ANALYSIS OF PROGNOSTIC AND DRUG RESISTANCE MARKERS IN LUNG CANCER

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Declaration

I, Scott Davidson, declare that this thesis represents my own work, except where acknowledged to others. The thesis does not include work presented successfully for another degree by either others or myself.

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Abbreviations

°C	degrees celsius
>	greater than
<	less than
μg	microgram
μl	microlitre
%	percentage
x g	times gravity
AAH	Atypical Adenomatous Hyperplasia
APC	Adenomatous Polyposis coli
ARI	Aberdeen Royal Infirmary
AUC	area under the curve
bp	base pairs
BTOG-2	British Thoracic Oncology Group – 2 study
CIMP	CpG Island Methylator Phenotype
CT	Computed Tomography
CT-PET	Computed Tomography-Positive Emission Tomography
CAV	Cyclophosphamide Doxorubicin Vincristine
DAB	diaminobenzidine tetrahydrochloride
DAC	5-deoxyazacytidine, 2'-deoxy-5-azacytidine, decitabine
DNA	deoxyribonucleic acid
EBUS-TBNA	endobronchial ultrasound trans bronchial needle aspirate
ECOG	Eastern Cooperative Oncology Group
EGFR	epidermal Growth factor receptor
ERCC1	excision repair cross complimentary 1
EDTA	ethylenediaminetetraacetic acid
EUS-TBNA	oesophageal guided ultrasound trans bronchial needle aspirate
GAPDH	glyceraldehyde-3 phosphate dehydrogenase
g	gram
G-CSF	granulocyte-colony stimulating factor
HNPCC	hereditary non-polyposis colorectal cancer

HCl	hydrochloric acid
HIC 1	hypermethylated in cancer 1
IHC	immunohistochemistry
kb	kilobase
kDa	kilodalton
LDH	lactate dehydrogenase
LRP	lung related protein
$MgCL_2$	magnesium chloride
MINT	methylated in tumour
MSI	microsatellite instability
mg	milligram
ml	millilitre
mm	millimeter
mmol/l	molar mass x 10^{-3} per litre
mol/l	molar mass per litre
MLH1	MutL homologue 1
MMR	mismatch repair
MRC	Medical Research Council
MRI	magnetic resonance imaging
MSH2	MutS homologue 2
MSP	methylation specific polymerase chain reaction
MVP	mitomycin vinblastine cisplatin
ng	nanogram
n	number
NER	nucleotide excision repair
N-I	non-informative
NSCLC	non small cell lung cancer
PBS	phosphate buffered saline
PCR	polymerase chain reaction
Rb	retinoblastoma
SCLC	small cell lung cancer
NaOH	sodium hydroxide
NSE	neurone specific enolase
PET	positive emission tomography

PS	performance status
QoL	quality of life
RCT	randomised controlled trial
SD	standard deviation
TBNA	transbronchial needle aspirate
TE	Tris-EDTA
TKEGFR	tyrosine kinase epidermal growth factor
Tris	tris(hydroxymethyl)aminomethane
U/S	ultrasound
uv	ultravioet
V	volt
VATS	video-assisted thoracoscopy
Vol.	volume
WHO	World Health Organisation
WIG	Western Infirmary Glasgow

Summary

Lung cancer continues to have by far the highest cancer mortality in the UK. Beyond stage of disease and the patient's Performance Status there are no other robust clinical or molecular markers of prognosis available today. One major reason for the high mortality rate of this disease is the significant proportion of patients who present with advanced incurable stage disease. A second significant problem in the management of patients with lung cancer is chemotherapy resistance. In patients with NSCLC (80% of cases) initial response rates to cisplatin-based chemotherapy are modest at best. In the case of SCLC (20% of cases) the initial response rates to cisplatin-based chemotherapy are high. However relapse, often with chemotherapy resistant disease, is all too common.

There is a significant body of evidence that demonstrates the role of the loss of mismatch repair activity in the mechanism of chemotherapy resistance. Studies to date have demonstrated that the loss of mismatch repair protein expression is a consequence of 2 main mechanisms: gene mutation and the epigenetic phenomenon of methylation of the MLH1 (a major mismatch repair protein) gene promoter region. Studies have shown that the loss of MLH1 expression is associated with acquired resistance in ovarian and breast cancers. Allelic imbalance (loss of heterozygosity) of chromosome 3p is common in lung cancer samples and the MLH1 gene locus resides here. This loss of heterozygosity (LOH) has been demonstrated to be a poor prognostic indicator in patients with primary adenocarcinoma of the lung. In this translational research project the role of mismatch repair (MMR) and LOH in patients with lung cancer has been further investigated.

In the first study using 2 separate cohorts of patients the potential role of mismatch repair proteins as a marker of prognosis in patients with NSCLC was investigated.

The first cohort of patients had received either cisplatin based or non-cisplatin based chemotherapy for predominantly advanced (stage IIIb/IV) disease at Stobhill Hospital in Glasgow. Pre-treatment paraffin-embedded bronchoscopic samples were collected retrospectively. The aims of this study were to examine the level of expression of the MLH1, MSH2, p53 and Ki67 proteins in these small samples and assess whether these results correlated with any clinicopathological variables or with prognosis. In addition differences in overall survival between the cisplatin and the non-cisplatin chemotherapy treated patients were assessed relative to the level of protein expression. Despite the small size of the biopsies good inter-observer kappa scores were demonstrated between 2 independent scorers for each protein immunohistochemistry score (IHC) (MLH1 - 0.6062, MSH2 – 0.4313, p53 – 0.591). Although this demonstrates that historic bronchoscopy samples can be used for such studies, the number of markers studied was limited due to small sample size and in this study there was insufficient tissue to assess expression levels of Ki67. No significant correlations were demonstrated between IHC score and overall survival for any of the proteins studied, nor was there any difference between the different chemotherapy regimens. Similarly there was no correlation between IHC score of the studied proteins and any clinicopathological variables.

The second cohort of patients all had resection of their primary NSCLC tumour at Aberdeen Royal Infirmary. Of these 50 patients, 10 had received pre-operative cisplatin-based chemotherapy. Fresh frozen surgical samples collected at the time of surgery were analysed retrospectively. The aims of this study were to examine the level

of expression of the MLH1, MSH2, p53 and Ki67 proteins in these samples and assess whether these results correlated with any clinicopathological variables or prognosis. Whether any difference existed in these variables between the patients receiving pre-operative chemotherapy and those that did not was also investigated. This study failed to show any significant correlation between the level of protein expression and overall survival or any other clinicopathological variable. Further there was no difference in the level of protein expression between those patients who had received pre-operative chemotherapy and those who had not. However given that only a small number of patients had received pre-operative chemotherapy, further large studies would be required to validate these results.

In the second study we investigated the status of CpG island methylation (using methylation specific PCR) and its role as a marker of prognosis in patients with NSCLC. The samples were the same surgical samples as described above as well as normal adjacent lung tissue. The markers studied were MLH1, p16, DAPK, TIMP 3, HIC 1, MINT 25, MINT 31 and RASSF1A. In this study 30 (60%) of samples exhibited methylation of at least one promoter site with 19 (38%) at 1 site, 5 (10%) at 2 sites, 2 (4%) at 3 sites and 4 (8%) at 4 sites. Twenty (40%) of the tumour samples exhibited no methylation at any promoter sites. Methylation rates in normal adjacent lung tissue were low. There was no significant correlation between the number of methylated sites and either overall survival or any other studied clinicopathological variable. The investigation of methylation at individual sites demonstrated an association between HIC 1 methylation and stage of disease ($p = 0.020$) and methylation of MINT 31 was associated with a better overall survival ($P = 0.030$). This remained the case when analysis was performed excluding those patients who had

received pre-operative chemotherapy. This is the first report of MINT 31 methylation being studied in NSCLC and therefore further independent studies would be required to validate these results and confirm that the associations had not occurred by chance due to multiple testing. No significant differences in any of the studied variables were demonstrated when comparing patients who had received pre-operative chemotherapy with those who had not.

A third study was performed to validate the findings of previous studies that loss of heterozygosity (LOH) of chromosome 3p is a common occurrence in patients with NSCLC (Mitsudomi et al., 1996). This study also aimed to investigate any correlations between 3p LOH (D3S1289, D3S1300, D3S1304) or *hMLH1* promoter methylation and level of MLH1 expression. In addition this study attempted to correlate the presence of any molecular changes found in the serum DNA taken preoperatively from patients with those of their primary tumour samples. For this study prospective collection of surgical tumour and normal adjacent lung tissue samples as well as a pre-operative whole blood sample was collected from patients undergoing resection of their primary disease at the Western Infirmary in Glasgow. Numbers in this study were small thus making any attempt at statistical analysis inappropriate. Observations demonstrated that 3p LOH was common in the primary tumour with 4/8 samples demonstrating LOH at D3S1289, 5/6 at D3S1300 and 5/9 at D3S1304. Corresponding changes were demonstrated in the preoperative serum samples in 2 of the 4 patients at D3S1289, 3/5 at D3S1300 and 2/5 at D3S1304. Loss of Heterozygosity at chromosome 3p did not appear to affect the level of MLH1 expression and *hMLH1* methylation was not demonstrated in any of the studied tumour samples.

A final study, in collaboration with Dr J Plumb, was performed to investigate the role of the mismatch repair proteins in the chemotherapy sensitivity of a panel of small cell lung cancer cell lines. In this study it was demonstrated that there was a high correlation between cisplatin sensitivity and the mismatch repair proteins MLH1 (r^2 = 0.83) and MSH2 (r^2 = 0.87) but not PMS2 (r^2 = 0.22). Two of the cell lines originated from metastatic biopsies from the same patient, one pre-chemotherapy treatment (LS274) and one post-chemotherapy (LS310). It was shown that LS310 is 2.3 times more resistant to cisplatin and shows a 50% reduction in MLH1 expression when compared to LS274 ($p < 0.001$). It was demonstrated that the *hMLH1* promoter region of LS310 exhibited methylation whereas the LS274 promoter region did not. Neither of these lines exhibited methylation of the p16, MINT 25 or DAPK loci suggesting that de novo methylation was not responsible for the methylation specific PCR results. Further work demonstrated that treatment of the LS310 cell line with the demethylating agent decitabine increased its cisplatin sensitivity as well as increasing the level of MLH1 expression of the cell line. No such changes were demonstrated in the LS274 cell line after treatment with decitabine.

In summary, this research project was limited by the availability of samples. However it has demonstrated that collaborative multidisciplinary prospective planned translational research can be done and emphasises the need for a translational component to be an integral part of future lung cancer studies.

1. Introduction

1.1 Lung Cancer: the clinical problem

Lung cancer remains the most common cancer in men in the United Kingdom. The incidence of lung cancer in males is greater than the next two most common male cancers (prostate and colon) taken together. The incidence of lung cancer in women continues to rise throughout the UK and in parts of the UK has overtaken the incidence of breast cancer (Gillis et al., 1992). Figures from Cancer Research UK (CRUK) show that approximately ninety per cent of patients with lung cancer survive less than 12 months and in 2003 33,436 deaths from lung cancer were reported in the UK (CRUK, 2006).

1.2 Pathogenesis and Classification

For a cell to become malignant it has to acquire several key properties (Hanahan and Weinberg, 2000) and these are:

1. Loss of senescence and the acquisition of the ability to replicate indefinitely
2. Independence from normal growth signals
3. Lack of sensitivity to anti-growth signals
4. Evasion of apoptosis
5. Independent angiogenesis
6. Ability to invade and metastasise

In the majority of cancers the initial insults are multi-factorial but in the case of lung cancer it has been unequivocally demonstrated that cigarette smoking is by far the most common causative factor in the development of lung cancer (Doll and Hill, 1950) and

accounts for approximately 90% of cases (Szabo and Mulshine, 1993). To date there are at least 60 known carcinogens within the 4000 known chemicals in cigarette smoke (Hecht, 2002), and these combine in susceptible individuals to cause the development of lung cancer.

Approximately 95% of primary lung cancers are epithelial in origin and these are categorised on the basis of histological appearance into Non-Small Cell Lung Cancer (NSCLC) and Small Cell Lung Cancer (SCLC). NSCLC diagnoses are then sub classified into squamous cell carcinoma, adenocarcinoma, and large cell carcinoma as well as undifferentiated NSCLC. There is no further histological sub-categorisation of SCLC (Wagenaar and Tazelaar, 1994). Each of these cancers however appears, after initial insult, to have a different developmental pathway. In squamous cell carcinoma there is a relatively well-understood developmental pathway from hyperplasia, dysplasia, carcinoma-in-situ through to invasive carcinoma (Hirsch et al., 2001). The developmental pathway for primary lung adenocarcinoma is less well understood. There is evidence that areas of Atypical Adenomatous Hyperplasia (AAH) are the precursor for the development of adenocarcinoma (Westra, 2000), these areas being detectable in the lungs of up to 40% of patients with adenocarcinoma as compared to 11% of those with squamous cell carcinoma. Further molecular evidence supports this hypothesis. In up to 39% of AAH K-ras mutations, a mutation characteristic in primary lung adenocarcinoma are found (Westra, 2000).

Loss of Heterozygosity (LOH) of chromosome 3p is another example of a molecular alteration characteristic in primary lung adenocarcinoma and this has been demonstrated in up to 18% of AAH areas (Kitaguchi et al., 1998). The difficulty in studying the

natural progression of areas of AAH is that they are often overlooked on usual imaging modalities (CT scanning) as they simply appear as areas of ground glass opacification with no specific characteristic features (Westra, 2000).

In SCLC there has been no characteristic pre-neoplastic sequence or morphological changes described however it has been demonstrated that there is a far higher rate of genetic instability (such as loss of heterozygosity at chromosome 3p) in SCLC compared to both primary squamous cell and adenocarcinoma of the lung (Carney, 1992).

A recent review which examined the histology of all lung cancer diagnoses in Scotland in 1995, recorded through the national cancer registry, found that for those cases with positive histology, NSCLC accounted for 72.5% with 38.7% of all cases being squamous cell, 19.5% adenocarcinoma and 14.3% classified as NSCLC only. SCLC accounted for 23.7% of the cases with 3.7% of cases in the Scottish population being unclassifiable histologically (Gregor et al., 2001).

Interestingly, this data differs somewhat from that reported in the United States where although NSCLC also accounts for approximately 80% of all Lung Cancer, adenocarcinoma is the predominant NSCLC accounting for 45% of cases with squamous only 20%. These differences must be taken into account when comparing international studies of NSCLC, particularly those evaluating treatment modalities, as adenocarcinoma in particular has an increased propensity to metastasise and there is evidence for example in early stage disease that the risk of relapse is higher with adenocarcinoma and that this correlates with a poorer survival (Moldvay et al., 2000).

1.3 Lung Cancer Staging

The accurate staging of lung cancer gives both important prognostic information and enables the most appropriate treatment plan for an individual patient to be made. The techniques used in the staging of lung cancer have evolved rapidly in recent years. Despite CT continuing to play a pivotal role in the process it has been shown to have both poor sensitivity and specificity when staging the mediastinum (sensitivity 57%, specificity 82%) (Toloza et al., 2003b). Accurate mediastinal staging is essential prognostic information when planning the appropriate treatment for individual patients, as well as comparing staging data between studies. More recent non-invasive staging techniques have demonstrated better sensitivity and specificity relating to the staging of the mediastinum and these include PET (Positive Emission Tomography) scanning (sensitivity 84%, specificity 89%) and CT-PET (where the CT and PET images are superimposed on one another) scanning (sensitivity 78-93%, specificity 82-95%) (Toloza et al., 2003b). In addition to these techniques there is an increasing use of minimally invasive diagnostic/staging techniques employed in the staging of the mediastinum. Initially TBNA (Trans-Bronchial Needle Aspiration) of lymph nodes seen on previous CT scanning was introduced. However this is a 'blind' technique and therefore has a sensitivity of only 76% although a better specificity of 96% (Toloza et al., 2003a).

Newer minimally invasive techniques involve the use of TBNA performed under Ultrasound guidance EBUS-TBNA (Endobronchial Ultrasound- TBNA) and EUS-TBNA (Oesophageal Ultrasound-TBNA) with reported sensitivities of 85% and 81% respectively and specificities of 100% and 91% respectively (Toloza et al., 2003a) are being introduced into routine clinical practice. However, the gold standard for staging

of the mediastinum continues to be the invasive technique of mediastinoscopy with a sensitivity of 81% and specificity of 100% (Toloza et al., 2003a).

Although the accurate staging of the mediastinum is essential for patients, so is evaluation of the possible presence of distant disease and non-invasive techniques used for this include U/S, CT, MRI, PET and CT-PET as well as invasive surgical techniques such as Video Assisted Thoracoscopy (VATS) and biopsy of possible metastatic lesions (e.g. adrenal, bone, skin and lung lesions).

NSCLC and SCLC are staged using 2 different systems. In NSCLC, the revised International Staging System (ISS) is used and details the anatomical extent of the disease, examining tumour size and position (T) as well as regional nodal involvement (N) and the presence of any distant disease (M). Figures 1a and 1b (Mountain, 1986) summarise this staging system. Approximately 65% of patients with NSCLC present with locally advanced, stage III or metastatic, stage IV disease and as such have incurable disease (Spiro and Silvestri, 2005).

Table 1.1: The TNM (revised International Staging System) classification

T	**Extent of primary tumour**
Tis	Carcinoma in situ
Tx	Positive cytology
T1	≤ 3cm
T2	> 3cm, main bronchus ≥ 2cm from carina, invades visceral pleura, partial atelectasis
T3	Chest wall, diaphragm, pericardium, mediastinal pleura, main bronchus < 2cm from carina, total atelectasis
T4	Mediastinum, heart, great vessels, carina, oesophagus, vertebra, separate nodules in same lobe, malignant effusion
N	**Condition of regional nodes**
N0	No regional lymph nodes
N1	Ipsilateral peribronchial, ipsilateral hilar
N2	Ipsilateral mediastinal, subcarinal
N3	Contralateral mediastinal or hilar, scalene or supraclavicular
M	**Presence or absence of distant metastases**
Mx	Distant metastasis cannot be assessed
M0	No distant metastasis
M1	Distant metastasis, includes separate nodules in different lobe

Source: UICC (International Union Against Cancer) TNM Classification of Malignant Tumours. Fifth Edition. Sobin LH, Wittekind Ch (editors). New York: Wiley-Liss, 1997

Table 1.2: Stage I –IV Lung Cancer System

Occult carcinoma	Tx	N0	M0
Stage 0	Tis	N0	M0
Stage IA	T1	N0	M0
Stage IB	T2	N0	M0
Stage IIA	T1	N1	M0
Stage IIB	T2 T3	N1 N0	M0
Stage IIIA	T1 T2 T3	N2 N2 N1, N2	M0
Stage IIIB	Any T T4	N3 Any N	M0
Stage IV	Any T	Any N	M1

Source: UICC (International Union Against Cancer) TNM Classification of Malignant Tumours. Fifth Edition. Sobin LH, Wittekind Ch (editors). New York: Wiley-Liss, 1997

In SCLC staging assesses only the extent of the disease and categorises it as either limited, confined to a hemithorax and regional lymph nodes that can be encompassable into a reasonable radiation port, or extensive, which is defined as any disease that is not limited (Mountain, 1986). Approximately two thirds of patients with SCLC will present with extensive disease.

For all patients presenting with lung cancer the primary question that needs to be answered is whether their disease is potentially curable? This will depend on various prognostic markers but none more so than the stage of disease at presentation. Other important prognostic factors are also taken into account when planning appropriate treatment options for the patient. These various other prognostic markers, as well as disease stage, are now discussed.

1.4 Non Small Cell Lung Cancer Management and Clinical Markers of Prognosis

1.4.1 Stage I / II

The single most important prognostic marker in patients with NSCLC is stage and whether the tumour is resectable or not, as to date surgery continues to be the treatment modality that offers the best potential of cure.

However accurate staging of the disease does not, on its own, predict operability. Other factors need to be taken into account including assessment of the likelihood that the patient will survive the operation. This will depend on the extent of the surgery required to achieve the best chance of cure and prediction of post-operative lung function. In addition to these factors the patients other co-morbidities must be taken

into account. It has been shown that Performance Status (PS) worse than 2 (figure 2) and weight loss >10% of pre-morbid body weight are poor prognostic indices, in patients being considered for resection (BTS Guidelines, 2001).

Table 1.3: WHO/ECOG Performance Status

0	Fully active. Able to carry on all pre-disease performance without restriction
1	Restricted in physically strenuous activities but ambulatory and able to carry out work of a light and sedentary nature
2	Ambulatory and capable of all self-care but unable to carry out many work activities; up and about more than 50% waking hours
3	Capable of only limited self-care; confined to bed or a chair for more than 50% of waking hours
4	Completely disabled; unable to carry out any self-care; totally confined to bed or chair

In the United Kingdom resection rates of only 11% have been achieved compared to 17% in the rest of Europe and 21% in North America (Fry et al., 1996). Several factors may account for this including the significant co-morbidities in patients with lung cancer in the United Kingdom as well as delays in patients seeking medical assessment. These delays are thought to occur predominantly due to the insidious onset of the disease. At present 65% of patients in the UK present with locally advanced stage III or metastatic stage IV disease (Spiro and Silvestri, 2005).

Surgery offers the best chance of cure in an individual with NSCLC. However, this only equates to five year survival figures of 67% for stage IA disease, 57% for stage IB, 55% for stage IIA, 39% for stage IIB (T2N1) and 38% for stage IIB (T3N0) (Mountain, 1997).

A recent MRC study (MRC LU22) investigating induction chemotherapy followed by surgery in resectable patients has recently been reported. This study of neo-adjuvant chemotherapy recruited 519 patients of whom 258 received pre-operative platinum-based chemotherapy and 261 who underwent surgery alone. Although chemotherapy led to a good response rate and indeed downstaging in approximately 20% of patients there was no benefit in progression free survival (282 events, HR 0.98, 95% CI 0.81, 1.23) or overall survival (232 deaths, HR 1.04, 95% CI 0.81, 1.35). In addition there were more brain metastases reported in patients receiving pre-operative chemotherapy than those that underwent surgery alone (30 versus 11) (Nicolson et al., 2007).

Several host factors such as poor lung function and significant comorbidity may combine to make a patient with clinically resectable disease medically inoperable. These patients are instead considered for potentially curative treatment with radical radiotherapy.

Although radical radiotherapy does offer the potential of cure there has been only one randomised trial comparing radical radiotherapy with surgery (Wood and Morrison, 1955). This study consisted of 58 patients randomised to either surgery or radical radiotherapy. The results showed a 4-year survival of 23% in the surgical arm versus 7% in the radiotherapy group. These differences were not statistically significant due to the small numbers involved in the study.

A systematic review of radical radiotherapy studies to treat early stage lung cancer reports survival figures ranging from 50-93% at 1 year, 22-72% at 2 years, 17-55% at 3

years and 0-42% at 5 years for patients with stage I/II disease (Rowell and Williams, 2001).

Therefore, current guidelines would suggest the use of radical radiotherapy for those patients with stage I or II disease who refuse operation or are deemed medically inoperable (Management of Patients with Lung Cancer, SIGN 80 February 2005).

A multi-centre randomised control study has demonstrated that the use of Continuous Hyperfractionated Accelerated RadioTherapy (CHART) in patients with NSCLC is associated with a 2 year survival of 30% compared to 21% in the control (conventional radical radiotherapy) arm, translating into an almost 50% improvement in 2 year-survival (Saunders et al., 1997). CHART is now recommended as the radical radiotherapy regime of choice.

None of the trials referred to above have examined the potential prognostic role of tumour specific molecular markers.

1.4.2 Stage IIIa

Two small trials showed encouraging results in terms of survival benefit with the use of neoadjuvant chemotherapy in patients with stage IIIa NSCLC (Rosell et al., 1994, Roth et al., 1994). Patients with stage IIIa disease remain potentially operable although it is recommended that only patients with proven early N2 disease may be considered for resection following neoadjuvant chemotherapy if there has been CT evidence of response (Management of patients with lung cancer, SIGN 80, February 2005). A recently published international multi-centre randomised control study has shown an

absolute survival benefit (4.1%) with cisplatin based chemotherapy post operatively with particular benefit for those patients with stage III disease (Arriagada et al., 2004).

As regards clinical prognostic markers, other than stage, it remains the case that the most important factor in delivering high intensity treatment is performance status.

1.4.3 Stage IIIb / IV

The majority of studies have combined patients with both locally advanced stage IIIb (any T4 or any N3) and metastatic (any M1) disease together when considering prognostic factors and management options. A significant proportion of patients with stage IIIB disease will have a poor performance status or weight loss. Recent advances in treatment options for this group suggest that the patients with a good performance status should be identified as there may be a role for intensive treatment regimes incorporating more than 1 treatment modality (Jett et al., 2003).

Several studies of treatment regimes which include induction chemotherapy followed by radical radiotherapy or surgery, and studies of concurrent chemoradiotherapy have demonstrated that good performance status patients benefit from combined modality therapy (Brundage et al., 2002).

In stage IV NSCLC, traditional management has been Best Supportive Care with radiotherapy for control of local symptoms. This approach does not impact on survival. In 1995, a meta-analysis was published evaluating the role of chemotherapy in NSCLC. This demonstrated that the use of cisplatin based chemotherapy results in a survival advantage when compared with the traditional treatment modalities alone, even in

patients with stage IV NSCLC. The survival benefit achieved with the addition of cisplatin based chemotherapy in stage IV NSCLC patients is an absolute survival benefit of 10% at 1 year, equating to a median survival improvement of six weeks, (Non-small cell lung cancer collaboration group, 1995).

Studies have attempted to identify other host prognostic indicators in patients with stage IV disease and have shown that female patients under 70 years of age have a better prognosis (Albain et al., 1991, Palomares et al., 1996). Clinical measurements including haemoglobin, LDH and albumin have been shown to be useful predictors of survival (Albain et al., 1991, Sugiura et al., 1997). However, throughout the published literature performance status, stage of disease and significant weight loss appear to be the strongest predictors of survival in patients with stage IV metastatic NSCLC (Brundage et al., 2002).

1.5 Small Cell Lung Cancer and Clinical Markers of Prognosis

The most important predictors of survival in Small Cell Lung Cancer (SCLC) are disease extent and performance status (PS) of the patient at presentation (Rawson and Peto, 1990). Patients presenting with limited stage disease have a median survival of 18 months with chemotherapy as compared to those with extensive disease who have a median survival of only 9 months. Long-term survival for patients with SCLC is uncommon with a 2-year survival of 15-20% for those with limited disease and only 2% for those with extensive disease (Simon and Wagner, 2003).

Other host related prognostic factors have been evaluated in SCLC and various groups have attempted to combine these into scoring systems in order to try and further predict survival. These include scoring systems from:

1. The Royal Marsden group using the variables of serum albumin, ALT levels and the ECOG/WHO performance status (Vincent et al., 1987).
2. The London Lung Cancer Group using the variables of serum albumin, sodium and alkaline phosphatase levels and the Karnofsky performance status (Souhami et al., 1988).
3. The Manchester score using the variables of serum LDH, sodium, alkaline phosphatase, bicarbonate in combination with the Karnofsky performance status and stage of disease (Cerny et al., 1987).

Unlike NSCLC, surgery is not a standard treatment for patients with SCLC due to its high propensity to metastasise. The treatment of choice for both limited and extensive disease is primarily chemotherapy, usually combined with radiotherapy, also having a role to play (Simon and Wagner, 2003).

1.6 Chemotherapy in the Management of Lung Cancer

Platinum based combination chemotherapy has a central role in the management of patients with SCLC and an ever-increasing role in the management of patients with NSCLC from early stage in the form of adjuvant therapy through to palliative chemotherapy in advanced disease.

'Peyrones Chloride' as it was originally known was first described in 1845 by Peyrone and its sterical configuration subsequently described in 1893 (Werner, 1893). Rosenberg observed the first reports of its anti-proliferative effects in 1965 (Rosenberg et al., 1965) and after successfully completing animal toxicology studies was first administered to a human in 1971 (Hill JM, 1971). It was routinely available in general oncology practice by 1978.

