it is very possible for methylation of a gene to have a different phenotypic effect at diagnosis compared to at relapse. For example methylation of BRCA1 can silence this gene (Teodoridis, Hall et al. 2005), resulting in a higher risk of ovarian cancer (Thompson and Easton 2002) but that same methylation later may result in an improved response to platinum agents and PARPi and hence perhaps a better PFS (Konstantinopoulos, Spentzos et al. 2010). This has been presented at ASCO this year, and reported in abstract form, in both the platinum-sensitive (J. A. Ledermann 2011) and platinum-resistant relapse setting (Birrer 2011).

6 Functional analysis of *SP5* and *KIAA1383*

6.1 Correlation between mRNA expression and DNA methylation OF *SP5* in cell lines by qRTPCR

The aim of the experiments described in this subchapter were to investigate whether increased methylation at the promoter of *SP5* resulted in decreased expression of the gene; in cell lines initially and then in tumours. Various mechanisms exist of down regulating gene expression so it was expected that expression may still be seen despite a change in methylation and that a change in expression could be seen without a change in methylation - however an increase in expression would not be predicted where an increase in methylation was seen.

The results in a panel of sensitive and resistant cell lines are shown in Figure 55 below. For A2780 sensitive cell lines (A2780, A2780 p3 and A2780 p6) vs. A2780 resistant cell lines (A2780 cp70, MCP1, MCP6. MCP9) a relative increase in methylation was seen to correlate with a decrease in *SP5* expression. For PEO1 and PEO4, one of the *in vivo* derived cell lines, no change in methylation was seen between the two cell lines but there was a decrease in expression. Between the PEO14 and PEO23 pairs the level of methylation remained high and a smaller impact on the already low levels of expression is seen. Results are shown with the y axis to the Log10.

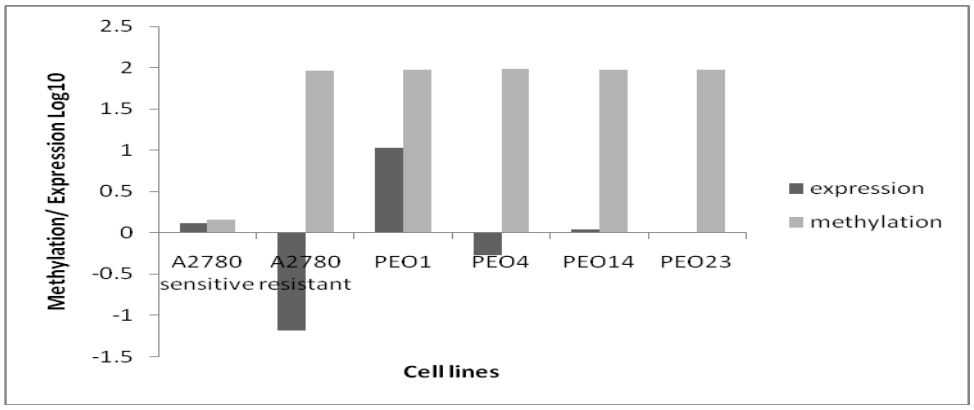


Figure 55. Effect of altered methylation of *SP5* on mRNA expression. A2780 sensitive (A2780, A2780 p6, A2780 p6), A2780 resistant (A2780 cp70, MCP 1, MCP6, MCP9), PEO1 (sensitive), PEO4 (resistant), PEO14 (sensitive), PEO23 (resistant). Methylation by pyrosequencing of bisulphite modified DNA (grey), expression by qRTPCR normalised to *GAPDH* and relative to A2780 (delta delta CT), (black). Experiments performed in duplicate.

As a decrease in expression was confirmed in the A2780 resistant cell lines the relationship between methylation and expression was next investigated in a panel of primary tumours for which DNA and RNA were both available.

6.2 Correlation between mRNA expression and DNA methylation OF *SP5* in primary ovarian cancer tumours by q RTPCR

The aim of this experiment was to establish if the same correlation between increased methylation and decreased expression (and decreased methylation and increased expression) was seen in primary ovarian cancer tumours. Figure 56 below demonstrates that for most samples this is the case.

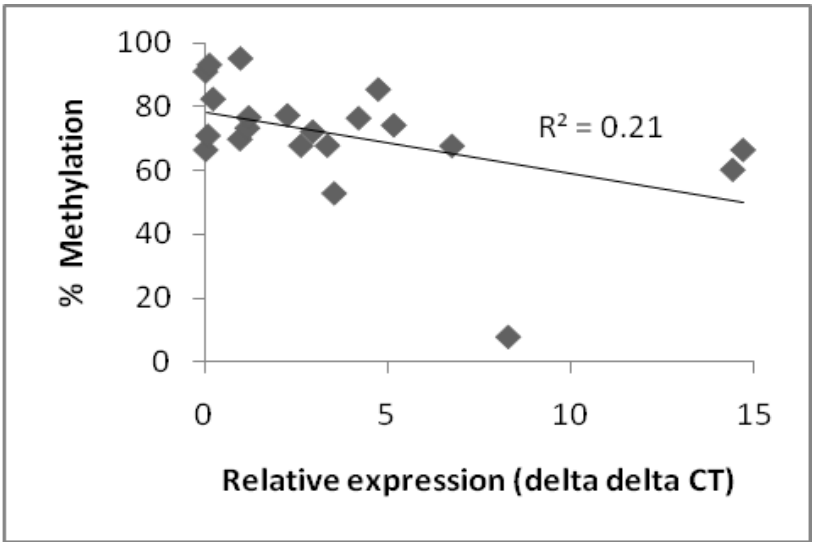
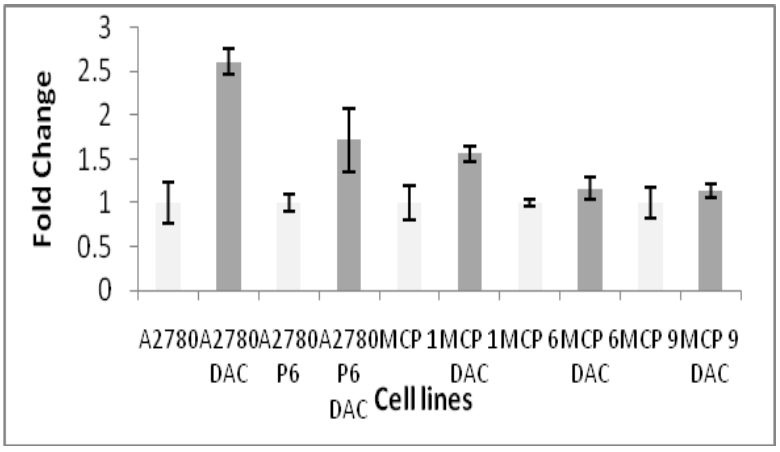


Figure 56. Scatter plot illustrating correlation between m RNA expression by qRTPCR and methylation by pyrosequencing for *SP5*. Diamonds represent each ovarian cancer tumour (n=21). RTPCR with triplicates, pyrosequencing duplicates.

As stated before given that DNA methylation is only one means of suppressing the expression of a gene these results were seen as very encouraging and lent weight to the hypothesis that decreased methylation of *SP5* could result in increased expression of *SP5* and a downstream phenotypic effect.

6.3 Re-expression of *SP5* in cancer cell lines following decitabine treatment

In order to address whether expression of *SP5* was related to the methylation status, cells were treated with a demethylating agent, decitabine (DAC), and RNA extracted. It was hypothesised that if methylation was relevant that treatment with a demethylating agent would result in re expression of the gene. The results are shown in Figure 57 below. For each of the cell line pairs an increase in expression is seen as a result of treatment with the demethylating agent although this was not statistically significant. Results of the student t-tests for each pair are shown to the right of the figure.



Cell line	Student t-Test
A2780 +/- DAC	0.09
P6 +/- DAC	0.24
MCP 1 +/- DAC	0.07
MCP 6 +/- DAC	0.19
MCP 9 +/- DAC	0.90

Figure 57. Relative Expression of *SP5* following decitabine treatment. Error bars show standard deviation. Light grey untreated cell lines, dark grey Decitabine (DAC) treated cell lines (DAC 0.5 μM for 4 days with daily media change). Two-sided Student t-test results shown to right.

6.4 Optimisation of siRNA knockdown of *SP5* in PEO14 and PEO23

In the next experiment I wished to assess whether knockdown of *SP5* had a phenotypic effect. I first planned to knock down *SP5* using siRNA and then perform an MTT experiment, and the first step was to optimise the conditions for this experiment; the results of which are now briefly presented.

Three siRNAs targeting expression of *SP5* were tried at two concentrations; 5 nM and 10 nM. The 2nd siRNA appeared to produce the best knockdown and as the results were similar between the 5 and 10 nM concentrations experiments were carried out using 5 nM to reduce the risk of off- target effects. The same results were seen for PEO14 (figure 58a) and PEO23 (Figure 58b). The effectiveness of the *SP5* knockdown was assessed by qRTPCR (delta delta CT) using the Taqman *SP5* primers normalised to *GAPDH*. The Allstars® scrambled control was used for relative quantitation. All experiments were performed in triplicate.

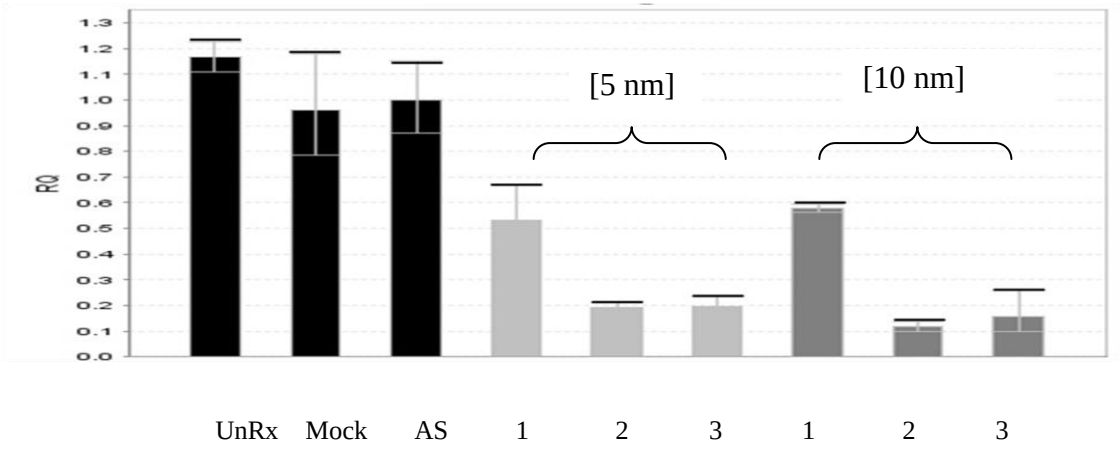


Figure 58a. Knockdown of *SP5* in PEO14 by siRNA measured by qRTPCR. UnRx untreated, Mock transfection reagent only, AS Allstars® scrambled control, 1-3 different siRNA's. Controls black, [5 nm] light grey, dark grey [10 nm].

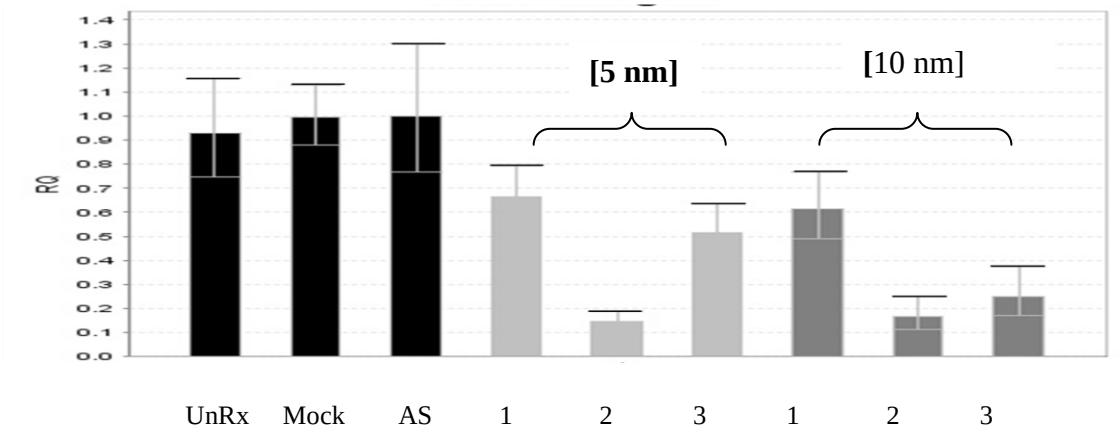


Figure 58b. Knockdown of *SP5* in PEO23 by siRNA measured by qRTPCR. UnRx untreated, Mock transfection reagent only, AS Allstars® scrambled control, 1-3 different siRNA's. Controls black, [5 nm] light grey, dark grey [10 nm].

The next experiment assessed the duration of *SP5* knockdown at mRNA level, again by taqman qRTPCR. Knockdown of *SP5* was observed from 24-96 hours in PEO14 and PEO23. In each experiment *SP5* knockdown is expressed relative to the scrambled control (delta delta CT). The results are shown in Figures 59a-d below.

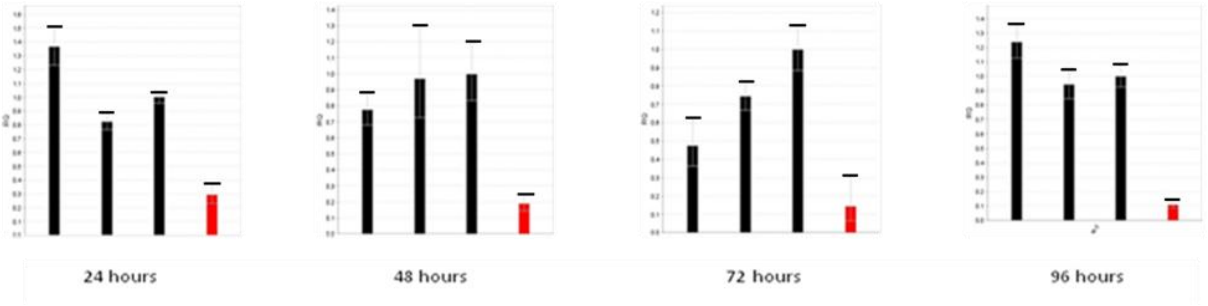


Figure 59a. *SP5* normalised to GAPDH in PEO14: 24-96 Hours. (For legend see 59d).

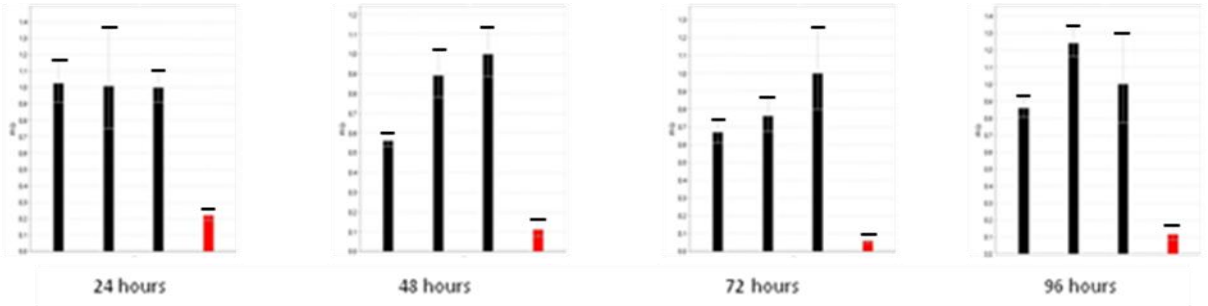


Figure 59b. *SP5* normalised to GAPDH in PE023: 24-96 Hours. (For legend see 59d).

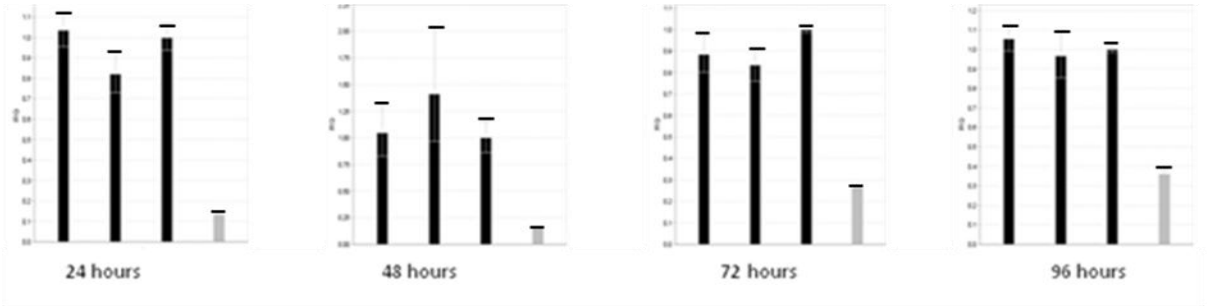


Figure 59c. *MAPK* normalised to GAPDH in PEO14: 24-96 Hours (For legend see 59d).

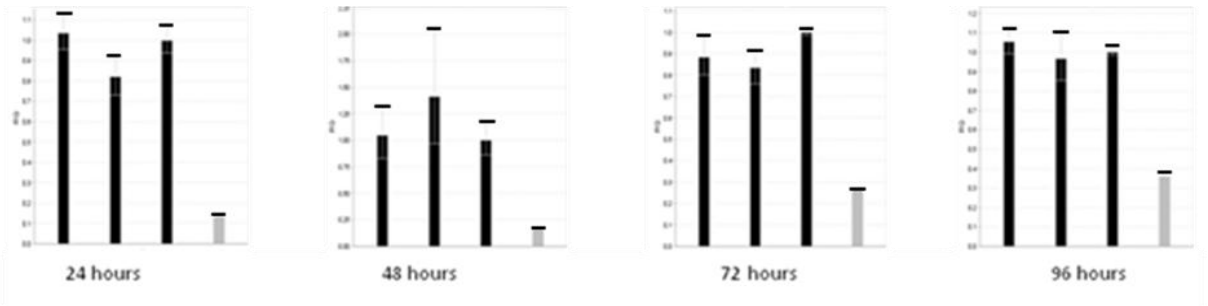


Figure 59d. *MAPK* normalised to GAPDH in PEO23 24-96 Hours. Controls black, *MAPK* grey. *SP5* Red UnRx untreated, Mock transfection reagent only, AS Allstars® scrambled control Results normalised to *GAPDH* & expressed relative to Allstars® scrambled control (delta delta CT).

6.5 **Effect of siRNA knockdown of *SP5* on chemosensitivity using the MTT assay**

Given that increased methylation appeared to correlate with decreased expression in the cancer cell lines and tumours it was hypothesised that this decreased expression could confer chemoresistance. In order to address this question *SP5* was knocked down by siRNA and then the cells were replated into a 96 well format in a variety of conditions and an MTT experiment performed where each condition was treated with five increasing concentrations of cisplatin (4X below IC_{50}, 2X below IC_{50}, IC_{50}, 2X above IC_{50} and 4X above IC_{50}).

The actual doses of cisplatin used for each cell line in the MTT part of the experiment are shown in table 34 below. These had been recently redetermined by a collegue (Dr Rizzo) for another study and I therefore did not repeat these experiments.

Table 34. Actual doses of cisplatin in μM which corresponded with each of the IC_{50} values

	PEO14	PEO23
IC_{50} X4	7.36	33.0
IC_{50} X2	3.68	16.5
IC_{50}	1.84	8.25
IC_{50}/2	0.92	4.13
IC_{50}/4	0.46	2.06

Experiment 1.

The first part of the experiment was to perform siRNA transfections in both cell lines, knocking down *SP5*, *MAPK* (positive control), and including the Allstars® negative control. This was performed as described in Chapter 2.18; initially $25x10^4$ cells were seeded in T25 flasks using the manufacturer's recommended quantities of transfection reagent and the optimised amount of siRNA. However after 48 hours most of the cells in the *SP5* knockdown flask were noted to have died. Efficient transfection (>85%) of *MAPK*

and the Allstars® scrambled control was confirmed by taking lysates and making RNA and subsequent cDNA and performing qRTPCR. There were not enough cells in the *SP5* flask to perform the qRTPCR.

Experiment 2

The reason for the especially low number of cells in the *SP5* flask was unclear however it was presumed that the transfection mastermix was too toxic and therefore the experiment was repeating using 50% of the recommended quantities of the transfection reagent. Again at 48 hours there were noticeably less cells in the *SP5* knockdown flask compared to the others. Again efficient transfection was confirmed by qRTPCR. It was therefore hypothesised that it was not necessarily the knockdown itself that was toxic (as the effect was so much more dramatic in the *SP5* knockdown flask) but that perhaps the *SP5* knockdown caused increased cell death (or less likely decreased proliferation).

Experiment 3-6

In order to increase the yield of cells at the end of the experiment (for the subsequent MTT) the next experiment was performed seeding $75x10^4$ cells into T75 flasks. Again there was a very high level of cell death in the *SP5* knockdown and not enough cells were left to continue the experiment. The last 3 experiments were performed in T75 flasks using 50% of the manufacturers recommended mastermix of transfection reagent and siRNA and using 3 flasks for the *SP5* knockdown and one for each of the others. For all experiments a high knockdown was consistently seen by RTPCR (≥90% for *SP5* and ≥85% for *MAPK*). Photographs of the cells in each of the flasks prior to trypsanisation for the various conditions for one of the experiments in PEO14 are shown in Figure 60 below.

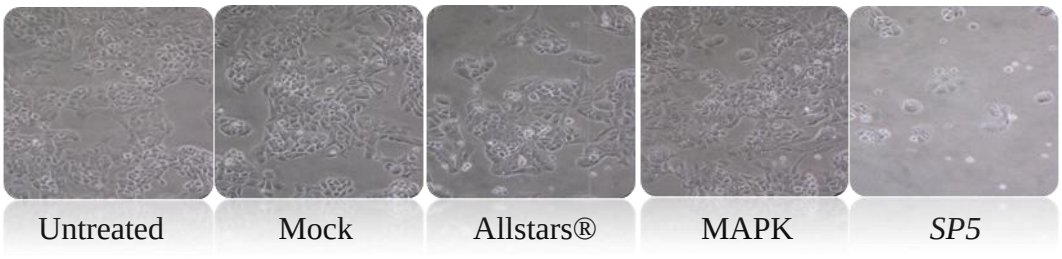


Figure 60. Photographs of cells in flasks representing each of the transfection conditions. Photos all taken at 72 hours.

In total, the experiment was performed five times in two cell lines (PEO14 and PEO23) using the smaller quantities of transfection reagent and on each occasion the number of cells alive at the end of the experiments in the *SP5* treated flask was much less. Dead cells in the media were washed off and after trypsanisation cells were counted, using the haemocytometer and the average percentage of *SP5* cells, compared to scrambled control for the 10 experiments is shown in Figure 61 below. Consistently there were >50% less cells in the *SP5* knockdown flasks than in the scrambled control. No formal test of viability was performed.

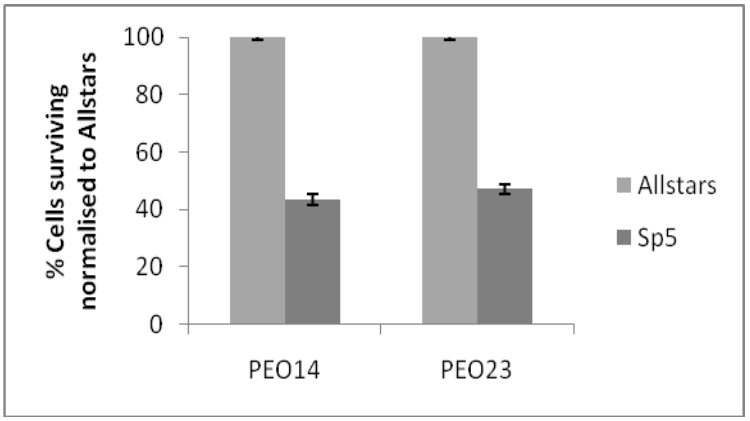


Figure 61. Assumed viable cell number expressed as percentage of *SP5* cells compared to scrambled control (Allstars®). Error bars show standard deviation over 5 experiments in each cell line.

After 48 hours cells were trypsinised and counted (and a lysate taken for later confirmation of knockdown by RTPCR) then reseeded onto 96 well plates for the MTT part of the

experiment. The plates were left for 24 hours to allow cells to adhere and then the varying doses of drug were added to the appropriate wells as shown for PEO23 in Figure 62 below:

Plate 1 of 2

	Untreated			Mock			All stars			*MAPK*	
B	0.0	4.13	16.5	0.0	4.13	16.5	0.0	4.13	16.5	0.0	4.13
B	0.0	4.13	16.5	0.0	4.13	16.5	0.0	4.13	16.5	0.0	4.13
B	0.0	4.13	16.5	0.0	4.13	16.5	0.0	4.13	16.5	0.0	4.13
B	0.0	4.13	16.5	0.0	4.13	16.5	0.0	4.13	16.5	0.0	4.13
	2.06	8.25	33.0	2.06	8.25	33.0	2.06	8.25	33.0	2.06	8.25
	2.06	8.25	33.0	2.06	8.25	33.0	2.06	8.25	33.0	2.06	8.25
	2.06	8.25	33.0	2.06	8.25	33.0	2.06	8.25	33.0	2.06	8.25
	2.06	8.25	33.0	2.06	8.25	33.0	2.06	8.25	33.0	2.06	8.25

Plate 2 of 2.

MAPK	*SP5*										
16.5	0.0	4.13	16.5								
16.5	0.0	4.13	16.5								
16.5	0.0	4.13	16.5								
16.5	0.0	4.13	16.5								
33.0	2.06	8.25	33.0								
33.0	2.06	8.25	33.0								
33.0	2.06	8.25	33.0								
33.0	2.06	8.25	33.0								

Figure 62. Layout for two plates for each MTT Experiment. Each condition shown in a different shade of grey. B is blank (no cells), untreated (cells in media only), Mock (includes transfection reagent). Numbers in each box represent the dose of cisplatin in µM.

After 24 hours the media +/- drug was removed and warm fresh media added. 48 hours later the MTT was added as described in chapter 2.20. The plate was read and the value given for each well represented the amount of proliferation in that well. The average of each of the quadruplicates was calculated. Next the value obtained from the wells which contained no cells (blanks) was removed from the other values. Then the mock cells were given a value of 1 and the other conditions converted to a number relative to this.

Consistently the MTT experiments showed the lowest proliferation for the *SP5* knock down cultures and there was only a small effect on proliferation with increasing dose of

cisplatin– examples of results obtained from the MTT experiments for PEO14 and PEO23 are shown below in Figure 63a & b:

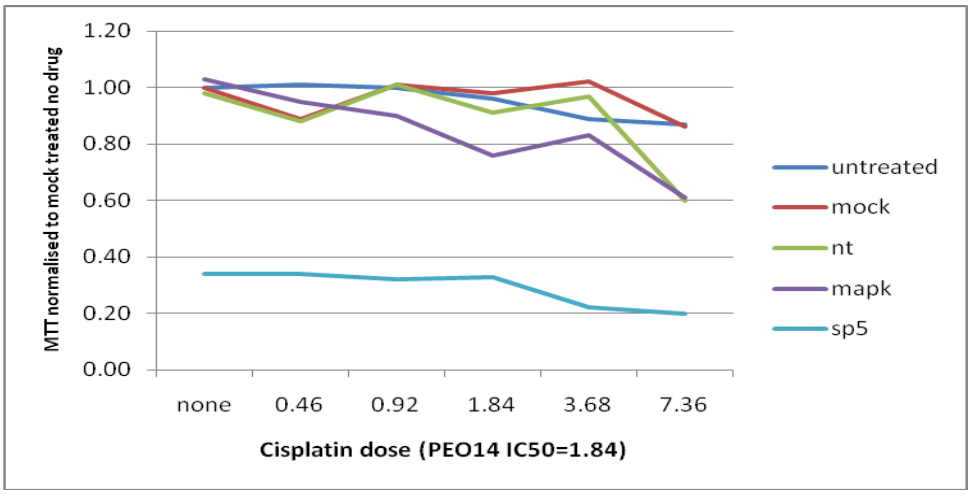


Figure 63a. Effect of a range of concentrations of cisplatin on proliferation of PEO14 under different experimental conditions, as measured by MTT (Figure represents n=1).

The same results were obtained for PEO23. The proliferation of *SP5* was again much lower for the *SP5* knockdown cells including in the samples treated with no drug and increasing the drug concentration appeared to have little effect, especially in the *SP5* knockdown cells.

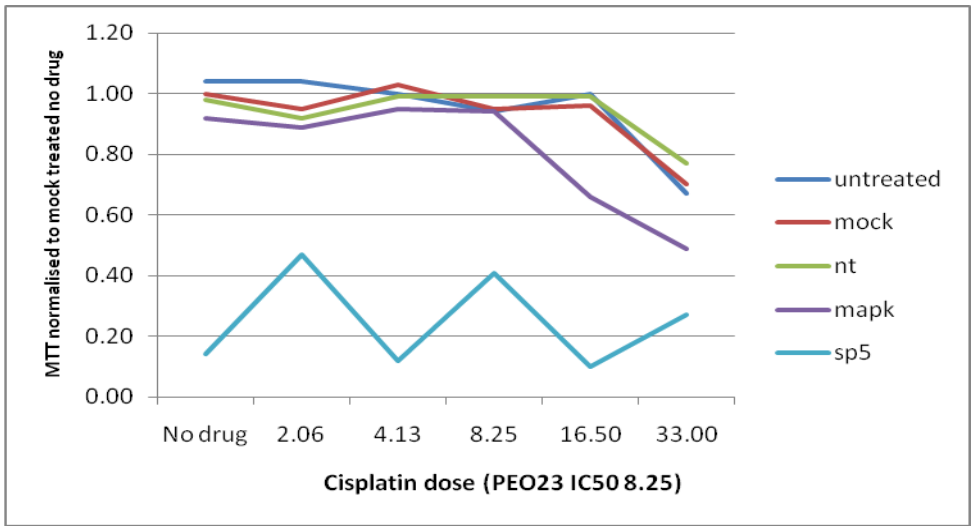


Figure 63b illustrating effect of a range of concentrations of cisplatin on proliferation of PEO23 under different experimental conditions, as measured by MTT (Figure represents n=1).

In order to assess whether the timescale of the transfection had affected chemsensitivity the experiment was repeated using a delayed transfection. Again the effect of increasing cisplatin dose was not seen to have a consistent effect on proliferation (data not shown).

Analysis of the graphs above indicates that it is likely that the cisplatin was not at a high enough dose however as knockdown of *SP5* expression appeared to reduce cell number it was not going to be possible to interpret the MTT assay. As the phenotypic effect in the *SP5* cells could be secondary to decreased proliferation or increased apoptosis an alternative approach was taken; namely to assess whether decreased expression of *SP5* resulted in induction of apoptosis.

6.6 Effect of siRNA knockdown of *SP5* on apoptosis using caspase-Glo® 3/7

In order to address whether knockdown of *SP5* resulted in increased apoptosis simultaneous experiments were performed where the impact of *SP5* on apoptosis was measured by induction of caspase 3 and 7 via caspase-Glo®, whilst normalising for cell proliferation (using MTT). As the biggest difference in methylation and expression had been in the A2780 series these experiments were initially performed in the A2780 cell line.

On this occasion transfection was performed directly into the 96 well plates as described in chapter 2.20. Cells were also kept for later lysis, RNA extraction, reverse transcription and assessment of knockdown and in addition cells were fixed and stained with crystal violet in an attempt to assess the effect of *SP5* knockdown on cell number. Experiments were repeated on three separate occasions and in each experiment triplicates were performed. The same controls were used as previously; namely untreated, mock, Allstars® scrambled

siRNA, *SP5* siRNA and *MAPK* siRNA. In the second two experiments an additional control for apoptosis, of cisplatin at a dose of 5 nm, was used. Experiments were performed at 24, 48 and 72 hours.

On each occasion the ability of *SP5* to induce apoptosis was maximal at 24 hours. This was despite a smaller degree of knockdown of *SP5* by qRTPCR and a less noticeable phenotypic effect on cell number by microscopy, than in previous experiments. Figure 64 below show the induction of caspase normalised for proliferation via the MTT assay. Results were all expressed compared to the Allstars® scrambled control.

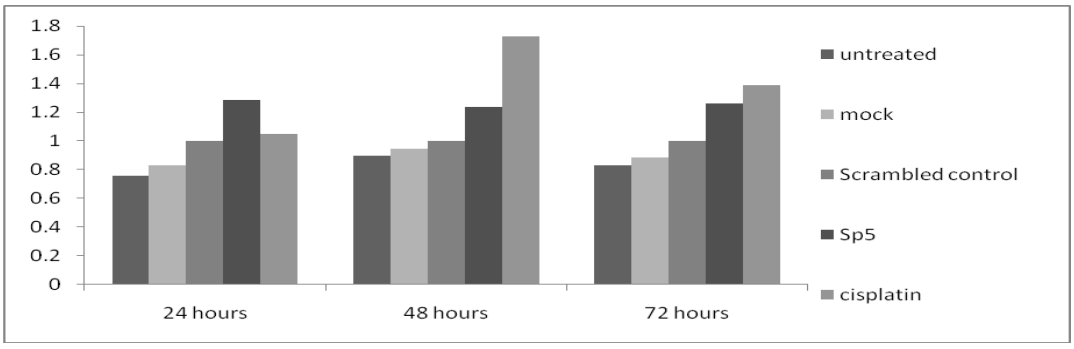


Figure 64. Induction of caspase following *SP5* knockdown in A2780. Samples normalised to MTT and then to Allstars® scrambled control (n=2 all conditions, n=3 for first 4 conditions ie cisplatin control not used in first set of experiments).

These results show maximal induction of apoptosis in the *SP5* knockdown at 24 hours. Cisplatin 5 µM was used as a positive control for the assay and as expected maximal apoptosis, as a result of platinum, was later at 48 hours. Cisplatin was causing apoptosis when we expected and we therefore believed these results to be credible and were therefore encouraged to see a 20% increase in apoptosis in the *SP5* knockdown. As the experiment was only performed twice with the full set of conditions, i.e. including the cisplatin controls error bars were not added but the results were seen to be consistent in all experiments and encouraged us to go further forward with characterisation of this locus.

6.7 Effect of over expression of *KIAA1383* on cell cycle in the presence of cisplatin

The effect of transient over expression of *KIAA1383* was examined in PEA2. *KIAA1383* was obtained already cloned into the pFN21A vector. Vector only control and *KIAA1383* were treated with 25 µM cisplatin 48 hours after transfection and the effect on the cell cycle analysed 24 hours later following addition of the fluorescent ligand and propidium iodide (PI) staining. Cisplatin was expected to cause S-phase stalling and a subsequent decrease of cells in G1 and G2. All experimental conditions were examined in triplicate and results were consistent between each replicate. A summary of the cell cycle data is shown graphically below in Figure 65. It shows that overexpression of *KIAA1383* causes a decrease in the effect of cisplatin on the cell cycle with less cells in S phase. However this result was not statistically significant and requires to be repeated and as no RTPCR primers or antibodies are available it is not possible to demonstrate definite over expression of *KIAA1383*.