Over the subsequent 30 years of clinical use cisplatin has continued to be the mainstay of many chemotherapeutic regimens, although success rates vary between tumour types. For example there are high cure rates for people with testicular cancer using cisplatin (Jones and Vasey, 2003) whereas although response rates are evident against cancer of the ovary, bladder, cervix, head and neck as well as SCLC and NSCLC, relapse and subsequent disease chemo-resistance are common. With other tumours such as colorectal and pancreatic cancer cisplatin based chemotherapy has been shown to have little impact on the disease (Haller, 2004, Lopes and Rocha Lima, 2005).

These differences demonstrate that the presence or acquisition of resistance by cancer cells to cisplatin is a major clinical problem that undermines the potential curative use of this drug. It is these issues, which are now addressed in relation to lung cancer.

There are significant problems with platinum based chemotherapy in both SCLC and NSCLC. In the case of SCLC relapse is almost certain despite an initial response rate of up to 90% and complete response rates in limited disease of 50% (Simon and Wagner, 2003). The relapse tumour is often resistant to further chemotherapy. In the case of NSCLC, despite the evidence that platinum based chemotherapy can lead to survival

advantage, initial overall response rates are at best 50% when given in combination with another cytotoxic agent and only 21% when given as a single agent (Bunn, 1989).

It has been suggested that there are several mechanisms involved with platinum chemotherapy resistance within the lung cancer population. Clinically some tumours exhibit intrinsic resistance whilst others appear to acquire resistance after initially responding to chemotherapy.

Several studies have been performed to try and identify the optimal treatment dose and scheduling of platinum based chemotherapy in order to try and maximise clinical response and survival for patients with all forms of lung cancer. The approaches most extensively studied have been conducted in patients with SCLC where the tumour is more chemosensitive than NSCLC and where chemotherapy plays an important role in the management of limited disease. In this situation, chemotherapy is given with curative intent.

In patients with SCLC these treatment approaches include the following:

A: Increasing the dose of chemotherapy.

Two reported studies have looked at increasing doses of cyclophosphamide and doxorubicin within the standard 3 weekly regime of cyclophosphamide, doxorubicin and vincristine (CAV). Despite demonstrating an increase in complete response rate from 22% compared to 12% there was a substantial increase in toxicity and no demonstrable survival advantage (Figueredo et al., 1985, Johnson DH, 1987).

A further study investigated increasing both the dose of cisplatin and etoposide for the first 2 cycles with standard doses for cycles 3 and 4. Those exhibiting a complete response continued with cisplatin etoposide to a total of 8 cycles and the remainder were converted to CAV for cycles 5 – 8. In this study there was no survival advantage for this dose intensified approach and there were problems with significant toxicities (Ihde et al., 1994).

B: Shortening treatment intervals between chemotherapy cycles, with or without the addition of haematopoietic growth factors.

Randomised controlled trials (RCT) have been reported examining the impact of shortening the interval (increased dose intensity) between chemotherapy treatments with or without haematopoietic support (G-CSF, granulocyte colony-stimulating factor) and the effect that this has on survival (Fukuoka et al., 1997, Steward et al., 1998, Thatcher et al., 2000, Woll et al., 1995). These studies did report a median and 2-year survival benefit with the addition of the haematopoietic support.

C: Alternate regimes

An alternating chemotherapy regimen has been evaluated in a non-randomised trial (Twelves et al., 1991). Twenty-three patients (16 limited stage disease) received ifosfamide and vindesine or vincristine in weeks 0, 2 and 4 with cisplatin and etoposide weeks 6, 9, 12 and doxorubicin and methotrexate weeks 15 and 17. This combination was well tolerated and demonstrated an overall response rate of 91% with a complete response rate of 43%. The median survival was 54 weeks.

The above studies in patients with SCLC have concluded that in terms of platinum based chemotherapy the optimum dosing schedule is 3 weekly and that the manipulation of chemotherapy approaches described above confer no significant survival advantage but add to patient toxicity.

In patients with NSCLC, the majority of chemotherapy is given to patients with locally advanced and metastatic disease with palliative intent. NSCLC exhibits relatively low chemosensitivity and this may explain the relative lack of studies evaluating dose intensification.

A number of approaches have been performed in patients with NSCLC being treated with chemotherapy and these approaches are similar to those attempted/evaluated in patients with SCLC. They include:

A: Giving the same total dose of cisplatin in different schedules.

A study by Gandara (Gandara et al., 1993) gave the same total dose of cisplatin but in different schedules (50mg/m2 every 28 days for 8 cycles versus 100mg/m2 every 28 days for 4 cycles). This study demonstrated no difference in clinical outcome with median survivals of 6.9 and 5.3 months respectively (p=0.53). However increased ototoxicity, emesis, and myelosuppression were seen in the 100 mg/m^2 cisplatin arm although rates of renal toxicities and neuropathy were similar.

Within this study there was a third arm where mitomycin was added to the high dose cisplatin but this showed no benefit in terms of median survival compared to the cisplatin alone arms.

At present there is a prospective randomised trial (BTOG-2) recruiting in the UK evaluating the optimum dose of cisplatin (50mg/m^2 versus 80mg/m^2, 3 weekly) when given in combination with gemcitabine as well as a third arm that investigates the effect of replacing the cisplatin with carboplatin (AUC 5). The primary endpoint of this study is overall survival.

B: Shortening treatment intervals between chemotherapy cycles, with or without the addition of haematopoietic growth factors.

A study by Font (Font et al., 1999) evaluated the same doses of cisplatin and etoposide (35mg/m2 and 200mg/m2) given on days 1-3 every 4 weeks or every 3 weeks with recombinant human granulocyte macrophage-colony stimulating factor (GM-CSF) given on days 4-13. This study showed no significant difference in terms of overall survival with the medians for the 2 groups being 7.2 and 9 months respectively (p=0.07).

What remains clear from all the previously published work is that, although cisplatin based chemotherapy can impact on patients with NSCLC as well as those with SCLC, a therapeutic plateau has been reached and in order to try and improve patient outcomes different approaches require to be investigated. One approach to try and improve survival in patients with lung cancer requiring systemic therapy would be to try and overcome or modulate the clinical problem of cisplatin resistance.

1.6.1 Platinum resistance mechanisms

The mechanism by which cisplatin enters the cell remains poorly understood but once inside the cell the active species is formed by aquation hydrolysis. This product reacts with many potential intracellular targets including RNA, proteins and genomic DNA. Approximately 1% reacts with genomic DNA and it is this reaction with DNA that leads most commonly to GG intra-strand crosslinks, although a variety of intra- and interstrand crosslinks are formed. There is good evidence to support that it is this DNA damage that is most relevant to the clinical cytotoxic effect of cisplatin (Siddik, 2003). A poor outcome has been shown where the level of DNA adducts is low and improved outcome where the level is high (Lawley and Phillips, 1996).

However, there appears to be no single common pathway that adequately explains the phenomenon of cisplatin resistance. Rather there is a complex interaction of many mechanisms that combine to produce the phenomenon of clinical resistance seen in the clinic in different patients and in different tumour types.

Examples of these mechanisms include:

Blood flow and drug delivery to the tumour:

A study by Stewart et al (Stewart DJ, 1995) demonstrated differing amounts of detectable platinum in resected samples from patients with different tumour types, with most drug being found in primary brain lymphoma and less in medulloblastomas, meningiomas, lung and head and neck cancers and the least in gliomas.

Drug uptake by the tumour:

Studies have shown that many cisplatin resistant cell lines, including lung, have reduced cisplatin accumulation that may account for that resistance (Siddik, 2003). There is evidence that this reduced drug uptake is not affected by drug dose but that it is affected by metabolic inhibitors without affecting drug efflux (Stewart DJ, 1995, Stewart DJ, 1996).

Drug efflux by the tumour:

Resistance can be associated with either increased drug efflux from the cell (Chau and Stewart, 1999) or from the nucleus to the cytoplasm (Wang et al., 2004b). Various cellular pumps have been implicated in these processes and include MRP2 (Peng et al., 2004), MRP1 (Yeh et al., 2005), p-glycoprotein (Peng et al., 2004, Yeh et al., 2005) and MVP/LRP (Peng et al., 2004).

Drug detoxification by the tumour:

It has been demonstrated that increased levels of glutathione can achieve this by a number of methods including the binding and inactivation of cisplatin, enhancing DNA repair or reducing cisplatin-induced oxidative stress (Siddik, 2003).

DNA repair mechanisms:

Cisplatin is highly effective against testicular cancers and it is known that these tumours have a very low capacity to repair platinum-induced DNA damage (Koberle et al., 1997). The primary mechanism by which platinum-damage is repaired is the nucleotide excision repair (NER) system (Reed, 1998). The ERCC1 gene is of significance in this regard as over-expression of the ERCC1 gene is associated with

reduced platinum efficacy in ovarian cancer as well as NSCLC (Dabholkar et al., 1992, Rosell et al., 2003).

Reduced apoptotic response:

It is has been demonstrated that cells with p53 deletions or mutations are often resistant to cisplatin (Kandioler-Eckersberger et al., 1999). Certain p53 missense mutations are associated with increased levels of p53 stability and therefore high protein levels (as measured using immunohistochemistry) of p53 and this is associated with poor outcome in platinum treated NSCLC (Kawasaki et al., 1998, Nakayama et al., 2003).

DNA mismatch repair system (MMR):

Cells deficient in MMR exhibit increased levels of cisplatin resistance and reduced apoptosis (Aebi et al., 1996). This is thought to be an important factor in clinical cisplatin resistance and will be reviewed in a later section.

1.7 Novel Therapies in the Management of Lung Cancer NSCLC): Epidermal Growth Factor Receptor (EGFR) Inhibitors

What is evident from the above examples of possible causes of cisplatin resistance is that, despite extensive research, the exact mechanisms involved and how these interact to produce the clinical problem remains unclear. This has led to studies exploring different approaches to the systemic based treatment of lung cancer. One such approach has been the study and introduction to the clinical arena of the Epidermal Growth Factor Receptor (EGFR) Inhibitors and the development of one such EGFR inhibitor, Gefitinib (Iressa, Astra Zeneca®) is discussed. Erlotinib (Tarceva, Roche®) is the only currently licensed EGFR inhibitor in use in the UK.

The development of EGFR inhibitors, using ZD1839 (Gefitinib Iressa, Astra Zeneca ®), the first to be investigated in the clinical setting as an example, is reviewed to illustrate the potential use of such novel agents in the management of patients with lung cancer.

The rationale for the clinical use of EGFR inhibitors is based on work reviewed by Salomon et al (Salomon et al., 1995) that showed the tyrosine kinase EGFR (TKEGFR) is over expressed in a wide variety of solid human cancers including NSCLC (as well as breast, head and neck, bladder and ovarian cancer). Moreover, in a number of studies, high levels of TKEGFR expression were associated with poor prognosis (Bartlett et al., 1996, Bucci et al., 1997). Tyrosine kinase appears to be an important component in signal transduction pathways, with mutated or over expressed tyrosine kinases frequently associated with tumour growth. EGFR has been shown not only to play a role in cell proliferation but also in processes important for tumour progression such as cell motility, cell adhesion, invasion, cell survival and angiogenesis. Various research groups supported by pharmaceutical companies have developed molecules that inhibit EGFR by blocking tyrosine kinase activity.

Gefitinib (Iressa, Astra Zeneca®) is a low molecular weight synthetic molecule (anilinoquinazoline). Preclinical work has shown Gefitinib (Iressa, Astra Zeneca®) to potently inhibit EGFR tyrosine kinase activity in vitro and inhibit the growth of EGF stimulated KB oral carcinoma cells in culture (Barker et al., 2001). In addition Gefitinib (Iressa, Astra Zeneca®) has shown good oral bioavailability and antitumour activity in a range of human tumour xenografts in nude mice (A431 vulval, A549 NSCLC, DU145 prostate, HX62 ovary as well as several colorectal tumours), treatment was tolerated for up to 3-4 months in these models and marked regression was seen in several tumour

types (Fry, 1999). These encouraging pre-clinical results took Gefitinib (Iressa, Astra Zeneca®) into clinical development.

A phase I study enrolled 64 patients at 8 dose levels of Gefitinib (Iressa, Astra Zeneca®). This study of toxicity included a pharmacokinetic component and demonstrated that Gefitinib (Iressa, Astra Zeneca®) was suitable for once daily oral administration. It was found to be well tolerated with dose limiting toxicity observed at a dose well above that at which antitumour activity had been demonstrated in the laboratory. Sixteen of the 64 patients enrolled into the phase I study had NSCLC. Four patients demonstrated a partial response (as defined by a reduction in measurable disease of greater than or equal to 50%) and all the responses were seen in patients with NSCLC. Eight patients demonstrated stable disease of whom 3 had NSCLC (Ranson et al. 2240-50). This study led directly on to the development of phase II and III studies in NSCLC. Two large phase II (IDEAL 1 and 2) and phase III (INTACT 1 and 2) trials have now been completed and reported.

IDEAL I and II (Fukuoka et al., 2003, Kris MG, 2002) were large, randomised, double blind, multicentre clinical trials. Patients were randomised to receive either a 250mg or 500mg once daily dose of Gefitinib (Iressa, Astra Zeneca®). The IDEAL I trial recruited 210 patients with stage IIIb (locally advanced) or stage IV disease who had recurrent or refractory disease following treatment with platinum based chemotherapy regimen. The IDEAL II trial recruited 216 patients with performance status 0-2. Eligible patients had stage IIIb or IV disease but in this trial had failed on two or more prior chemotherapy regimes containing platinum and docetaxel given separately or in

combination. All patients were of PS 0-2. EGFR status was not an inclusion criterion for entry into the trials.

Both trials assessed tumour response, symptom response and quality of life as well as toxicity/tolerability and survival. The results are shown in table 1.4 and demonstrate no difference between the 2 studies.

Table 1.4: Comparison of IDEAL I and II Results

	IDEAL I		IDEAL II	
Endpoint	**250mg/day (n=104)**	**500mg/day (n=106)**	**250mg/day (n=102)**	**500mg/day (n=114)**
Tumour Response Rate	18.4%	19.0%	11.8%	8.8%
Stable Disease	36.0%	32.4%	31%	27%
Disease Control Rate	54.4%	51.4%	42.8%	35.8%
Progression Free Survival	2.7 months	2.8 months	1.9 months	-
Overall (median) Survival	7.6 months	8.0 months	7.0 months	6.0 months
1 year Survival Rate	35.0%	29.0%	27.0%	24.0%

Examination of the Quality of Life (QoL) data for evaluable patients in these trials demonstrated a trend toward better QoL in the 250mg dose arms of both studies.

The toxicity data had previously shown Gefitinib to be well tolerated with the most common side effects being diarrhoea and skin rashes. The phase II studies demonstrated that adverse events were less frequent and less severe at the 250mg/day dose of Gefitinib.

These results encouraged the development of 2 large randomised, double blind, phase III trials (INTACT I and II) investigating the use of Gefitinib (Iressa, Astra Zeneca®) as first line treatment for patients with NSCLC in combination with chemotherapy (Giaccone et al., 2004, Herbst et al., 2004). There were 3 arms to each study: chemotherapy plus 250mg/day Iressa versus chemotherapy plus 500mg/day Iressa versus chemotherapy plus placebo. In INTACT I the combination chemotherapy was cisplatin plus gemcitabine and in INTACT II the combination chemotherapy was carboplatin plus paclitaxel. The primary endpoint in both studies was overall survival with secondary endpoints of progression free survival and time to worsening of disease related symptoms. The results did not demonstrate any benefit in overall survival with Gefitinib (Iressa, Astra Zeneca®) when added to standard platinum based chemotherapy versus chemotherapy alone in advanced Non Small Cell Lung Cancer. The authors of these papers have cited several reasons to explain these negative results including the fact that patients were neither recruited nor randomised on the basis of EGFR status. EGFR status was not known in either of the phase II trials above where encouraging response rates were demonstrated. The phase II study patients were different in that they had all been previously treated with chemotherapy and had either refractory or recurrent disease. Thus despite initial promising results from this translational research approach, the phase III studies were negative and Gefitinib (Iressa, Astra Zeneca®) has been withdrawn from clinical use in the United Kingdom. However, another EGFR Inhibitor Erlotinib (Tarceva, Roche®) has gone through the same development process and has been shown to prolong survival in phase III studies of the same patient population. Erlotinib (Tarceva, Roche®) is now used both alone and in combination with traditional chemotherapy (Shepherd et al., 2005). There is also currently a trial evaluating the role of Erlotinib as a first line monotherapy in locally advanced and metastatic NSCLC.

In summary the EGFR tyrosine kinase inhibitors are a new class of drug that have been developed in a truly translational manner and one of these drugs, Erlotinib (Tarceva, Roche®) is now being used in clinical practice in the United Kingdom.

There remain various unanswered questions in the chemotherapy of lung cancer and these include, how to predict in advance which patients are likely to benefit from cytotoxic drugs, as well as how to determine the optimal combination of conventional cytotoxic agents. Future studies answering these questions could prove clinically useful in terms of improving patient survival.

However it is likely that problems in relation to cytotoxic chemo-resistance will persist and as such new biological/molecular agents such as Erlotinib (Tarceva, Roche®) will need to be developed, based on a thorough understanding of the molecular biology of lung cancer.

1.8 NSCLC and Tumour Related Molecular Prognostic Markers

1.8.1 An overview of selected markers

A review by Brundage et al in 2002 identified 887 published studies examining both patient (host) and tumour specific prognostic factors that were predictive of patient survival (Brundage et al., 2002). Within these studies 169 separate prognostic factors were identified as predictors of survival, although individual studies evaluated only a few of these. The tumour markers studied have included:

Markers of proliferation such as Ki67:

Conflicting evidence has been published as to any potential role Ki67 may have as a prognostic marker. One small retrospective study of 61 surgically resected patients demonstrated a significant inverse association between patient survival and proliferation index (Ki67 expression) independent of any clinicopathological factor (Pence et al., 1993). The results of this work were corroborated in a larger study of surgical patients where a high Ki67 score at diagnosis correlated with a worse disease free survival ($p<0.03$) (Scagliotti et al., 1993). However a further large retrospective study by Pujol et al failed to show any relationship between Ki67 expression and prognosis (Pujol et al., 1996).

p53 status:

Alterations in the p53 – p21 pathway, controlling G1/S transition, are amongst the most commonly observed aberrations in NSCLC. Studies performed have used both immunohistochemistry and direct sequencing to assess any effect these changes may have on prognosis and have given conflicting results. Passlick et al (Passlick et al., 1995) reported on 73 patients using immunohistochemistry where the tumour was deemed p53 positive if > 1% of the cells stained positive. In this study 45% of cases were positive and this correlated with increased disease free survival in patients with early stage disease ($p=0.004$) and in men ($p=0.023$). However no significance was identified in advanced stage of disease. A further study by Lee reported on 156 patients (Lee et al., 1995). In this study tumours were deemed to be p53 negative if <0.1% of them stained, low if 0.1 – 50% of cells stained and high if >50% stained p53 positive. The authors reported that patients with high p53 positive tumours survived longer ($p=0.002$) with this significance relating predominantly to non-squamous cell cancers

(p=0.008) but not squamous cell cancers (p=0.17). However, the studies of the prognostic significance of p53 have produced conflicting results. A study by Quinal et al demonstrated that p53 positivity was associated with a worse prognosis ($p<0.001$) (Quinlan et al., 1992). In this study of 114 patients with stage I/II disease tumours were scored positive on the basis of even a few cells staining positive for p53. Likewise in a study of 85 patients with NSCLC, 64% of tumours stained positive for p53 (on this occasion positivity being those tumours where > 10% of cells stained) and these patients had a worse prognosis (borderline statistical significance) (Carbone et al., 1994). The fact that these studies all use differing scoring systems to define p53 positivity may have produced conflicting results.

Another method of evaluating the status of p53 is to assess gene mutation. In the study by Carbone et al., the researchers examined the mutational status as well as immunohistochemistry. Interestingly the mutational status results differed within the study population compared to those results obtained using immunohistochemistry. Considering mutational status, 53% of the tumours exhibited DNA abnormalities but there was no survival difference demonstrated between this and the group with no demonstrable mutation (Carbone et al., 1994). However, two further studies demonstrated p53 mutations were related with a poor overall survival (Horio et al., 1993, Mitsudomi et al., 1993). Horio et al. examined the tumours from 71 patients who had undergone potentially curative resection and identified a p53 mutation in 49% of these. Furthermore they demonstrated a correlation between p53 mutation and poor overall survival (p=0.014). This correlation was also seen in those patients with stage I/II disease (p=0.016) and on multivariate analysis p53 mutation was seen to be an independent adverse prognostic marker (p=0.013). Mitsudomi et al. studied 120

patients and demonstrated p53 mutations in 43% of their tumours. Mutation did not correlate with age, sex or stage of disease but did appear to be more frequently associated with squamous cell differentiation, and on univariate analysis it was seen to be a poor prognostic marker (p=0.01). In this study p53 mutation was a particularly poor marker for survival in stage III/IV disease (p=0.0091) but not stage I/II disease (p=0.2837). On multivariate analysis p53 mutation was an independent poor marker of prognosis (p=0.018).

K-ras mutation:

The Ras proteins are pivotal regulators of cellular proliferation, differentiation, motility and apoptosis. As in the case of p53 there are conflicting reports in the literature as to whether mutations of K-ras represent a negative prognostic marker or not. Most recently Camps et al., reported on the presence or not of K-ras mutations in the circulating DNA from patients with stage III/IV NSCLC (Camps et al., 2005). Thirty percent of these patients exhibited mutations of K-ras but there was no difference statistically in respect of patient characteristics, response rates (p=0.37), progression free survival (p=0.23) or overall survival (p=0.28). Schiller et al., reported on 184 patients that had undergone surgical resection of their primary tumour. Of these, 24% had K-ras mutation and although the median survival in this group was shorter at 30 versus 42 months for those with no K-ras mutation this did not reach statistical significance (Schiller et al., 2001). In this study there was no association between K-ras mutation and baseline patient characteristics. However, Rosell et al., in a study of 112 surgically resected patients reported the potential prognostic significance of the K-ras mutation (Rosell et al., 1995). In this study the mutation rate was 27%. When analysed by stage of disease those with stage I disease and no K-ras mutation had a median

survival of 46 months compared to 21 months for those with mutations present. Interestingly those patients with stage IIIA disease and no K-ras mutation had a statistically similar survival to those with stage I disease at 16 months, whereas those with mutation had a median survival of only 7 months.

Neuron Specific Enolase (NSE) expression:

A study by Carles et al., looked at 97 NSCLC tumours immunohistochemically, 46% of them demonstrated NSE expression (Carles et al., 1993). The NSE negative patients had a poorer prognosis than those who expressed NSE and this correlated well on multivariate analysis along with Performance Status as an independent marker of prognosis. Another study by Diez et al., examined NSE levels in the serum of 84 patients with NSCLC, 40 healthy controls and 20 patients with benign pulmonary disease (Diez et al., 1993). The level of NSE did not correlate with either the TNM stage or histological subtype of the tumour. However a level of >15ng/ml had a significantly worse prognosis than those with a level < 15ng/ml a 24 months ($p<0.05$).

ERCC1:

The excision repair cross complementing (ERCC) genes are an integral part of the nucleotide excision repair pathway that repairs DNA damage. ERCC1, in particular, has a critical role in this pathway (Soria, 2007). In some recent studies it has been shown to be an important marker of prognosis in patients with NSCLC in certain clinical situations although the results are conflicting.

In the surgical setting Simon et al., reported that tumour ERCC1 expression levels of more than 50 (Taqman quantitation) correlated statistically significantly with survival

(Simon et al., 2005). Those patients with ERCC1 <50 had a median survival of 35.5 months compared to 94.6 months in those with ERCC1 >50 (p=0.01).

However, in a study of patients who received adjuvant chemotherapy it was reported that loss of expression of ERCC1 correlates with both a better response to cisplatin based chemotherapy as well as improved median survival (Olaussen et al., 2006). This study retrospectively evaluated 761 patients enrolled in the International Adjuvant Lung Cancer Trial (Arriagada et al., 2004) and using IHC; showed that 335 (44%) of samples were ERCC1 negative and 426 (56%) were ERCC1 positive. A benefit from cisplatin-based chemotherapy was associated with ERCC1 negative status (test for interaction, p=0.009), with the study reporting that patients with ERCC1 negative tumours who received adjuvant chemotherapy had a significantly better survival than those in the observation arm (p=0.002) but no such survival advantage was found in those patients with ERCC1 positive tumours who received adjuvant chemotherapy compared to those in the observation arm (p=0.40) (Olaussen et al., 2006). However, in keeping with the findings of Simon et al it was shown that in those patients who did not receive chemotherapy that survival was better in those with ERCC1 positive tumours than those with ERCC1 negative tumours (p=0.009) (Simon et al., 2005).

Despite the wealth of prognostic studies described above, there remains limited information regarding specific factors that would allow stratification of patients into different prognostic groups prior to receiving systemic chemotherapy. These studies would be of significant benefit to the multi-disciplinary team when planning treatment options and could impact significantly on survival. Specifically the current widespread

use of chemotherapy, including adjuvant chemotherapy, could be rationalised and targeted on an individual patient basis.

1.8.2 DNA Mismatch Repair (MMR) and Lung Cancer

Within the human genome there are hundreds of thousands of regions where a single nucleotide or short DNA sequences are repeated and these are termed microsatellites. They occur in both coding and non-coding regions of the gene and are of a constant length. During DNA replication there is often misalignment of bases within these microsatellite regions. This is due to slippage during replication of repetitive sequences or during strand recombination resulting in base-to-base mismatch, as well as insertion/deletion loops (ranging from one to ten or more bases) if they escape DNA polymerase proofreading. Within the normal phenotype this does not represent a significant problem as there is a DNA repair mechanism known as DNA mismatch repair (MMR) first described in 1975 (Wildenberg and Meselson, 1975). A highly conserved post-replicative process MMR plays an integral role in maintaining genomic stability following DNA damage or during DNA replication by recognising newly synthesised daughter strands containing nucleotide sequencing errors and repairing it. DNA MMR is present not only in mammalian DNA but also in that of yeasts and bacteria (Prolla et al., 1994).

In humans the mismatch repair system is known to be made up of at least 6 different proteins (hMLH1, hMSH2, hPMS1, hPMS2, hMSH6 and hMLH3) (Bignami et al., 2003). The initial step is for the DNA mismatch to be recognised by heterodimers containing hMSH2: hMSH6 complexes and the subsequent repair step occurs when an hMLH1: hPMS2 complex interacts with this by some, as yet undefined, mechanism

(Fishel, 1999), although it is known that DNA heliclases, nucleases and polymerases are involved in the process (Genschel et al., 2002).

Some tumours however display alterations in the length of microsatellites and this leads to genetic instability more commonly termed Microsatellite Instability (MSI) or allelic imbalance/shift (Hoeijmakers, 2001, Katz and Kaestner, 2002, Loeb, 1994). It has been demonstrated that Microsatellite Instability is due to defects in the mismatch repair pathway (Karran, 1996) where replication errors within the microsatellite sequences are not repaired properly leading to the MSI. Therefore the presence of MSI in tumours reflects an abnormality within the mismatch repair system.

The significance of a defect in the mismatch repair mechanism is that it results in a spectacular increase in the DNA mutation rate - up to 100 times that observed in normal cells (Herman et al., 1998), and this propagates carcinogenesis. There are well described human cancers, in particular Hereditary Non-Polyposis Colonic Cancer (HNPCC) where the hallmark of the tumour is microsatellite instability (Hemminki et al., 1994). HNPCC (Lynch Syndrome) is the most common hereditary (autosomal dominant) form of colorectal cancer and affects many generations at an early age (mean age 45 years). It has preponderance for right-sided colonic tumours. In addition, patients with HNPCC display an increased incidence of certain extra-colonic cancers. These include endometrial, ovarian, stomach, small bowel, pancreas, hepatobiliary tract, brain and upper uroepithelial tract cancers (Lynch et al., 2003). The germ-line mutations of DNA mismatch repair proteins responsible for HNPCC have been identified as predominantly hMLH1 (33%) and hMSH2 (31%) (Herman et al., 1998). The relevance of MMR deficiency in sporadic colorectal cancers is also of interest as

Microsatellite Instability (MSI+) has been observed in approximately 13% of these cancers.

Because of the recognition that MSI plays an important role in the pathogenesis of colorectal cancers, criteria for the definition of MSI were agreed (Boland et al., 1998, Dietmaier et al., 1997). The presence of high-frequency MSI has been defined as the presence of instability in 2 of a panel of 5 markers and low frequency of MSI if only 1 marker demonstrates instability. The markers used for this in colorectal cancer are D2S123, APC, p53 and mfd15CA. This panel has been applied to other tumours when studying the presence of MSI. With regard to HNPCC the panel has been recently updated, to include on occasion more than 5 markers to improve sensitivity (Umar et al., 2004).

It has been shown that within this MSI+ population of colorectal cancers there is a significant subset where no mutation of MMR genes could be identified within the cancer, despite a decrease in expression of the protein (expression assessed by immunohistochemistry) (Aaltonen et al., 1993). In a study by Herman et al samples of sporadic colorectal cancers demonstrated hypermethylation of hMLH1 in 84% of cancers (Herman et al., 1998). This finding suggests that the transcriptional silencing of MMR genes might come about by more than one route.

With respect to lung cancer a review article by Lawes et al., reported that MSI is not seen in SCLC but with rates varying between 0 – 68% in NSCLC (Lawes et al., 2003) and these differences are presumably due to the differing number of and loci of the microsatellite markers tested. A study by Adachi et al demonstrated a statistically

significant increase in MSI in stage III and IV tumours compared to early stage disease (p=0.0021) (Adachi et al., 1995). Several studies have shown a worse prognosis for those with MSI+ tumours (Rosell et al., 1997, Zhou et al., 2000).

The MMR gene *hMLH1* resides on the short arm of chromosome 3p and it has been demonstrated in NSCLC that loss of heterozygosity at 3p is an independent adverse prognostic marker for survival in adenocarcinoma (p=0.052) but not squamous cell carcinoma (Mitsudomi et al., 1996). There was however no association between loss of 3p heterozygosity with gender, disease stage or grade of differentiation.

Work done by Xinarianos et al. (Xinarianos et al., 2000), has demonstrated that expression of the Mismatch Repair (MMR) proteins MLH1 and MSH2 was reduced in primary lung cancer and that MLH1 was more frequently reduced in primary squamous cancer ($p < 0.006$) and MSH2 was more frequently reduced in primary adenocarcinoma of the lung ($p < 0.003$). Previous work has already identified the potential prognostic role for the MLH1 protein in patients with breast cancer, where it was found that a correlation existed between reduced MLH1 expression after neo-adjuvant chemotherapy (compared to pre-chemotherapy levels of expression) and poorer survival (Mackay et al., 2000). No such studies have been performed in patients with lung cancer.

1.8.2.1 Loss of Mismatch Repair function and Platinum Resistance

There is a substantial body of preclinical and clinical evidence, demonstrating that alterations in mismatch repair proteins play a role in clinical cytotoxic chemotherapy resistance mechanisms. How lack of MMR activity directly affects response to DNA damage as caused by cytotoxics drugs remains ill understood.

Preclinical studies examining cell lines (HEC59) deficient in MMR proteins (MLH1 and MSH2) and resistant to 6-thioguanine and N-methyl-N'-nitro-N-nitrosoguanidine, have demonstrated these lines have low level resistance to cisplatin, carboplatin and etoposide but are sensitive to mephalan, 5-fluorouracil, doxorubicin, perfosfamide or paclitaxel (Aebi et al., 1996).

Further work on the HCT116 cell line, where chromosome 3, incorporating the *hMLH1* gene locus has been transfected into the cell line, demonstrates a resensitisation to the cytotoxic agents (Vikhanskaya et al., 1999) thus supporting that it is the loss of MMR activity that leads to this platinum resistance.

In the ovarian cancer cell line A2780 it has been shown that in 9 of 10 cisplatin resistant derivatives that, although there is complete loss of MLH1 and PMS2 expression, there is no apparent loss of the *hMLH1* gene (Brown et al., 1997). A study by Durant et al reported that A2780/cp70 (an in-vitro-derived cisplatin resistant derivative cell line) demonstrated re-expression of the MLH1 protein when chromosome 3, containing a wild-type *hMLH1* gene was re-inserted into the A2780/cp70 line and this correlated with a partial restoration of cisplatin sensitivity (Durant et al., 1999). Further work has reported that ovarian samples taken at laparotomy from chemotherapy treated patients show a significant increase in loss of MLH1 expression (4/11, 36%) compared to samples from patients who had not had chemotherapy before surgery (4/39, 10%) (Brown et al., 1997). This result was of borderline statistical significance ($p=0.059$, Fisher Exact test). No statistically significant difference was demonstrated for MSH2, MSH6 or PMS2.

Mackay et al. reported a study in which samples were taken from 36 patients with breast cancer undergoing neoadjuvant chemotherapy. Paired samples before and after neoadjuvant chemotherapy were obtained from 28 patients and there was a significant reduction in the percentage of cells expressing MLH1 in the samples obtained after chemotherapy (p=0.001, n=28). Moreover a reduction in the intensity of staining for MLH1 within cells after chemotherapy was identified (p=0.068, n=28). Cox regression analysis showed there was a highly significant correlation between low levels of MLH1 expression in the post-chemotherapy samples and worse disease free survival (p=0.0022, n=28). In addition there was a difference between the pre- and post-chemotherapy scores of MLH1 expression that correlated with a poor disease free survival (p=0.0025, n=27) (Mackay et al., 2000).

Further work on the A2780 ovarian cell line reported that the cisplatin sensitive parental cell line had methylation of only one *hMLH1* allele whereas all 9 resistant cell lines derived from parental A2780 had methylation of both *hMLH1* promoter regions and this resulted in a complete loss of MLH1 expression (Strathdee et al., 1999).

These studies taken together further support the hypothesis that mismatch repair proteins play an important role in both intrinsic and acquired platinum resistance.

1.8.3 DNA Methylation

DNA methylation is a post-replicative enzyme-mediated chemical modification and is the only known naturally occurring DNA modification process. Unlike MMR, there is little or no methylation in simple organisms such as yeasts and bacteria and this process is limited to mammals and humans (Bird, 1986). DNA methylation is a very specific

process that occurs only on cytosines that are followed by a guanine (CpG dinucleotides) in the DNA sequence (Herman and Baylin, 2003). The function of this modification with particular reference to lung cancer as well as chemotherapy resistance is now described.

1.8.3.1 DNA Methylation within the Normal Cell

The CpG dinucleotide frequency within the human genome is lower than that which would be expected from mathematical models. The majority of these (70-80%) are methylated (Bird, 1996). The majority of this methylated DNA is found in the non-coding regions of DNA (figure 1.1) and is associated with delayed transcription of these regions, which facilitates transcriptional silencing of these regions. This, in itself, may play a protective role for the normal cell as it may prevent transcription of inserted viral sequences and transposons (DNA sequences that have moved from their usual location into a new region of the genome). In the normal cell, specific roles of DNA methylation include X-chromosome inactivation, control of imprinted genes, suppression of testis specific genes as well as cell type specific repression (Jaenisch and Bird, 2003).

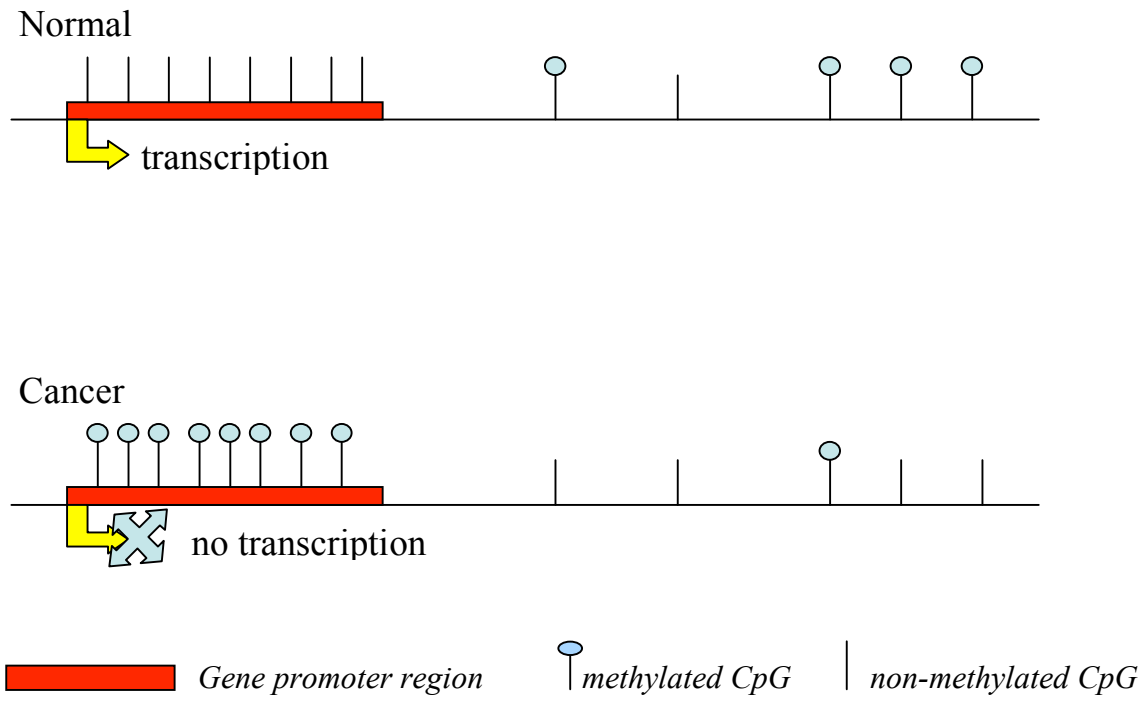
However there is an exception to this and relates to the so-called CpG Island (Bird, 1996). CpG islands are areas of 300 – 3000 base pairs, which in total make up approximately 1% of the human genome. Here the CpG frequency is of the order that would be expected from mathematical modelling and is much higher than the CpG frequency throughout the remainder of the genome. However, in comparison to the highly methylated CpG dinucleotide regions found in these non-coding regions, these CpG islands are predominantly methylation free thus allowing transcription to take

place (Bird, 1996, Jones and Laird, 1999). Further it is known that more than 60% of human gene promoters are located within these CpG islands (Bird, 1986, Gardiner-Garden and Frommer, 1987, Jones and Laird, 1999, Larsen et al., 1992, Strathdee et al., 2001b).

1.8.3.2 DNA Methylation within the Cancer Cell

DNA methylation is an epigenetic phenomenon leading to an alteration in gene expression without altering the nucleotide sequence. The cancer cell differs from the normal cell as regards methylation profile. There is a global hypomethylation of the genome but despite there being a loss of methylation of the CpG dinucleotides found within the non-coding regions of the DNA in cancer cells, there is a gain in the methylation of the CpG dinucleotides found within the so-called CpG islands in cancer cells (Figure 1.1) (Esteller, 2000, Herman and Baylin, 2003, Toyota et al., 1999a). Both of these mechanisms are thought to play a role in carcinogenesis.

Figure 1.1: **DNA methylation patterns in the normal and cancer cell**



When a cancer cell loses methylation within the non-coding regions of DNA there is the possibility of loss of transcriptional repression of normally silent genome regions, which could cause potentially harmful expression of viral genes or normally silenced genes. Alternatively loss of methylation may result in loss of functional stability of chromosomes in the cancer cell.

Methylation of the CpG islands within the promoter regions of genes is associated with transcriptional silencing. This is important in carcinogenesis, as it is a mechanism by which Tumour Suppressor Genes such as p16, APC, *hMLH1*, Rb and BRCA1, may be inactivated (Herman and Baylin, 2003). Methylation can therefore be considered an epigenetic phenomenon that may be responsible for either the first or second hit in the

Knudson Hypothesis of transcriptional silencing of tumour suppressor genes (Knudson, 2001). What remains unclear is the timing of DNA methylation in carcinogenesis. Age related methylation in normal tissues is well recognised and this increase in methylation with age may partly explain the increased incidence of many tumours in older patients (Ahuja and Issa, 2000).

It has been demonstrated that certain tumours exhibit a so-called CpG Island Methylator Phenotype (CIMP) where the tumour is characterised by a number of methylated genes (Strathdee et al., 2001a, Toyota et al., 1999a). What again remains unclear in many cancers, including lung cancer, is whether the cancer possesses a CIMP and if they do is it the number/pattern of gene methylation or merely the loss of expression of a critical gene that leads to carcinogenesis?

1.8.3.3 DNA Methylation, Mismatch Repair and Chemotherapy Resistance

As described earlier Brown et al., (Brown et al., 1997) were able to demonstrate that selection for cisplatin resistance in the human ovarian cancer cell line A2780 resulted in the loss of mismatch repair protein MLH1 expression in 90% of the resultant cisplatin resistant cell lines and that re-introduction of the *hMLH1* gene by direct chromosome transfer led to at least partial restoration of cisplatin sensitivity (Durant et al., 1999). This has also been reported to be the case in colorectal cancer cell lines (Fink et al., 1998, Sammalkorpi et al., 2007).

It has subsequently been shown in several tumour cell lines that this loss of MLH1, and subsequent cisplatin resistance, often relates to methylation of the *hMLH1* gene

promoter. The study by Strathdee et al, demonstrated that *hMLH1* promoter hypermethylation was invariably associated with loss of MLH1 expression in the MMR deficient A2780/Cp70 ovarian cancer cell line and work by this group demonstrated that when 2 of the resistant cell lines were treated with 5-azacytidine, which acts as a demethylating agent, there was also re-expression of the MLH1 protein (Strathdee et al., 1999).

It has been demonstrated also that the demethylating agent 2'-deoxy-5-azacytidine (DAC) can lead to resensitisation of MLH1 negative (secondary to gene promoter hypermethylation) MMR deficient drug resistant tumour (ovarian cell line A2780/cp70 and colon SW48 xenografts in vivo (Plumb et al., 2000). In this study, MLH1 expression was estimated using immunohistochemistry and the methylation status of the *hMLH1* gene promoter assessed by Southern blotting. Re-expression of the MLH1 protein in vivo was achieved using DAC at doses that were non-toxic to the mice. DAC at these doses had no effect on tumour xenograft size (even at the maximum tolerated dose 15mg/kg), which is an important finding in this study as DAC itself is a known cytotoxic agent. However when the mice bearing xenografts were treated with a lower dose of cisplatin (6 mg/kg) after they had been treated with DAC, the xenografts showed a clear growth delay, indicating a restoration of cisplatin sensitivity (Table 1.5).

Table 1.5: Analysis of the effects of DAC pre-treatment on the drug sensitivity of MMR-deficient A2780/cp70 and MMR-proficient A2780/cp70-ch3 xenografts

Treatment	**Time to double initial tumour volume (days)** (mean 6 mice)			
	A2780/cp70	**p-value**	**A2780/Cp70-chr3**	**p-value**
Control DAC	2.4 ± 0.2 2.5 ± 0.3	NS	2.9 ± 0.2 3.3 ± 0.4	NS
Carboplatin DAC+Carboplatin	2.9 ± 0.2 6.1 ± 0.5	$p < 0.001$	5.4 ± 0.2 5.6 ± 0.2	NS
Cisplatin DAC+Cisplatin	2.9 ± 0.2 6.0 ± 0.3	$p < 0.001$	5.1 ± 0.2 6.1 ± 0.2	$p < 0.05$
Temozolomide DAC+Temozolomide	2.1 ± 0.2 3.6 ± 0.2	$p < 0.001$	4.7 ± 0.4 4.6 ± 0.4	NS
Epirubicin DAC+Epirubicin	4.3 ± 0.4 6.0 ± 0.6	$p < 0.05$	4.8 ± 0.5 5.3 ± 0.7	NS
Taxol DAC+Taxol	4.5 ± 0.2 4.8 ± 0.6	NS	5.2 ± 0.4 5.2 ± 003	NS

Modified from Plumb et al (Plumb et al., 2000)

This finding is of potential clinical significance as it raises the possibility that a demethylating agent, such as decitabine (DAC) may be used to overcome methylation induced drug resistance in the clinical setting.

Examples of tumours taken from cancer patients that have been studied and found to have significant levels of methylation of the *hMLH1* promoter with subsequent reduction in MLH1 expression are ovary, gastric and breast cancer (Mackay et al., 2000, Strathdee et al., 1999, Toyota et al., 1999a). In ovarian cancer it is known that majority of tumours will respond to platinum based chemotherapy but that 20-30% will be intrinsically resistant to platinum and in chemosensitive tumours, despite initial

response, relapse with platinum resistant disease is common (Kaye, 1996). It may be that methylation of *hMLH1* plays a significant role in the acquisition of this resistance as it has been demonstrated that there is a loss of expression of MLH1 in post-chemotherapy tumour samples in comparison to pre-chemotherapy samples (Brown et al., 1997).

1.8.3.4 DNA Methylation patterns in NSCLC

The first report of gene promoter methylation in lung cancer was by Merlo et al in 1995 (Merlo et al., 1995). These authors demonstrated that the methylation of the p16 CpG Island was associated with transcriptional silencing of the gene and treatment of the monosomic cell lines with 5-deoxyazacytidine led to reversal of this genetic silencing. This is an important finding because these cell lines do not exhibit any mutations within the p16 gene and therefore gene silencing was attributable entirely to methylation. This demonstrates that epigenetic silencing of genes by methylation is possible without the need for another genetic 'hit' (Merlo et al., 1995).

Herman et al first described the technique of Methylation Specific PCR (MSP) (Herman et al., 1996a). The technique differentiates between methylated and unmethylated DNA sequences and has allowed the study of specific genes or patterns of gene methylation in tumours and how these may relate to tumour behaviour. The research into the role that methylation of specific genes may play in lung cancer, is highlighted in table 1.6. The majority of these studies have examined methylation of specific genes in isolation and varied in sample number from 3 to 126 with a median of 35.

One of the largest studies reported is that by Zochbauer-Muller at al, which examined abberant methylation of 8 gene promoter regions in 107 primary NSCLC tumour samples (Zochbauer-Muller et al., 2001). The promoter regions studied were RARB, TIMP-3, p16, DAPK, MGMT, ECAD, p14 and GSTP1 and correlation was made with some clinicopathological factors, namely gender, age, smoking status, TNM stage, histology and overall survival. It was demonstrated that 82% of tumours had methylation of at least one of the studied gene promoters, with 37% one gene, 22% two genes, 13% three genes, 8% four genes and 2% five genes. There was no correlation between the number of methylated genes and any of the clinicopathological variables. The authors demonstrated that methylation of ECAD (19 of 107 [18%] samples) correlated with a better overall survival, particularly in stage I disease ($p = 0.005$, Kaplan-Meier log rank test). In addition, it was reported that 41% of tumours exhibiting methylation of at least one promoter region had lymph node involvement whereas only 11% of tumours exhibiting no methylation had lymph node involvement ($p = 0.012$). This study also reported that gene promoter methylation correlated significantly with loss of gene expression assessed immunohistochemically (p16 gene, $p = 0.009$) (Zochbauer-Muller et al., 2001).

Table 1.6: MSP studies of gene promoter hypermethylation in lung cancer

Gene	Reported methylation frequency (%)	References
ARF / p14	0 – 8	(Esteller et al., 2001, Kim et al., 2001b, Zochbauer-Muller et al., 2001)
APC	0 – 46	(Esteller et al., 2001, Virmani et al., 2001)
BRCA1	4	(Esteller et al., 2001)
ECAD	18	(Zochbauer-Muller et al., 2001)
HCAD	43 - 50	(Toyooka et al., 2001)
p16	17 – 43	(Ahrendt et al., 1999, Esteller et al., 1999, Kashiwabara et al., 1998, Kim et al., 2001b, Ng et al., 2002, Sanchez-Cespedes et al., 1999, Zochbauer-Muller et al., 2001)
p15	0 – 5	(Esteller et al., 2001, Hamada et al., 1998, Herman et al., 1996b)
p19	0	(Zhu et al., 2001)
DAPK	16 – 23	(Esteller et al., 2001, Esteller et al., 1999, Zochbauer-Muller et al., 2001)
FHIT	37 – 64	(Zochbauer-Muller et al., 2001)
GSTP1	7 – 9	(Esteller et al., 2001, Esteller et al., 1999, Zochbauer-Muller et al., 2001)
HOXB	75	(Flagiello et al., 1996)
MGMT	21 – 29	(Esteller et al., 2001, Esteller et al., 1999, Palmisano et al., 2000, Wolf et al., 2001, Zochbauer-Muller et al., 2001)
hMLH1	0 – 2	(Esteller et al., 2001, Virmani et al., 2002)
RARB	40 – 76	(Virmani et al., 2000, Zochbauer-Muller et al., 2001)
RASSF1A	30	(Burbee et al., 2001)
S100A2	89	(Feng et al., 2001)
TGFBR2	0 – 12	(Hougaard et al., 1999, Osada et al., 2001, Virmani et al., 2002)
TIMP3	19 – 50	(Bachman et al., 1999, Esteller et al., 2001, Zochbauer-Muller et al., 2001)
TP73	0	(Esteller et al., 2001)

Table adapted from Tsou et al (Tsou et al., 2002)

Only 4 genes from the above table have demonstrated significant correlation with clinicopathological variables, DAPK (Death Associated Protein Kinase), p16, MGMT (O^6-Methylguanine-DNA-methyltransferase) and RASSF1A (Ras Association Domain Family 1). DAPK is a positive mediator of apoptosis and methylation of this gene has been shown to be associated with advanced NSCLC stage ($p = 0.003$), increased tumour size ($p = 0.009$) and lymph node involvement ($p = 0.04$) and although stage I patients had a worse overall survival this did not reach statistical significance (Kim et al., 2001a). As discussed in section 1.8.4 below, methylation of the p16 (a cyclin-dependant kinase inhibitor) gene may be present in the sputum of high-risk individuals prior to their developing NSCLC. More recently p16 methylation in patients with early stage (I/II) disease has been reported to be associated with a worse overall survival ($p = 0.002$) (Wang et al., 2004a). Methylation of the RASSF1A gene has been shown to be associated with a worse overall survival in stage IIIa disease ($p < 0.0001$) (Wang et al., 2004a). No correlation was demonstrated between RASSF1A gene methylation and overall survival by Toyooka et al (Toyooka et al., 2004).

With regard to chemotherapy response, methylation of the HIC 1 and RASSF1A loci has been associated with chemotherapy resistance in male germ cell tumours (Koul et al., 2004). This has not been demonstrated in patients with lung cancer.

1.8.4 Prognostic markers in the serum DNA of lung cancer patients

The presence of circulating tumour DNA in the serum of patients with cancer was first described in 1977 (Leon et al., 1977). The presence of tumour DNA out-with the tumour sample in patients with lung cancer has been reported in sputum (Mao et al., 1994, Miozzo et al., 1996) and more recent work has demonstrated methylation of lung

cancer related genes such as p16 in the sputum of lung cancer patients (Palmisano et al., 2000). However sputum is an inconsistent and unreliable source of material from patients. Not all patients with lung cancer produce sputum and sputum induction is an uncomfortable, sometimes unpleasant, experience for patients, as well as the resultant samples differing markedly in quality.

The possibility of being able to isolate sufficient amounts of circulating tumour DNA in patients with cancer is attractive as it could allow the molecular profiling of a tumour without requiring a further tissue sample.

At present there are no clinical examples of tumours in which the molecular DNA profile of the tumour is reflected in the serum of the cancer patient with sufficient robustness that can be used to predict response to therapy or likely prognosis.

1.8.4.1 Allelic imbalance in the serum of lung cancer patients

The detection of circulating tumour DNA in patients with lung cancer was first described by Chen et al (Chen et al., 1996). In this study at least one microsatellite alteration was detectable in 16 of 21 (76%) of SCLC samples and 15 of 21 (71%) of the corresponding serum samples. One serum sample exhibited a microsatellite alteration when there was no microsatellite alteration in the primary tumour sample.

Sozzi et al in 2001 in a series of 84 patients with NSCLC demonstrated that the mean concentration of DNA in serum was higher than that found in the serum of 43 control patients. It was also reported in this study that 20 of 33 (61%) informative analysed patients displayed loss of heterozygosity at the 3p locus (6 markers studied) and that 9

(45%) of these 20 informative samples demonstrated the same allelic imbalance as in the tumour (Sozzi et al., 2001). This was one of the first studies of circulating DNA found in the serum of cancer patients to be confirmed as originating from the tumour, by virtue of it's molecular profile.

1.8.4.2 DNA methylation in serum of patients with lung cancer

The feasibility of detecting tumour DNA methylation in the serum of NSCLC patients was first described by Esteller et al (Esteller et al., 1999). Moreover it has been demonstrated in ovarian cancer patients that detection of DNA methylation in the serum is predictive for poor overall survival (Gifford et al., 2004).

p16 is one of the most studied genes in patients with NSCLC and it has been shown that methylation of p16 can be detected in the sputum of patients with lung cancer and that these changes may predate the clinical diagnosis of NSCLC by up to 35 months (Kersting et al., 2000, Palmisano et al., 2000). Two recent studies have demonstrated the presence of p16 methylation in the circulating serum DNA in patients with NSCLC using methylation-specific PCR. Bearzatto et al demonstrated that 22 of 35 (63%) NSCLC samples demonstrated p16 methylation and that of these 22, 12 (55%) demonstrated methylation of p16 in the corresponding serum sample (Bearzatto et al., 2002). A further study reported at the same time demonstrated the presence of p16 methylation in 73 of 92 (79.3%) NSCLC samples, with corresponding p16 methylation in the serum of 64 (87.8%) of these patients (An et al., 2002).

DAPK and GSTP1 methylation has also been detected in 4/5 and 1/2 serum samples from methylated tumours respectively (Esteller et al., 1999).

Despite all of the work that has been performed to date lung cancer mortality remains high and in the majority of patients treatment intent is palliative. In this situation the development of molecular markers that would allow the stratification of patients into tailored management pathways would be a major advance for this patient population.

1.9 Aims of the Study

The results of studies of molecular markers in patients with lung cancer are conflicting and the relative importance of many molecular markers is still unclear. In particular, there remains little evidence for molecular markers, other than possibly ERCC1, being useful in predicting which patients may benefit from systemic chemotherapy. Patients may require additional new approaches such as the enhancement of the response to cisplatin-based chemotherapy using demethylating agents. This is already the case in other tumour types. Alternatively molecular markers may predict sensitivity to novel biological agents such as EGFR tyrosine kinase inhibitors, either alone or in combination with cytotoxic drugs.

Several techniques now exist that will allow further evaluation of molecular markers as potential prognostic indicators, with the aim of providing tailored therapeutic strategies for patients with lung cancer.

The purpose of this study is therefore to investigate the role of MMR proteins and other molecular markers as prognostic indicators in lung cancer, in relation to chemotherapy.

The aims of this study, in 3 separate cohorts of patients, are as follows:

1. Investigate the role of mismatch repair proteins as a possible marker of prognosis and predictor of chemotherapy response in patients with NSCLC.

2. Investigate CpG island methylation status and its role as a possible marker of prognosis in patients with NSCLC

3. Investigate the feasibility of examining the presence of allelic imbalance at a variety of loci both in tumour samples from and in the serum of patients with NSCLC and comparing this with the investigation of mismatch repair function in the tumour as well as the methylation profile in the tumour.

The first cohort of patients (Stobhill Hospital, Glasgow) all received systemic chemotherapy, either cisplatin or non-platinum based, and from these patients historical paraffin-embedded tumour samples taken at the time of diagnostic bronchoscopy were available. From these samples the immunohistochemical study of MLH1, MSH2 and p53 expression was undertaken. The level of expression of these molecular markers was correlated with survival data as well as tumour stage and histology. In addition any correlation between these molecular markers and survival according to the various chemotherapy regimes used was analysed.

The second cohort of patients (Aberdeen Royal Infirmary, Aberdeen) all underwent surgical resection for their primary tumour and 20% (10) of these received preoperative cisplatin based chemotherapy. From these patients banked, fresh, frozen tumour and normal adjacent lung was collected. In addition to the fresh samples, corresponding paraffin embedded samples were also available. From these paraffin embedded samples the expression of MLH1, MSH2, p53 and also Ki67 was studied. These results were correlated with survival, stage and histology as well as comparing these results between those who had received preoperative chemotherapy and those who had not. From the fresh tumour samples the presence of methylation at multiple loci and the existence of a CpG island methylator phenotype was investigated. The results of this methylation profile were correlated with survival, tumour stage and histology as well as

investigating any potential differences between the results from those patients who received preoperative chemotherapy from those who had not.