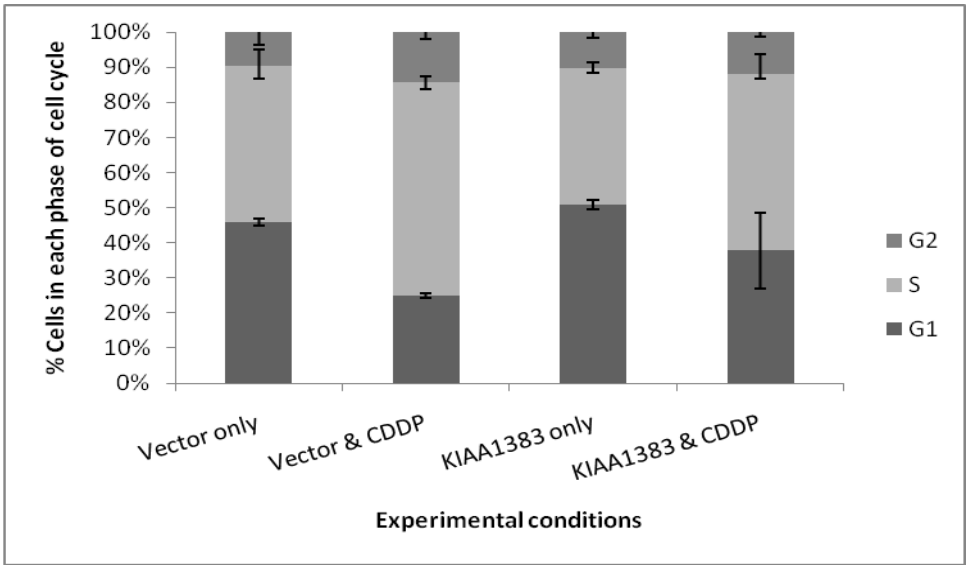
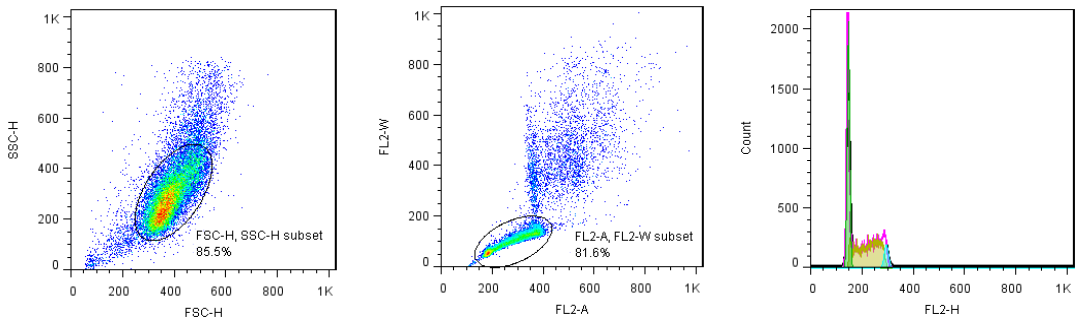
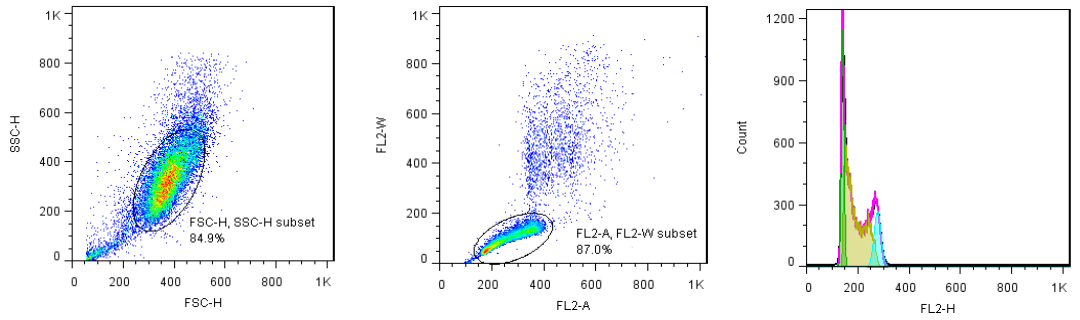


Figure 65. Effect of over expression and cisplatin treatment on the cell cycle. Error bars show standard deviation of triplicate results.

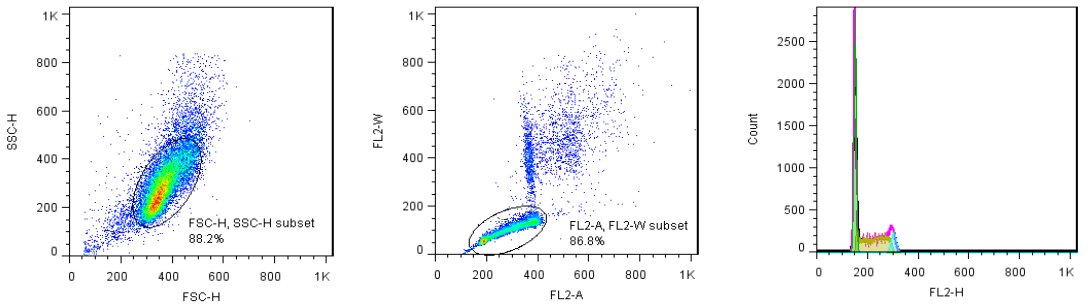
The raw data for the cell cycle analysis is also shown below in Figure 66 and confirms an increase in S Phase following cisplatin treatment which is reduced by *KIAA1383* overexpression.



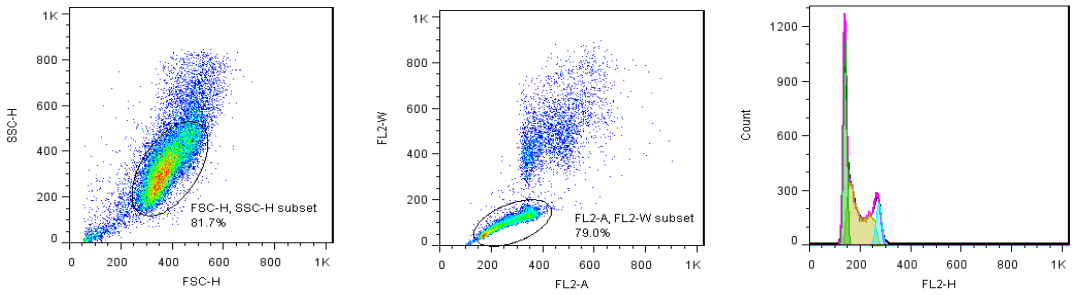
Vector only



Vector and cisplatin treated



***KIAA1383* over expressing only**



***KIAA1383* over expressing & cisplatin treated**

Figure 66. FACS analysis showing initially gating and final cell cycle results for *KIAA1383*. SSC side scatter, FSC forward scatter, FLT2 green/transfected cells. W width or transit time, A area or total fluorescence of the particle, H height or maximal fluorescent intensity, Count is number of nuclei so cells in G2/M have twice the DNA of that in G0/ G1 (first peak) and are represented in the second peak. S phase is the area between the two peaks.

6.8 Effect of over expression of *SP5* on chemosensitivity in the presence of cisplatin (MTT)

In a collaboration with Jenny Hersey at the Institute of Cancer Research the effect of overexpression of *SP5* was examined in A2780 cp70. This vector system allowed for stable over transfection and it was therefore possible to use the MTT assay to more directly measure chemosensitivity. The vector map is shown in Figure 67 below:

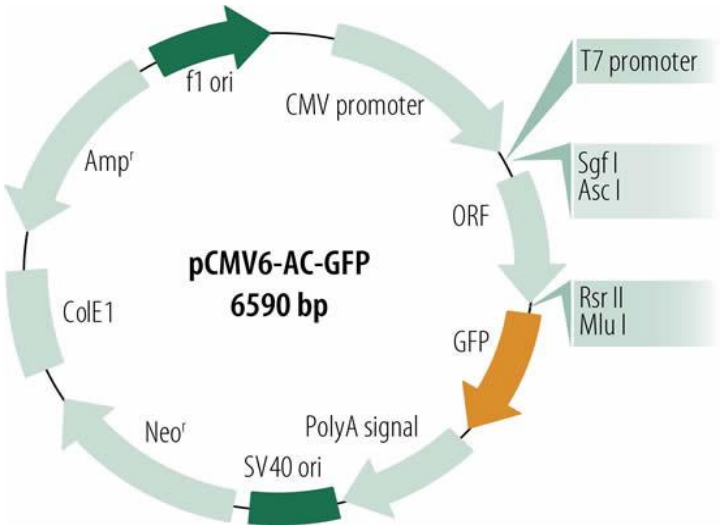


Figure 67. Vector map for pCMV-AC-GFP allowing stable over expression of *SP5*

Experiment 1.

Cells were plated at 3 different densities in duplicate in 6 well plates: 0.5 x 10^5, 1 x 10^5 and 2 x 10^5. These were then incubated overnight (~22hrs) prior to transfection. Transfection was performed and clones selected which showed persistent expression of *SP5;* as described in chapter 2.23. qRTPCR was then performed as described in Chapter 2.14.3 to assess expression of *SP5* in transfected cell lines. This analysis was carried out between passage 2 and 3 and the results are shown in Figure 68 below.

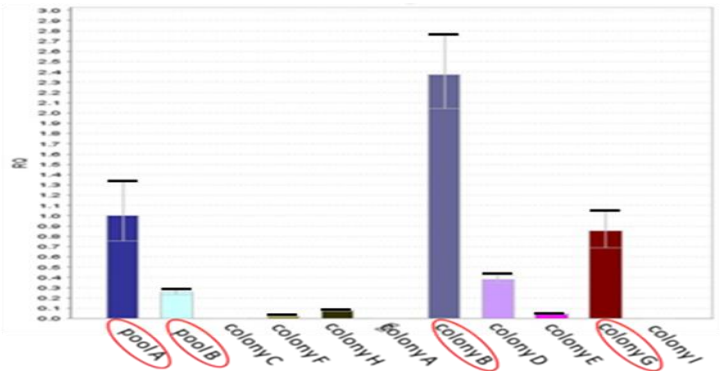


Figure 68. qRTPCR showing expression of *SP5* in 9 separate colonies (A-H) and 2 pools of all colonies. Results normalised to pool A. Colonies circled in red were chosen for future experiments. RQ relative expression. Error bars standard deviation of replicates. Top of error bar accentuated as black line.

Next an MTT assay was performed as described in Chapter 2.20 in order to assess whether cisplatin had a differential effect on chemosensitivity when *SP5* was over expressed. The IC50 of cisplatin in A2780 cp70 is 5.7 uM and 6 doses reflecting a range of concentrations from ¼ of the IC50 to 4 times the IC50 were examined (1/4 IC50, ½ IC50, IC50, 1.5 IC50, 2XIC50, 4XIC50) in addition to wells with media only and cells and media only. Results were variable between the 5 transfected groups of cells. The table below shows the percentage of viable cells when compared to untreated control at each drug concentration.

Table 35. Percentage of viable cells when compared to untreated control in MTT experiment.

Cisplatin	0 uM	1.425 uM	2.85 uM	5.7 uM	8.55 uM	11.4 uM	22.8 uM
vector only	100.0	94.1	95.7	72.6	62.2	47.9	30.3
***SP5* pool A**	100.0	92.3	82.1	63.2	50.3	45.7	26.5
***SP5* pool B**	100.0	114.7	105.4	76.3	75.8	56.8	39.7
***SP5* colony B**	100.0	96.7	91.3	61.9	60.3	48.1	39.6
***SP5* colony G**	100.0	99.4	65.2	54.0	51.8	47.3	36.7

These results are plotted onto growth curves below (Figure 69 below) and are shown in order (a to d) of increasing *SP5* expression.

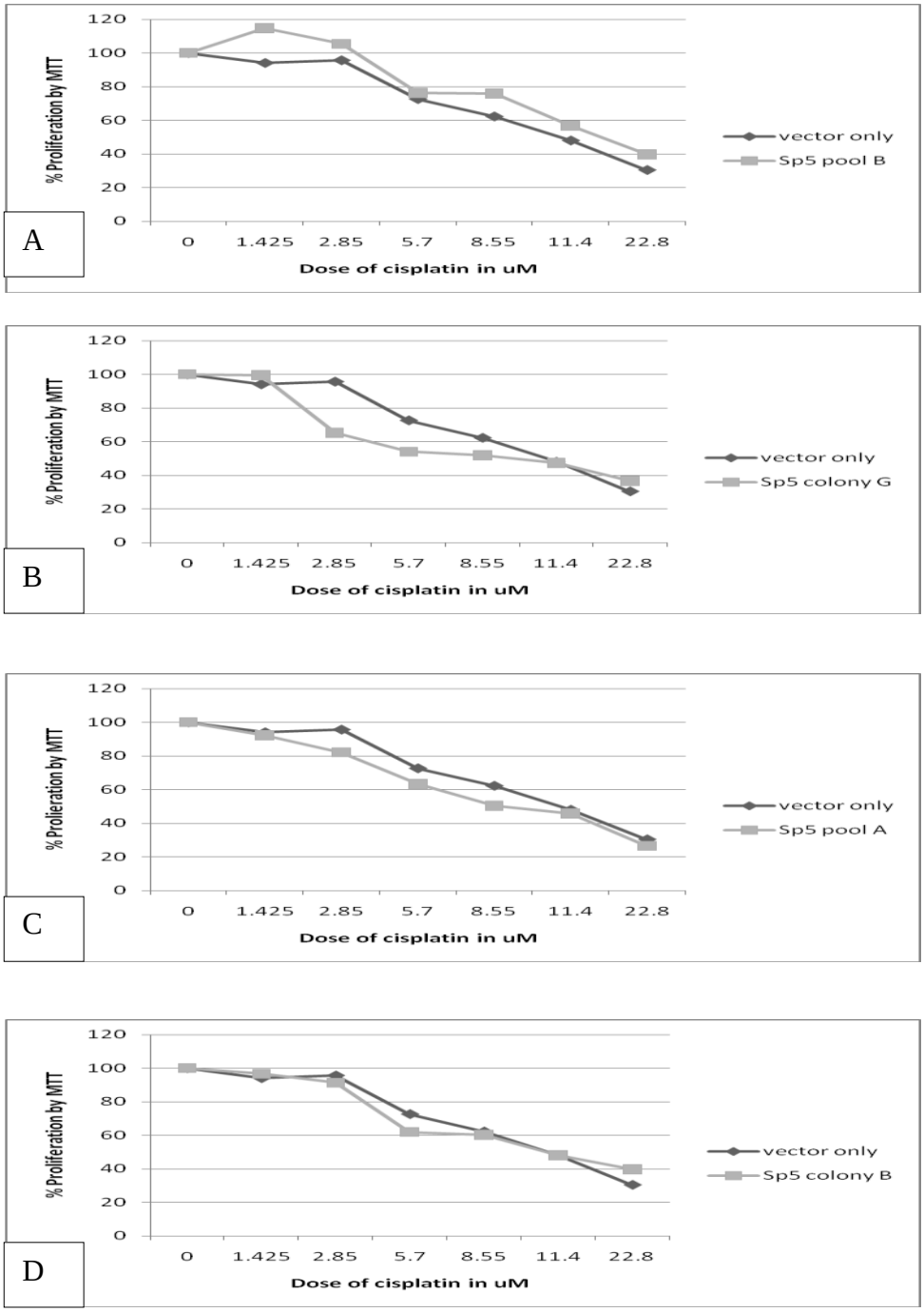


Figure 69. Effect of *SP5* overexpression on proliferation by MTT assay. (vector only shown by qRTPCR not to express *SP5*; A: *SP5* pool B , B: *SP5* colony G, C: *SP5* pool A, D: *SP5* colony B) (1 experiment, each condition in triplicate, averaged results shown).

During plating of cells for MTT assay $4x10^5$ cells were taken and lysed for RNA so that *SP5* expression could be determined. Results show that all 4 *SP5* transfected lines continue to express *SP5* at high levels and results show a similar pattern to original analysis. There is a small decrease in expression of *SP5* in pool A.

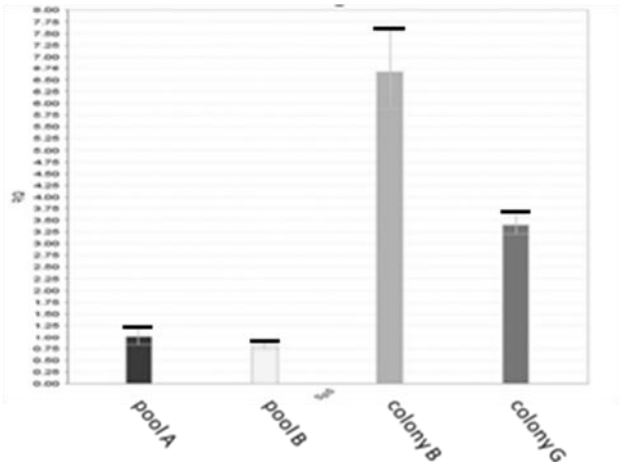


Figure 70. *SP5* expression after second passage confirming ongoing expression. RQ relative expression. Error bars standard deviation of triplicates in one experiment. Top of error bar accentuated as black line.

In conclusion, no effect of over expression on proliferation of cp70 cells, in the presence of cisplatin, was observed. The experiment was therefore repeated using a wider range of cisplatin doses. It was done twice independently and the results are shown below:

Experiments 2 and 3.

In the next two experiments the dose range included higher doses of cisplatin. All other experimental conditions were unchanged. Again qRTPCR was first examined to confirm expression of *SP5* in the various colonies. This time it was shown relative to the empty vector. qRTPCR results are shown firstly below for experiment 2 (71a) and experiment 3 (71b).

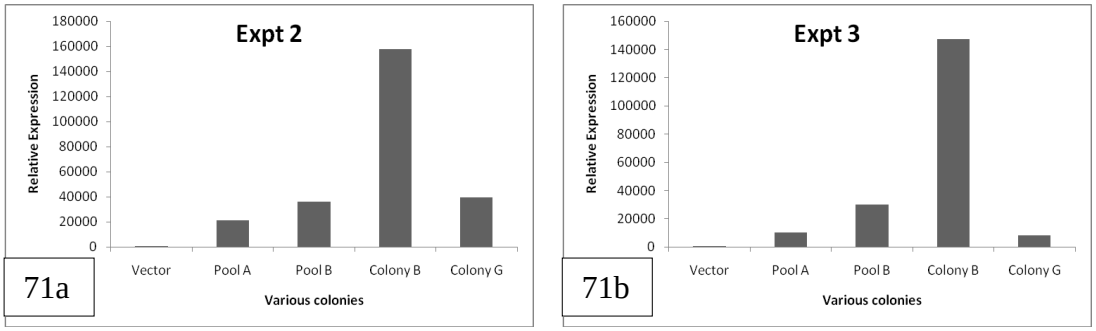


Figure 71a and b. Expression of SP5 by qRTPCR relative to empty vector. (Error bars not shown but samples were done in triplicate with standard error of mean less than 0.09).

As was observed in the first experiment colony B showed very high levels of *SP5* expression. Again pool A and B and colony G over express *SP5* – although the degree of overexpression in colony G is lower in Expt 3. Next the growth curves are shown for each separate colony. Again with each compared to the empty vector. Figure 72 illustrates the average of both experiments.