The third cohort of patients (Western Infirmary, Glasgow) underwent surgical resection of their primary NSCLC and in addition to tumour and normal adjacent samples being collected prospectively, a whole blood sample was collected prior to thoracotomy. In this study the protein expression of MLH1, MSH2 and p53 was measured using immunohistochemistry as well as the study of the CpG island methylation profile. In addition the presence of allelic imbalance at a panel of loci was examined in the tumour sample as well as in the lymphocyte and serum samples.

Finally, a cell line study was undertaken in SCLC cell lines to evaluate the role of the mismatch repair proteins and assess the significance of methylation of these in relation to chemotherapy sensitivity. For this a panel of small cell lung cancer lines were examined for methylation of their *hMLH1* promoter region and this was correlated with chemosensitivity.

2. Materials and Methods

2.1 Immunohistochemistry

2.1.1 Materials

2.1.1.1 Stable solutions for immunostaining

These solutions were stable at 20°C.

1. Phosphate buffered saline (PBS)
2. Buffer for autostainer (PBS with 0.05% Tween: 0.5ml Tween in 1 litre PBS).
3. 10-mmol/l citrate buffer (5.88g trisodium citrate in 2 litre distilled water, adjusted to pH 6.0 with concentrated hydrochloric acid).
4. 0.1 % hydrogen peroxide: 1ml 100 vol. Hydrogen peroxide + 1 litre distilled water.
5. Antidote to diamminobenzidine tetrahydrochloride (DAB): 3g potassium permanganate + 2 g sodium carbonate in 100 ml distilled water.

2.1.1.2 Unstable solutions for immunostaining

This quantity was made for 45 slides. Solutions were made freshly and kept on wet ice. The Vectastain® Elite ABC Kit, Mouse IgG (Vector Laboratories) was used throughout.

1. Blocking serum (Vectastain® Yellow). 20 ml PBS + 6 drops yellow block (300µl normal horse serum).
2. Biotinylated antibody (Vectastain® Blue). 20 ml PBS + 6 drops yellow block + 2 drops blue block (100µl biotinylated anti-mouse IgG).
3. ABC reagent (Vectastain® Grey). 10 ml PBS + 4 drops reagent A (Avidin DH solution) + 4 drops Reagent B (biotinylated enzyme).

4. Diaminobenzidine tetrahydrochloride (DAB). 10 ml distilled water + 4 drops Vectastain® buffer + 4 drops 0.1% hydrogen peroxide + 8 drops Vectastain® DAB reagent.
5. DAB antidote (potassium permanganate solution)

2.1.1.3 Primary Antibodies

1. Anti-hMLH1. 210 μl MLH1 antibody (500 μg/ml purified anti-human mouse monoclonal IgG, clone G168-15, Pharmingen®) + 2079 μl PBS.
2. Anti-MSH2. 110 μl MSH2 antibody (100 μg/ml purified anti-human mouse monoclonal IgG, Ab-2 clone FE11, Calbiochem®) + 2189 μl PBS.
3. Anti-p53. 22 μl p53 antibody (100 μg/ml purified anti-human mouse monoclonal IgG, DO-1 clone FE11, Oncogene®) + 2198 μl PBS.
4. Anti-Ki67. 111 μl Ki67 antibody (100 μg/ml purified anti-human mouse monoclonal IgG, B56 clone, BD Pharmingen ®) + 2198 μl PBS

2.1.1.4 Positive and negative controls

Sectioned paraffin-embedded samples of cell lines with known positivity for each antibody were included in each immunohistochemistry run. Cell lines used are indicated in table 2.1. Also included in each run was a negative control cell line slide to which primary antibody was not added. This was to ensure no residual endogenous peroxidase activity, which should have been removed during the 0.1 % hydrogen peroxidase step. On completion of the immunostaining run, this slide should stain blue (negative). If it did not, then the run was repeated. With a new antibody, dilutions of the positive and negative controls were run to ensure the best antibody concentration was used. The antibody concentration chosen was the one, which gave the best

definition between positive (brown) and negative (blue) controls but minimal background staining.

Table 2.1: Immunohistochemistry cell line controls

	POSITIVE CONTROL	NEGATIVE CONTROL
MLH1	A2780	A2780/cp70
MSH2	A2780	LoVo
P53	A2780/cp70	A2780
Ki67	A2780	No negative

2.1.1.5 Solutions for counterstaining

1. Haematoxylin; Harris formula filtered before use, Surgipath®
2. 100 % ethanol.
3. 70 % ethanol.
4. Histo-clear®, Fisher Scientific Ltd.
5. Scott's Tap Water: 1 in 10 dilution in distilled water of Surgipath® Scott's Tap Water substitute.
6. Acid alcohol: 10 ml concentrated HCl (specific gravity 1.16 g/ml – approximately 33 %) + 990 ml ethanol.
7. Hystomount®, Hughes and Hughes Ltd.

2.1.2 Methodology for Immunohistochemistry

The department developed and validated the use of an automated immunohistochemistry staining technique (DAKO Autostainer®) and this technique was used for all the immunohistochemistry performed in this project.

The DAKO Autostainer® protocol was developed using samples with known immunohistochemistry scores to ensure reliability and reproducibility of results.

The immunohistochemistry technique employed in this project involved the use of streptavidin-biotin coupled to peroxidase activity as a label, which utilises the high affinity of avidin for biotin. Because this affinity is more than one million times that of an antibody to most antigens, the binding of avidin to biotin is fixed and irreversible. Furthermore, avidin has four binding sites for biotin. These properties mean that macromolecular complexes can form between avidin and biotinylated enzymes.

Initially, unlabelled primary antibody binds to the antigen of interest in the tissue. This subsequently binds to a preformed avidin and biotinylated horseradish peroxidase macromolecular complex. The bound antibody complex turns brown after oxidation, which can be visualised using a light microscope. Diamminobenzidine is added as the substrate for the bound antibody complex to produce the colour change. If, however, no primary antibody is bound to the antigen of interest, there will be no peroxidase activity and no brown colour will form. Such negative samples are visualised by counterstaining with haematoxylin, producing a blue colour. Endogenous peroxidase activity, which would give a false positive result, is removed using hydrogen peroxide.

In this study, microwaving was used to expose antigens to antibodies where the energy from heating in a microwave disrupts the protein-protein crosslinks that may occur in tissue sections fixed in formaldehyde and then embedded in paraffin and this increases their immunoreactivity. The buffer used is important (Shi et al., 1995) and our group has found consistent results using microwave heating in citrate buffer at pH 6. Before

proceeding with an immunohistochemistry run, the optimum antibody concentration was determined by testing various antibody dilutions on samples of known positivity. In this way, positive staining was optimised and background staining reduced to a minimum.

2.1.2.1 DAKO® Autostainer protocol

A. Dewaxing of slides (in a laminar flow hood)

1. Slides were placed in a metal rack and immersed in Histo-Clear® for 20 minutes.
2. Slides were then rinsed in a bath of 100 % ethanol for 1 minute.
3. Slides were then rinsed in a bath of 70 % ethanol for 1 minute.
4. Slides were then rinsed in tap water for 1 minute.
5. Slides were then washed in a bath of PBS for 5 minutes.

B. Antigen retrieval

1. Slides were placed in a plastic rack and then into a microwave box with 750 ml citrate buffer. The box was covered with cling film and the cling film then pierced.
2. The box was microwaved at 650-Watt power for 15 minutes.
3. The box was then left to cool for 20 minutes and the slides then transferred to PBS prior to loading onto the autostainer.

C. Setting up the autostainer run and preparing reagents

1. The software instructions were followed to enter the number and identification of each slide. This also calculated the volume of each reagent required.
2. The reagents were prepared as per the autostainer instructions and stored on ice until ready.
3. The slides were loaded in order, the reagents were inserted into the correct position and the autostainer run commenced.

2.1.2.2 Counterstaining with haematoxylin (in a laminar flow hood)

A. Counterstaining

1. Slides were placed in a metal rack in tap water.
2. Slides were washed in haematoxylin for 1 minute and then rinsed in tap water.
3. Slides were then rinsed in acid alcohol for 5 seconds and then rinsed in tap water.
4. Slides were then washed in Scott's Tap Water for 1 minute and then rinsed in tap water.
5. Slides were then washed in 70 % ethanol for 1 minute.
6. Slides were then washed in 100 % ethanol for 1 minute.
7. Finally, slides were washed in Histo-Clear® for 5 minutes.

B. Mounting slides

1. Each slide was mounted using Hystomount® and cover slips
2. Slides were left to set for at least 1 hour. Care was taken to ensure all areas of the section were covered by Hystomount® to prevent drying out.

2.1.2.3 Immunohistochemistry scoring

Slides were scored by two observers independently and blinded to all clinical information, thus allowing for variation between observers (inter-observer variability). The first observer, KK, is a Professor of Pathology with a particular interest in lung cancer pathology and the second is the author of the thesis, who received specific training in lung cancer pathology and immunohistochemistry scoring. To allow for variability in staining two slides from each patient were stained for each antibody. Relevant positive and negative controls (table 2.1) were checked for each run prior to scoring to try to minimise variability.

Sections in this study were therefore scored using the multiplicative 'quickscore' and the scoring system below. The overall immunohistochemistry score (IHC-score) was calculated by multiplication of the percentage score and the intensity score, giving a final score between 0 and 9. When calculating the percentage score, a representative area of the section was chosen using light microscopy at low power and then counted at higher power. The intensity score was calculated by assessing the score that most accurately represented the majority of the cells counted.

Table 2.2: Immunohistochemistry scoring system: Intensity staining

Intensity Score (I-score)	
0	No stain
1	Weakly positive
2	Positive
3	Strongly Positive

Table 2.3: Immunohistochemistry scoring: Percentage staining

Percentage Score (%-score)	
0	0 – 4% of cells positive
1	5 – 19% of cells positive
2	20 – 79% of cells positive
3	80 – 100% of cells positive

2.1.2.4 Validation of immunohistochemistry

Our group, to investigate variability during immunohistochemical staining and scoring, has undertaken extensive work, predominantly by Dr M Mackean (Centre for Oncology and Applied Pharmacology, University of Glasgow), and to validate the methods used in our group. The key objective of using immunohistochemistry is to identify variability in staining due to differences in expression of the protein under investigation. However, variation in results may occur for other reasons including the staining methodology used, inter-observer variability, intra-observer variability and the variation in protein expression within a heterogeneous tissue section.

Dr Mackean investigated the variability of their immunohistochemistry scoring system used in our group by calculating kappa scores. The kappa score is a method that assesses the difference between scores by giving a weighting to the difference in the score. The kappa statistic is the observed agreement, corrected for a chance, as a fraction of the maximum agreement between observers above that due to pure chance. When analysed by Dr Mackean (unpublished observations), the variability in the immunohistochemistry 'quickscore' scoring system was found to show high reproducibility,

2.2 DNA Extraction from blood and cultured cell lines

2.2.1 Materials

The QIAamp® DNA Blood Mini Kit (QIAGEN®) was used. This kit is specifically designed to purify up to 6 μg of total DNA from 200 μl of whole blood. Qiagen has not disclosed the exact constituents of the reagents supplied with this kit.

Materials supplied with the QIAGEN® DNA Blood Mini Kit:

1. QIAGEN® protease stock solution (stored at 4°C).
2. Buffer AL (lysis buffer). This contains guanidine hydrochloride and a chaotropic salt.
3. Buffer AW1 (wash buffer). This contains guanidine hydrochloride and has high ethanol content.
4. Buffer AW2 (wash buffer). This has high ethanol content.
5. Buffer AE (elution buffer). This contains Tris-EDTA.
6. Spin columns and collection tubes.

Materials not supplied:

1. Rnase A (20mg/ml)
2. Heating block
3. Centrifuge
4. 100 % ethanol (added to buffers AW1 and AW2 as per kit instructions)

2.2.2 Methodology

Whole blood samples were received by taxi or collected in person from Stobhill Hospital and collected in person from the Western Infirmary in Glasgow. They were immediately centrifuged at 5000g for 10 minutes to separate the serum. This was then removed with a pipette and placed in a separate sterile tube and labelled with the study number. Samples were then stored at 4°C before performing DNA extraction. Samples were equilibrated to room temperature prior to proceeding with DNA extraction.

Cultured cell lines were obtained from Dr J Plumb (Centre for Oncology and Applied Pharmacology, University of Glasgow) and DNA extraction methodology was the same as for the whole blood sample.

All extractions were performed in a room designated for Category 2 work and in a laminar flow cupboard. Precautions were taken to avoid cross-contamination between sample preparations, including the use of aerosol-barrier pipette tips, changing pipette tips between all liquid transfers and regular glove changes during the procedure.

1. Heating block prewarmed to 56°C.
2. Lysis and DNA Precipitation.

		Blood/ Cell Cultures 1.5ml eppendorf		**Serum** 15ml falcon tube
		200 µl		1 ml
Rnase A (P20 pipette)		4 µl		20 µl
Qiagen Protease (P200 pipette)		25 µl		125 µl
Vortex				
Buffer AL (Lysis Buffer)		200 µl		1 ml
Vortex				
Incubate on Heating Block @ 56°C for 10 minutes				
100% Ethanol (P1000 pipette)		210 µl		1050 µl
Vortex				
Centrifuge Eppendorfs so contents are at base of tube				

3. Spin columns were labelled for each sample.
4. 635µl of each blood and serum was pipetted into the appropriate spin column and then placed into a collection tube.
5. Samples were then centrifuge at 8000 x g for 1 minute.

6. The elute was discarded and each spin column was placed into a fresh collecting tube.
7. Steps 4 - 6 was repeated a total of 5 times for each serum sample, due to the original volume of each serum sample.
8. 500µl of buffer AW1 was added to each spin column without wetting the rim of the column.
9. The samples were then centrifuged at 8000 x g for 1 minute.
10. The collection tubes were then discarded and each spin column placed in a fresh one. 500µl of buffer AW2 was then added into each spin column, without wetting the rim of the column.
11. Samples were then centrifuged at 8000 x g for 3 minutes to ensure that all buffer AW2 was eluted through the spin column.
12. The collection tubes containing the buffer AW2 were discarded and the spin columns placed into a clean 1.5 ml microcentrifuge tube. 200 µl buffer AE was added to each spin column and these were incubated at room temperature for 5 minutes.
13. The samples were centrifuged at 8000 x g for 1 minute. Each elute was passed through the spin column again, incubated for 5 minutes and centrifuged at 8000 x g for 1 minute again. In total, this step was performed twice for serum-depleted blood and 5 times for serum.
14. The DNA concentration in the elute was quantified using a spectrophotometer. Three repeat samples were measured and the mean of these taken as the DNA quantity.
15. Each sample was labelled and stored at -70°C

2.3 DNA Extraction from tissue

2.3.1 Materials

The QIAGEN® Genomic DNA extraction kit was used throughout. This kit is specifically designed to purify DNA from tissue.

Materials supplied with the kit:

1. Buffer G2 (lyses nuclei and denatures proteins). This contains 800mmol/l guanidine hydrochloride; 30 mmol/l tris-hydrochloride (Tris-Cl), pH 8.0; 30 mmol/l EDTA, pH 8.0; 5 % Tween-20 and 0.5 % Triton X-100.
2. Buffer QF (high-salt elution buffer). This contains 1.25 mol/l sodium chloride; 50 mmol/l Tris-Cl, pH 8.5 and 15 % isopropranolol.
3. Buffer QBT (equilibration buffer). This contains 750 mmol/l MOPS, pH 7.0; 15 % isopropranolol and 0.15 % Triton X-100.
4. Buffer QC (medium-salt wash buffer). This contains 1.0 mol/l sodium chloride; 50 mmol/l MOPS, pH 7.0 and 15 % isopropranolol.
5. Qiagen® genomic-tips.

Materials not supplied with the kit:

1. Qiagen® protease (1 × 125 mg protease in 6.25 ml distilled water).
2. Rnase A stock (100mg/ml)
3. 100 % ethanol
4. 70 % ethanol.
5. Liquid nitrogen.
6. Microdismembranation chamber
7. Heating block and centrifuge.
8. Isopropranolol.

2.3.2 Methodology

Fresh tumour and normal lung tissue was identified macroscopically by consultant pathologists and frozen at -70°C immediately after surgery. Care was taken to remove normal lung tissue from as far from the tumour as possible to reduce the risk of contamination of the normal tissue with tumour cells. Once frozen at -70°C, samples were batched and DNA extracted at a later date. The samples collected from the Western Infirmary in Glasgow were collected prospectively, whereas the samples from Aberdeen Royal Infirmary were collected retrospectively from Dr Keith Kerr, consultant pathologist. Both sample sites had collected and processed the samples as described above.

All utensils were washed in 100 % ethanol prior to use and also in between each sample to ensure no contamination. All utensils were cooled with liquid nitrogen prior to handling each tissue sample in order to keep the samples as cold as possible and ensure maximum DNA extraction. All extractions were performed in a laminar flow cupboard.

1. A pestle and mortar was used to break each tissue sample into small pieces, keeping the samples cold with liquid nitrogen. The tissue sample was placed in the microdismembranation chamber on full amplitude for 5 seconds. This was repeated twice more, ensuring that the sample remained cold in liquid nitrogen in between.
2. The resulting fine powder was added to 19 ml G2 buffer, 38 µl Rnase A and 1 ml Qiagen protease (prepared earlier). This was then vortexed and incubated at 50°C overnight.

3. The lysate should be clear after incubation. Any particulate matter was removed by centrifugation at 5000 × g for 10 minutes at 4°C.
4. The Qiagen Genomic-tips were equilibrated with 10 ml buffer QBT. The buffer QF was incubated at 50°C.
5. The lysate was vortexed for 10 seconds and then poured through the equilibrated genomic-tip. If the DNA lysate was particularly concentrated, then gentle positive pressure was applied to increase the flow rate through the genomic-tip.
6. The tip was washed with 15 ml buffer QC. This was repeated one more time.
7. The DNA was eluted with 15 ml buffer QF into a clean Sorvall tube.
8. The DNA was precipitated with 10.5 ml isopropranonol and mixed.
9. The sample was centrifuged at 10 000 × g for 15 minutes at 4°C to precipitate the DNA.
10. The supernatant was removed and the DNA pellet was washed with 500 μl of cold 70 % ethanol. The sample was then vortexed.
11. The DNA and ethanol were then pipetted into a fresh Eppendorf tube and centrifuged at 13 000 × g for 10 minutes.
12. The supernatant was removed and the DNA was air-dried at 37°C for 30 minutes. The pellet was re-suspended in 200 μl distilled water and the DNA concentration was measured using a spectrophotometer.

2.4 Allelic imbalance analysis

Fluorescently labelled primers were used in a PCR reaction, followed by analysis of the fluorescently labelled PCR products using an automated DNA sequencer and appropriate software. In this study, the Applied Biosystems ABI PRISM® DNA Sequencer was used throughout.

2.4.1 Materials

2.4.1.1 Polymerase chain reaction (PCR)

1. Taq DNA polymerase Amplitaq gold, 5 U/µl supplied with Geneamp 10 times PCR buffer II: 150 mmol/l KCl, pH 8.0 (applied Biosystems).
2. MgCl2 solution supplied at 25 mmol/l (Applied Biosystems).
3. dNTP mixture containing dATP, dCTP, dGTP and dTTP, 10 mmol/l each (Applied Biosystems).
4. Microsatellite primers fluorescently labelled with either 5'-FAM or HEX (Oswel DNA service, Southampton, UK).
5. DNA samples.
6. Sterile water.

2.4.1.2 Sample preparation prior to polyacrylamide gel electrophoresis

1. 100 % ethanol.
2. Glycogen (20mg/ml), molecular biology grade (Roche).

2.4.1.3 Polyacrylamide gel electrophoresis

1. Sequagel 6 monomer solution supplied with Sequagel Complete Buffer solution (Flowgen).

2. 10 % ammonium persulphate freshly made with distilled water as required (Fisher).
3. Deionised formamide sample loading buffer: 1 ml deionised formamide, 200 μl dextran blue, 50 mmol/l EDTA pH 8.0.
4. Genescan DNA internal lane size standard GS500XL ROX (Applied Biosystems).
5. TBE: 21.6 g Tris, 11 g boric acid, 1.69 g EDTA in 2 litres distilled water.
6. Glass plates for Applied Biosystems ABI PRISM® 373 DNA Sequencer, appropriate spacers (0.3 mm), bulldog clips, duck-billed pipette tips and a sharkstooth comb.

2.4.2 Methodology

2.4.2.1 PCR

1. All PCR was performed under conditions designed to prevent cross contamination. Separate areas were designated for pre- and post- PCR and all PCR preparation was carried out in PCR hoods with UV sterilisation facilities. Equipment was kept separate from that used for other techniques within the laboratory.
2. The quantity and recipe for the mastermix required depended on the primer used and the number of samples. For each PCR run, the quantity (number of samples) + (negative control) + 1 was made.
3. The DNA samples and reagents were thawed at room temperature and transferred to wet ice. The mastermix was made up as above and then vortexed briefly in a benchtop centrifuge. The mastermix was stored on wet ice until use. 2 μl of each DNA sample was added to the base of a well in a 96 well plate. The

appropriate amount of mastermix was added to each well and the plate was then covered with an adhesive plate sealer. The plate was centrifuged briefly.

4. The PCR reactions were performed using the protocol: 95°C, 10 minutes, 1 cycle; 95°C, 45 seconds, T_m, 45 seconds, 72°C, 45 seconds, for a total of 30 cycles; 72°C, 5 minutes, 1 cycle; 4°C hold. The annealing temperature (T_m) was adjusted dependant upon the locus being amplified (table 2.4). The PCR products were stored at -20°C until required.

Table 2.4: Allelic imbalance / shift PCR primer conditions

Primer (chromosome)	Primer sequence (5' – 3')	Tm (°C)	Size (bp)
APC (5q)	ACTCACTCTAGTGATAAATCG AGCAGATAAGACAGTATTACTAGTT	55	96-122
D2S123 (2p)	AAACAGGATGCCTGCCTTTA GGACTTTCCACCTATGGGAC	60	197-227
p53 (17p)	GAATCCGGGAGGAGGAGGTTG AACAGCTCCTTTAATGGCAGCGGGAGGAGGTTG	55	140-175
MfD15CA (17q)	GGAAGAATCAAATAGACAAT GCTGGCATATATATATTTAAACC	52	150
D3S1289 (3p)	AAAGCAACTTGTAAGAGAGCA CTCCTAGATATAATCACTGGCA	51	197-215
D3S1300 (3p)	AGCTCACATTCTAGTCAGCCT GCCAATTCCCCAGATG	48	217-241
D3S1304 (3p)	TTCGCTCTTTGATAGGC ATTTCATTTGTAATTTACTAGCAG	47	253-269

2.4.2.2 PCR product preparation

1. 1 μl of each PCR sample was transferred to another 96 well plate and 0.5 μl glycogen, molecular biology grade and 3 μl 100 % ethanol was added to each well. The plate was covered with adhesive film, centrifuged briefly and stored at -20°C overnight.
2. The following day, the plate was centrifuged at 1500 × g for 10 minutes, then the adhesive cover was removed and the samples air-dried for several hours until all the ethanol had evaporated.
3. Once dry, 1 μl deionised formamide running dye and 0.5 μl Genescan size standard GS500XL were added to each sample to resuspend the DNA. The size standard is an internal lane marker added to account for lane-to-lane differences across the gel during electrophoresis. Following this, the samples were denatured at 95°C for 5 minutes and then placed on wet ice prior to gel loading.

2.4.2.3 Polyacrylamide gel electrophoresis

1. The glass plates were cleaned so that they were free from dust and the cleaned plate surfaces were not touched. To assemble the plates, two notched gel spacers were positioned on the plate at each edge and the second plate was aligned, ensuring that the edges were flush. The plates were clamped together with bulldog clips.
2. 40 ml Sequagel 6 monomer solution, 10 ml Sequagel complete buffer and 400 μl fresh ammonium persulphate solution were mixed and the gel was cast using a 50 ml syringe. The plates were tapped at the same time to ensure no air bubbles were trapped between the plates. A sharkstooth comb was inserted between the plates to form a well for loading. The gel was allowed to set for 2 hours.

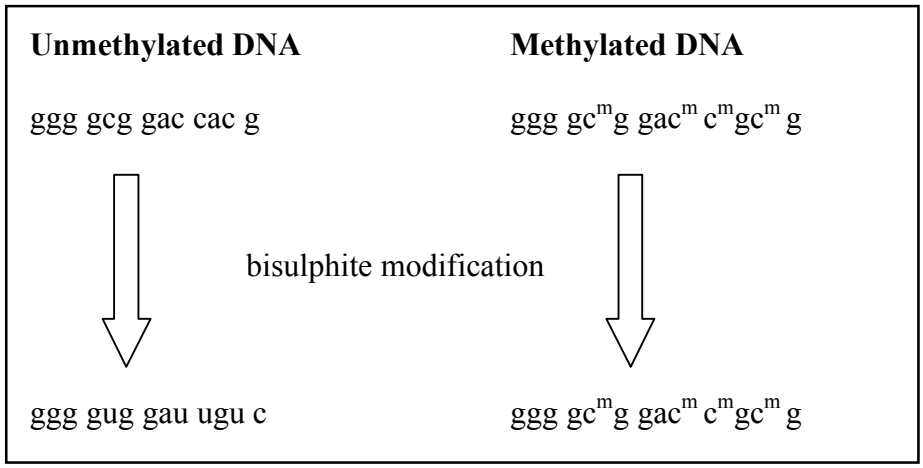
3. All 1.5 μl of the resuspended PCR product (see sections 2.2 and 2.3) was loaded into each well using duck-billed pipette tips and electrophoresis was commenced for 12 hours at 2500 V.
4. The virtual gel produced was analysed using software to compare the size of the product bands with known sizes of bands in the internal lane size standard. The electropherograms produced displayed the height of each peak, this being the relative amount of each allele present.
5. Results were calculated by comparing alleles in tumour/serum DNA with the corresponding alleles in lymphocyte DNA. The formula $(n_1)(t_2)/(n_2)(t_1)$ was used to calculate results for heterozygous alleles, where n_1 = lymphocyte samples larger allele, t_2 = tumour (serum) samples smaller allele, n_2 = lymphocyte samples smaller allele, t_1 = tumour (serum) samples larger allele. In this study, allelic imbalance was defined as an imbalance of allele intensities > 30%, i.e. < 0.7.

   Where this calculation could not be performed, for example when the sample was homozygous at the locus investigated in the lymphocyte DNA then the PCR result was termed as non-informative.

2.5 Methylation specific PCR

Methylation specific PCR is dependent upon modification of the DNA samples by sodium bisulphite. The purpose of this modification is to deaminate all unmethylated cytosines such that they are converted to uracil, whilst the 5-methylcytosines remain unchanged, leading to a sequence change between the methylated and unmethylated DNA and this is shown in figure 2.1. This sequence change then allows the development of specifically designed PCR primers for the different sequences between the originally methylated and unmethylated DNA. This methodology was first described by Herman (Herman et al., 1996) and is termed methylation specific PCR (MSP). Subsequent PCR products can be further analysed either on an agarose gel or by DNA sequencing.

Figure 2.1: DNA bisulphite modification



2.5.1 Materials

2.5.1.1 DNA Modification

In this study all modifications performed were done so using the Intergen CpGenome^{TM} DNA Modification kit (Intergen) and is done over 2 days. The exact constituents of the reagents supplied with the kit have not been disclosed by Intergen.

The methodology of the modification will be discussed in detail, but in essence day 1 consists of reagent preparation and commencement of the modification process with completion of the modification-taking place on day 2. The DNA samples are then stored at -20°C ready for Methylation Specific PCR, the modified samples being stable for up 1-month post modification.

Materials supplied with the kit:

1. DNA Modification Reagent I: this contains a sodium salt of the bisulphite ion which causes unmethylated cytosines to be sulfonated and hydrolytically deaminated, yielding a uracil sulfonate intermediate.
2. DNA Modification Reagent II: This contains another salt, which acts with Reagent III to bind DNA.
3. DNA Modification Reagent III: This contains a micro-particulate carrier, which binds the DNA in the presence of Reagent II.
4. DNA Modification Reagent IV: This improves the yield if the starting sample contains less than 1 μg DNA.

Reagents I, II and IV were stored at -20°C and reagent III at 4°C

Materials not supplied with the kit:

1. Water bath incubator set at 37°C and 50°C as appropriate.
2. Microcentrifuge.
3. PH indicator paper.
4. Screwcap centrifuge tubes.
5. NaOH pellets.
6. 70 %, 90 % and 100 % EtOH

7. β-mercaptoethanol.
8. TE Buffer (10mmol/l Tris-HCl; 0.1mmol/l EDTA, pH 7.5).

2.5.1.2 Methylation-Specific PCR

1. Taq DNA polymerase FastStart Taq (Roche Diagnostics).
2. FastStart Taq buffer (no $MgCl_2$ added) (Roche Diagnostics).
3. $MgCl_2$ solution supplied at 25 mmol/l (Applied Biosystems).
4. dNTP mixture containing dATP, dCTP, dGTP, 10 mmol/l each (Applied Biosystems).
5. Microsatellite primers.
6. Template DNA sequences.
7. Sterile water.
8. *In vitro* methylated DNA (IVM) (Intergen).
9. Genomic human DNA.
10. Mineral oil.

2.5.1.3 Agarose gel electrophoresis

1. Ethidium bromide (10 mg/ml).
2. Agarose.
3. TBE: 21.6 g Tris, 11 g boric acid, 1.69 g EDTA in 2 litres distilled water.
4. 123bp DNA ladder (Life Technologies).

2.5.2 Methodology

The environment in which modification and subsequent DNA amplification is performed is critical to achieving accurate, reliable results. All modifications were

therefore carried out in CAT 1 (pre-PCR DNA) facilities. This area is separate from both the PCR and post-amplification areas. All materials such as pipettes, pipette tips etc. were separated from other laboratory equipment and all reagents were made freshly for each modification procedure. 1 μg of DNA was used for each modification. If less than 1 μg of DNA was available, as near to 1 μg as possible was used and Reagent IV added. The DNA concentration was quantified using a spectrophotometer, with the mean of three repeat samples being taken. Control samples were modified together with the samples of interest. In vitro methylated DNA and distilled water were included in each batch of samples to be modified as positive and negative controls respectively.

2.5.2.1 Reagent Preparation

All reagents were made freshly for each modification procedure.

1. DNA Modification Reagent II: 1 μl of B-Mercaptoethanol was added to 20 ml of deionised water. 750 μl of this solution and 30 μl of 3mM NaOH was added to 1.35 g of DNA Modification Reagent II for each sample to be modified. This mixture was then shaken well to ensure complete dissolution.
2. 3 M NaOH Stock: 1g of NaOH pellets was dissolved in 8.3 ml of water.
3. 20 mM NaOH/90% EtOH: To prepare 1ml of this solution 900 μl of 100% EtOH was combined with 93.4 μl of H_2O and 6.6 μl of 3 mM NaOH.
4. DNA Modification Reagent I: 571 μl of water was added to 227 mg of DNA Modification reagent I for each sample to be modified. The pH was adjusted to 5.0 with 30 μl mol/l NaOH.

2.5.2.2 DNA Modification Procedure

In microcentrifuge tubes

1. 2 μl of DNA Modification Reagent IV was added to each DNA sample containing less than 1 μg DNA. the total volume was brought up to 100 μl with sterile water.
2. 7.0 μl 3M NaOH was added and mixed.
3. Samples were incubated at 37°C for 10 minutes in a waterbath.
4. 550 μl of freshly prepared DNA Modification Reagent I was then added and each sample vortexed.
5. Samples were then incubated at 50°C for 16 to 20 hours in a waterbath.

2.5.2.3 Completion of Chemical Modification and DNA Clean-up

1. DNA Modification Reagent III was re-suspended by vigorous vortexing.
2. 5 μl of DNA Modification Reagent III (well-suspended) was added to the DNA solutions in each tube.
3. 750 μl of DNA Modification Reagent II was added to each tube and mixed briefly.
4. Samples were incubated at room temperature for 10 minutes.
5. Samples were then spun at 5000×g to pellet DNA Reagent III. The supernatant was discarded.
6. 800 μl of 70% EtOH was added to each sample, vortexed and centrifuged at 5000 × g for 10 seconds. The supernatant was discarded. This step was performed 3 times in total.
7. The tubes were then centrifuged at 12000×g for 3 minutes and the remaining supernatant removed with a pipette.

8. 50μl 20 mM NaOH/90% EtOH was added to each of the samples.
9. The pellets were then resuspended by vortexing and the samples were then incubated at room temperature for 5 minutes.
10. The samples were then spun at 5000 × g for 10 seconds to pellet the sample. The pellets were then washed by adding 800μl of 90% EtOH and vortexing. The samples were spun again at 5000 × g and supernatant removed. This step was repeated once.
11. After the supernatant from the second wash was removed, the samples were centrifuged at 12000×g for 5 minutes.
12. All the remaining supernatant was removed with a pipette and 40 μl of TE (10mmol/l Tris/EDTA, pH 7.5) added. The pellet was resuspended by vortexing again.
13. The samples were incubated for 15 minutes at 55°C in order to elute the DNA.
14. The samples were then centrifuged at 12000 × g for 3 minutes and the supernatant (modified DNA) transferred to a new-labelled tube prior to PCR.
15. Samples then stored at -20°C for up to 2 months and used for Methylation Specific PCR

2.5.2.4 Methylation Specific PCR (MSP)

1. Methylation-specific PCR was performed in a similar manner to conventional PCR with standard precautions taken to prevent contamination. PCR was performed in dedicated hoods with dedicated pipettes, pipette tips and PCR tubes.

2. The quantity and recipe for the PCR mastermix required depended upon the primer being used and the number of samples. The quantity made for each PCR run was (number of samples) + (negative control) + 1.
3. Initially, optimal PCR conditions for each primer were determined using in vitro methylated bisulphite modified DNA (IVM) as a positive control and genomic (lymphocyte) DNA as a negative control. The annealing temperature and magnesium concentrations were varied using a gradient block cycler until the optimal conditions were obtained. Optimal conditions were then tested on a panel of positive and negative samples, together with IVM DNA, genomic DNA, unmodified DNA and distilled water as controls. Once the optimal conditions were confirmed, the same PCR block was used for all the PCR reactions performed with that primer.
4. Initially, the success of a modification was confirmed by a PCR reaction using a primer specific for unmethylated DNA, such as GAPDH. With such a primer, all modified samples should give a positive band using agarose gel electrophoresis but unmodified samples should be negative. Subsequently, the samples were amplified using primers specific for methylated DNA at the loci being analysed.
5. The DNA samples and reagents were thawed at room temperature and then transferred to wet ice. The recipe for each PCR reaction was a 25 μl reaction volume containing 1 μl modified template DNA, 1 × PCR buffer, 0.1 mmol/l dNTPs, 0.5 μl each forward and reverse primer, 1 U FastStart® Taq DNA polymerase and varying magnesium concentrations for each primer, as shown in table 2.5, each sample was overlaid with 1 drop of mineral oil.

6. PCR reactions were performed using the protocol: 95°6 minutes, I cycle; 95°C, 30 seconds, T_m 30 seconds, for a total of 35 cycles; 72°C, 5 minutes, 1 cycle; 4°C, hold. Specific PCR conditions for each primer are given in table 2.5.

2.5.2.5 Agarose gel electrophoresis

1. Following methylation-specific PCR, products were separated on a standard 2 % agarose gel. 2 g agarose was added to 100 ml 1 % TBE and this was microwaved on high power for 1 minute. To visualise the products, 30 μl ethidium bromide (10mg/ml) was added before pouring the gel.
2. 1 μl loading dye was added to 5 μl PCR product and this was then loaded into each well. Appropriate size DNA ladders were used to determine the product size.
3. PCR products were visualised using the Bio-Rad Gel Documentation system. IVM DNA showing a positive signal. Modified and unmodified human (lymphocyte) DNA and water should show a negative or a very low signal. Samples were scored positive if the PCR signal was approximately equivalent to IVM (score 3), intermediate if equal to IVM 1:5 dilution (score 2), weak if equal to IVM 1:25 dilution (score 1) and negative if no signal or equivalent to human (lymphocyte) DNA (score 0). Samples were deemed positive if score 2 or 3 was obtained in two separate PCR reactions.

Table 2.5: Methylation-specific PCR primer conditions

Primer	Primer sequence 5' 3' F: R:	Tm (°C)	Magnesium Conc. (mmol/l)	Product size (bp)
hMLH1	ACGTAGACGTTTTATTAGGGTCGC CCTCATCGTAACTACCCGCG	64	2	115
P16	TTATTAGAGGGTGGGGCGGATCGC CCACCTAAATCGACCTCCGACCG	65	2	234
DAPK	GGATAGTCGGATCGAGTTAACGTC CCCTCCCAAACGCCGA	64	2	98
TIMP-3	CGTTTCGTTATTTTTTGTTTTCGGTTTC CCGAAAACCCCGCCTCG	59	3	116
HIC-1	TTCGGGTTAGGGTCGTAGTC CTAACCGAAAACTATCAACCCTCG	57	2.5	243
MINT 25	GCGAAAGCGAAAGTCGTT CCCAACGCACATAACGAACC	57	3	213
MINT 31	AGGGTAATTAGGGAGACGAC AAAACGCTTACGCCACTACG	58	2	252

3. The role of MMR proteins as a marker of prognosis in patients with NSCLC

3.1 Introduction

In the UK, approximately 80% of patients with NSCLC present in the UK with locally advanced or incurable metastatic disease. Moreover approximately 50% of patients with surgically resected disease will develop tumour recurrence. There is a clearly established role for chemotherapy in all stages of NSCLC (Non-small cell lung cancer collaboration group, 1995, Arriagada et al., 2004). Other than the recently reported studies of ERCC1 (Simon et al., 2005, Olaussen et al., 2006) there is limited information on potential molecular markers of response to chemotherapy and survival in patients with NSCLC. The markers studied were the mismatch repair proteins MLH1 and MSH2 as well as p53 and the marker of proliferation Ki67.

The objectives of this study in 2 distinct cohorts of patients were therefore to address the following questions:

- Does the level of expression of a panel of molecular markers correlate with prognosis in patients with NSCLC?
- Does the level of expression of these markers correlate with other clinicopathological variables?
- Can small diagnostic bronchial biopsies be used to study this panel of molecular markers?

- Does neoadjuvant chemotherapy alter expression levels of the molecular markers and has this any affect on overall survival?
- Can these molecular markers help to predict in advance treatment response to platinum versus non-platinum containing chemotherapy?

3.2 Patients

Patient samples within this study came from 2 distinct groups:

Group 1:

Archived paraffin blocks of tissue taken at the time of diagnostic bronchoscopy in 67 patients were obtained. All of these patients had received chemotherapy as first line treatment for their NSCLC between 1995 and 2000 at Stobhill Hospital in Glasgow. Clinical data including histology and stage was available for all these patients and thirty-six were male. Mean age for all patients was 65.9 years (range 44 – 78 years). A summary of patient characteristics and clinicopathological data obtained from a review of the patient case notes as well as the pathology reports taken from the time of the diagnostic bronchoscopy is given in table 3.1.

Table 3.1: Patient characteristics – The Stobhill Bronchoscopy Population

Patient characteristics	Cisplatin based chemotherapy (n = 45)	Non-Cisplatin based chemotherapy (n = 22)
Sex		
Male	27	9
Female	18	13
Age		
Range	44 – 83	56 – 78
Mean	64.3	65.9
Performance Status		
0	1	0
1	34	12
2	7	10
Not documented	3	0
Histology		
Squamous	19	11
Adenocarcinoma	6	3
NSCLC – not specified	16	6
Large cell	3	1
Undifferentiated	1	1
Stage		
I/II	5	2
III	22	12
IV	12	4
Not documented	6	4
Chemotherapy regimes	MVP (35) gemcitabine/cisplatin (10)	ifosfamide (15) gemcitabine (7)
Survival		
median (months)	9	10
range	0.5 - 38	2 - 28

Group 2:

Archived paraffin embedded tumour samples were collected retrospectively from 50 patients who had undergone surgical resection of their tumour between 1996 and 2000. All patients were treated at Aberdeen Royal Infirmary and of this group 10 had received neo-adjuvant cisplatin based chemotherapy (prior to surgery). In addition to the paraffin embedded samples, fresh frozen tumour and normal lung samples were also collected from this cohort of 50 patients at time of surgery and frozen for future

analysis. Thirty-eight were male and the mean age for all patients was 65 years (range 46 – 80 years). A summary of patient characteristics is given in table 3.2. Again clinicopathological data was collected from a review of the patient case notes as well as the pathology reports taken from the time of the surgery.

Table 3.2: Patient characteristics – The ARI Surgical Cohort

Patient characteristics	Surgery alone (n = 40)	Neoadjuvant cisplatin chemotherapy + surgery (n = 10)
Sex		
Male	33	5
Female	7	5
Age		
Range	49 – 79	43 – 73
Mean	65.5	59.7
Histology		
Squamous	32	5
Adenocarcinoma	0	3
NSCLC – not specified	8	2
Large cell	0	0
Undifferentiated	0	0
Stage		
I	15	2
II	16	3
III	7	3
IV	1	0
Not documented	1	2
Survival		
Median (months)	42	23
Range	1 - 63	1 - 58

3.3 Methods

Ethical approval for this study was obtained through local Ethics committees in both Glasgow and Aberdeen. Immunohistochemical studies were performed as described in detail in chapter 2.1. Two individuals as described in chapter 2.1.2.3 scored sections

from the bronchoscopy cohort independently. Statistical analysis was performed using Statistical Package for the Social Sciences (SPSS).

3.4 Results

3.4.1 Inter-observer variability

Table 3.3 shows inter-observer Kappa scores for the proteins analysed in group 1 patients. This confirms good correlation between the 2 observers for immunohistochemistry scoring with only 1 comparative result less than 0.4. These results confirm a close correlation between the 2 observers.

Table 3.3: Inter-observer Kappa scores for proteins analysed

Protein	Kappa score	Standard Error	Number of valid samples
MLH1			
Intensity	0.5233	0.0694	61
Percentage	0.6386	0.0658	
IHC score	0.6062	0.059	
MSH2			
Intensity	0.4242	0.0748	57
Percentage	0.3852	0.0908	
IHC score	0.4313	0.0718	
pP53			
Intensity	0.5966	0.0703	60
Percentage	0.4942	0.0781	
IHC score	0.591	0.0679	

3.4.2 Correlation of protein expression with overall survival

Possible correlation between IHC score and overall survival was evaluated in both cohorts of patient. The univariate examination of this association was made using Cox regression analysis. Table 3.4 shows a summary of these results for group 1 patients. No correlation between the IHC score from any of the proteins studied and overall survival was demonstrated in this cohort of patients.

Possible correlation between IHC score and overall survival between those patients in group 1 who had cisplatin based chemotherapy versus those that had non-cisplatin based chemotherapy were also evaluated and these results are tabulated in table 3.5. No correlation between the IHC score and overall survival was demonstrated in either the group that had platinum based chemotherapy or the group that received non-platinum based chemotherapy.

Table 3.4: Correlation between IHC scores and overall survival in bronchoscopic samples (Group 1)

Protein	p-value	Standard Error	Number of valid samples
MLH1	0.554	0.04	67
MSH2	0.121	0.044	67
p53	0.121	0.039	66

Table 3.5: Correlation between IHC score, chemotherapy regime and overall survival in bronchoscopic samples (Group 1)

Protein	p-value	Standard Error	Number of valid samples
MLH1			
Cisplatin	0.539	0.056	43
Non-cisplatin	0.913	0.081	22
MSH2			
Cisplatin	0.183	0.057	43
Non-Cisplatin	0.913	0.076	22
p53			
Cisplatin	0.576	0.046	42
Non-cisplatin	0.062	0.078	22

Table 3.6 shows a summary of these results for the group 2 patients. No correlation between the IHC score from any of the proteins studied and overall survival was demonstrated in this cohort of patients.

Possible correlation between IHC score and overall survival between those patients in group 2 who had cisplatin based chemotherapy preoperatively versus those that had surgery alone were also evaluated and these results are tabulated in table 3.7. No correlation between the IHC score and overall survival was demonstrated in either the group that had platinum based chemotherapy preoperatively or the group that underwent surgery alone.

Table 3.6: Correlation between IHC scores and survival in surgical samples (Group 2)

Protein	p-value	Standard Error	Number of valid samples
MLH1	0.308	0.052	50
MSH2	0.154	0.065	50
p53	0.497	0.046	50
Ki67	0.968	0.070	50

Table 3.7: Correlation between IHC score, chemotherapy regime and overall survival in surgical samples (Group 2)

Protein	p-value	Standard Error	Number of valid samples
MLH1			
no chemotherapy	0.428	0.054	40
chemotherapy	0.632	0.187	10
MSH2			
no chemotherapy	0.161	0.073	40
chemotherapy	0.986	0.143	10
p53			
no chemotherapy	0.553	0.052	40
chemotherapy	0.886	0.107	10
Ki67			
No chemotherapy	0.935	0.080	40
chemotherapy	0.661	0.260	10

3.4.3 Correlation of protein expression with tumour histology and stage

Possible correlation between protein expression and either histological subtype or tumour stage in both bronchoscopic and surgical samples was investigated by Kruskal-Wallis test. This failed to demonstrate any relationship between IHC score for any of the proteins examined and histological subtype or tumour stage in both cohorts of patients.

3.5 Discussion

Group 1:

Sixty-seven archived paraffin embedded samples were obtained from 67 patients who received chemotherapy for NSCLC at Stobhill Hospital, Glasgow between 1995 and 2000. This represents 60.9% (67/110) of all patients who received chemotherapy for NSCLC over the same time period. Difficulties in obtaining bronchoscopic samples from all of these 110 patients related to the fact that the local pathology department was in the process of relocating making tracing of the samples difficult. Moreover the storage facilities for some of the older archived samples had been affected by damp and subsequent mould, rendering the samples unsuitable for analysis. Despite these difficulties 67 of 110 samples were obtained, processed and analysed.

The pathology reports indicated a diagnosis of NSCLC in 22 of 67 (32.8%) of these samples with no further subclassification possible. This is unfortunate as there are separate pathways for tumour initiation and progression for squamous cell carcinoma and adenocarcinoma. These two histological subtypes account for the vast majority of NSCLC and without accurate histological diagnosis potentially significant differences in molecular markers between these two sub-types may be masked.

A broad range of chemotherapeutic agents and regimens were used in this cohort of patients. This relates to the fact that during the study time period (1995 – 2000) the use of chemotherapy in NSCLC was growing and the significant role of cisplatin as an important cytotoxic drug in NSCLC was being established (Non-small cell lung cancer collaboration group, 1995). Moreover new drugs were being introduced over the time

period (e.g. gemcitabine). In this study 22.4% (15/67) patients received single agent ifosfamide as part of a clinical study ongoing at the time of their treatment.

In retrospective studies it is often difficult to collect accurate data on the patient population, which is dependant on the quality of the records kept for the patients in relation to disease extent, stage, detailed pathology and treatment. This was the case in this patient cohort where stage was documented for only 57 of 67 (85.1%) patients in the study population. Moreover 7 patients (10.4%) were documented as having stage 1 or 2 disease and patients with such early stage disease would usually be regarded as potentially radically treatable by means of either surgery or radical radiotherapy. Ten patients (14.9%) had no stage documented.

Within the sample population the majority of patients had Performance Status (PS) documented and the majority were of PS 0 – 2. This finding demonstrates that patients who need to be fit enough for chemotherapy to treat their NSCLC had this important prognostic indicator considered when planning their treatment. Only 3 patients had no record of their PS documented.

Group 2:

The number of patients undergoing resection for NSCLC in Scotland remains low with an average resection rate of 10.7% (Gregor et al., 2001).

At the time that this research project was carried out there were national concerns pertaining to the ownership of tissue samples as well as retained organs in pathology departments and this severely limited our ability to obtain fresh tissue prospectively. In

the past it had not been felt necessary to ask patients or relatives for their consent for research to be carried out on tissue removed as part of their surgical procedure. National guidelines regarding consent and use of tissue and organs for research have now been published (Human Tissue Act, 2004).

On account of the insurmountable problems (logistical as well as ethical) in relation to obtaining fresh tumour prospectively, we studied previously collected and banked fresh frozen surgical samples. We obtained samples retrospectively from patients who had lung cancer resection surgery in Aberdeen Royal Infirmary. The local ethics committee approved the collection and use of these samples without requiring the patients or the relatives of the patients to be informed as it was felt that the research would have no bearing on the future health or treatment options of these individuals. All patients were deceased at the time of this study-taking place.

The Pathology department in Aberdeen at the time had a particular interest in studying primary adenocarcinoma of the lung. Thus the primary adenocarcinoma samples had already been studied in other research projects and as a consequence the tissue collected for this study was predominantly primary squamous cell carcinoma of the lung (n=37, 74%).

Within this group there were patients that were pathologically staged as having stage III disease (10 patients, 20%) and stage IV in 1 patient (2%). Three of the stage III patients had preoperative chemotherapy whilst 7 (14%) of these patients had no neoadjuvant chemotherapy and there is no record of adjuvant therapy having been given. These 7

patients were presumably clinically under-staged as currently in the UK operative therapy for stage III disease is limited to the context of a clinical trial.

Within this cohort the PS of the patient was not documented but it is assumed that the majority if not all the patients will have had a PS less than 2 to be considered medically fit for thoracotomy.

With regard to immunohistochemical scoring several methods of scoring both intensity (I-score) and percentage of cells scored (%-score) have been described and the area remains controversial with different authors using various methods to calculate the overall score. Some authors use the 'H-score' (McClelland et al., 1990), which gives a result between 0 and 300 using a complicated calculation; (% of cells stained at intensity 1 × 1) + (% of cells stained at intensity 2 × 2) + (% of cell stained at intensity 3 × 3), where the intensity score is defined as in table 2.2.

However, it has been demonstrated that more simple calculations produce comparable results. In one study (Detre et al., 1995), it was found that similar results could be obtained with either an additive (I + %) 'quickscore' or a multiplicative (I x %) 'quickscore', where the I-score and the %-score are defined as in tables 2.2 and 2.3 respectively. Importantly scoring took one quarter of the time when compared with the H-score technique.

The use of an immunohistochemistry 'quickscore' for calculating both intensity and percentage of cells stained is well established (Kinsel et al., 1989) and Dr. M. Mackean (Centre for Oncology and Applied Pharmacology, University of Glasgow) has

previously validated the multiplicative 'quickscore' technique. Using this technique, Dr. Mackean has also shown that it is possible to achieve a high level of correlation in inter-observer, intra-observer and intra-slide scores and therefore this scoring method was used in the current study.

There remains a paucity of data regarding possible correlations between the level of mismatch repair expression and clinicopathological variables in patients with lung cancer. Moreover the studies that have been performed in lung cancer also used the different scoring techniques described above, and this makes interpretation and comparison of study results difficult.

A study by Xinarianos et al demonstrated a correlation between reduced MLH1 expression levels and the presence of nodal metastases in surgically resected primary squamous cell carcinoma of lung but not adenocarcinoma (Xinarianos et al., 2000). Brooks et al reported that reduced expression of MSH2 correlated with a poor overall survival in a cohort of patients where the nodal tissue examined was obtained at mediastinoscopy (Brooks et al., 2003). This study also demonstrated that an intense level of p53 expression correlated with a poor overall survival. In this study immunohistochemistry was measured using a semiquantative scale where it was the percentage of cells staining rather than the intensity that constituted the positive or negative score for protein expression. A study by Skarda et al demonstrated no correlation between mismatch repair protein expression (MLH1 and MSH2) and either disease free or overall survival in a cohort of patients where the study samples were taken at time of surgery (Skarda et al., 2006).

Nonetheless all of the studies described above demonstrate the potential importance of examining molecular markers and were all carried out on either surgically resected samples or samples taken at mediastinoscopy. It would be of significant advantage to have prognostic information obtained from examination of the diagnostic sample. In the majority of patients this means examining diagnostic tissue taken at time of bronchoscopy. Thus it is important to validate studies in bronchoscopic specimens and this is what we attempted to do in Group 1 patients. The bronchoscopy samples used in this study were truly representative of the tissue available for study by the pathologists having been used to obtain the initial diagnosis of lung cancer.

The principle question in these 2 cohorts of patients was does a correlation exist between a panel of molecular markers and prognosis in patients with NSCLC?

In our study of samples taken at both the time of diagnostic bronchoscopy (patient group 1) and at surgery (patient group 2) there was no correlation demonstrated between MMR protein expression and survival (tables 3.4 and 3.6) in keeping with the study performed by Skarda (Skarda et al., 2006). However our results conflict those reported by Brooks (Brooks et al., 2003).

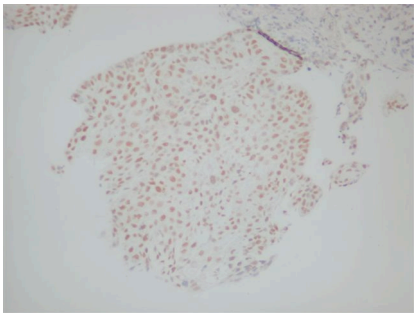
An obvious criticism of using bronchoscopic samples is the relatively small size of the tissue sample, often with only a few cells present, and an example of this is that we were unable to study the expression of Ki67 in these bronchoscopic samples due to lack of tissue. Despite small sample size good correlation between independent observers was demonstrated (table 3.1) when scoring the immunohistochemistry samples. However, when the study sample is relatively small, as with the bronchoscopy samples,

counting the percentage of cells is integral to the IHC score and there is the potential for bias dependant on the number of cells present in any given sample. In an attempt to confirm our findings in relation to immunohistochemistry in the bronchoscopic cohort of patients (group 1) we repeated the study, in the cohort of patients (group 2) where surgical samples were studied. In this latter study the expression levels of a panel of molecular markers and lack of correlation with clinicopathological variables were found to be the same. Figure 3.1 shows staining for each protein demonstrating the size difference between bronchoscopy and surgical obtained samples.

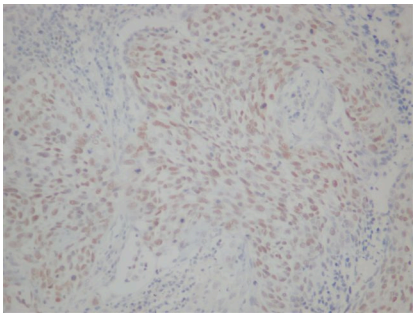
This does not confirm that there was no bias present in the bronchoscopy study. In a study by Taillade et al comparison was made between the immunohistochemical expression of 5 markers (EGFR, Ki67, ERCC1, phospho-Akt and hTERT) in both bronchoscopy samples and resected surgical samples from the same patient (Taillade et al., 2007). In the case of Ki67, ERCC1 and hTERT there was significant correlation between the bronchial biopsies and corresponding surgical sample (0.64 [$p < 0.0001$], 0.83 [$p < 0.0001$] and 0.55 [$p < 0.001$] respectively) but poor correlation for the markers EGFR and phospho-Akt (0.24 [$p = 0.17$] and 0.29 [$p = 0.09$] respectively), demonstrating that for certain previously validated markers the use of the bronchoscopy samples is a valid and important study method, when the number of patients undergoing resection for NSCLC in Scotland remains low with an average resection rate of 10.7% (Gregor et al., 2001).

Figure 3.1: IHC staining for individual proteins; bronchoscopy and surgical samples

MLH1

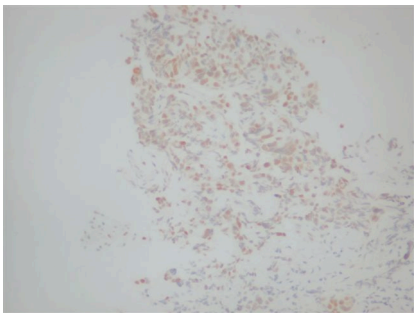


bronchoscopy

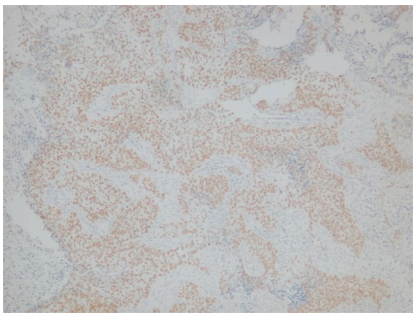


surgical

MSH2

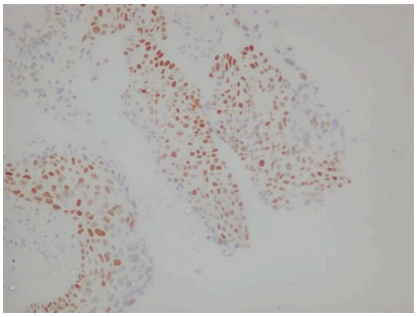


bronchoscopy

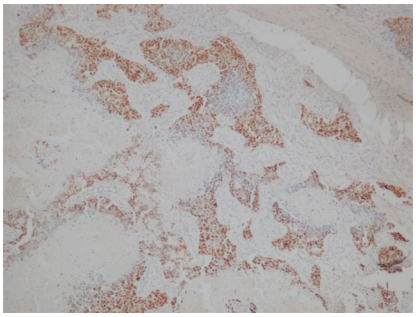


surgical

p53



bronchoscopy



surgical

All sections photographed at low power.