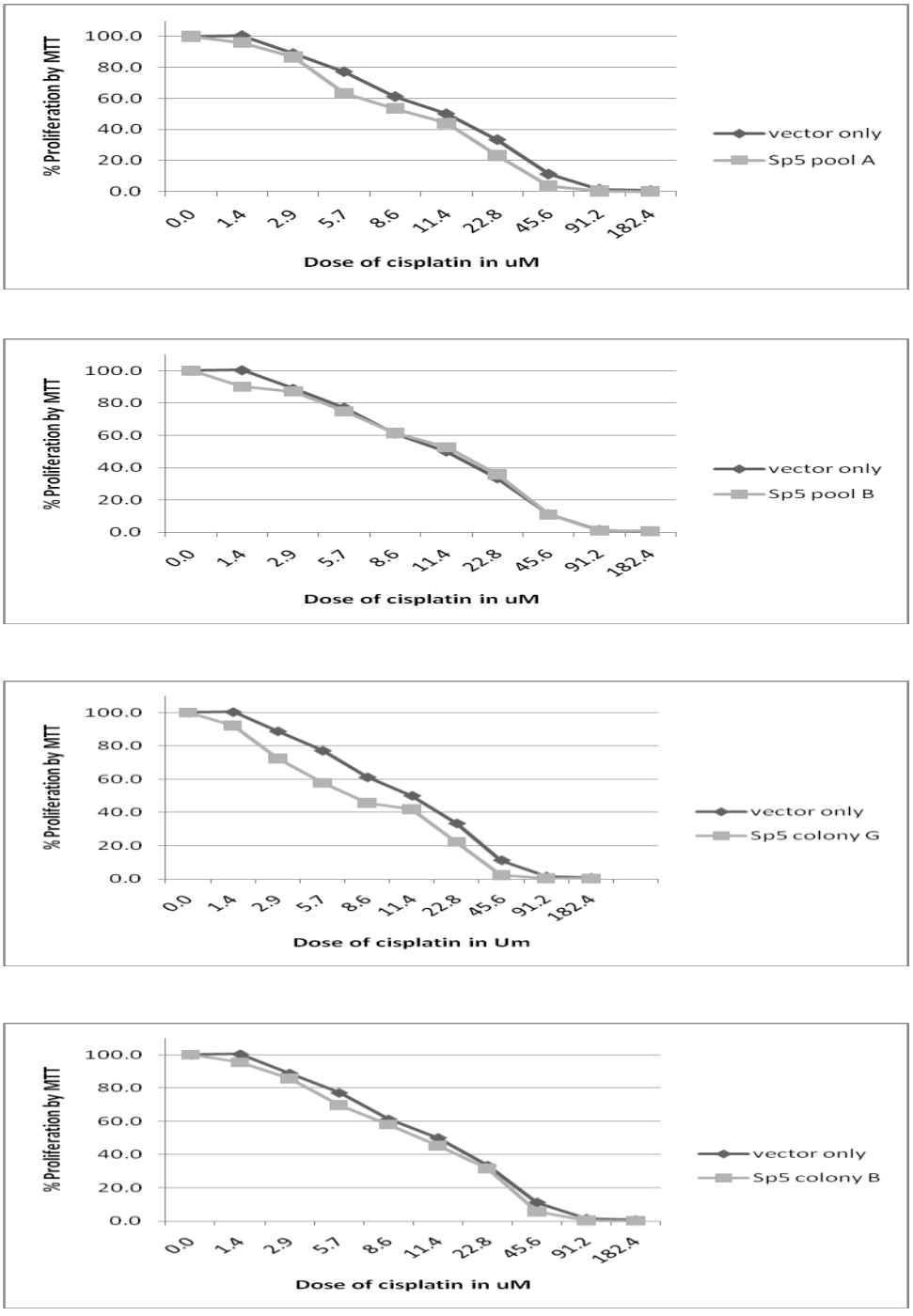


Figure 72 a-d. Effect of *SP5* over expression on proliferation as measured by MTT (n=2).

Again over expression of *SP5* was not shown to effect chemosensitivity when assessed using the MTT assay and these experiments have therefore not been taken any further.

6.9 Discussion

6.9.1 SP5

SP5 is known to be a transcription factor which antagonises *SP1* (Harrison, Houzelstein et al. 2000) and is a downstream target of Wnt signalling (Takahashi, Nakamura et al. 2005; Weidinger, Thorpe et al. 2005; Chen, Guo et al. 2006; Fujimura, Vacik et al. 2007). As Wnt is known to be dysregulated in EOC and has also been implicated in the pathogenesis of tumour initiating or sustaining cells and this makes loss of methylation in tumours which regain expression of *SP5* an important novel observation. As discussed in the conclusion of chapter 4 we have recently shown that methylation of key genes in the Wnt pathway have an impact on PFS on ovarian cancer (Dai, Teodoridis et al. 2010) and this adds further weight to the notion that methylation plays an important role in ovarian cancer drug resistance.

SP5 has been shown to be dynamically expressed during CNS development (Harrison, Houzelstein et al. 2000; Treichel, Becker et al. 2001; Weidinger, Thorpe et al. 2005) but it was only recently noted to show increased expression in colorectal, gastric and hepatocellular cancers with a negative impact (Chen, Guo et al. 2006). Our data suggest that demethylation of *SP5* results in an increase in expression of *SP5* with a subsequent decrease in apoptosis. Although we hypothesised that this could result in resistance to chemotherapy we have not been able to demonstrate this when over expressing *SP5* and assessing chemosensitivity using the MTT assay.

Chen et al, using an inducible gene expression system combined with microarray analysis found that over expression of *SP5* in MCF7 cells resulted in significant growth promotion

supporting our results (Chen, Guo et al. 2006). This fits with the high levels of methylation seen in the cell lines and majority of primary tumours. No association with PFS or OS was observed but it is possible if it is only those tumours with decreased methylation that have a worse outcome that the numbers in the study would therefore be underpowered to demonstrate this. These experiments contribute additional information regarding the possible importance of decreased methylation and increased expression of the *SP5* transcription factor, and the Wnt pathway more generally in ovarian cancer.

6.9.2 KIAA1383

Given the statistically robust correlation between increased *KIAA1383* methylation and PFS we were very keen to pursue further functional analysis of this gene. Transient over expression of *KIAA1383* could reduce the effect of cisplatin chemotherapy and this would fit with decreased methylation correlating with a poorer PFS. My results show that over expression of *KIAA1383* mediates the effect of cisplatin on the cell cycle and next we aim to assess this more directly using the GFP stable expression system (described for *SP5*) so that we can go on to do an MTT experiment with cisplatin. This would provide a more direct measure of any mediation of chemosensitivity (than the inferred results from a change in cell cycle).

6.10 **Conclusion**

6.10.1 SP5

In this chapter I have demonstrated that an increase in *SP5* methylation correlates with a decrease in gene expression, in A2780 based cell lines and ovarian cancer primary tumours. I have also shown that gene silencing can be reversed using a demethylating agent, decitabine. siRNA-induced gene silencing appeared to cause increased cell death which was shown to be secondary to apoptosis, using the caspase assay. Since I left the

laboratory, my colleague has since managed to optimise stable over expression of this gene and perform an MTT assay but this did not demonstrate an effect of over expression on chemosensitivity. It is possible that knock down of this gene has a critical effect on cells whereas the converse is not true with over expression. In addition over expression was checked at the mRNA level using qRTPCR and it is possible that this did not reflect an effect at the protein level.

6.10.2 KIAA1383

In the previous chapter I showed that methylation of *KIAA1383* correlates with PFS. Using flow cytometry cell cycle analysis following transient over expression of *KIAA1383* in this chapter I have shown that over expression of *KIAA1383* results in reduced S phase stalling, following treatment with cisplatin, in the PEA2 cell line. This is with the caveat though that RTPCR primers and antibodies were not available to confirm over expression at either the m RNA or protein level.

Further plans for both loci are discussed in the final thesis conclusion/ future plans (Chapters 8 and 9).

7 Validation of candidate loci in the OGT array

7.1 Aims and background

7.1.1 OGT (Phase I and II)

Oxford Gene Technology (OGT) offer a customised arrays service, whereby samples can be treated by DMH (as described in Chapter 2.9) and then hybridised to CGI arrays, where the loci to be tested have been chosen by the investigator. This does not limit examination to the promoter only and the arrays can be customised in any way required. As this was the first time such an approach had been used by our group the study was divided into an exploratory Phase I study and confirmatory Phase II study. The aim of the Phase I was to check the precision of this approach and to ask some initial exploratory questions regarding genes with differential methylation in the resistant cell lines. The aim of the Phase II was to take genes identified in the Phase I (and previous 12k array, see earlier Chapters (Heisler, Torti et al. 2005)) and validate them in a large panel of primary tumours. In addition in the Phase II experiment genes relating to key pathways in EOC were investigated.

This focused approach offers the advantage of being high throughput but with the ability to ask more directed questions, for example the role of methylation in key pathways, or to validate genes discovered from other approaches, for example the loci identified from the experiments described in the previous chapters of this thesis.

A diagram outlining the various validations that were performed is shown below in Figure 73: Briefly the candidate loci identified from Chapters 3-5 were examined in the relevant cell lines using the OGT I array. These loci were then examined in primary tumours in OGT Phase II experiment.

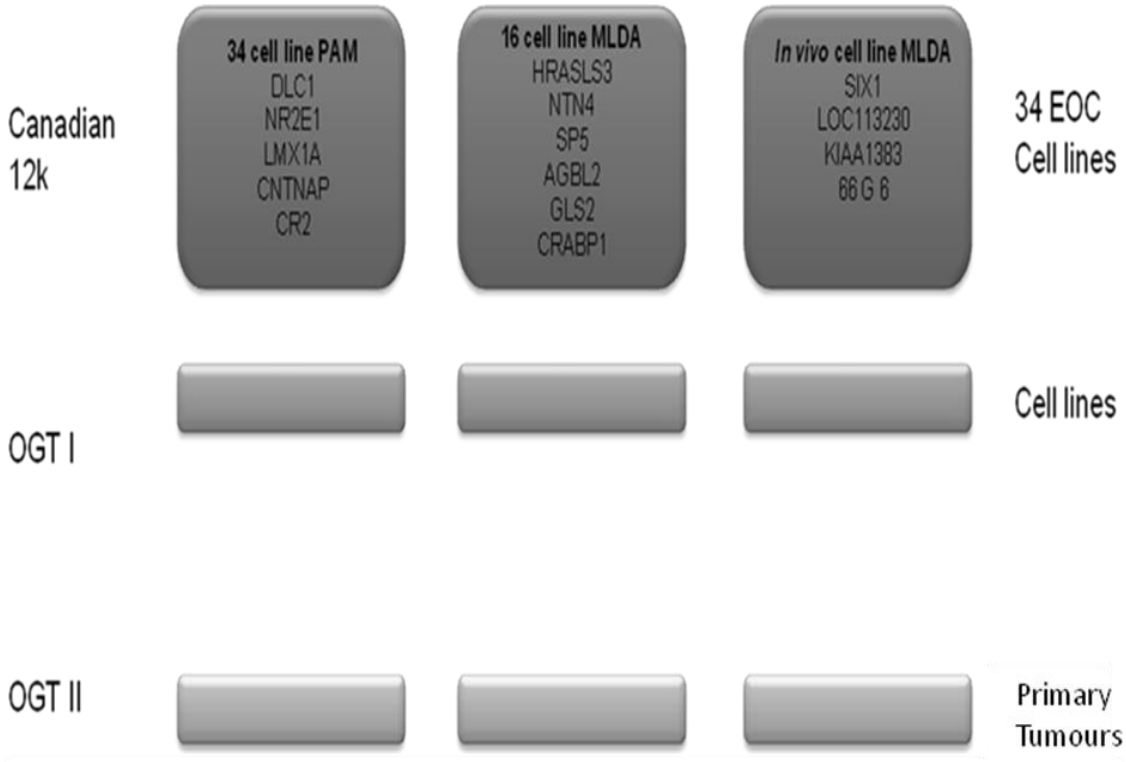


Figure 73 Summary of the validation in cell lines and primary tumours (of loci identified from the original 12K array). 34 cell line PAM refers to candidates from chapter 3, 16 cell lines MLDA refers to candidates from Chapter 4 and in vivo cell lines MLDA refers to candidates from Chapter 5. Canadian 12k (Heisler, Torti et al. 2005), OGT Oxford Gene Technology. EOC epithelial ovarian cancer.

7.2 OGT Phase I

7.2.1 Introduction

The aim of the Phase I experiment was to assess the reproducibility of technical and biological replicates and to assess the effect of dye swap experiments on results. Biological replicates describe separate DMH preparation of the DNA (see chapter 2.10) and technical replicates describes separate spotting of the same DMH preparation onto different areas of the array. In addition we planned to validate whether candidates discovered in previous experiments showed differential methylation using this alternative approach, and identify novel targets which could then be tested in a more comprehensive panel of tumours in the Phase II experiment. The source of the genes/ loci that were used in this array are summarised in Figure 74 below:

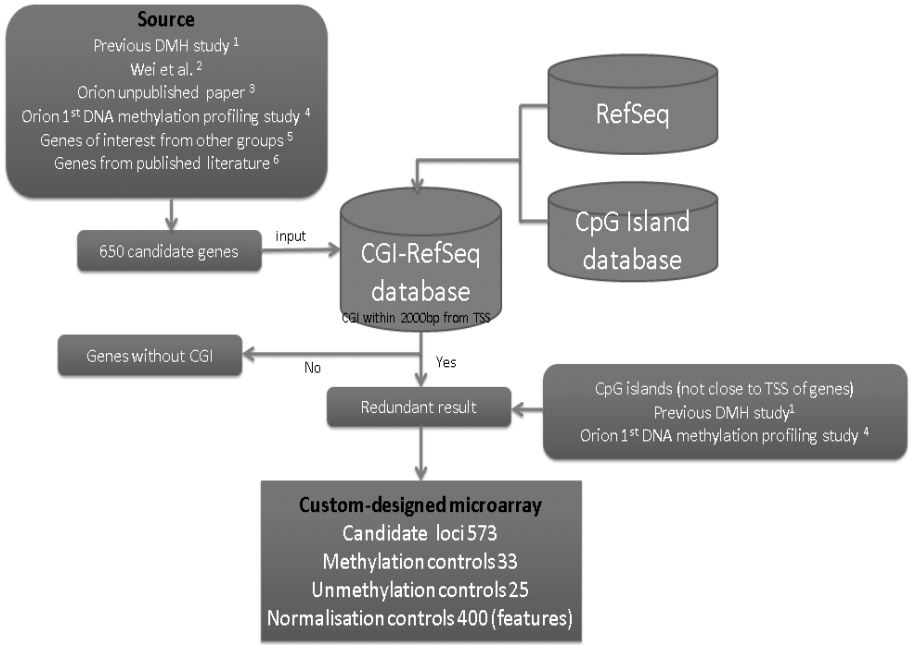


Figure 74. Source of loci hybridised in the OGT Phase I study. CGIs identified from DNA methylation profiling study by DMH assay on HCGI12K microarray between A2780 sensitive and resistant derivatives generated as well as between in vivo generated cell line pairs (PEO1 vs. PEO4, PEA1 vs. PEA2, PEO14 vs. PEO23) performed by Dr. Jens M. Teodoridis; 2) Prognostic DNA methylation signature associated with progression free survival in ovarian cancer (Wei, Balch et al. 2006); 3) and 4) CGIs identified from DNA methylation profiling study on Orion MethylScope two-channel microarray based on McrBC restriction enzyme to detect differential methylation between A2780 sensitive and resistant cell lines (data not published, lists available from Wei Dai or Robert Brown); 5) candidate genes collected from the studies about acquired drug resistance in ovarian cancer through our collaborations (List available from Professor Hani Gabra or Dr. Euan A. Stonach from Ovarian Cancer Action); 6) other loci/genes differentially expressed before and after cisplatin treatment from gene expression profiling studies published from 2001 to 2008 (List available from Epigenetics O drive via Wei Dai or Robert Brown). Figure from Wei Dai and further details can be found in her thesis published in 2011. Also see Dai et al, (Dai, Teodoridis et al. 2008).

The origins of the cell lines used in this experiment can be thought of in three parts (see figure 75 below). Firstly a detailed comparison of PEO1 vs. PEO4 was made which investigated the effects of dye swap, independent DNA preparation and repeat hybridisation. Secondly the EOC A2780 sensitive cell lines (A2780, A2780p3, A2780p5 and A2780p6) vs. A2780 resistant cell lines (A2780cp70, MCP1 and MCP6) were compared and here dye swap replicates only were performed. In the third comparison differences in methylation between the *in vivo* derived sensitive (PEO1, PEA1, PEO14) and resistant (PEO4, PEA2, PEO23) were sought and again dye swaps were performed. This is summarised in figure 75 below: (Further details of the cell lines in Chapter 2.17.4).

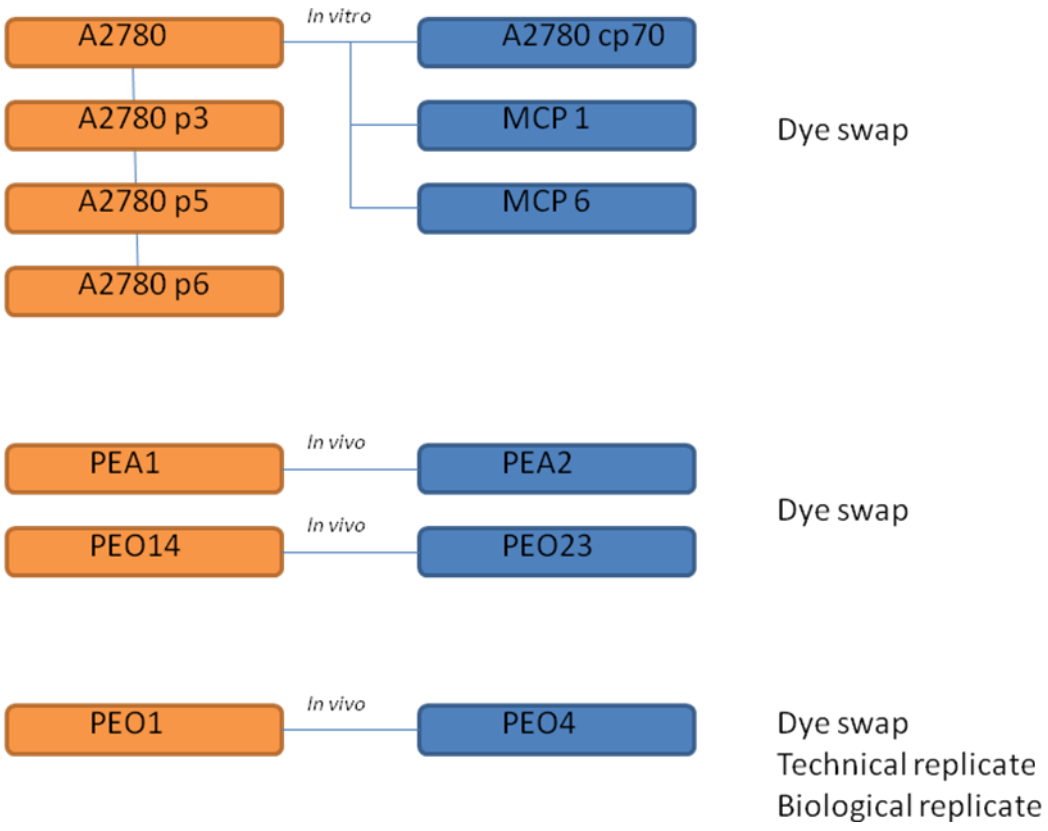


Figure 75. Cell lines used for OGT Phase I Expt. Cell lines as outlined in Chapter 2.17.4. Peach sensitive cell lines, Blue resistant cell lines. For the A2780-based cell lines and PEA1 vs. 2, and PEO14 vs. PEO23 hybridisation with a dye swap was performed. For PEO1 vs. PEO4 the effect of dye swap, technical replicates and biological replicates was assessed (For a more detailed explanation see text above figure).