At the time of this research project the role of neoadjuvant chemotherapy was the subject of intense interest in patients with NSCLC. In this study we also examined if there was any difference between MLH1 expression levels in those samples obtained from patients treated with chemotherapy pre-operatively and those samples obtained from patients that had surgery alone and if these were predictive of any differences seen in relation to overall survival. Previously published work by Mackay et al in breast cancer demonstrated a reduction in MLH1 expression in tumour samples obtained from patients who had been treated with preoperative chemotherapy. This reduced MLH1 expression correlated with a poor disease free survival (Mackay et al., 2000).

We demonstrated in the surgical cohort of patients (group 2) no difference in expression level of MLH1 (IHC score) and no survival difference between those patients that had preoperative chemotherapy versus those that underwent surgery alone. This was also the case for MSH2 and p53. These findings are similar to those reported in a recent study by Skarda et al which also described no difference in expression levels of MLH1 or MSH2 and survival, irrespective of treatment with preoperative platinum based chemotherapy or not (Skarda et al., 2006).

A further objective of this study was to evaluate the role of the mismatch repair proteins (MLH1 and MSH2) as well as p53 in treatment naive patients diagnosed at bronchoscopy who proceeded to have either platinum based or non-platinum containing chemotherapy (Group 1). The hypothesis being is there reduced level of MMR expression (IHC score) pre-treatment and if so does this reduced expression level allow you to predict chemotherapy response to chemotherapy and overall survival. If this is

the case it opens the possibility in the future to tailor chemotherapy regimes dependant on molecular markers.

In our study we demonstrated no difference in survival rates when correlated with level of protein expression and chemotherapy regime (table 3.5, group 1).

The only other published study that has examined protein expression in chemonaive patients before going on to receive chemotherapy was the study by Brooks et al. In this study there were 2 distinct treatment groups. The first received single agent vinorelbine chemotherapy plus concurrent radiotherapy. The second group were treated with vinorelbine plus carboplatin and concurrent radiotherapy. In this study patients with a reduced expression of the mismatch repair protein MSH2 had a significantly reduce response to therapy and a worse cancer-free and overall survival (Brooks et al., 2003). However it is not clear whether the significantly reduced response to therapy relates to chemotherapy or radiotherapy or both.

In summary the results of this study do not support the findings from other studies that demonstrate the potential role of a panel of molecular markers in predicting response to treatments as well as important prognostic information. However it is clear when comparing all these studies that there is no well defined mechanism for scoring in immunohistochemistry studies making direct comparison often difficult in addition to all of the studies evaluation samples taken form different sites at differing points in their cancer management journey.

Future studies should centre on the collection and prospective study of a large panel of molecular markers using diagnostic samples collected pre-treatment. These results should be correlated with a variety of clinicopathological variables, including response to chemotherapy, the majority of which will still be platinum based in the UK and most importantly survival.

In addition a large prospective study where the primary objective is to correlate the findings in small samples such as bronchoscopy biopsies with larger surgical samples to confirm the study by Taillade et al is required (Taillade et al., 2007).

4. A study into CpG Island methylation status and its role as a marker of prognosis in patients with NSCLC

4.1 Introduction

Methylation is known to be an important epigenetic mechanism leading to gene suppression (Jones and Laird, 1999). Studies indicate that there may be concordant patterns of methylation present in certain tumour types including ovarian cancer (Strathdee et al., 2001a), prostate cancer (Jeronimo et al., 2004), gastric cancer (Toyota et al., 1999a) and gallbladder cancer (Roa et al., 2006). In each of these studies the number of loci examined and the endpoints assessed have varied, from early molecular detection in prostate cancer to survival in the study of gallbladder cancer. A recent paper by Park et al has demonstrated distinct methylation patterns (13 loci studied) in 9 common cancers (lung, breast, prostate, larynx, liver, colon, stomach and cervix) but did not report any significant correlations with clinicopathological data and methylation profile (Park et al., 2007).

In the case of lung cancer there have been a number of studies investigating the methylation profiles of both NSCLC and SCLC (Safar et al., 2005, Toyooka et al., 2001), but the clinical relevance of these methylation profiles remains unclear. This includes possible use of methylation profiles to predict response to specific cytotoxics drugs.

The objectives of this study were therefore to try and answer the following questions:

- Does the methylation profile in NSCLC correlate with overall survival?
- Does the methylation profile correlate with any other clinicopathological variables?
- Is there any difference in survival between patients who have had neoadjuvant chemotherapy plus surgery versus patients who had surgery alone in relation to the methylation profile of the tumour sample?

4.2 Methods

Ethical approval for this study was obtained from the local Ethics committee in Aberdeen. The patient population was the same as that described in chapter 3, group 2 and their clinicopathological data is shown in table 3.2. Methylation Specific PCR (MSP) was performed as described in detail, chapter 2.5 with DNA extracted from the cancer and normal lung sample as per the protocol described in chapter 2.3. Statistical analysis was performed using SPSS.

The methylation profile of these 50 tumours was analysed using 8 gene promoter regions. Using methylation-specific PCR the primers analysed were MLH1, p16, DAPK, TIMP3, HIC1, MINT 25, MINT 31 and RASSF1A (the MSP for RASSF1A gene promoter region was carried out by another student in the lab).

The methylation status of the same 8 gene promoter loci were analysed in normal adjacent lung removed at the time of the cancer resection.

4.3 Results

Of these 50 samples the mean DNA concentration from the tumour samples was 145.8 μg/ml (range 23 – 673) and for normal lung 88.1 μg/ml (range 0 – 242). MSP was successfully performed on all. All patients had died at the time of analysis and so definitive survival data is available for all patients. Results for the MSP carried out on the tumour samples are shown in table 4.1. The results of MSP in adjacent normal lung are shown in table 4.2. In tables 4.1 and 4.2 the presence of methylation is indicated by an X and no evidence of methylation with a 0. Table 4.3 summarises the methylation at each locus in both tumour and normal tissue.

Table 4.1: Methylation status of lung cancer samples at eight loci

Primer	hMLH1	p16	DAPK	TIMP3	HIC1	MINT25	MINT31	RASSF1A
1	0	0	0	0	0	0	0	X
2	0	X	0	0	0	0	0	0
3	0	0	0	0	X	0	X	0
4	0	0	0	0	0	0	0	X
5	0	0	0	0	0	0	X	0
6	0	0	0	0	0	0	0	0
7	0	0	0	0	0	0	0	0
8	0	0	0	0	0	0	X	0
9	0	0	0	0	0	0	0	0
10	0	0	0	0	0	0	0	X
11	0	X	0	0	0	0	0	0
12	0	0	0	0	0	0	0	0
13	0	0	0	0	X	0	0	0
14	0	0	0	0	0	0	0	X
15	0	X	X	0	0	0	X	X
16	0	0	0	0	0	0	0	0
17	0	0	0	0	0	0	0	X
18	X	0	0	0	0	X	X	X
19	0	0	0	0	0	0	0	0
20	0	X	0	0	0	0	0	0
21	0	0	0	0	0	0	0	0
22	0	0	0	0	0	0	0	X
23	0	0	0	0	0	0	0	0
24	0	0	0	0	0	0	0	0
25	0	X	X	0	0	0	X	X

26	0	X	0	0	X	0	X	X
27	0	X	0	0	X	X	0	0
28	0	X	X	0	0	0	0	0
29	0	X	0	0	0	0	0	0
30	0	0	0	0	0	0	0	0
31	0	0	0	0	0	0	X	0
32	0	X	0	0	0	0	0	X
33	0	X	0	0	0	0	0	0
34	0	0	X	0	0	0	0	0
35	0	0	0	0	0	0	0	0
36	0	X	0	0	0	0	0	0
37	0	0	0	0	0	0	0	0
38	0	X	0	0	0	0	X	X
39	0	X	X	0	0	0	0	0
40	0	0	0	0	0	0	0	0
41	0	0	0	0	X	0	0	0
42	0	0	0	0	0	0	0	0
43	0	0	0	0	0	0	0	0
44	0	0	0	0	0	0	0	0
45	0	0	0	0	0	0	0	0
46	0	0	0	0	0	0	0	0
47	0	0	0	0	0	0	X	0
48	0	0	0	0	0	0	0	0
49	0	0	0	0	0	0	0	0
50	0	0	X	0	0	0	0	X

Table 4.2: Methylated and unmethylated normal lung samples at eight loci

Primer	hMLH1	p16	DAPK	TIMP3	HIC1	MINT25	MINT31	RASSF1A
1	0	0	0	0	0	0	0	0
2	0	0	0	0	0	0	0	0
3	0	0	0	0	X	0	0	0
4	0	0	0	0	0	0	0	0
5	0	0	0	0	0	0	0	X
6	0	0	0	0	0	0	0	0
7	0	0	0	0	0	0	0	0
8	0	0	0	0	0	0	0	0
9	0	0	0	0	0	0	0	0
10	0	0	0	0	0	0	0	0
11	0	0	0	0	0	0	0	0
12	0	0	0	0	0	0	0	0
13	0	0	0	0	0	0	0	0
14	0	0	0	0	0	0	0	0
15	0	0	0	0	0	0	0	0
16	0	0	0	0	0	0	0	0

17	0	0	0	0	0	0	0	0
18	0	0	0	0	0	0	0	0
19	0	0	0	0	0	0	0	0
20	0	0	0	0	0	0	0	0
21	0	X	0	0	0	0	0	0
22	0	0	0	0	0	0	0	0
23	0	X	0	0	0	0	0	0
24	0	0	0	0	0	0	0	0
25	0	X	0	0	0	0	0	0
26	0	0	0	0	0	0	0	0
27	0	X	0	0	0	0	0	0
28	0	0	0	0	0	0	0	0
29	0	0	0	0	0	0	0	0
30	0	0	0	0	0	0	0	0
31	0	0	0	0	0	0	0	0
32	0	0	0	0	0	0	0	0
33	0	0	0	0	0	0	0	0
34	0	0	0	0	0	0	0	0
35	0	0	0	0	0	0	0	0
36	0	0	0	0	0	0	0	0
37	0	0	0	0	0	0	0	0
38	0	X	0	0	0	0	0	0
39	0	0	0	0	0	0	0	0
40	0	0	0	0	0	0	0	0
41	0	0	0	0	0	0	0	0
42	0	0	0	0	0	0	0	0
43	0	0	0	0	0	0	0	0
44	0	0	0	0	0	0	0	0
45	0	0	0	0	0	0	0	0
46	0	0	0	0	0	0	0	0
47	0	0	0	0	0	0	0	0
48	0	0	0	0	0	0	0	0
49	0	0	0	0	0	0	0	0
50	0	0	0	0	0	0	0	0

Table 4.3 **Summary of tumour and normal tissue methylation at each locus**

Primer	Tumour (n)	Tumour (%)	Normal (n)	Normal (%)
hMLH1	1	2	0	0
p16	14	28	5	10
DAPK	6	12	0	0
TIMP3	0	0	0	0
HIC 1	5	10	1	2
MINT 25	2	4	0	0
MINT 31	9	18	0	0
RASSF1A	13	26	2	4

In total 60% (30) of the tumours demonstrated methylation of at least one primer site whilst 40% (20) of the tumours demonstrated no methylation at any primer site. Looking at number of methylated primers per tumour 38% (19) demonstrated methylation at 1 site, 10% (5) at 2 sites, 4% (2) at 3 sites and 8% (4) at 4 sites. This is illustrated graphically in figure 4.1. Examples of MSP gels demonstrating successful bisulfite modification and methylation at the MINT31 locus are shown in figures 4.2 and 4.3 respectively.

Figure 4.1: **Number of methylated loci per cancer sample**

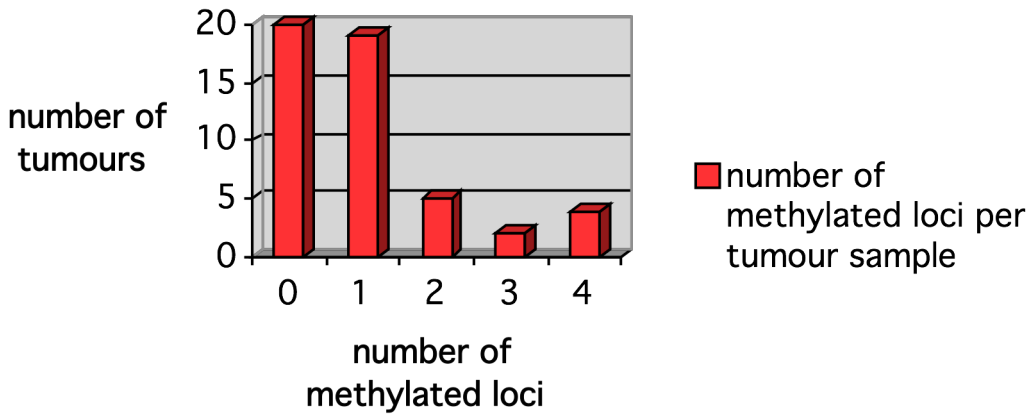
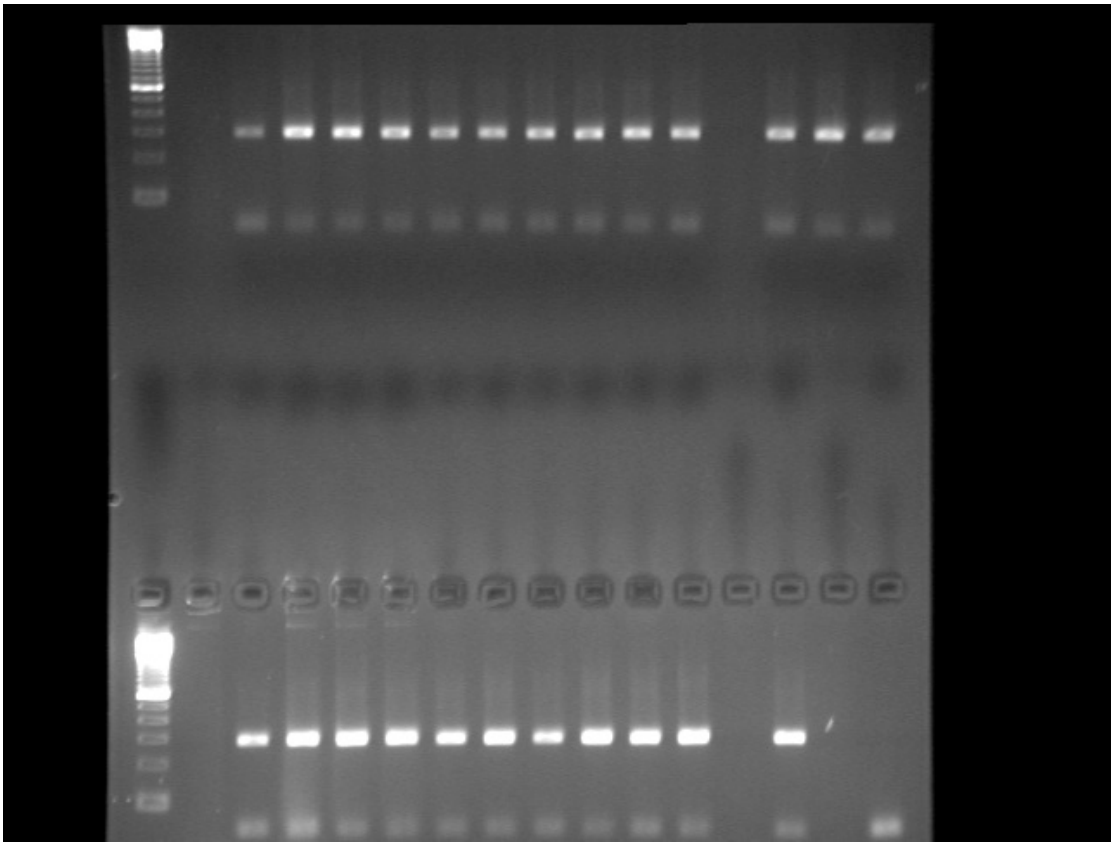


Figure 4.2: <u>GAPDH primer to analyse DNA modification; 2% agarose gel</u>

Ladder 1 2 3 4 5 6 7 8 9 10 A B C



123 bp ladder

Lanes 1 – 10: tumour (top) and normal adjacent lung samples (bottom)

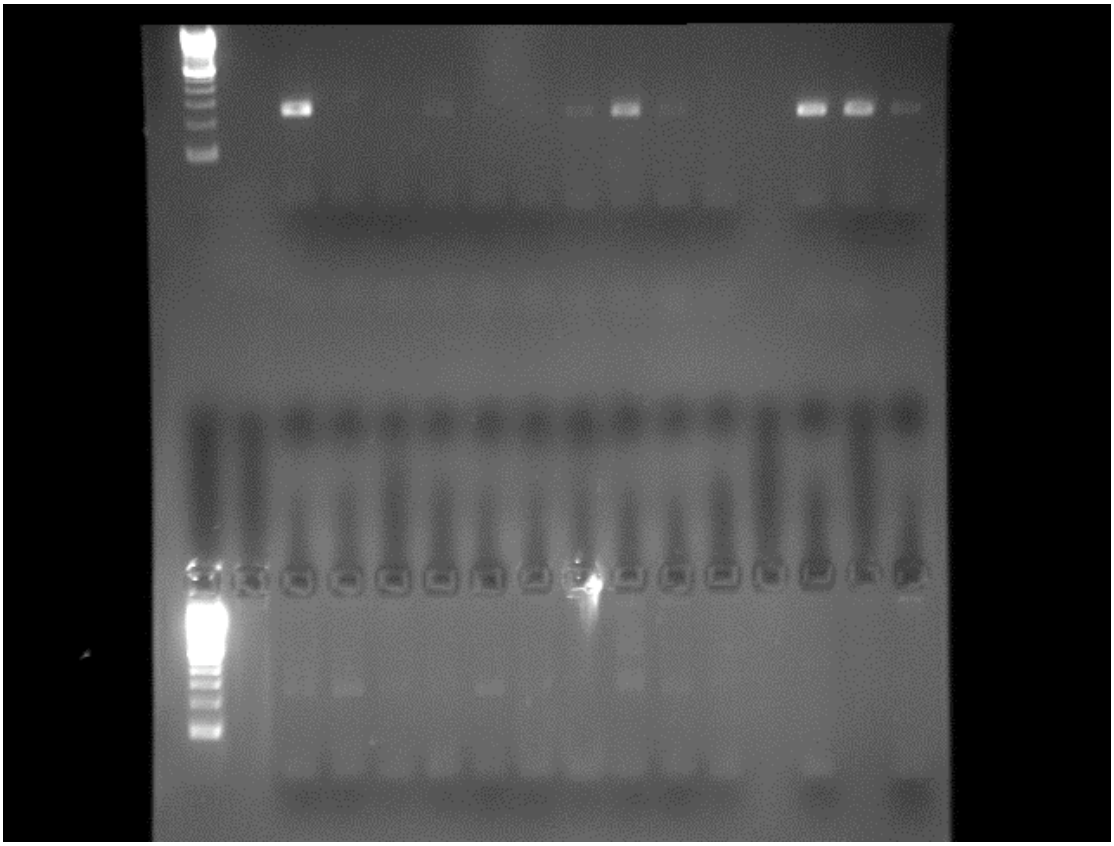
Lane A: ivm DNA (top) and lymphocyte DNA (bottom)

Lane B: ivm DNA, 1 in 5 dilution (top) and no sample (bottom)

Lane C: ivm DNA, 1 in 25 dilution (top) and distilled H_20 (bottom)

Figure 4.3: <u>MSP MINT31 primer; Samples 31 - 40; 2% agarose gel</u>

Ladder 1 2 3 4 5 6 7 8 9 10 A B C



123 bp ladder

Lanes 1 – 10:	tumour (top) and normal adjacent lung samples (bottom)
	Positive results are seen in lanes 1 and 8,
	(tumour samples 31 and 38)
Lane A:	ivm DNA (top) and lymphocyte DNA (bottom)
Lane B:	ivm DNA, 1 in 5 dilution (top) and no sample (bottom)
Lane C:	ivm DNA, 1 in 25 dilution (top) and distilled H_20 (bottom)

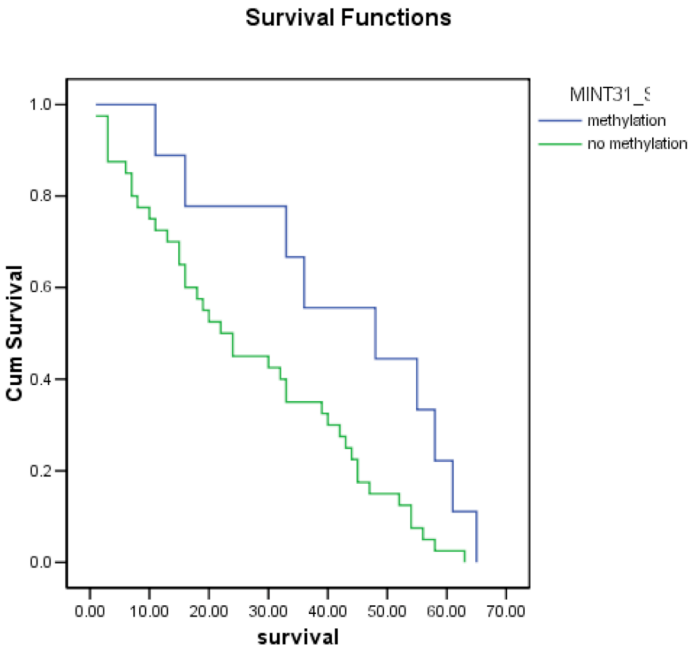
4.3.1 Correlation between methylation at individual sites and overall survival

Univariate analysis of any association between methylation at individual sites and overall survival was performed using Cox regression analysis. Univariate analysis demonstrated a significant association between the methylation of the MINT 31 gene promoter region and improved overall survival (p=0.03) but no other statistically significant associations with the primers studied was seen, table 4.4. This is demonstrated in the Kaplan-Meier survival curve shown in figure 4.4.

Table 4.4: Correlation between methylation at individual sites and overall survival

		Number (%) of samples	
Primer	**p-value**	**Methylated**	**Non-methylated**
hMLH1	0.196	1	48
p16	0.323	14	35
DAPK	0.924	6	43
TIMP3	NA	0	49
HIC1	0.475	5	44
MINT25	0.171	2	47
MINT31	0.030	9	40
RASSF1A	0.06	13	36

Figure 4.4: **Kaplan-Meier survival curve (months); methylated versus non-methylated MINT 31: all patients**



4.3.2 Correlation of methylation at multiple sites and overall survival

Univariate analysis of any association between overall survival and methylation at multiple sites was examined using Cox regression analysis. Samples demonstrating the presence of 2 or more methylated sites were considered to be 'multiple' compared to those with methylation at only 1 site. There was no significant correlation demonstrated with overall survival dependant on the number of sites demonstrating methylation of the primers examined ($p=0.252$, $n=29$).

4.3.3 Correlation of methylation at both individual and multiple sites and clinicopathological variables

Assessment of any potential correlations between the methylation at individual sites and the clinicopathological variables of stage and histology was performed using Kruskal-Wallis statistics. A statistically significant relationship between methylation of the HIC 1 primer with stage was identified (p=0.02, table 4.5). All the samples exhibiting methylation of this primer were obtained from patients with stage IIb disease.

Analysis of whether there was a correlation between the presence of methylation at multiple sites and stage or histology was performed using Cox regression analysis. Multiple methylated sites were defined as the presence of methylation at 2 or more sites. There was no statistically significant correlation between the number of methylated sites with either stage or histology (data not shown).

Table 4.5: Correlation between methylation at individual sites with stage and histology

	Clinicopathological Variable (p value)	
Primer	**Stage**	**Histology**
hMLH1	0.426	0.893
p16	0.446	0.544
DAPK	0.893	0.772
TIMP 3	1.000	1.000
HIC 1	0.020	0.382
MINT 25	0.750	0.699
MINT 31	0.503	0.672
RASSF1A	0.527	0.477

4.3.4 Correlation between methylation status and survival between those patients undergoing chemotherapy and surgery with those having surgery alone

To perform this analysis Cox regression analysis was used and the cohorts of patients receiving chemotherapy preoperatively were examined independently from those that underwent surgery alone. The number of patients receiving neoadjuvant chemotherapy was small and failed to show any statistically significant survival data in relation to methylated primers, (table 4.6).

Table 4.6: Correlation between methylation at individual sites and overall survival; neoadjuvant chemotherapy patient samples

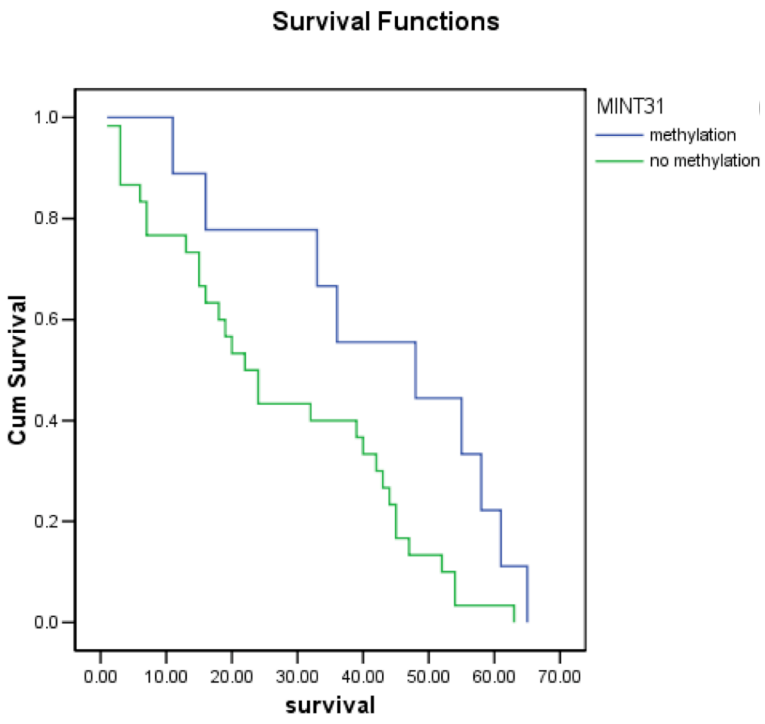
		Number (%) of samples	
Primer	**p-value**	**Methylated**	**Non-methylated**
hMLH1	NA	0	10
p16	0.437	1	9
DAPK	NA	0	10
TIMP3	NA	0	10
HIC1	0.994	2	8
MINT25	NA	0	10
MINT31	NA	0	10
RASSF1A	0.963	2	8

Performing the same analysis on those patients receiving no preoperative chemotherapy again demonstrated a statistically significant relationship between the presence of methylation at the MINT 31 primer and overall survival, table 4.7 and figure 4.5.

Table 4.7: Correlation between methylation at individual sites and overall survival; no chemotherapy patient samples

		Number (%) of samples	
Primer	**p-value**	**Methylated**	**Non-methylated**
hMLH1	0.207	1	38
p16	0.761	13	26
DAPK	0.920	6	33
TIMP3	NA	0	39
HIC1	0.591	3	36
MINT25	0.187	2	37
MINT31	0.035	9	30
RASSF1A	0.071	11	28

Figure 4.5: Kaplan-Meier survival curve (months); methylated versus non-methylated MINT 31: no chemotherapy



4.4 Discussion

Methylation-specific PCR (MSP) is a now well-established technique for evaluating the presence of methylation in tumours as well as other clinical samples. It is a 2-step procedure that relies firstly on successful (bisulphite) modification of the study DNA before the PCR amplification stage.