7.2.2 Results

7.2.2.1 Reproducibility: dye swap, biological replicates and technical replicates

Reproducibility was found to be high when investigating the effect of technical or biological replicates. However this was not the case when investigating the effect of dye swap experiments where the R^2 results were lower. The variability was found to be less when the digested samples were labelled in red and undigested samples were labelled in green. A summary of this data is shown in Figure 76 below:

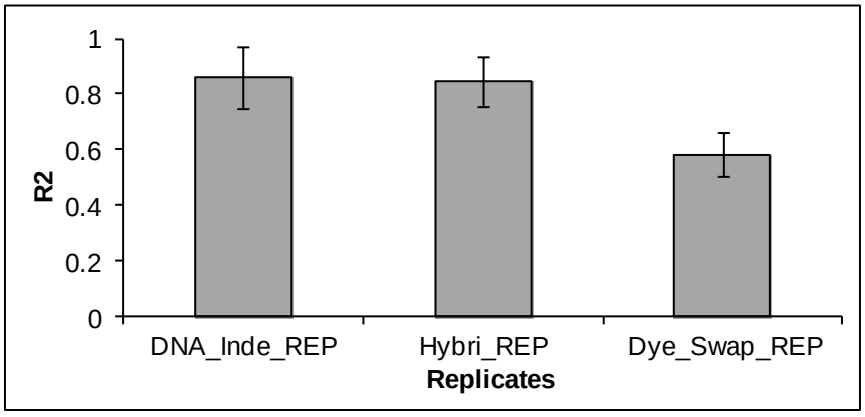


Figure 76. Bar chart showing R^2 values for technical and biological replicates hybridised to the OGTI array. Independent DNA bisulphite modification (DNA_inde_REP), separate hybridisations of the same bisulphite modified DNA (Hybri_REP) and dye swap. Error bars represent standard error of mean (SEM). (Figure from Wei Dai).

7.2.2.2 Ability to accurately detect previously validated loci

The ability of MLDA vs. SAM or PAM to identify candidate genes was next examined. Among 51 CGIs identified by MLDA on the Human CGI 12k array in the A2780 series cell lines previously, 32 CGI are validated by MLDA, 30 CGI by SAM and 26 by PAM on the OGT array. Further details of this are supplied in the publication by Dai et al, (Dai,

Teodoridis et al. 2008). We next assessed whether MLDA could identify the 14 candidates which had been identified using the Canadian 12k array (Heisler, Torti et al. 2005) and independently validated by MSP +/- pyrosequencing (Dai, Teodoridis et al. 2008). The results are illustrated in Figure 77 below and demonstrate that we could detect 13 out of 14 of the previously validated candidates (92.9% sensitivity). This box plot was developed in R by Wei Dai and plots the differences/ DMH log ratios (a measure of methylation) and corresponding chromosome locations of the 14 loci ($p=2.79x10^{-4}$) compared to the unmethylated controls on chromosome 16.

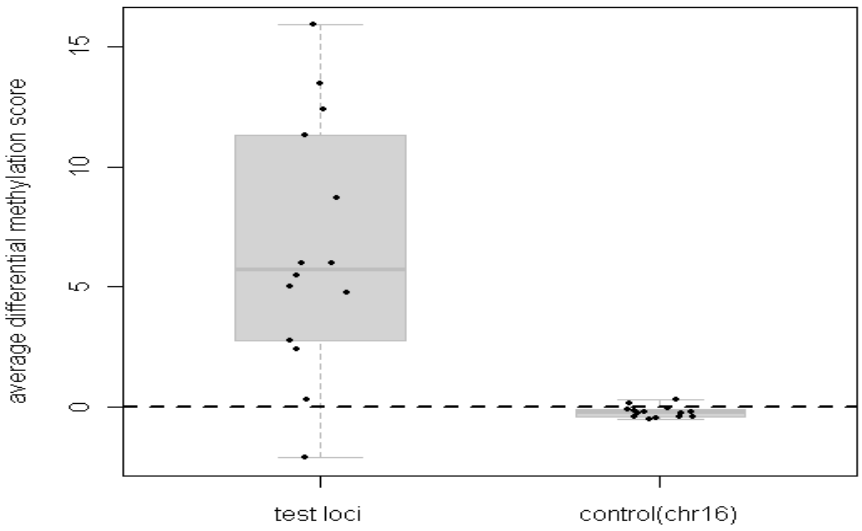


Figure 77. Box Plot showing ability of MLDA to detect candidates in the OGT Phase I experiment that had been detected in previous methylation hybridisation experiments as differentially methylated (left) compared to the unmethylated controls (right). (Figure from Wei Dai).

7.2.2.3 Correlation between array results (DMH ratio) and pyrosequencing results (% methylation of bisulphite modified samples)

The final test was to examine the correlation between methylation as detected by the DMH ratio as compared to pyrosequencing for individual loci and an example of this is shown in figure 78 below: This showed a high correlation and added confidence that the probes were covering the area of interest.

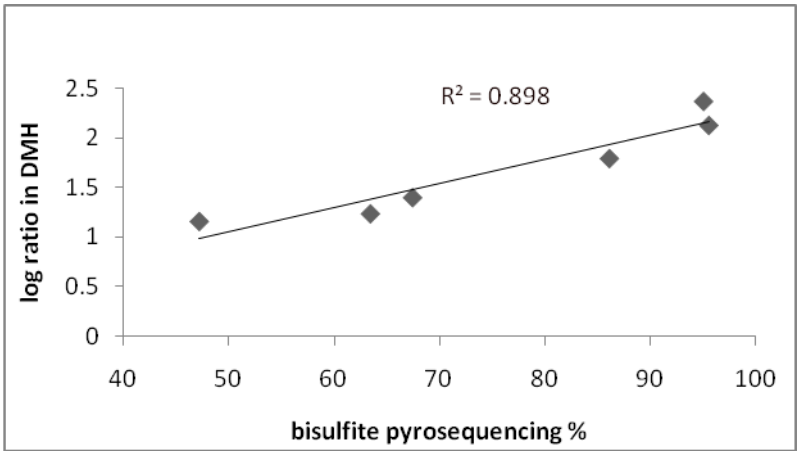


Figure 78. Correlation between DMH log ratio and methylation as detected by pyrosequencing of bisulphite modified DNA for 17 G 11.

7.2.2.4 Validation of loci identified from previous Chapters

MLDA was used to investigate whether the genes that had been identified previously from the 12K array showed differential methylation in this independent array. The results in the previous chapters have shown that a relatively low number of the loci that are differentially methylated in cell lines also show this characteristic in the primary tumours or matched tumour pairs (chapters 3-5). The matched pairs especially are a very scarce and finite resource of tumour DNA; however if a locus is identified in the cell lines and then differential methylation is confirmed in a heterogeneous panel of primary tumours (OGT II) then it is much more likely that this candidate could be of biological relevance.

We were able to ask two key questions following our initial tests of robustness of the data. Firstly to validate the loci identified in the 12K DMH experiment (Heisler, Torti et al. 2005) in an independent platform and secondly to use the cell lines as a discovery set to identify new potential biomarkers in ovarian cancer. In this study candidates were identified from each of the comparisons (see Figure 73, beginning of the chapter) and below I will focus on the validation of candidates identified in Chapters 3-5.

7.2.2.4.1 Examination of genes identified previously in this thesis, in the A2780 cell lines

Using MLDA we analysed the OGT I data and detected 105 loci that gained methylation in the resistant cell lines and 6 that lost methylation. All 6 of the previously identified genes (chapter 4) were identified and given that the two arrays are entirely independent that was seen as highly reassuring. In addition 66G6 from the *in vivo* comparison (chapter 5) and *DLC1, LMX1A* and *NR2E1* (Chapter 3) were all observed to show increased methylation in the resistant cell lines. A table showing the results for the loci identified from previous chapters is shown below:

Table 36 Candidates validated in the OGT array that had been identified from the 12K array

Gene	Start	End	chro	txstart	txend	hit	total	hit/total %
34 Cell line PAM (3/3)								
DLC1	13034461	13035285	8	12985242	13035180	7	16	43.75
LMX1A	163590110	163590435	1	163437728	163591641	5	14	35.71
LMX1A	163590815	163592952	1	163437728	163591641	5	34	14.71
NR2E1	108592364	108597232	6	108593954	108616704	21	74	28.38
A2780 MLDA (6/6)								
CRABP1	76420377	76421144	15	76419757	76427620	10	20	50.00
GLS2	55167668	55168969	12	55151003	55168448	7	26	26.92
HRASLS3	63137719	63138672	11	63098824	63138469	6	26	23.08
AGBL2	47693315	47693682	11	47637720	47692878	3	18	16.67
SP5	171278123	171282150	2	171280106	171282743	2	40	5.00
NTN4	94708004	94709330	12	94575714	94708667	1	32	3.13
***IN VIVO* MLDA (1/5)**								
66_G_6	95747937	95748308	9	NA	NA	6	14	42.86

Start and end refer to chromosome locations of the probes, chro chromosome, txstart and txend are the start and end of the transcription start site. Hit is number of probes showing increased methylation in resistant cell lines, total is total number of probes examining that gene, hit/total is the % of hits compared to the number of probes for the gene. 34 cell lines PAM refers to the genes identified in Chapter 3, A2780 MLDA refers to the genes identified in Chapter 4, *in vivo* MLDA refers to the genes identified in Chapter 5.

Our laboratory has previously worked extensively on MLH1 which is known to be associated with a gain in methylation in the resistant cell lines and as a check of the data we examined whether it appeared on this list of genes also, which it did, with 8 out of 22

probes showing significantly increased methylation in the resistant cell lines – adding further weight to the credibility of the data.

The original 12K array had 12000 CGIs spotted, although these were not limited to CGIs at the 5' end of the gene and many of the probes also covered non-CG sites (as shown in the high level of false positives or 'noise' seen in the table in chapter 3). In contrast the OGT Phase I used a 15k array of 573 loci which spanned the promoter region more comprehensively. In addition the genes were identified using PAM for the 34 cell comparison whereas MLDA was used on this occasion. To see such a high number of genes being detected by these two independent methods was therefore highly reassuring.

As stated before it would be important for a gene to show differential methylation in tumour samples as opposed to just cell lines if it was to be of important in ovarian cancer initiation or maintenance. This question was addressed when these genes were examined in the OGT Phase II data set (later in Chapter 7.3).

Next I examined whether any of the loci which had previously been identified could be detected as differentially methylated, by comparing the *in vivo* pairs, in the new data set (Chapter 5).

7.2.2.4.2 Examination of thesis candidate genes in 'in vivo' cell lines.

In this comparison we examined genes that showed increased methylation between the *in vivo* sensitive and resistant cell lines; PEO1, PEA1 and PEO14 vs. PEO4, PEA2 and PEO23. Using MLDA, 20 loci which corresponded to 17 genes were identified as gaining methylation in the resistant cell lines. Two of five of the previous identified candidates were validated (*KIAA1383* and *LOC113230*) and in addition *CRABP1* which had been identified in the A2780 comparison (Chapter 4) also showed differential methylation in these independent cell lines. The results for the three genes are shown in Table 37 below:

Table 37. Validation of genes identified in the 12k array in the *in vivo* cell lines on the OGT I array

Gene	Chro	Start	End	Txstart	Txend	Hit	total	% hits/total
CRABP1	15	76419724	76420163	76419757	76427620	5	18	27.78
KIAA1383	1	231007677	231008330	231007260	231012715	3	22	13.64
LOC113230	19	14046107	14046601	14044820	14046874	2	16	12.50

Start and end refer to chromosome locations of the probes, chro chromosome, txstart and txend are the start and end of the transcription start site. Hit is number of probes showing significantly increased methylation in resistant cell lines identified by MLDA in a pairwise comparison (3 pairs, FDR<5%), total is total number of probes examining that gene, hit/total is the % of hits compared to the number of probes for the gene.

In this comparison more weight was lent to the PEO1 and PEO4 comparison because of the number of replicates of this cell line on the array. (PEO1 and PEO4 had biological, technical and dye-swap comparisons whereas PEA1 and 2 and PEO14 and PEO23 had dye swaps only). Given this fact it was reassuring to see 2 of 3 of the candidates validating by this independent method (6 loci identified, 4 mapped to 5' and had a CGI, then 66G6 abandoned secondary to lack of pyrosequencing primers leaving *SIX1*, *KIAA1383* and *LOC113230*).

It is also very interesting to see differential methylation of *CRABP1* given it had originally been identified in a comparison of the A2780 based cell lines (Chapter 4). Again the next step was to examine these loci in the primary tumours in the OGT Phase II experiment.

7.2.3 OGT I Summary

In this pilot study we were able to use DMH and subsequent MLDA to detect differential methylation in resistant cell lines with high sensitivity. We demonstrated that dye bias reduces the reproducibility of dye swapped replicates and therefore excluded the use of dye swap in the Phase II of the study. The variability of DNA independent replicates and hybridisation replicates were similar and smaller when the digested samples were labelled in red and undigested samples labelled with green.

Many of the genes identified in Chapters 3-5 were shown to demonstrate increased methylation in resistant cell lines using this independent method. These loci could then be further characterised in the larger panel of ovarian cancer primary tumours (OGT Phase II). In this chapter I have been able to demonstrate that loci that could have been disregarded (because it was difficult to design primer or optimise primers for MSP or pyrosequencing) were able to be validated using this high throughput technique.

Overall it was thought that the OGT focussed array had proved to be a robust way of validating known candidates and also had the potential to be used to discover new targets. MLDA was confirmed to be a highly sensitive method for detecting differential methylation (Dai, Teodoridis et al. 2008).

7.3 OGT Phase II (Examination in primary tumours).

7.3.1 Introduction

The first aim of the OGT Phase II was to examine genes that had been identified in the 12K array, in primary tumours. The primary tumours are known to be very heterogeneous and this was therefore done with two caveats – firstly that a gene could be important in acquired drug resistance and be detected in the resistant cell lines but not necessarily be present at a detectable level at the outset of disease and therefore might not be picked up in the primary tumours and secondly that a negative result might not mean that a gene was not of relevance biologically – just that due to tumour heterogeneity the study was underpowered to detect the difference.

The second aim of the OGT Phase II was as a discovery platform – i.e. to identify new genes that were important in ovarian cancer at the outset of disease. This work is not discussed in this thesis but has recently been published, (Dai, Teodoridis et al. 2010).

Genes from the previous 12K and OGT Phase I experiments were included along with other genes relating to key pathways that are known to be important in ovarian cancer (for example BRCA1/2, p53, MMR, mTOR and WNT). In total 824 loci were examined in 111 primary tumour samples, the majority of which represented advanced disease. The origin of the loci/genes that were investigated is illustrated in Figure 79 and table 37 below:

1. Dai W et al.: **Methylation Linear Discriminant Analysis (MLDA) for identifying differentially methylated CpG islands.** *BMC Bioinformatics* 2008, **9**:337.
2. Wei SH, Balch C, Paik HH, Kim YS, Baldwin RL, Liyanarachchi S, Li L, Wang Z, Wan JC, Davuluri RV, et al: **Prognostic DNA methylation biomarkers in ovarian cancer.** *Clin Cancer Res* 2006, **12**:2788-2794.
3. http://gspd.biocompare.com/Gene/Pathway.aspx?gp=55
4. http://www.genome.jp/kegg/pathway.html

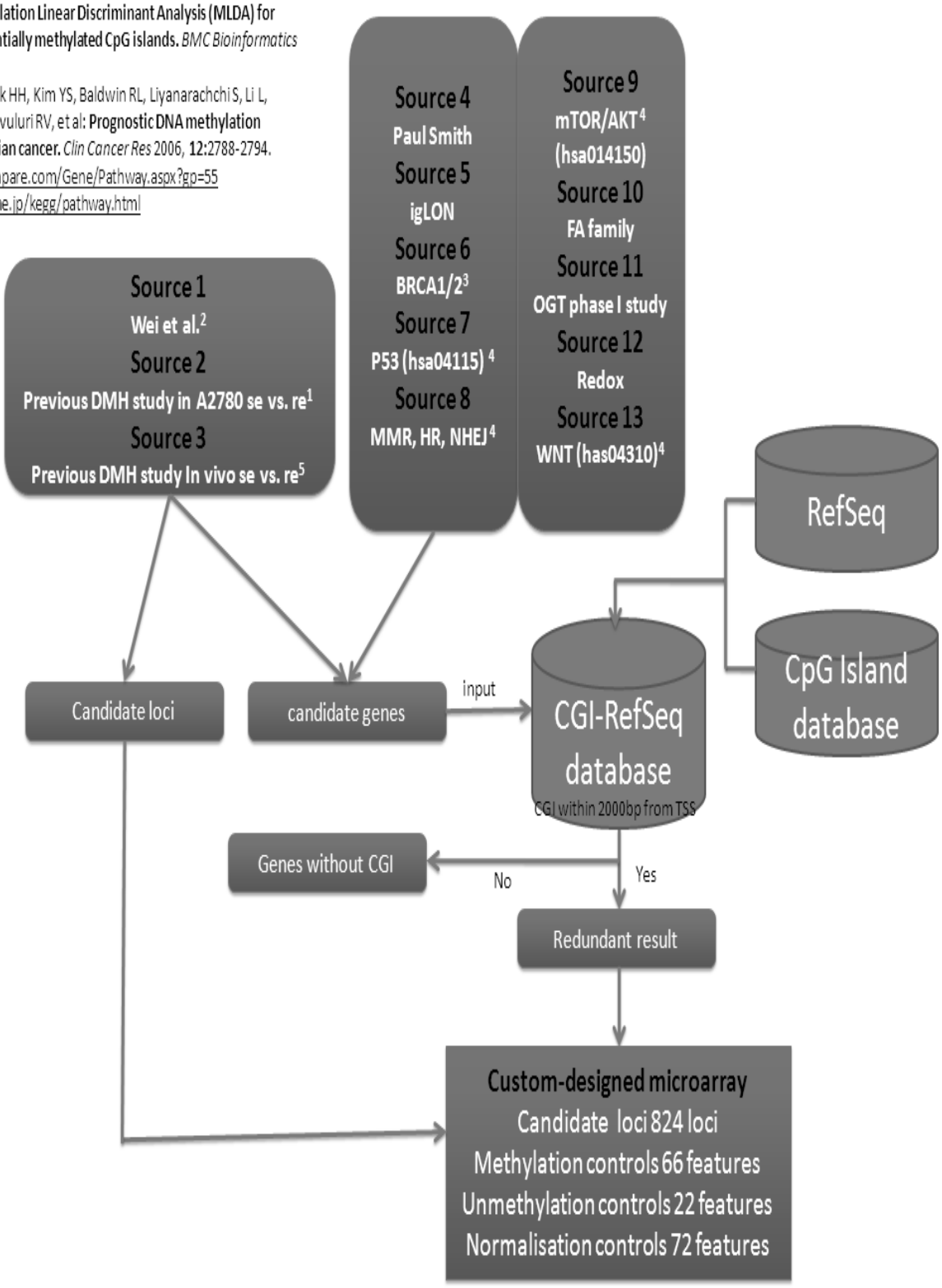


Figure 79. Flow diagram of origin of candidate loci for the OGT Phase II. Data pre-processing data and controls are published as a supplementary table in the recent Wnt paper (see 'Supplementary Method 1_Pre-processing of DMH data') (Dai, Teodoridis et al. 2010) or sources 3-13 genes (excluding sources 1,2 and 5 which are published) see table 38 below. Source 1 (Dai, Teodoridis et al. 2008), source 2 (Wei, Balch et al. 2006), source 5 (Ntougkos, Rush et al. 2005).

Table 38. List of genes investigated in the OGT II Experiment.

Source 3	**Source 4**	**Source 6**	**Source 7**	**Source 8**	**Source 9**	**Source 10**	**Source 11**	**Source 12**	**Source 13**	**Source 13**
***in vivo* DMH**	**Paul Smith**	**BRCA1**	**P53**	**MMR Etc**	**mTOR/ AKT**	**FA**	**OGT I**	**Redox**	**WNT**	**WNT**
AK094166	AKT1S1	ATF1	APAF1	BLM	AKT1	BRCA2		APEX1	APC	RAC1
AX747809	AKTIP	ATM	ATM	BRCA1	AKT2	C17orf70	ADM	CAT	APC2	RAC2
C12orf60	ASCL1	ATR	ATR	BRCA2	BRAF	C19orf40	AGBL2	FOXO1	AXIN1	RAC3
C15orf37	ASL	BACH1	BAI1	DCLRE1C	CAB39	FANCA	AK124699	FOXO3	AXIN2	RBX1
C6orf159	ASS1	BARD1	BAX	DMC1	DDIT4	FANCB	AREG	FXN	BTRC	RHOA
CYP27B1	BAPX1	BLM	BBC3	EME1	EIF4B	FANCC	ATF3	GLRX	CACYBP	ROCK1
KIAA0859	BARX1	BRCA1	BID	EME2	EIF4E	FANCD2	BDNF	GPX1	CAMK2B	ROCK2
KIAA1383	BASP1	BRCA2	CASP3	EXO1	EIF4E1B	FANCE	C1orf190	GPX3	CAMK2D	RUVBL1
LOC113230	BTG2	CDKN1A	CASP8	HMGB1	EIF4E2	FANCG	C1orf88	GPX4	CAMK2G	SENP2
NOLA1	BTG3	CHEK1	CASP9	LIG1	EIF4EBP1	FANCI	CD47	GPX7	CCND1	SFRP1
OPCML	DAB2	CHEK2	CCNB1	LIG4	FRAP1	FANCM	CDH13	GSTM1	CCND2	SFRP2
TMEM86A	DUSP1	E2F1	CCNB2	MLH1	GBL	PALB2	CDS1	GSTM2	CCND3	SFRP4
TRDMT1	DUSP16	E2F2	CCND1	MLH3	HIF1A		CKLF	GSTM4	CHP	SFRP5
VPS13B	DUSP2	E2F3	CCND2	MRE11A	KIAA1303		*CNTNAP*5	GSTM5	CREBBP	SIAH1
	DUSP6	E2F4	CCND3	MSH2	LYK5		COL14A1	GSTP1	CSNK1A1	SKP1
	DUSP7	E2F5	CCNE1	MSH3	MAPK1		CRABP1	GSTT1	CSNK1E	SMAD2
	DUSP8	E2F6	CCNE2	MSH4	MAPK3		CTSL1	KEAP1	CSNK2A1	SMAD3
	GFRP	FANCA	CCNG1	MSH5	PDPK1		CXCL2	MPO	CSNK2A2	SMAD4
	GLUL	FANCC	CCNG2	MSH6	PGF		*DLC1*	NFE2L2	CSNK2B	SOX17
	IGFBP1	FANCD2	CD82	MUS81	PIK3CA		DNAJC11	NOX4	CTBP1	TBL1X
	IGFBP2	FANCE	CDC2	NBN	PIK3CD		DNAJC6	NOX5	CTBP2	TBL1XR1
	IGFBP3	FANCF	CDK2	NHEJ1	PIK3R1		DPH1	NRF1	CTNNB1	TBL1Y
	IGFBP4	FANCG	CDK4	PCNA	PIK3R2		DUOXA2	PRDX1	CTNNBIP1	TCF7
	IGFBP5	FANCL	CDK6	PMS1	PIK3R3		DUSP1	PRDX2	CUL1	TCF7L1
	IGFBP6	GADD45A	CDKN1A	PMS2	PRKAA1		DUSP3	PRDX3	CXXC4	TCF7L2
	IGFBP7	GADD45B	CDKN2A	POLB	PRKAA2		ENSG00000172268	PRDX4	DAAM1	TP53

	IGFBPL1	GADD45G	CHEK1	POLD1	RHEB	EPM2AIP1	PRDX5	DAAM2	VANGL1
	LEPRE	HDAC1	CHEK2	POLD2	RICTOR	ERBB2	PRDX6	DKK1	VANGL2
	LEPREL1	HDAC10	CYCS	POLD3	RPS6	F3	SOD1	DKK2	WIF1
	LEPREL2	HDAC11	DDB2	POLD4	RPS6KA1	FANCF	SOD2	DVL1	WNT1
	LRIG1	HDAC2	EI24	POLL	RPS6KA2	FEZF2	SRXN1	DVL2	WNT10A
	LRIG2	HDAC3	FAS	POLM	RPS6KA3	FOXD4	TXN2	DVL3	WNT10B
	LRIG3	HDAC4	GADD45A	PRKDC	RPS6KB1	FOXE1	TXN2	EP300	WNT11
	MSX1	HDAC5	GADD45B	RAD50	RPS6KB2	GLS2	TXNRD1	FBXW11	WNT16
	PHLPP1	HDAC6	GADD45B		STK11	GPM6B	TXNRD2	FOSL1	WNT2
	PROTOR2	HDAC7	GADD45G	RAD51	TSC1	GSTM3	WWOX	FRAT1	WNT2B
	PRR5	HDAC8	GTSE1	RAD51C	TSC2	HK1		FRAT2	WNT3
	RASL12	HELLS	IGFBP3	RAD51L3	ULK1	HLX1		FZD1	WNT3A
	REDD1	MRE11A	LRDD	RAD52	ULK1	HOXB13		FZD10	WNT4
	REDD2	MSH2	MDM2	RAD54B	ULK2	HOXB5		FZD2	WNT5A
	REELIN	MSH6	MDM4	RAD54L	ULK2	HOXD11		FZD3	WNT5B
	RHEB	NBN	PERP	RDM1	ULK3	HPSE		FZD4	WNT6
	RHOB	PLK1	PMAIP1	RECQL	VEGFA	HPSE2		FZD5	WNT7A
	SPINT1	RAD50	PPM1D	RECQL4	VEGFB	HRASLS3		FZD6	WNT7B
	SPINT2	RAD51	PTEN	RECQL5	VEGFC	HSPB1		FZD7	WNT9A
	TLE3	RB1	RCHY1	RFC1		IGSF4B		FZD8	WNT9B
	ZNF655	RBBP8	RFWD2	RFC2		IL17RD		FZD9	
		SMARCA1	RPRM	RFC3		IL1F8		GSK3B	
		SMARCA2	RRM2	RFC4		JAG1		JUN	
		SMARCA4	RRM2B	RFC5		*KIAA1383*		LEF1	
		SMARCA5	SESN1	RPA1		LBH		LRP5	
		SMARCAD1	SESN2	RPA2		LBX1		LRP6	
		SMARCB1	SESN3	SHFM1		LHX2		MAP3K7	
		SMARCC1	SFN	SMARCC1		LHX5		MAPK9	
		SMARCC2	SHISA5			LIPG		MYC	
		SMARCD1	SIAH1	SPO11		*LMX1A*		NFAT5	
		SMARCD2	STEAP3	TOP3A		*LOC113230*		NFATC1	
		STAT1	THBS1	WRN		MAGEA6		NFATC2	
		TP53	TNFRSF10B	XRCC2		MAGEC7		NFATC3	
		UBB	TP53	XRCC3		MAGEG1		NFATC4	

UBC	TP53I3	XRCC4	MAL	NKD1
	TP73	XRCC5	MAMDC2	NKD2
	TSC2	XRCC6	MLH1	NLK
	ZMAT3		MLLT6	PLCB1
			MSX2	PLCB3
			MT2A	PORCN
			MYOD1	PPARD
			NLF2	PPP2CA
			NR2E1	PPP2CB
			NTN4	PPP2R1A
			NUDT4	PPP2R1B
			P2RY8	PPP2R2A
			PCDH15	PPP2R2B
			PDX1	PPP2R2C
			PEG10	PPP3CA
			PIK3R1	PPP3CA
			SFRP1	PPP3CB
			SLC27A1	PPP3CC
			SOCS1	PPP3R1
			SP5	PPP3R2
			TFAP2C	PRICKLE1
			TMEM16E	PRKACA
			TP53	PRKACB
			TP53INP1	PRKACG
			TRAP1	PRKCA
			TSPAN13	PRKCB
			TUBB2B	PRKCG
			UBE2S	PRKX
			UNCX4.1	PRKY
			WNT1	PSEN1

The list of loci corresponding with these genes is held on the O drive of the Epigenetics group at Imeprial College London and can be accessed via Wei Dai or Robert Brown or via NCBI (Dai, Teodoridis et al. 2010)

7.3.2 Results

7.3.2.1 Correlation between DMH ratio and % methylation by pyrosequencing

As before, in order to assess the quality/ reliability of the data, the correlation between the DMH ratio and the results obtained from previous pyrosequencing of 17G11 in primary tumours (chapter 5.3.1) was investigated. The results are shown in Figure 80 below (this was not done for all loci).

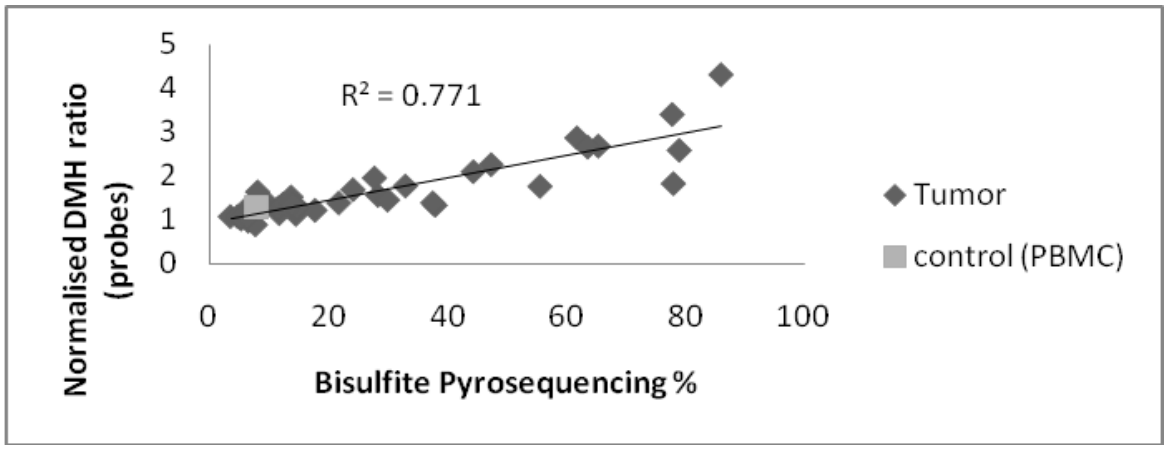


Figure 80. Scatter plot of DMH ratio of individual probes vs. pyrosequencing of *SIX1* in primary tumours (chapter 5.3.1).

Using *SIX1* as an example, the chromosome locations for the pyrosequencing primers, the DMH probes and the locus are shown in Table 39 below:

Table 39 Chromosome locations for DMH probes and pyrosequencing for *SIX1*

	SIX1 (17G11)	
	Start	End
Pyrosequencing	60174234	60174273
DMH probe	60174197	60174329
MSE1 fragment	60173731	60174416

7.3.2.2 Candidates genes from Chapters 3-4

The genes that had been identified as showing increased methylation in the resistant cell lines in Chapters 3-4 were first investigated to see if any of these showed increased methylation in the panel of 111 primary EOC tumours (tumour and patient characteristics are described in methods section 2.5.5).

It was not possible to design a probe for *CR2* and for *SP5* the probe could not be designed to overlap with the exact location of the locus from the 12k array (24D3). Of the 9 genes that were investigated one showed a correlation with PFS when analysed using the Cox regression model; *CNTNAP*. This result is highlighted in bold in table 40 below:

Table 40 Summary of effect of methylation of genes from chapters 3-4 on PFS in OGT II.

12K ID	Gene	HR	HR Lower 95	HR Upper 95	p value	FDR
Chapter 3						
119_A_6	*NR2E1*	0.83	0.37	1.86	0.65	0.91
5_D_4	*LMX1A*	0.89	0.72	1.08	0.23	0.71
127_F_12	*DLC1*	0.40	0.06	2.79	0.35	0.71
114_E_4	***CNTNAP***	**0.49**	**0.27**	**0.86**	**0.01**	**0.14**
chapter 4						
17_H_9	*HRASLS3*	0.65	0.27	1.52	0.30	0.71
20_F_11	*NTN4*	1.26	0.26	6.02	0.77	0.91
38_D_7	*AGBL2*	1.81	0.05	55.85	0.73	0.91
41_D_12	*GLS2*	1.22	0.13	11.25	0.86	0.93
121_D_9	*CRABP1*	1.08	0.19	6.27	0.93	0.93

12K ID 12K array identity/name, HR hazard ratio, HR Lower and higher 95 are 95% confidence intervals, *P value is two-sided score test of the univariate Cox model. HR: hazard ratio. HR>1 indicates the increased risk of disease progression associated with the increase of methylation. HR<1 indicates the decreased risk of disease progression/death associated with the increase of methylation. FDR: False discovery rate estimated using the method described before (Benjamini & Hochberg, 1995).

It was interesting that a gene that appears to have a role in acquired drug resistance, where increased methylation was seen in the resistant cell lines, could also have a role at the outset of disease – where an increase in methylation correlates with a better PFS. From the Uni Prot Swiss Prot data base http://www.uniprot.org/uniprot/Q9UHC6) it seems that *CNTNAP* may play a role in the formation of functional distinct domains critical for saltatory conduction of nerve impulses in myelinated nerve fibres and seems to demarcate the paranodal region of the axo-glial junction. In association with contactin it may have a role in the signalling between axons and myelinating glial cells; a role in cancer has not been established and nor is their documentation that this gene is epigenetically regulated.

Next the same loci were examined in terms of the effect of methylation on overall survival and we did not observe any correlation between methylation and overall survival; the results are summarised in table 40 below.

Table 41. Summary of correlation between methylation and OS for each CGI and its corresponding gene

12K ID	Gene	HR	HR Lower 95	HR Upper 95	p value	FDR
Chapter 3						
119_A_6	*NR2E1*	0.98	0.33	2.98	0.98	0.98
5_D_4	*LMX1A*	0.95	0.76	1.20	0.67	0.94
127_F_12	*DLC1*	0.94	0.17	5.25	0.95	0.98
114_E_4	*CNTNAP*	0.61	0.32	1.16	0.13	0.39
Chapter 4						
17_H_9	*HRASLS3*	0.87	0.42	1.84	0.73	0.94
20_F_11	*NTN4*	2.48	0.33	18.45	0.38	0.75
41_D_12	*GLS2*	0.79	0.05	12.98	0.87	0.98
38_D_7	*AGBL2*	13.99	0.38	511.70	0.15	0.39
121_D_9	*CRABP1*	2.36	0.31	17.75	0.41	0.75

12K ID 12K array identity/name, HR hazard ratio, HR Lower and higher 95 are 95% confidence intervals. *P value is two-sided score test of the univariate Cox model. HR: hazard ratio. HR>1 indicates the increased risk of death associated with the increase of methylation. HR<1 indicates the decreased risk of disease progression/death associated with the increase of methylation. FDR: False discovery rate estimated using the method described before (Benjamini & Hochberg, 1995).