It has now become apparent that the technique can on occasion be associated with false positive results and a review of these issues and how they may relate to this study in particular follows because false positive results could lead to an overestimate of the DNA methylation frequency.

Two reviews highlight the potential of false positives when using MSP and relate to the bisulphite modification of the DNA as well as the annealing temperature and the number of cycles within the PCR amplification step (Derks et al., 2004, Rand et al., 2002). Modification must be complete as the design of the primers often ends with a cytosine of a CpG site at the 3' site and this in itself can lead to amplification of unconverted sequences. In this study modification was checked by a PCR reaction using a primer specific for unmethylated DNA, such as glyceraldehide-3-phosphate dehydrogenase (GAPDH). With this primer, all modified samples give a positive band (2% agarose gel electrophoresis) but unmodified samples are negative.

Both the annealing temperature and the number of cycles within the PCR reaction are important steps as it has been shown that a low annealing temperature and the use of an excessive number of PCR cycles can lead to mismatching within the reaction that then manifests as a false positive result and within this study no more than 35 cycles per

primer reaction was used and the annealing temperature for each primer is detailed in table 2.5, page 103.

Rand et al (Rand et al., 2002) has also suggested further controls are required to confirm MSP results and one such technique is COBRA (Combined Bisulphite Restriction Analysis) whereby restriction digests determine DNA methylation, a technique first described by Xiong et al. (Xiong and Laird, 1997). Whilst the use of COBRA was not available during this research study subsequent work by others on the study samples with a HIC1 primer have confirmed the MSP results for that primer, (personal communication).

In this study we have demonstrated methylation of at least 1 gene promoter region in 60% (n=30) of the cancer samples. This is less than in the published literature where the percentage appears to be higher (approximately 80%) (Kim et al., 2001a, Zochbauer-Muller et al., 2001). Both of these studies used MSP and analysed the methylation status of 8 promoter loci. In both of these studies there was a higher proportion of adenocarcinoma than in this study. In the paper by Kim et al there is evidence of a higher percentage of methylation of RASSF1A in the adenocarcinoma samples compared to the squamous (47% versus 36%), and their findings would thus be consistent with the findings in this study.

Individual genes were methylated in varying amounts in this study with p16 the highest, which demonstrated methylation in 28% of the samples through to TIMP3, which showed no evidence of methylation in any of the studied tumour samples. A review of lung cancer methylation studies by Tsou et al published after the completion of our

MSP study shows our data to be similar to that in the published literature when comparison is made only with studies involving clinical tumour samples assessed by MSP for the gene promoters *hMLH1* (2% observed versus 0% published), p16 (28% versus 17-43%), DAPK (12% versus 16-44%) and RASSF1A (26% versus 30%) (Tsou et al., 2002). The only locus studied that differs significantly from the published literature is TIMP3 where we found no evidence of methylation in the cancer samples studied. This compares with results in the published literature where the rates of methylation varied between 19 – 26%. In relation to the other loci studied there are no published studies of MSP for HIC1 or MINT sequences using clinical tumour samples.

When comparing the lung cancer tissue with normal adjacent lung resected at the time of surgery there was very little evidence of methylation within the normal lung other than in the case of p16 where 10% (n=5) of the normal lung demonstrated methylation of the p16 promoter. Interestingly 2 of these patients did not demonstrate methylation of the p16 promoter in the corresponding cancer sample. A possible explanation for this would be experimental contamination. However, if this were the case, then one would expect similar results across all of the studied loci and this is not the case. However p16 is one of the most commonly studied genes in lung cancer methylation changes and it may be the case that the methylation represents field change perhaps secondary to the effects of cigarette smoking. In that regard it is worthy of note that methylation of p16 has been studied in the sputum of high risk smoking individuals and also in patients with lung cancer and 2 separate studies have demonstrated the presence of p16 methylation in the sputum of individuals with no evidence of active cancer at the time (Kersting et al., 2000, Palmisano et al., 2000). In one of these studies p16 methylation

was found in the sputum of patients 34 and 35 months prior to the diagnosis of lung cancer being made.

The primary objective of this study was to investigate any correlation between methylation of the panel of genes with overall survival. In 7 of the 8 studied genes there was no statistically significant correlation. This is not in agreement with a study of DAPK which reported poor overall survival in those patients with stage I disease who had methylation of the DAPK promoter (p=0.007) (Tang et al., 2000). However, there was no such correlation between DAPK promoter methylation and survival in another published study despite them demonstrating an association between DAPK methylation and larger tumour size (p=0.009) as well as more advanced stage (p=0.003) (Kim et al., 2001a). In the study by Tang et al the reported rate of DAPK methylation was 44% and in the Kim paper the rate of DAPK methylation was 24%. This is more in keeping with the results of the present study.

The significance of the MINT sequences has not previously been reported in lung cancer. It has been shown in this study that methylation of the MINT 31 promoter is associated significantly with overall improved survival (p=0.03). This was the case on univariate analysis for all patients and this remained the case when the patients who received preoperative chemotherapy were removed from the analysis (p=0.035). The methylation status of the MINT sequences has not previously been studied in lung cancer and their function remains unknown.

MINT 31 is situated on chromosome 17q21 where recently a t-type calcium channel gene was identified (CACNA1G). It has been proposed that this t-type channel could

be involved in cancer development by modulating calcium signalling, potentially affecting cell proliferation and apoptosis (Toyota et al., 1999b). However a paper by Ogi et al did not show a significant correlation between the methylation of these 2 gene promoter regions and it will require further study to elucidate the role of the genes controlled by the MINT 31 and the other MINT sequences (Ogi et al., 2002).

Other studies have reported that methylation of the MINT31 locus is associated with better prognosis in other solid tumour types, for example gastric cancer ($p=0.04$) (An et al., 2005). Others have reported it to be associated with a poorer prognosis, for example oral squamous cell carcinoma ($p=0.041$) (Ogi et al., 2002).

With the small numbers involved in the current study it is difficult to comment further on the potential prognostic role of methylation of these promoters in NSCLC. Similarly it is difficult to determine the effect that the methylation of any one single gene has on clinical behaviour. The current findings require further study in a larger cohort of patients.

Within this study cohort of patients the methylation of the HIC1 gene was the only individual gene to show a correlation with stage of disease. In this study methylation of HIC 1 was present only in those patients with stage IIb squamous cell lung cancer and more specifically those tumours that were more than 3cm in size and had cancer positive hilar lymphadenopathy. However there was no evidence that survival was affected by the methylation of this gene making it unlikely that the methylation of this gene could relate to the clinical aggressiveness of the tumour. There is little published evidence pertaining to the methylation status of HIC 1 in NSCLC. Eguchi et al

published a paper in 2001 (Eguchi et al., 1997), which reported the presence of methylation at multiple sites in 22% of patients with lung cancer. Similar to our results this was evident more in patients with squamous cell cancer than adenocarcinoma and in those samples obtained from patients with tumours greater than 3cm and with evidence of lymph node involvement. However, these results did not reach statistical significance (p value not given). Further large-scale clinical trials are required to evaluate further the potential clinical relevance of this finding. Interestingly it has been recently suggested that methylation of HIC 1 in germ cell cancer cell lines may indicate cisplatin resistance and this would have potential significance as regards the chemotherapy treatment for patients with NSCLC. It is worth noting that treatment of these cell lines with the demethylating agent 2-deoxy-5-azacytidine did not improve chemotherapy sensitivity in all of the resistant lines (Koul et al., 2004).

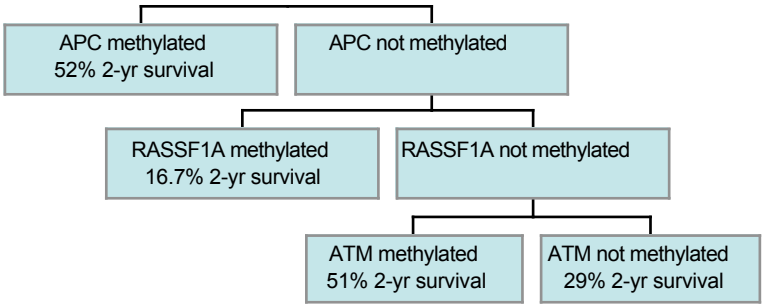
In this study the presence of multiple methylated sites did not correlate with either overall survival, stage of disease or histological subtype. This would suggest that assessing the methylation status of multiple gene promoter sites in patients with NSCLC does not offer any advantage over the prognostic factors that are currently in use today (performance status and stage of disease). However, this study suffers from several limitations, not least of which is sample size with a total population of only 50 patients. Of these only 11 patients demonstrated methylation at multiple sites. This makes it impossible to draw any definite conclusions.

There are a number of published studies, which have investigated the presence of multiple methylated loci in patients with lung cancer. Zochbauer-Muller in 2001 reported the methylation profile across a panel of 8 genes and concluded those tumours,

which exhibited multiple sites of methylation, might represent a subgroup of lung cancers with a unique pathogenesis. However, they failed to show any correlation between the methylation profile and clinicopathological variables (Zochbauer-Muller et al., 2001).

Toyooka et al reported that there were differences in methylation profiles between NSCLC and SCLC tumour samples but within these groups there was no association reported between the methylation profile and survival (Toyooka et al., 2001). Two more recent papers have also studied methylation at multiple sites and both used a panel consisting of 8 genes. In the first of these by Safar et al it has been reported that by using the technique of recursive partitioning, precise patterns of gene methylation with prognostic significance can be identified. Moreover these remain significant when other recognised prognostic factors are included in a multivariate analysis. The genes found to have prognostic significance within this study using recursive partitioning were APC, ATM and RASSF1A, (figure 4.6). However, MLH1, DAPK, p16, MGMT nor ECAD did not have any bearing on this model. In this study it was observed that, if the methylation status of RASSF1A was considered in isolation, Cox regression analysis demonstrated no association with overall survival.

Figure 4.6: Illustration of recursive partitioning in NSCLC (data, Safar et al)



One further article in press at present again examined the methylation status of a panel of 8 genes and again failed to show any statistically significant correlation between the number of genes methylated and either tumour subtype (adenocarcinoma versus squamous cell) or tumour stage (Kim et al., 2007).

In summary the presence of and clinical relevance of the methylation status in NSCLC is potentially great with many studies including our own highlighting this. However the studies to date are relatively small with the majority looking at samples from less than 100 patients. The next step must therefore be for a consensus to be reached as a result of these studies on which genes are important and how many of these should be included in future studies.

5. A study into the expression of the mismatch repair proteins in a cohort of surgically resected patients with NSCLC

5.1 Introduction

Microsatellite Instability has a reported frequency in NSCLC of between 0 – 67% (Lawes et al., 2003) and Loss of Heterozygosity (LOH) at chromosome 3p has been shown to be an independent adverse prognostic marker for survival in primary adenocarcinoma of lung ($p = 0.052$) (Mitsudomi et al., 1996). These studies did not compare LOH at chromosome 3p with MLH1 protein expression (chromosome 3p locus). A more recent paper has demonstrated a correlation between MLH1 expression and allelic imbalance at chromosome 3p (Xinarianos et al., 2000). It is now recognised that circulating DNA in the serum of lung cancer patients originating from tumour cells can be isolated and often demonstrates the same allelic imbalances as those of the primary tumour (Sozzi et al., 2001).

To further investigate the above a study was designed to prospectively collect surgically resected tumour and normal adjacent lung samples from patients with lung cancer undergoing surgical resection of their tumours. In addition a preoperative (and where available a postoperative) whole blood sample was also obtained.

The objectives of this study were:

- To examine for the presence of allelic imbalance in fresh frozen lung cancer samples and compare the findings with those in normal adjacent lung and lymphocyte DNA obtained from circulating blood.
- To establish if any allelic imbalance identified in fresh tumour were present in the pre-operative serum sample taken from the same patient.
- To assess where possible if any allelic imbalance identified in the serum DNA of patients pre-operatively persisted in a post-operative serum sample.
- To evaluate if allelic imbalance at chromosome 3p or methylation of the *hMLH1* promoter (if identified) correlated with loss of expression of MLH1 as measured by immunohistochemistry.

5.2 Methods

Ethical approval for this study was obtained through the local Ethics committee in North Glasgow. Samples were collected from 10 patients undergoing resection of NSCLC at the Western Infirmary in Glasgow. This involved obtaining consent from the patient the night prior to operation and then personal attendance during the patient's thoracotomy. The author then took the surgical sample immediately to the Pathology department where a consultant pathologist localised and resected two tumour samples (in such a way as to not affect the pathological staging of the tumour) for the study. In addition 2 macroscopically normal adjacent lung samples were also collected. A single tumour and normal adjacent lung sample were then immediately frozen to -70°C and stored for DNA extraction at a later date. The second tumour and normal samples were paraffin embedded and slides cut from these by Dr Colin Nixon (University of Glasgow Veterinary School, Department of Pathology). Formal pathology reports were collected

at a later date. For each patient there was a whole blood sample collected pre-operatively and the serum immediately separated from this by centrifuge at 5000 × g for 10 minutes. The samples were then stored at 4°C prior to DNA extraction. Where possible a second blood sample was collected and processed (as described for the pre-operative sample) by myself from the patient approximately 3 months post surgery. The patient demographics are shown in table 5.1.

Table 5.1: Patient demographics

Patient characteristics	Surgery (n = 10)
Sex	
Male	4
Female	6
Age	
Range	49 – 75
Mean	59.4
Histology	
Squamous	6
Adenocarcinoma	1
Large cell	1
Bronchoalveolar	1
NSCLC unspecified	1
Stage	
IA	4
IB	4
IIA	2

Immunohistochemical studies were carried out as per the methodology described in chapter 2.1. DNA extraction from whole blood and serum was performed using the protocols detailed in chapter 2.2 and from tissue in chapter 2.3. Allelic imbalance studies were performed following the protocol described in chapter 2.4. The loci examined in this prospective observational study were, mfd15CA, APC, p53, D2S123, D3S1289, D3S1300 and D3S1304. The MSP studies were performed as per the methodology described in chapter 2.5. The promoter regions analysed were MLH1, p16,

DAPK, TIMP 3, HIC 1, MINT 25 and MINT 31. Due to the small number of samples available statistical analyses have not been performed but observations made.

5.3 Results

5.3.1 Allelic Imbalance in NSCLC

Table 5.2 demonstrates the proportion of homozygous (non-informative) alleles for each locus studied in the tumour samples. Of the remaining heterozygous (informative) tumour samples table 5.3 demonstrates the number of tumours exhibiting Loss of Heterozygosity (LOH) for each studied locus with results ranging from 25% (1/4, p53) to 83% (5/6, D3S1300). Figures 5.1 – 5.7 illustrate the distribution of allelic imbalance ratio results for the lung cancer tumour samples.

Table 5.3 illustrates those serum samples that demonstrated LOH when compared with the corresponding tumour, with Figure 5.8 showing an example of LOH. A range of results was again demonstrated from 0% (both p53 and D2S123) to 67% (2/3, APC).

Table 5.2: Proportion of homozygous, heterozygous and non-informative alleles for each locus

Primer	Number of homozygous alleles	% of homozygous alleles	Number of heterozygous alleles	% of heterozygous alleles	Unsuccessful PCR
Mfd15CA	2	20	8	80	-
APC	2	25	6	75	2
D2S123	1	17	5	83	4
p53	6	60	4	40	-
D3S1289	0	0	8	100	2
D3S1300	3	33	6	67	1
D3S1304	0	0	9	100	1

Figure 5.1: **Frequency of allelic imbalance ratio in tumour DNA: mfd15CA**

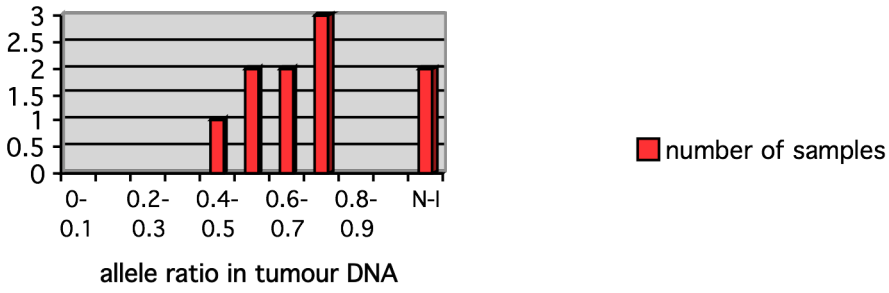


Figure 5.2 **Frequency of allelic imbalance ratio in tumour DNA: APC**

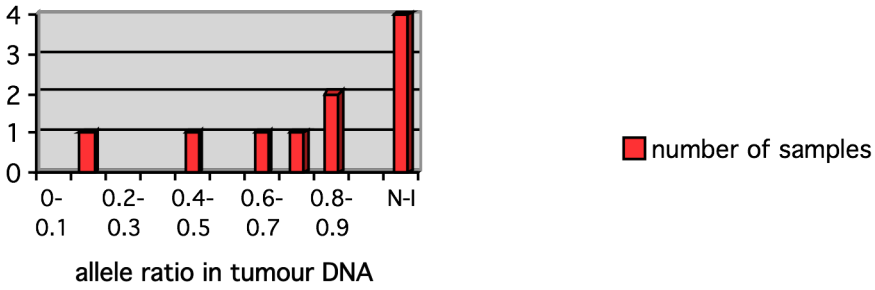


Figure 5.3 **Frequency of allelic imbalance ratio in tumour DNA: D2S123**

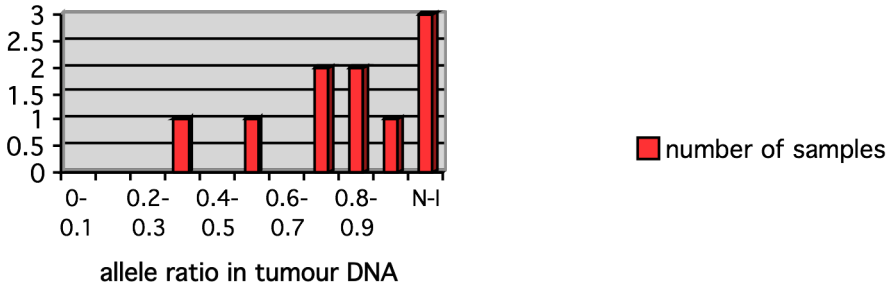


Figure 5.4: **Frequency of allelic imbalance ratio in tumour DNA: p53**

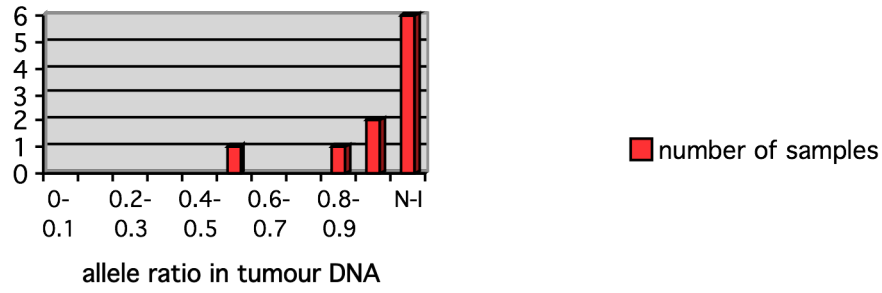


Figure 5.5: **Frequency of allelic imbalance ratio in tumour DNA: D3S1289**

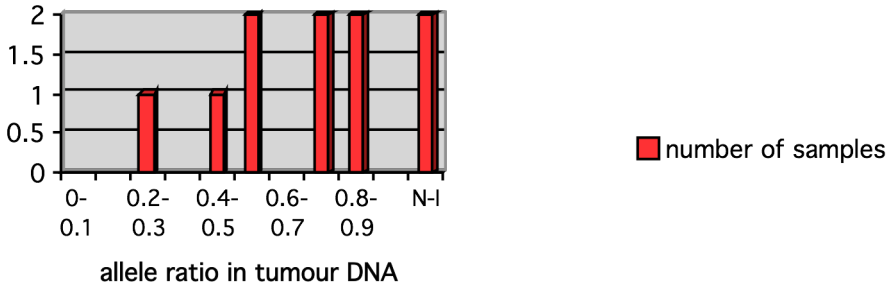


Figure 5.6: **Frequency of allelic imbalance ratio in tumour DNA: D3S1300**

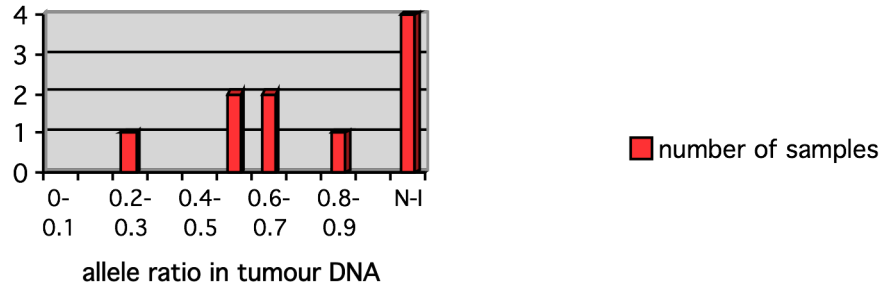


Figure 5.7: Frequency of allelic imbalance ratio in tumour DNA: D3S1304

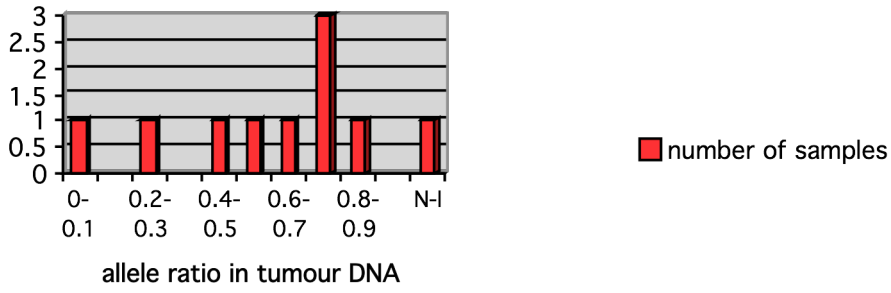
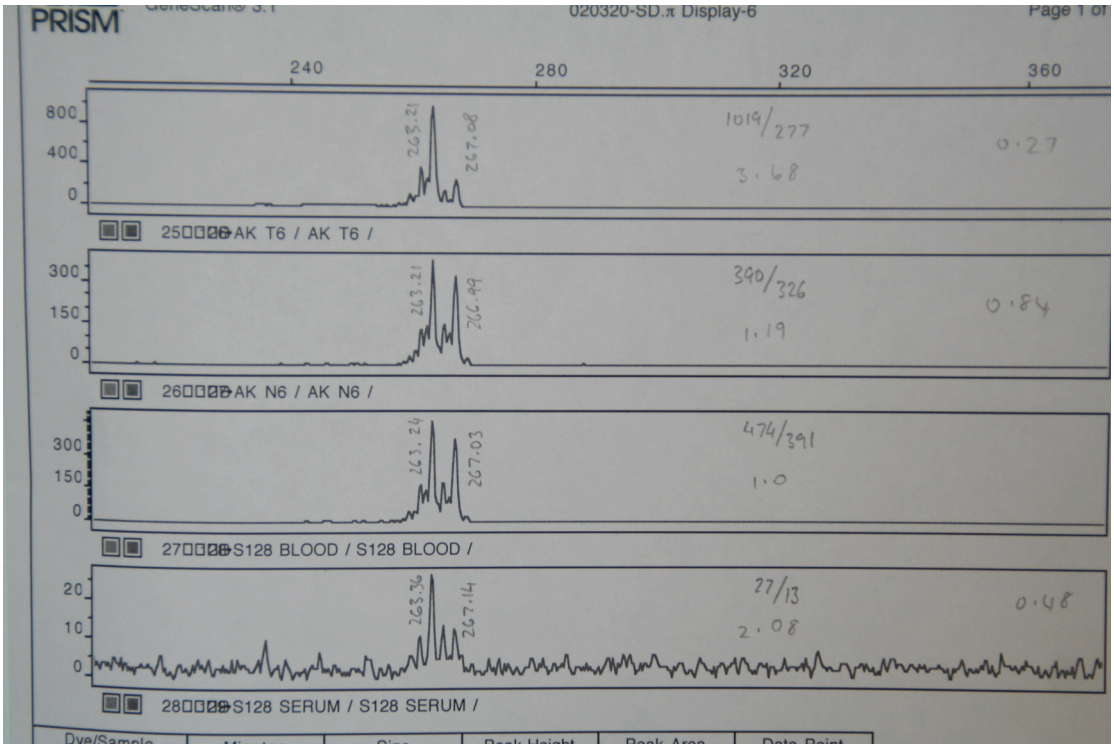


Table 5.3: Summary of the frequency of allelic imbalance in tumour and serum DNA at each locus

Primer	Number of tumour samples with allelic imbalance	% of tumours with allelic imbalance	Number of corresponding serum samples with allelic imbalance	% of corresponding serum samples with allelic imbalance
Mfd15CA	5/8	62	3/5	60
APC	3/6	50	2/3	67
D2S123	2/5	40	0/2	0
p53	1/4	25	0/1	0
D3S1289	4/8	50	2/4	50
D3S1300	5/6	83	3/5	60
D3S1304	5/9	56	2/5	40

Post-operative serum samples were available for only 3 of the 10 patients. One patient's postoperative serum sample continued to demonstrate LOH in 2 of the 4 markers demonstrating LOH in their pre-operative serum sample. The other 2 patients post-operative serum samples demonstrated novel LOH at 1 and 2 markers respectively compared to the pre-operative serum samples whilst the pre-operative LOH was no longer evident.

Figure 5.8: **LOH at D3S104 loci: Virtual fluorescent polyacrylamide gel analysis**



Horizontal axis: *Allele size in base pairs as measured against a size standard*

Vertical axis: *Allele peak heights as measure against a size standard*

Fig 5.8 demonstrates LOH of the DNA at loci D3S1304 in both the tumour and serum DNA taken from the same patient. No LOH is demonstrated in the normal adjacent lung tissue sample

5.3.2 Methylation profile in early stage (I/IIa) NSCLC and normal adjacent lung samples

Of these 10 samples studied MSP was successfully performed on all. Results for the MSP carried out on the tumour samples are shown in table 5.4. The results of MSP in adjacent normal lung are shown in table 5.5. In these tables the presence of methylation is indicated by an X and no evidence of methylation with a 0.

Table 5.4: Methylation status of lung cancer samples at seven loci

	Primer						
Sample	**hMLH1**	**p16**	**DAPK**	**TIMP 3**	**HIC 1**	**MINT 25**	**MINT 31**
1	0	X	0	0	0	0	0
2	0	0	0	0	0	0	0
3	0	0	X	0	0	0	0
4	0	0	0	0	0	0	0
5	0	0	X	0	0	0	0
6	0	0	0	0	0	0	X
7	0	0	0	0	0	0	0
8	0	0	0	0	0	0	X
9	0	0	0	0	0	0	0
10	0	X	0	0	0	0	0

In total 60% (6/10) of the tumours demonstrated methylation of one primer site whilst 40% (4/10) of the tumours demonstrated no methylation at any primer site.

Table 5.5: Methylation status of normal adjacent lung samples at seven loci

	Primer						
Sample	**hMLH1**	**p16**	**DAPK**	**TIMP 3**	**HIC 1**	**MINT 25**	**MINT 31**
1	0	0	0	0	0	0	0
2	0	0	0	0	X	0	0
3	X	0	0	0	0	0	0
4	0	0	0	0	0	0	0
5	0	0	0	0	0	0	0
6	0	0	0	0	0	0	0
7	0	0	0	0	0	0	X
8	0	0	0	0	0	0	0
9	0	0	0	0	0	0	0
10	0	0	0	0	0	0	0

The majority of normal lung samples (7/10) showed no methylation at any of the studied primer sites whilst three demonstrated methylation at one primer site, these were *hMLH1*, HIC 1 and MINT 31. Interestingly adjacent normal lung samples 2 and 7 did not demonstrate methylation of any primer site in the corresponding primary tumour sample. In adjacent normal lung sample 3, which demonstrated methylation of the *hMLH1* promoter site, the corresponding tumour did not demonstrate methylation of this site. However tumour sample 3 did show methylation of the DAPK locus.