7.3.2.3 Candidate genes from Chapter 5

Next the CGIs within the five genes that had been identified by comparing PEA1 and PEA2 and PEO14 and PEO23 (chapter 5) were examined to see whether these correlated with survival in the 111 primary tumour pairs. *KIAA1383* had been found to correlate with PFS in the primary tumours examined in Chapter 5.3.4 but the relationship between *LOC113230* (85B2) methylation and survival had not been examined previously. This was because by MSP and pyrosequencing, *LOC113230* had been thought to represent a false positive result as it was very methylated in all controls, sensitive and resistant cell lines - however it is possible that the primers were simply not designed to detect the area that showed maximal differential methylation and this should be reinvestigated (Chapter 5).

In this experiment *LOC113230* was found to correlate with PFS and *KIAA1383* and *LOC113230* were found to correlate with OS. A summary of the results for PFS and OS are shown below (Tables 42 and 43).

Table 42 Effect of methylation on PFS in OGT II data set

		Progression Free Survival (PFS) (N=111)				
12K ID	**Gene**	**HR**	**HR Lower 95**	**HR Upper 95**	**p value**	**FDR**
85_B_2	***LOC113230***	**0.04**	**0.003**	**0.68**	**0.02**	**0.14**
21_G_5	*KIAA1383*	0.68	0.40	1.15	0.15	0.66
66_G_6		2.23	0.37	13.48	0.38	0.71
17_G_11	*SIX1*	0.86	0.57	1.31	0.49	0.79

HR hazard ratio, HRLower and higher 95 represent 95% confidence intervals, *P value is two-sided score test of the univariate Cox model. HR: hazard ratio. HR>1 indicates the increased risk of disease progression associated with the increase of methylation. HR<1 indicates the decreased risk of disease progression/death associated with the increase of methylation. FDR: False discovery rate estimated using the method described before (Benjamini & Hochberg, 1995).

Table 43 Effect of methylation on OS in OGT II data set

		Overall Survival (OS) (N=111)				
12K ID	**Gene**	**HR**	**HR Lower 95**	**HR Upper 95**	**p value**	**FDR**
85_B_2	***LOC113230***	**0.01**	**<0.01**	**0.60**	**0.04**	**0.24**
21_G_5	***KIAA1383***	**0.45**	**0.23**	**0.89**	**0.02**	**0.24**
66_G_6		5.92	0.83	42.02	0.08	0.33
17_G_11	*SIX1*	1.12	0.70	1.81	0.63	0.94

HR hazard ratio, HRLower and higher 95 represent 95% confidence intervals, *P value is two-sided score test of the univariate Cox model. HR: hazard ratio. HR>1 indicates the increased risk of death associated with the increase of methylation. HR<1 indicates the decreased risk of disease progression/death associated with the increase of methylation. FDR: False discovery rate estimated using the method described before (Benjamini & Hochberg, 1995).

LOC113230 and *KIAA1383* were also examined in a multivariate analysis which included the usual confounding factors such as age, stage, grade and histological subtype. PFS remained significant for *LOC113230* at 'p'=0.04 (two-sided score test) and the result for overall survival approached significance (p=0.06) (two-sided score test). For *KIAA1383* the correlation with OS was also upheld (p=0.01) (two-sided score test). As these loci were shown to validate on a different platform to their discovery platform these results were deemed to be highly significant and lent further weight to the ongoing functional analysis of *KIAA1383* (Chapter 6).

7.3.2.4 OGT II Summary

The purpose of the OGT Phase II was to examine whether loci identified as showing differential methylation in ovarian cancer sensitive and resistant cell lines had a role at the outset of disease. One of 11 genes derived from chapters 3-5, *CNTNAP*, showed a statistically significant relationship with PFS. This was a gene that I had not been able to optimise MSP primers for and this shows the benefit of examining loci using different methods.

Two of four loci identified from the *in vivo* comparison, *KIAA1383* and *LOC113230* correlated with survival and this would support the notion that the *in vivo* derived cell lines may be of more use when attempting to identify biologically relevant targets. In addition because the multiple probes are designed to investigate an individual gene it is possible to detect differential methylation that could be missed by pyrosequencing – as is likely to be the case for *LOC113230* and so DMH combined with array analysis has been demonstrated to be useful for both candidate validation and the identification of novel genes.

This data set consisted of tumours which were mostly late stage and excluded more unusual subtypes such as clear cell and mucinous tumours. It would be very interesting to investigate whether any of these loci had *predictive* as well as *prognostic* ability as biomarkers and also whether methylation of these genes is required in some types of ovarian cancer but not others – as this could lead to a better understanding of the biology underpinning the different biological subtypes.

Increasingly it will be possible to assess the efficacy of predictive biomarkers as increasing numbers of patients are treated with biological agents in combination with standard chemotherapy. For example numerous studies reported over the last 18 months have shown the benefit of antiangiogenic agents (Burger, Sill et al. 2007; Cannistra, Matulonis et al. 2007; Nimeiri, Oza et al. 2008) and the recent abstract at ASCO by Gourley *et al* has demonstrated that patients with a subtype of high grade serous tumours, expressing markers associating with angiogenesis, can be identified by RNA profiling of formalin-fixed paraffin-embedded tumour samples (C. Gourley 2011).

It would also be of interest to assess whether these changes in methylation could be detected in serum and whether the methylation of any of these genes is tied in with response to debulking surgery as this is another factor that could affect PFS and OS. The

prognostic benefit of methylation of these markers in early stage disease has not been assessed in this study but this would be especially useful. Most clinicians would agree that a large area of concern is the patients with early stage disease who are treated with chemotherapy - where a smaller proportion benefit from it (compared to those with advanced disease).

In conclusion, this focussed approach which analysed the methylation status of previously identified candidates (from cell lines) has identified 3 genes that appear to have a role at the outset of disease and confer prognostic information. The next important step was therefore to test these loci in the acquired resistance setting and for this we have used the Agilent 244 array of 10 matched pre- and post- relapse tumour pairs and work is ongoing.

8 Thesis Conclusion and outline of future work

8.1 Summary of thesis findings

One of the first aims of this project was to validate pyrosequencing as an improved method, compared to MSP, of assessing methylation of candidate genes. MSP is a highly sensitive technique (Herman, Graff et al. 1996) but does not allow for quantification and therefore makes correlations with survival more difficult. I have demonstrated throughout this thesis that pyrosequencing (Tost and Gut 2007) provides highly reproducible quantitative data and information on a number of adjacent CpGs. In addition in Chapter 7.2.2.2 I have demonstrated that for one candidate gene, *SIX1*, that MLDA results correlate well with pyrosequencing results.

During the course of my studies MLDA was developed by our group as an alternative method of biostatistically analysing differential methylation from DMH experiments (Dai, Teodoridis et al. 2008). In order to validate the ability of MLDA to identify significant differences in differential methylation between sensitive and resistant cell lines and validate this novel statistical technique (Dai, Teodoridis et al. 2008), the experiments outlined at the beginning of Chapter 4 were undertaken. I demonstrated that the loci that were predicted by MLDA to show increased methylation in resistant cell lines did indeed do so (all except one, *MLLT6*). MLDA is therefore a highly sensitive method for identifying genes which show differential methylation between cisplatin-sensitive and -resistant cell lines.

In the original PAM analysis in Chapter 3 several genes did not map to the 5' end or contain a CGI, close to a known gene. In contrast in the MLDA analysis of Chapter 4

although candidates were chosen at random and ranked from 2-75 by MLDA score (highest ranking representing largest difference in methylation between sensitive and resistant cell lines) many of those that could be investigated individually, by pyrosequencing, did show increased methylation in the resistant cell lines and most mapped to the 5' end and contained CGIs. In addition MLDA takes into account the unique biological differences that are seen when examining methylation as opposed to expression changes (a larger number of assymetrical changes not following a normal distribution and without 'over-normalisation' of the dataset with the loss of valuable information).

In addition it was proposed that the *in vivo* generated cell line pairs (Langdon, Lawrie et al. 1988) could be more biologically relevant than the *in vitro* generated cell lines and could therefore be useful models for identifying clinically relevant biomarkers. I have been able to show that although a comparison of this small number of available paired lines generates a smaller number of candidates than the *in vitro* derived pairs, that these do indeed seem to be biologically relevant – as at least two of four genes in Chapter 5 showed a correlation with PFS or OS in ovarian cancer patients. This is important as although criticisms are often raised with regards to the artificial nature of cell line models compared to experiments using patient samples they are a less finite resource and may well be more appropriate at the discover stage of biomarker development. In addition whereas information is not always available on whether matched tumours represent platinum sensitive or resistant relapse this information is available for the *in vivo* derived cell lines (Langdon, Lawrie et al. 1988).

In Chapter 6 functional experiments were undertaken in an attempt to further characterise the role of potential candidate genes in acquired drug resistance. We chose *SP5* and *KIAA1383* to focus on for these experiements. *SP5* was selected because of its high level

of methylation in most of the cell lines and also the majority of primary tumours. We hypothesised that instead of a gain of methylation being relevant here it may be a loss of methylation in a sub-group of tumours. We predicted that this loss of methylation could result in an increase in expression and if this was under epigenetic regulation that treatment with decitabine, a demethylating agent, would reverse this gene repression. This was indeed the case; albeit in a small number of samples and with the proviso that although the trend was always in the correct direction (more methylation = less expression) that this was not always statistically significant. Following on from this experiment we hypothesised that if a small number of primary tumour samples were losing methylation and that this was resulting in an increase in expression with a negative outcome that if we knocked down the gene, using siRNA that we should see a phenotypic effect. In the experiments outlined in Chapter 5.10 I showed that it was not possible to perform a valid MTT experiment to investigate the effect of *SP5* knockdown on chemosensitivity, because when *SP5* was knocked down a high proportion of the cells died.

Given this interesting observation I went onto perform an apoptosis assay where I was able to demonstrate that knockdown of *SP5* caused an approximately 20% increase in apoptosis (Chapter 6.6). As a result we were interested to know whether if *SP5* knockdown caused increased apoptosis whether the reverse would be true – and we predicted if this was the case that over expressing *SP5* would cause increased proliferation and possibly a reduction in chemosensitivity. These experiments were then performed by my colleague, Jenny Hersey, who successfully demonstrated that she could stably over express *SP5* in the A2780 CP70 cell line. The stable clones which over expressed *SP5* emerged quickly compared to the vector only control– within less than a week – and this encouraged us that *SP5* might indeed promote proliferation. However, using an MTT experiment we did not see any alternation in proliferation in the presence of cisplatin, a surrogate for modulation of chemosensitivity. The limitations of this experiment and future plans are discussed later.

For KIAA1383, our plans to investigate the phenotypic effect, were more hampered. For nearly all experiments which involve under- or over-expressing a gene of interest, and assessing the phenotypic effect of this, it is necessary to show that one has indeed modulated the target – either at the mRNA or protein level. The fact that I was unable to optimise the *KIAA1383* RTPCR primers therefore caused huge limitations.

With this in mind we felt the best first experiment would be to over express *KIAA1383* transiently and investigate whether there was any effect on cell cycle modulation. At least with this method there was the ability to gate our cells (FLT2 High vs. FLT2 low) and ensure we had green fluorescing cells in the over expressing populations and this should be a surrogate for over expression of the gene of interest. In addition in these experiments controls were included which included the vector alone so that if we did see a positive result it would be more likely it was coming from the over expression of *KIAA1383*, rather than the vector alone.

We were encouraged to observe that *KIAA1383* did seem to attenuate the effect of cisplatin on the PEA2 cell line, resulting in less S phase stalling. These results require to be confirmed and ideally in experiments that include proof of overexpression either at the mRNA or better protein level. Again this will be discussed in the later section; outline of future work (Chapter 8).

The work that is described in Chapter 7 was carried out in parallel to the other experiments, and given the time taken to design the probes, perform the DMH, hybridise the samples and extract the results using bioinformatic approaches, was only recently analysed.

It showed that genes that had been disregarded in early chapters because of technical difficulties with primer design or which had not looked interesting in terms of their pattern of methylation (*CNTNAP* and *LOC113230*) according to our hypothesis (biomarkers of acquired platinum resistance would show increased methylation in resistant cell lines or matched tumour pairs, and a heterogeneous pattern of methylation in primary tumours) were in fact correlated with survival. It also confirmed that *KIAA1383* appeared to be a possible prognostic biomarker. This was an independent and substantially better annotated array so these results were seen as very encouraging. It should be noted though that there is overlap between the primary tumour samples used in Chapters 3-5 and those hybridised in Chapter 7 so this is not a truly independent validation. Plans for further work on these candidate loci are again discussed later in Chapter 8.10.

8.2 **Tumour heterogeneity in EOC**

In the longer term it is the aim of our group to develop further cell lines where the resistance is generated within patients rather than by repeated drug treatment in the laboratory. Clinically the different underlying biology in ovarian cancer is important, and if matched sensitive and resistant cell line models could be generated in patients reflecting clear cell vs. mucinous vs. HGS vs. LGS for example this would be extremely useful.

As an example it is suspected clinically that mucinous tumours behave more like colorectal cancers and hence, as discussed in the introduction, the current clinical trials are examining the use of colorectal regimens in such patients – if this hypothesis could be formally tested in the laboratory this would be very relevant and could direct which therapies are guided towards which patients – true tailoring of patients treatment.

Patient samples are clearly hugely important, especially matched pre- and post- samples however the examinations in matched tumour pairs in this thesis generated disappointing results and were limited by both the small number of samples and the lack of clinical annotation for the samples. As discussed in the introduction, the platinum free interval (Markman 2001) is a critical surrogate for response to chemotherapy, and therefore it is crucial to know whether the relapse samples represented platinum sensitive or resistant relapse. In addition when using the residual disease pairs the assumption is that disease removed at the time of 'second look surgery' represents resistant disease, however there is no way of knowing this for sure, with the current samples.

As discussed in the introduction it is becoming increasingly clear that ovarian cancer is highly heterogeneous from the outset of disease with quite different biological pathways involved, and genes over- and under- expressed, in different subtypes. This has huge implications when using a relatively small panel of matched pairs as it is possible they all represented, for example, HGS, which is relatively platinum sensitive or the clear cell or mucinous subtypes, which are more platinum resistant (Mackay, Brady et al. 2010).

In addition it has been noted that although stage *per se* is important that the distribution of stage at presentation is different for the different histological subtypes. HGS tumours more commonly present with later stage disease (35.5% of stage I/II cancers vs. 87.7% of stage III/IV cancers) whereas endometriod (26.6% early stage vs. 2.5% late stage) and clear cell sub types (26.2% vs. 4.5%) are enriched within early stage disease (Gilks, Ionescu et al. 2008; Kobel, Kalloger et al. 2010). These results suggest that the different biological subtypes do not progress through the stages in an advancing manner and instead that the differences in stage from the outset are determined by the underlying histological subtype.

This clearly has implications when attempting to derive prognostic biomarkers. One would predict that the earlier disease stage that the cancer is detected at the better the prognosis however given the enrichment of patients with HGS pathology who present in the later stages this represents a huge technical challenge. Some small hope is provided by the fact it does appear that some of these changes may be detectable at very early stages. The precursor lesion in Type II tumours is less clearly understood (see Chapter 1.1 and table 1) and their origin unknown until recently when studies suggested that a large proportion originate in the fallopian tube seccretory epithelial cell (FTSEC) – This was uncovered following pathological analysis of tissue from BRCA patients with germline mutations who were having prophylactic surgery. This putative serous carcinoma precursor lesion was observed in the fimbriated end adjacent to the ovary and if >12 FTSECs were observed this was termed 'the p53 signature' and although it was concerningly morphologically benigh on H&E staining it showed intense nuclear p53 immunostaining (Karst, Levanon et al. 2011). These 'p53 signatures' were frequently the same as the patients somatic mutations suggesting a common origin for both (Lee, Miron et al. 2007).

In addition matters are further complicated because although patients with mucinous and have a favourable prognosis when diagnosed with early stage disease the opposite is the case when the disease is diagnosed when Stage III or IV. In an analysis of patients treated in the Gynaecologic Cancer InterGroup study of approaching 9000 women, with advanced ovarian cancer, median survival was noted to be the following; endometriod (50.9 months), HGS (40.8 months), clear cell (21.3 months), mucinous (14.6 months) (Mackay, Brady et al. 2010). This is presumably because these patients are more resistant to the standard treatment of carboplatin and paclitaxel and highlights the need for predictive biomarkers.

Another challenge with ovarian cancer, compared to some of the other solid tumours, is that the targeted agents that have been showing promise in the clinic in other cancers are likely to only be effective in small sub-groups – and this may explain the disappointing results of trials of targeted agents until recently. For example patients with LGS tumours have activating mutations in KRAS and BRAF which could be targeted with MEK or BRAF inhibitors but these mutations are only found in approximately 20% of patients and LGS only make up around 5% of EOCs (see table 1 in introduction). Similarly it is suspected that clear cell tumours of the ovary may behave more like clear cell tumours of the kidney (Zorn, Bonome et al. 2005) and therefore may respond to drugs such like sunitinib. Again clear cell tumours make up a small proportion of ovarian cancers and therefore if this agent is to be fairly assessed it is critical that this sub group is selected out.

Some encouragement can be taken from the study presented at ASCO this year (2011) by Ledermann and colleagues. In a Phase II randomized placebo-controlled study they treated patients who had platinum sensitive relapsed HGS cancer with the PARP inhibitor, olaparib (AZD2281) (J. A. Ledermann 2011). They were selecting out platinum sensitive patients in order to test the existence of the 'BRCAness phenotype' and its predicted response to synthetic lethality using a PARP inhibitor (Konstantinopoulos, Spentzos et al. 2010). The results were very encouraging with a 4 month improvement in PFS (J. A. Ledermann 2011) and given that HGS is the most prevalent subtype this makes the results of huge utility. The limitation to this approach being that, like the PFI, one has to treat patients and assess the length of time to relapse ie their "platinum sensitivity" in order to know whether these agents might be helpful. What would be more clinically useful would be if a gene expression profile or methylation profile could identify patients prospectively who had the BRCAness phenotype and then these patients could be given the relevant agents upfront (Konstantinopoulos, Spentzos et al. 2010). This approach may be feasible in the next few years. As discussed previously, David Bowtells laboratory recently

presented a gene expression profile which divided patients into 6 subgroups and 4 of these validated in an independent data set (Tothill, Tinker et al. 2008) and more recently, again this year at ASCO, the Gourley laboratory have been able to prospectively identify a sub group of patients with HGS cancer that over expression angiogenesis markers (C. Gourley 2011). The most encouraging results seen this year in ovarian cancer were with the use of the angiogenesis inhibitor, bevacizumab, and it does therefore seem feasible that in the next few years predictive biomarkers really could become 'real-time'. There is potential to investigate whether changes in methylation also differentiate tumours into these distinct sub groups and if this was the case this would be of clinical utility as DNA methylation is well suited to clinical studies (see chapter 8.5 and 8.6).

In order to address the issue of a lack of matched samples in more homogenous sub-groups, during the course of this project, I wrote a clinical trial protocol (under Dr Glasspools supervision in Glasgow) to collect matched samples from patients. Several studies have shown the ability to use surrogate tissues such as plasma (Gifford, Paul et al. 2004), serum and ascites (Ibanez de Caceres, Battagli et al. 2004), and in this protocol we are collecting plasma +/- ascites +/- tumour from patients at diagnosis and relapse. Over the last two years we have collected approximately 40 samples although unfortunately few of them are matched, highlighting the difficulties in trying to collect these very valuable samples.

Despite the concerns outlined above it still seems likely that genes that show differential methylation between 'sensitive' and 'resistant' tumour pairs could be more biologically relevant than those derived from cell lines. And although the A2780 series represent 16 sensitive and resistant cell lines it is possible that in reality we are only testing 3 variables as the cell lines are isogenically matched (A2780 sensitive, A2780 CP70 single high dose of cisplatin, and the MCPs multiple doses of cisplatin). Therefore in work which is

ongoing the loci identified in this thesis are being examined in a small number of matched tumour pairs which have been prepared by DMH by myself and hybridised to the Agilent 244k array. Clearly this could be repeated using a larger number of matched samples if it proves successful and in the meantime we will endeavour to obtain more clinical information with regard to theses samples.

8.3 The increasing availability of publically available datasets for validation

Examining the relationship between methylation and expression for each individual locus can be time consuming and if parallel experiments could be preformed examining differential methylation and expression this could be an efficient means of better identifying interesting candidates. In addition, in several recent studies (Noushmehr, Weisenberger et al. 2010), (Tothill, Tinker et al. 2008) investigators have begun to test their candidate genes in publically available large well annotated data sets such as the TCGA resource (cancergenome.nih.gov).

The Cancer Genome Atlas (TCGA) began as a three-year pilot in 2006 with an investment of $50 million each from the National Cancer Institute (NCI) and National Human Genome Research Institute (NHGRI). The TCGA pilot project showed that a national network of research and technology teams working on distinct but related projects could pool the results of their efforts and develop an infrastructure for making the data publicly accessible. Importantly, it proved that making the data freely available would enable researchers anywhere around the world to make and validate important discoveries. The success of the pilot led the National Institutes of Health to commit major resources to TCGA to collect and characterize more than 20 additional tumour types. Various

platforms exist including those investigating gene, exon, miRNA, and protein expression, copy number variation, single nucleotide polymorphisms (SNPs), loss of heterozygosity (LOH) and especially relevant to this thesis, DNA methylation (https://wiki.nci.nih.gov/display/TCGA/Array-Based+Data).

Investigators used the infinium 27k array, which utilises bisulphite modified DNA (www.illumina.com/.../infinium_humanmethylation27_beadchip_kits.ilmn) and allows the opportunity to investigate methylation changes focussed around the promoter and elsewhere in the genome. In addition correlations between methylation and expression can be made a much larger scale basis than the approach taken in this thesis.

8.4 Validation of the OGT CGI focussed array.

An additional important task was to begin to perform experiments using customised arrays; both for biomarker validation and discovery. Our group has now gained a substantial amount of experience using the Agilent technology in conjunction with the OGT focussed platforms and a further collaborative experiment is planned. This experience spans from the bioinformatics utilised to design probes and the statistics used to identify loci right through to the validation in the wet laboratory. Given that our group is focussed on DNA changes around the promoter there is a benefit of designing more probes to focus around this particular area of interest (as opposed to an array like the infinium 27k array which has broader coverage of the genome but less detail around the promoter and therefore may miss changes in differential methylation in this area). In collaboration with two post doctoral fellows in our laboratory I have gained experience in the technical preparation of the samples for such arrays. (Dr Teodoridis and Dr Zeller).

8.5 Using methylation or expression arrays to identify key pathways and driving (epi)mutations.

One of the primary aims of our group is to use differential methylation to identify key genes, or biological processes or pathways. In work not focussed on this thesis and driven by one of my colleagues, Wei Dai, we have recently identified that methlyation plays a key role in the Wnt pathway and that this has prognostic significance (Dai, Teodoridis et al. 2010) and work is ongoing assessing other pathways that are associated with ovarian cancer and drug resistance (As per Figure 78 and table 38). Similar approaches have been succesfully utilised by others to demonstrate pathways and genes with critical dysregulation; either by assessment of epigenetic changes (Anderton, Lindsey et al. 2008; C. Gourley 2011) or more commonly gene expression profiles (Tothill, Tinker et al. 2008; C. Gourley 2011).

8.6 The benefits to using methylation as a biomarker

Methylation is a stable change and DNA lends itself well to clinical studies. In the development of a biomarker it is critical that a test can be performed with highly reproducible results – and if a test is to be 'rolled out' to a large number of patients then it is important that the assay is not too sensitive to small differences in how it is handled. For example DNA is more stable than RNA and less prone to degradation through, for example

, temperature changes. This means that if DNA is to be extracted from a tumour that the handling of that sample from the time of surgical resection to processing by the pathologist is less critical than for example if RNA was required from fresh frozen tissue. The release of DNA into body fluids and again its relative stability compared to other sources of biomarkers, means that relatively non-invasive means of monitoring epigenetic changes in tumour can be used. Since normal tissues tend not to have CGIs methylated, then the sensitivity of detecting tumour specific changes is increased and is less prone to be being confounded by normal cell contamination. In addition in terms of then analysing individual candidate, techniques such as pyrosequencing once optimised are relatively less operator and institution dependent and relatively higher through put compared to RTPCR. As discussed earlier DNA has the benefit of being able to be extracted from various tissues including blood, plasma, ascites and tumour and can be stored for relatively long periods of time at -20°C without degradation.

8.7 The interaction between DNA methylation and other epigenetic mechanisms and the role of tumour initiating cells

In the introduction to this thesis the interplay between chromatin, methylation and micro RNAs is discussed and it is likely that over the next few years that this will be increasingly well understood and may better explain the function of differential methylation. In addition this thesis has focussed on changes in methylation at the promoter but it is becoming increasingly clear that changes within the gene body are also important (Ball, Li et al. 2009).

During the course of this project the existence of cancer stem cells or tumour initiating cells has been increasingly debated. This was not the focus of this project but it is very interesting to note that many of the candidates that were identified as potential biomarkers of acquired drug resistance had links to stem cells – such as *NR2E1* and *LMX1A*, as discussed in Chapter 3. It does seem increasingly likely that researchers working in drug development in ovarian cancer will attempt to target the stem cell component and if methylation is demonstrated to have a role in altering the expression of stem cell genes then methylation could have a role both as a biomarker of stem cell phenotype and in terms of the utilisation of epigenetic therapies.

The table below summarises the key findings relating to genes in this thesis that showed differential methylation between cisplatin sensitive and resistant cell lines or correlated with survival in primary ovarian cancer tumours. In addition the final column documents any speculative roles in developmental biology or stem cell function. In this small number of candidates there does appear to be an enrichment of transcription factors involved in developmental biology.

Table 44. Key findings from the literature for genes outlined in this thesis.

Relevant reference(s)	Drug resistance/ general cancer role	Putative stem cell/ developmental biology role
LMX1A. (LIM homeobox transcription factor 1, alpha). Transcriptional factor. (Chapter 3)		
(Liu, Chao et al. 2009) (Chizhikov and Millen 2004)	Metastasis suppressor in cervical cancer. Methylated 89.9% of squamous cell carcinomas of cervix compared to 6.7% in normal cervix. Over-expression reduces colony formation and a less invasive phenotype is observed using a matrigel invasion assay.	Development of the roof plate and specification of dorsal cell fates in the CNS and developing vertebrae. In the chick developing spinal cord *LMX1A* has been shown to induce expression of *WNT1*. *LMX1A* as having a role in the differentiation of human embryonic stem cells into midbrain dopamine neurons in culture and after transplantation into a Parkinson's disease model.
N2RE1(nuclear receptor subfamily 2, group E, member 1) (tlx is mouse homologue.) Transcription factor. (Chapter 3)		
(Yu, Chiang et al. 2000) (Dziarmaga, Hueber et al. 2006; Hueber, Waters et al. 2006)	*tlx*, is an upstream regulator of *PAX2* and suppresses its expression in mice. Downregulation of *PAX2* can enhance cisplatin sensitivity.	*PAX2* activates *WNT4* gene expression. In the adult is expressed exclusively in the astrocyte-like B cells of the subventricular zone - acts as a key regulator of neural stem cell expansion and brain tumour initiation. The area of over expressed *tlx* corresponds with overexpression of Nestin (neural stem cell marker). *tlx* induced NSC expansion is associated with increased angiogenesis and migration.
CRABP1 (cellular retinoic acid binding protein 1). Retinoic acid mediated differentiation and proliferation. (Chapter 4)		
(Huang, de la Chapelle et al. 2003; Lind, Kleivi et al. 2006; Ogino, Brahmandam et al. 2006; Tanaka, Imoto et al. 2007; Wu, Lothe et al. 2007).	Frequently methylated tumour. suppressor gene in oesophageal cancer, colorectal cancer, ovarian cancer and thyroid cancer.	SAGE databases demonstrate it is expressed in the brain, retina and spinal cord and also in skin, breast and ovarian cancers although to a lesser extent (www.genecards.org/cgi-bin/carddisp.pl?gene=CRABP1).
CNTNAP (contactin-associated protein 1). An innate immunity gene (Chapter 4)		
(Lee et al 2009).	Not known.	Role in the formation of functional distinct domains critical for saltatory conduction of nerve impulses in myelinated nerve fibers. Demarcates the paranodal region of the axo-glial junction. In association with contactin may have a role in the signaling between axons and myelinating glial cells
SP5. Transcriptional activator (Chapter 4)		
(Harrison, Houzelstein et al. 2000; Treichel, Becker et al. 2001; Weidinger, Thorpe et al. 2005) .	Increased expression in colorectal, gastric and hepatocellular cancers. Over expression in MCF7 cells resulted in growth	Role in the coordination of changes in transcription required to generate the developmental pattern in the developing embryo. *A*ntagonises *SP1* and is a downstream target of Wnt signalling

(Takahashi, Nakamura et al. 2005; Weidinger, Thorpe et al. 2005; Chen, Guo et al. 2006; Fujimura, Vacik et al. 2007).	promotion. Downstream targets of *SP5* implicated in ovarian cancer and drug resistance include *p21, TGFB1, MDM2, ABCG2* and *ABCC3* (see Chapter 1.3).	Dynamically expressed during CNS development
LOC113230 hypothetical protein. (Chapter 5))		
None	Unknown.	Unknown.
KIAA1383 hypothetical protein. (Chapter 5)		
None	Unknown.	Unknown.
SIX1 (sine oculis homeobox 1). Transcription factor. (Chapter 5)		
Coletta, Christensen et al 2008. McCoy, Iwanaga et al 2009	DNA dependent regulation of transcription. Over expression induces genomic instability and is sufficient for malignant transformation. Can induce mammary tumours to undergo epithelial – mesenchymal transition.	Role in limb development, ureteric bud development, kidney development and in expansion of the mouse mammary epithelial stem/ progenitor cell pool.

8.8 Difficulties encountered/ limitations of the experiments described within this project

One difficulty in the present study is when a candidate is identified which is "unknown" - for example in the case of *KIAA1383*. If the decreased methylation correlates with increased expression then there is the ability to investigate the functional effect of over and under expression but this does not get to the bottom of what the function of the gene is and which other genes it is associated with or which pathway it is important in. In the case of *KIAA1383* it was not found to be expressed in any cancers on a wide variety of publically available data bases and nor was it known to show homology to any other known genes. Due to the small size of this gene which only spans one intron it has thus far not been possible to optimise RTPCR primers and there are no commercially available antibodies in order to examine this protein by western blotting. It then becomes very difficult to know where to go next with such a candidate – but given the consistent correlation between differential methylation and PFS it is frustrating not to be able to take characterisation of this locus further.

It could be argued that if a change in methylation correlates with a significant difference in, for example, OS or PFS, that whether or not there are known functional consequences of this change is irrelevant and that this finding alone is enough to make it a valuable biomarker. But in the longer term most would argue that more can be gained by trying to identify the functional consequences of a change in methylation.

Throughout this thesis MLDA has proved sensitive at detecting differential methylation between cisplatin-sensitive and resistant cell lines. However candidates that show differential methylation in the cell lines do not necessarily show this in the matched pairs and there is a lack of an adequate number of well annotated samples to investigate whether a change seen *in vitro* ie in the cell lines represents a genuinely important gene *in vivo* – in the patient. In addition two of the genes that correlated with survival in ovarian cancer, including in the validation experiment of Chapter 7, did so in the 'opposite direction' to that which would have been predicted from the cell lines experiments; namely an increase in methylation correlated with improved survival.

8.9 Alternative approaches that could have been utilised

Various alternative approaches could have been taken in order to identify genes or pathways which are predictive or prognostic biomarkers in epithelial ovarian cancer. In the years following the start of this project much larger and better annotated microarrays have become available and in 2011 one would not use the 12k array which was used in Chapters 3 and 4.

In addition the DMH experiments were performed in a limited number of cell line pairs given that the many of the A2780 series are isogenically matched. These experiments could be refined by using a larger number of cell line pairs – and preferably using a larger number of the *in vivo* derived cell lines.

Another alternative, especially in terms of identifying prognostic markers, would be to do the initial experiments in a panel of well annotated primary tumours as opposed to cell lines; or to perform the DMH in an adequately powered set of paired samples which are known to represent truly platinum –resistant disease. Validating individual candidates, by

methods such as MSP or pyrosequencing, is time-consuming and as described earlier in this chapter increasingly well annotated public data bases, such as the TCGA, are becoming available, which can be utilised to validate candidates in an independent dataset.

In terms of identifying key pathways, as described throughout this thesis, it is becoming increasingly clear that the pathways which are dysregulated are very different depending on the biological subtype. Therefore a more robust way of identifying key pathways or genes is to ensure that an adequate number of all kinds of epithelial ovarian cancer are represented on the microarray – in the experiments outlined in this thesis I tended to try and use primary tumour samples that represented high stage and grade – assuming that these are the tumours in which candidates of acquired drug resistance would be most enriched – and in retrospective probably selecting out the HGS sub-population (as clear cell and mucinous tumours were generally excluded).

An additional refinement would be to investigate whether groups of genes rather than an individual candidate correlates with survival and also to test formally whether there is an enrichment for particular types of genes, or groups of genes within a particular key pathway – for example using a Gene Ontology (GO) analysis (http://www.geneontology.org/GO.tools.microarray.shtml).

8.10 Outline of Future Work

8.10.1 KIAA1383

In the immediate term one of my colleagues is going to continue the work on over-expression of *KIAA1383* and *SP5*. Providing *KIAA1383* can be stably over-expressed in cells, the next plan would be to perform an MTT assay.As referenced earlier in the thesis, *KIAA1383* has been noted to be over expressed as a result of DZNep (a histone methyltransferase inhibitor) treatment and it would therefore be worth investigating the

role of histone methylation in the regulation of this gene. It would appear that its main scientific merit currently is as a prognostic biomarker and if we could confirm some preliminary phenotypic effect we could consider investigating this prospectively in a clinical trial setting. Additionally we plan to investigate whether it has been shown to be either over expressed or hypermethylated in the TCGA publically available data sets.

8.10.2 CNTNAP, NR2E1, LMX1A

In addition some other genes which were identified in the cell lines and then later validated in the experiments described in Chapter 7 should be further investigated. *CNTNAP* was initially observed to show increased methylation in the resistant cell lines, in the DMH experiment outlined in Chapter 3. In the OGT Phase II experiment it was also shown to correlate with PFS in a panel of primary ovarian cancer tumours. This gene has very little published in relation to its function and is a novel candidate in terms of a relationship to cancer. Similarly nothing has been published with regards to the potential role for methylation in modulating gene expression. Like *NR2E1, LMX1A* and *DLC1* it appears to be involved in neural development. It seems to demarcate the paranodal region of the axo-glial junction and in association with contactin may have a role in the signalling between axons and myelinating glial cells (www.genecards.org/cgi-bin/carddisp.pl?gene=CNTNAP1).

8.10.3 LOC113230

LOC113230 (85B2), which was identified from the comparison of the *in vivo* generated cell lines, in the experiments described in Chapter 5 also showed a correlation with survival in the OGT Phase II experiments. It correlated with PFS (HR 0.04, ‘p’=0.02, FDR 0.14) and OS (HR 0.01, ‘p’=0.04, FDR 0.24) by univariate analysis and PFS

('p'=0.04) in a multivariate analysis; with the results for OS ('p'=0.06) also approaching significance (for full details of results see chapter 7.3.2.3).

When this candidate was examined by pyrosequencing a high level of methylation including in normal controls was observed but it is possible that the primers could be redesigned. We plan to optimise RTPCR primers to investigate whether although there doesn't appear to be a large change in methylation whether there is a larger change in expression – and also to see whether the use of a demethylating agent reverses this. In addition we are planning over expression experiments involving this candidate and if successful an MTT experiment; as was described for SP5 and KIAA1383 in Chapter 6.7 and 6.8.

In the longer term MLDA has now been validated as a sensitive method of detecting differential methylation. The work done by my colleague Wei Dai has validated our approach of probe design and statistical interrogation in the OGT arrays. It seems likely that future work will involve high throughput detection methods with ongoing use of pyrosequencing to validate individual loci in the laboratory. For loci such as *LOC113230* and *KIAA1383* which appear to have a real correlation with survival then prospective validation of these biomarkers in clinical samples may be warranted. How far our laboratory goes in terms of trying to characterise and define the function of these unknown candidates is difficult to predict at this stage and the first step will be to see the results of the over expression experiments which are ongoing.

Now that we have shown that we can correlate methylation in the primary tumours with survival or response to chemotherapy in selected candidates, we plan to perform further experiments examining changes in methylation in samples from patients at diagnosis and relapse; including ascites samples. If DNA of a high enough quality can be obtained from surrogate tissues then this opens up several opportunities for future work.

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