5.3.3 Protein expression in early stage (I/IIa) NSCLC and normal adjacent lung samples

Of the 10 patient samples immunohistochemistry for MLH1, MSH2 and p53 was successfully performed on all of the tumour and normal adjacent lung samples. The results are summarised in table 5.6.

Table 5.6 **Protein expression levels in early stage (I/IIa) lung cancer and normal adjacent lung samples**

	MLH1			MSH2			p53		
Sample	**% T/N**	**I T/N**	**IHC T/N**	**% T/N**	**I T/N**	**IHC T/N**	**% T/N**	**I T/N**	**IHC T/N**
1	0/3	0/2	0/6	0/3	0/2	0/6	0/3	0/2	0/6
2	0/1	0/1	0/0	1/2	1/1	1/2	0/2	0/2	0/4
3	2/2	1/1	2/2	1/1	1/1	1/1	0/0	0/0	0/0
4	2/2	1/1	2/2	2/0	1/0	2/0	0/0	0/0	0/0
5	2/2	1/1	2/2	0/1	0/1	0/1	0/0	0/0	0/0
6	3/2	2/1	6/2	3/0	2/0	6/0	3/1	2/1	6/1
7	2/2	1/1	2/2	1/1	1/1	1/1	0/1	0/1	0/1
8	2/1	1/1	2/1	2/0	1/0	2/0	3/0	2/0	6/0
9	1/1	1/1	1/1	2/0	2/0	4/0	3/0	3/0	9/0
10	2/1	1/1	2/1	1/0	1/0	1/0	2/0	2/0	4/0

% = percentage score of cells staining; I = Intensity score for cell stain

T = tumour sample; N = normal adjacent lung sample

Nine of the 10 samples studied demonstrated LOH at least one chromosome 3p locus (D3S1289, D3S1300, D3S1304). No correlation between IHC score and LOH at chromosome 3p was identified. Likewise no tumour samples exhibited methylation of the *hMLH1* gene promoter and therefore no correlation could be recognised between methylation of this locus and MLH1 protein expression.

5.4 Discussion

As previously discussed this research project was carried out at a time when there were national concerns pertaining to the ownership of tissue samples as well as retained organs in pathology departments and this severely limited our ability to obtain fresh tissue prospectively. As a direct consequence the number of samples collected and studied in this chapter is significantly less than had been originally calculated during the planning of the project. Despite this it has been clearly demonstrated that the prospective collection of tissue and blood is possible by integrated multidisciplinary cooperation with the cardiothoracic team and the Pathology Departments at the Western Infirmary in Glasgow as well as the Pathology Department at the University of Glasgow Veterinary School. By the end of the research period a further 10 samples had been collected and stored for future analysis. Due to the small numbers statistical analyses were not performed but useful observations were made.

The ratio of heterozygous alleles in normal tissue DNA (normal adjacent lung, lymphocytes) should be equal to 1.0, although slight variation is common. In respect to the allelic imbalance studies the allelic ratio of the DNA from the heterozygous (informative) samples from all sources was compared with the allelic ratio in lymphocyte DNA. The equation used for the calculation of LOH is given in chapter 2.4.2.3. Lymphocyte DNA was used as the normal control as it is understood that macroscopically and indeed microscopically normal lung may be affected by either contamination with cancer cells or genetic field change due to exposure to the inhaled smoke carcinogens (Hittelman et al., 1996). A ratio of 0.7 was used to define LOH in this study. One of the difficulties in comparing studies of LOH is that the definition of LOH as well as the study technique varies significantly in the literature between tumour

types as well as between studies looking at the same tumour types. Examples of this in lung cancer include the study by Liloglou et al where a ratio of 0.77 was used in comparison to the study by Wong et al where LOH was defined as 'a relative reduction in one allele in the tumour sample' (Liloglou et al., 2000, Wong et al., 2002). A more recent study by Woenckhaus et al using tumour DNA from pleural fluid has demonstrated that using a ratio of 0.7 and comparing this with a ratio of 0.5 in the same samples leads to an increase in the sensitivity of the test but a decrease in specificity (Woenckhaus et al., 2005). This decrease in specificity would be of concern if taking such a molecular assay into the clinical setting. These differences will only be overcome if there is an internationally agreed definition for LOH. Figures 5.1 to 5.7 illustrate the spectrum of results that could be obtained depending on the definitions used to define LOH.

Although it was not possible to make statistical comparisons and draw firm conclusions in our study due to the small numbers it has been clearly demonstrated that allelic imbalance is a common occurrence in patients with NSCLC, however microsatellite instability was not evident in the studied samples.

Although similar allelic imbalance was demonstrable in the serum DNA when compared with the corresponding tumour sample this was not a consistent finding. Again the small numbers involved in the study limit any firm conclusions been drawn, but a study by Sozzi et al has demonstrated a similar incidence of allelic imbalance at chromosome 3p in primary tumour DNA and the corresponding serum DNA sample (Sozzi et al., 2001).

It is the case however that if serum DNA were to be a reliable substitute tool for analysing molecular changes in the primary tumour then the results would require significantly more consistency between the 2 DNA sources.

In this study no correlation between the changes demonstrable in the pre-operative sample with the post-operative sample were identified. This may simply be a reflection of the small study numbers but the analysis may be compounded by the serum DNA reflecting changes in putatively 'normal' tissue affected by field change. Unless a large clinical trial was to show significant results it might unfortunately be the case that serum DNA is not a reliable tool for the analysis of tumour molecular DNA changes.

Due to difficulties in tissue collection described above and the resultant time constraints of the research project analysis of the gene promoter regions in the serum DNA with correlation with the corresponding changes in the primary tumour was not possible. However 10 further samples (tumour, normal, serum, lymphocyte) have been collected and added to the original 10 samples and it is envisaged that this resource will be made use of and studied by others in the group, in particular the study of DNA methylation changes in serum DNA.

With the number of samples available no statistical correlation with clinicopathological variables was made but in the samples tested there was no observational correlation between allelic imbalance at chromosome 3p, or methylation of the *hMLH1* promoter region with the expression level of MLH1 measured by immunohistochemistry.

Until a large clinical trial has been undertaken to identify if serum DNA is a reliable tool for the analysis of DNA changes demonstrated in the primary tumour, the results of this work suggest that this is not the case.

6. A study into the role of the mismatch repair proteins in the chemotherapy sensitivity of a panel of SCLC cell lines

6.1 Introduction

Unlike NSCLC, Small Cell Lung Cancer (SCLC) is a highly chemo-sensitive cancer with platinum based chemotherapy in patients with limited stage disease given with curative intent. In patients with extensive disease overall response rates of 60 - 70% are seen with complete responses achieved in 20 - 30% of patients (Simon and Wagner, 2003). However, despite these initial high response rates, relapse is common and indicates a uniformly poor prognosis with the median survival for those patients with limited disease only 12 - 18 months and for extensive stage disease only 6 - 9 months, table 6.1.

Table 6.1 Chemotherapy response rates and survival in SCLC

	Limited Disease	**Extensive Disease**
Complete response	50%	20 – 30%
Partial response	40%	30 – 40%
Overall response	90%	60%
Median survival	12 – 18 months	6 months

Published studies have investigated the potential role of mismatch repair mechanisms in SCLC as measured by microsatellite instability (MSI). In these studies differing results were obtained with reported MSI rates of 0 – 76% (Chen et al., 1996, Mao et al., 1994, Merlo et al., 1994, Pylkkanen et al., 1997).

Only the study by Merlo et al examined any correlation that may exist between MSI and clinicopathological factors. Tumour samples were collected from archived paraffin embedded tissue derived from autopsy studies. No differences were identified in the rate of MSI and either stage of disease or between patients who received chemotherapy and those who did not (Merlo et al., 1994).

There has to date been only one study published which investigated the methylation profile of SCLC. Moreover this study did not examine the methylation status of the *hMLH1* promoter (Toyooka et al., 2001).

The objectives of this study were in a panel of small cell lung cancer cell lines were to:

- Examine if there was any correlation between the level of mismatch repair protein expression and level of chemotherapy sensitivity.
- Evaluate the possible role of methylation of *hMLH1* in relation to chemosensitivity

6.2 Methods

Cell lines established by Dr R Milroy in the Centre for Oncology and Applied Pharmacology, University of Glasgow were used in this study and the source of each cell line is detailed in table 6.2. Cell lines LS274 and LS310 were derived from the same patient pre and post chemotherapy respectively. Both the original biopsies were taken from sites of metastasis.

Table 6.2 Source of biopsy and treatment history of the cell line panel

Cell Line	Source	History
LCPH3	Mouse xenograft	Post-therapy relapse
LS106	Bronchial biopsy	Pre-therapy
LS111	Neck node	Pre-therapy
LS112	Skin metastasis	Pre-therapy
LS274	Skin nodule	Pre-therapy
LS277	Skin nodule	Pre-therapy
LS310	Axillary node	Post-therapy

Drug sensitivity studies were performed by Dr J Plumb using methodology previously published (Plumb et al., 1994). Methylation Specific PCR (MSP) was performed as described in detail, chapter 2.5 with DNA extracted from the cultured cell lines as per the protocol described in chapter 2.3

6.3 Results

6.3.1 Correlation between MMR protein expression and chemotherapy sensitivity

Figure 6.1 demonstrates a high correlation between cisplatin sensitivity and the mismatch repair proteins MLH1 ($r^2 = 0.83$) and MSH2 ($r^2 = 0.87$) but not PMS2 ($r^2 = 0.22$). Figure 6.2 demonstrates no such correlation between doxorubicin sensitivity and MLH1, MSH2 expression. Drug sensitivities and protein expression levels are shown in table 6.3. A clear correlation between etoposide sensitivity and MLH1 ($r^2 = 0.66$) and MSH2 ($r^2 = 0.59$) but not PMS2 was also identified (figure not shown).

Table 6.3: MMR and chemosensitivity levels in a panel of SCLC cell lines (courtesy of Dr J Plumb)

Cell line	MLH1 expression	MSH2 expression	PMS2 expression	Cisplatin IC50 (μM)	Doxorubicin IC50 (nM)	Etoposide IC50 (μM)
LS106	0.53	0.77	0.22	7.16	33	5.69
LS111	2.27	1.61	0.19	0.07	284	1.70
LS112	0.88	0.58	0.08	5.73	64	3.61
LS277	1.91	1.32	0.40	0.41	0.1	0.22
LCPH3	1.42	1.13	0.08	0.87	60	0.79
LS274	0.63	0.72	0.20	4.53	144	11.17
LS310	0.30	0.25	0.06	10.6	172	10.71

Levels of MMR protein expression measured by Western blot and quantified by densitometry.

Drug sensitivity does not relate to the proliferating cell population (Ki67) or to the population doubling times of the cell line

Figure 6.1: Assessment of possible correlation of MMR protein expression with cisplatin sensitivity (courtesy of Dr J Plumb)

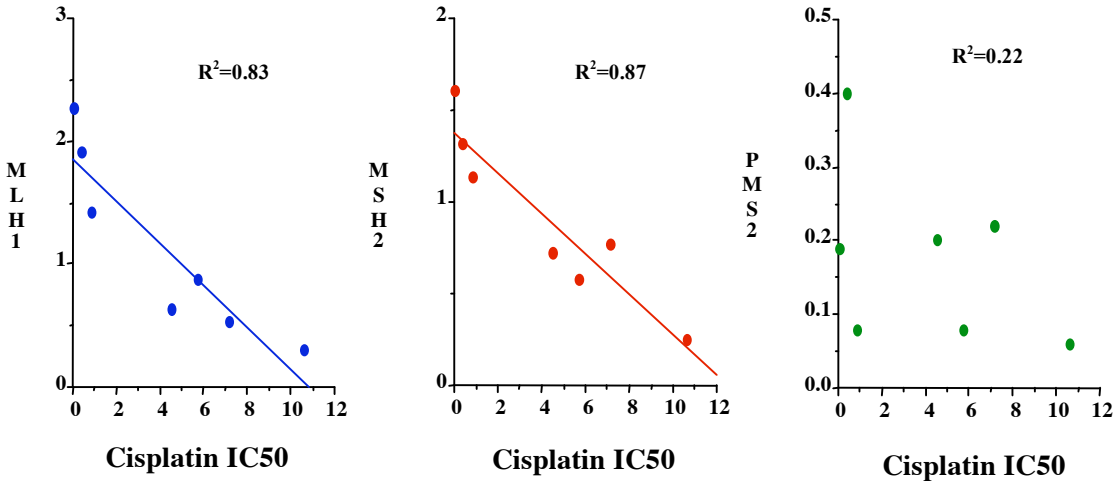
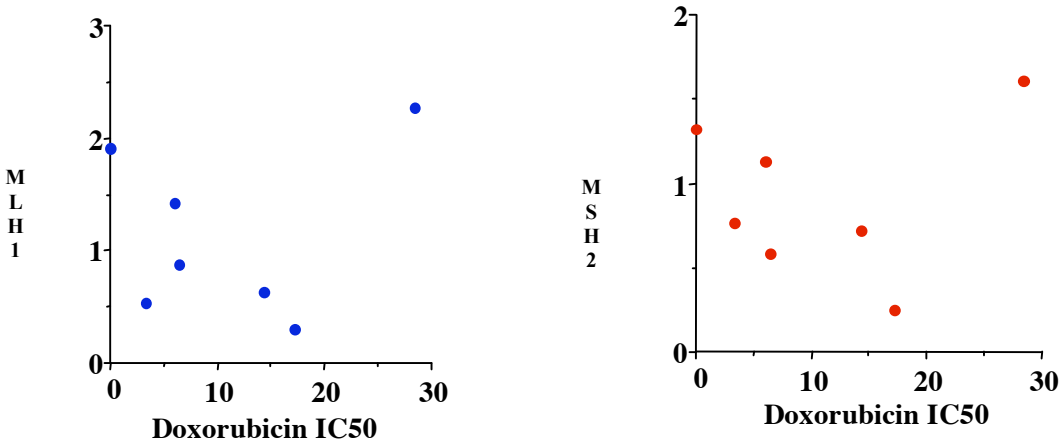


Figure 6.2: **Assessment of possible correlation of MMR protein expression with doxorubicin sensitivity (courtesy of Dr J Plumb)**



6.3.2 Does methylation of the *hMLH1* promoter play a role in cisplatin sensitivity in a panel of SCLC cell lines

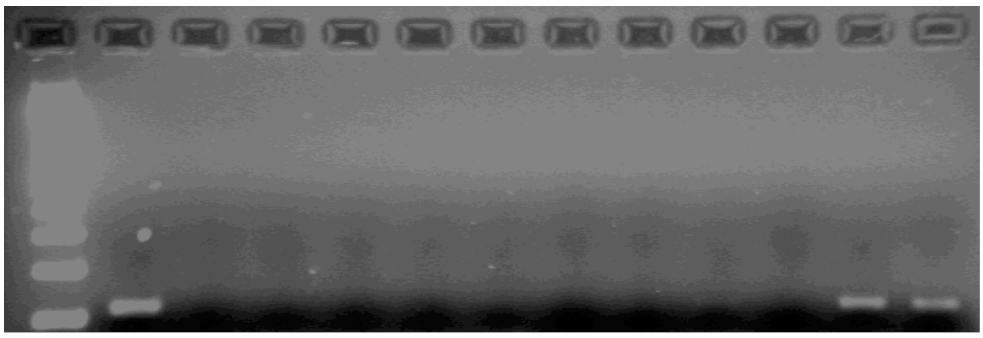
Figure 6.3 demonstrates that there is only methylation of the *hMLH1* promoter in 1 of the cell lines examined, LS310 this is a post-chemotherapy cell line compared with LS274 that is a pre-chemotherapy cell line derived from the same patient . There was no evidence of methylation of the p16, DAPK and MINT 25 loci in any of the cell lines studied.

Table 6.1 demonstrates that LS310 is 2.3 fold more resistant to cisplatin and shows a 50% reduction in MLH1 expression when compared to the LS274 cell line ($p < 0.001$).

Figure 6.4 demonstrates that treatment of the LS310 cell line with the demethylating agent 2-deoxy-5-azacytidine results in a 1.5 fold increase in sensitivity to cisplatin ($p < 0.01$). This was accompanied by a 1.4 increase in MLH1 expression.

Figure 6.3: Methylation of the *hMLH1* promoter in a panel of SCLC cell lines

Ladder 1 2 3 4 5 6 7 8 9 10 11 12



Ladder: 123bp

Lane 1: ivm DNA – positive control

Lane 3: dH_2O – negative control

Lane 5: LCPH3

Lane 6: LS106

Lane 7: LS111

Lane 8: LS112

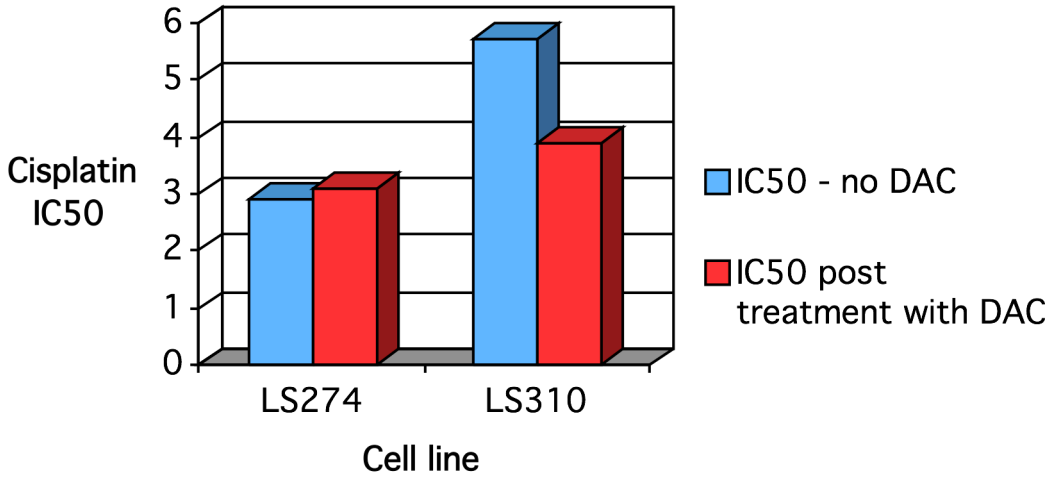
Lane 9: LS274

Lane 10: LS277

Lane 11: LS310

Lane 12: LS310

Figure 6.4: **The effect of 2-deoxy-5-azacytidine on cisplatin sensitivity of the SCLC cell lines, LS274 and LS310 (courtesy of Dr J Plumb)**



6.4 Discussion

In this study we have demonstrated that in paired cell lines derived from the same patient pre and post chemotherapy that there is increased cellular drug resistance in the post-chemotherapy cell line (LS310). We have also demonstrated methylation of the *hMLH1* gene promoter in the cell line established post-chemotherapy (LS310).

Although this finding has been demonstrated by Strathdee et al. in the A2780 ovarian cancer cell line where cisplatin resistance is associated with loss of MLH1 expression it has never previously been reported in small cell lung cancer studies. In Strathdee's study it was shown that the loss of MLH1 expression correlated with hypermethylation of the *hMLH1* gene promoter and that treatment of the resistant cell lines with 5-azacytidine led to both restoration of MLH1 expression and an increase in cisplatin sensitivity (Strathdee et al., 1999). We have also demonstrated significantly increased

sensitivity to cisplatin following treatment of the cell line with the demethylating agent decitabine.

A recent study by Hansen et al examined the role of mismatch repair in a panel of small cell lines (Hansen et al., 2003) and evaluated the possible role methylation of *hMLH1* may play in any MMR deficiency demonstrated. In this study 1 cell line (86MI) was found to be deficient in MMR and this cell line demonstrated resistance to the alkylating agent methylnitronitrosoguanidine (MNNG). Although this cell line was found to be deficient in MMR using a heteroduplex repair assay that measures strand specific repair in M13mp2 DNA (Thomas DC, 1995) this was very much an unexpected result as all the MMR core proteins appeared to be expressed normally, as measured by both Northern and Western blotting analyses (Hansen et al., 2003). There was no evidence of MMR protein methylation as measured by MSP in this study. It was concluded that the phenotype of 86MI could be accounted for by one of two possibilities, either a mutation in one of the four MMR genes (MLH1, MSH2, MSH6 and PMS2) or inactivation of a secondary, known or as yet unknown MMR protein.

A further study by Mackay et al. investigated the expression of the MLH1 protein in a cohort of patients with locally advanced breast cancer receiving chemotherapy. Samples were obtained from the same patient pre and post chemotherapy. This study demonstrated that the level of MLH1 expression pre-chemotherapy did not predict response to chemotherapy or disease-free survival. However primary chemotherapy did result in a significant reduction in the percentage of cells expressing MLH1 ($p=0.010$). This reduction in MLH1 expression after chemotherapy was shown to be strongly associated with poorer disease-free survival ($p=0.0025$) (Mackay et al., 2000). It thus

appears that in patients with locally advanced breast cancer, a decreased cellular MLH1 expression is associated with a survival advantage in patients treated with combination chemotherapy. This further supports the role of the MMR protein *hMLH1* in chemotherapy resistance.

Due to the current process of managing lung cancer prospectively collected small cell lung cancer samples are not readily available for study and much of the published work has been performed using archived historical samples and cell lines (Chen et al., 1996, Mao et al., 1994, Merlo et al., 1994, Pylkkanen et al., 1997). This has particular relevance when investigating the possible impact of the methylation of specific gene promoters in studies of cultured cell lines. There is evidence of high levels of de novo methylation at CpG islands in cell lines at sites that would, in the clinical setting be methylation free, however the reasons for this phenomenon remain unclear (Antequera et al., 1990). Within our study only the cell line established post-chemotherapy, LS310, exhibited methylation at the MLH1 gene promoter and none of the cell lines studied demonstrated methylation of either the DAPK, p16 or MINT 25 loci suggesting that de novo methylation in these cell lines does not occur. A study by Toyooka et al demonstrated no methylation in a panel of small cancer cell lines of p16 and DAPK but did not study the *hMLH1* locus (Toyooka et al., 2001).

Despite the fact that fresh clinical SCLC samples are difficult to obtain the results of our study taken in conjunction with the studies by both Strathdee and Mackay highlight the importance of and need for future SCLC clinical studies to incorporate the collection of fresh tumour samples for study as an integral part of the protocol.

Potential future studies include the study of any alterations in the methylation profile of a group of patients before and following chemotherapy and if this would need to be correlated with clinicopathological variables. Including patients with SCLC into studies examining the role of decitabine given along with chemotherapy is worthy of study in relation to our study results.

The possibility of a clinical study in patients with NSCLC to examine the role of decitabine given in conjunction with chemotherapy might be another area of research.

Thus the future holds the exciting prospect of well planned collaborative prospective combined clinical and translational research, which could yield important and fruitful interventions in the management of patients with lung cancer.

7. Conclusions

The association between methylation of the MINT31 locus and an overall improved survival in NSCLC has never previously been reported and requires verification in a large clinical trial, as does the finding that only patients with stage II disease demonstrated methylation of the HIC1 locus. With regard to this an important success of this project was to establish cooperation between the Beatson Laboratories and the Cardiothoracic Unit at the Western Infirmary, Glasgow.

Successful multidisciplinary collaboration was achieved and the collection of fresh frozen tumour and normal adjacent lung samples along with a corresponding blood sample was undertaken. The aim was to enrol as many patients as possible undergoing resection of their primary lung cancer through the Unit. Unfortunately our ability to do this was hindered by national concerns at the time of this research regarding tissue collection and retention for use in clinical trials. As a result of these concerns all sample collections at the University of Glasgow were discontinued whilst a review of all projects involving tissue collection was undertaken. At the end of the study period an additional 10 surgical samples with corresponding blood samples had been collected. These samples are stored and will be available for future research, although much larger trials involving appropriate sample collection will be required to answer all of the questions raised in this thesis.

Other aspects of this research project were also limited by the availability of samples. In the case of our retrospective study of protein expression in bronchoscopic samples the number of samples available was disappointing. Only 67 of a possible 110 samples were collected. The major limiting factors were that a number of samples could not be

located due to the laboratory moving as well as a significant number of samples being unusable secondary to damage by damp whilst in storage. However in the case of the retrospectively collected surgical samples all the possible available samples were identified, collected and studied.

In addition to the sample collections described our local ethics approval in Glasgow allowed the prospective collection of pre-treatment blood samples and diagnostic tissue samples from patients as well as a post-treatment blood samples. The aim of this study was to investigate possible molecular changes (allelic imbalance/CpG Island Methylation) that might predict response to chemotherapy and/or prognosis. Moreover we intended to monitor these changes in the serum during/after treatment. To date samples have been collected from 75 patients of whom 20 underwent surgery and 55 received treatment with chemotherapy. In addition 44 samples were collected from patients after treatment. All these samples have been stored and now that the technology and methodology for reliable assessment of methylation of serum DNA is well established, these specimens will be available for study in the future.

As a consequence of the above problems of sample collection the results in all of the studies undertaken are based on small study numbers, despite significant efforts at sample collection during the study period. Therefore their statistical significance cannot be established with confidence and in order to validate these results, and in particular to validate the positive findings of the relationship between methylation of the MINT 31 promoter region and improved overall survival, further large-scale trials are needed.

It will be important to investigate further the question raised in the SCLC cell line study described in this thesis (chapter 6) as to whether resistance (acquired or intrinsic) to chemotherapy secondary to the methylation of the *hMLH1* promoter is a phenomenon that is actually seen in clinical practice. There is a growing body of literature investigating the use of the demethylating agent, decitabine, in the clinical arena and future translational studies of its effects are essential. These studies will require the measurement of serum DNA changes, as this source of DNA presents a potentially very attractive non-invasive means of monitoring/assessing the DNA molecular profile of tumours.

In order for these goals to be achieved it will be essential that future clinical trials in patients with lung cancer have a translational component incorporated into the protocol allowing the collection and study of important clinical samples in conjunction with robustly collected clinical data.

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9. Appendix

9.1 Patient Information Sheets – Collection of Blood samples

As you know you have recently been told that you have lung cancer. The doctors looking after you will have discussed your treatment options with you and your treatment will now go ahead as planned. Although we hope that this treatment will help you, as you know there is no guarantee of cure. This is because lung cancer may recur or prove resistant to the various treatments. It is important to find out more about resistance to treatment in lung cancer and previous studies have suggested that this might be related to a specific marker in the tumour. We would like to invite you to take part in a research study, which is looking at this.

The aim of this study, is to look at blood samples from lung cancer patients in the laboratory to see if we can identify a genetic abnormality in the tumour which shows up in the blood. If it is detected then this study may help us to plan treatment decisions in lung cancer patients in the future.

If you agree to take part in the study then an extra blood sample will be taken (about 2 teaspoonfuls) along with your routine blood samples, before, during and after the course of your treatment. It is important for you to understand that the information obtained will not benefit yourself but may be of use in planning treatment for patients with lung cancer in the future.

You can of course decide not to take part in the study and if this is so it will in no way affect your relationship with the medical and nursing staff looking after you. If you agree to take part in the study then your GP will be informed. You can also at any time change your mind and withdraw form the study. As stated earlier your treatment will be unaffected by this study.

If you would like further information please contact:

Dr S Davidson	0141 201 3715
Mr J McPhelim	0141 201 3718
Dr R Milroy	0141 201 3715

9.2 Patient Information Sheets – Collection of Tumour/Normal Lung Samples

As it will have been explained to you, you are about to undergo an operation to remove all, or part of, your lung. Doctors in the Pathology Department will perform a number of tests on the tissue that is removed during the operation. This will allow your surgeon to give you a diagnosis and prognosis and guide you if any other treatment is required. Once they have completed their examination, the tissue is normally discarded.

Because we are constantly engaged in research ourselves and in collaboration with others, we are seeking your permission to use some of this tissue that is normally discarded. This would be used in a number of ongoing projects designed to investigate cancer and in detailing the anatomy and physiology of the blood vessels within the lung.

If you wish your resected tissue to be used in this way, the following guarantees will be given:

- No tissue will be removed apart from what is deemed necessary for your operation as decided by the surgeon.
- Sampling of tissue will not prejudice in any way the results of your operation or affect the ability of the pathologist to produce an accurate report.
- Samples used for research will be removed from tissue that would normally be discarded.
- In cases of cancer research, small samples may be frozen and stored to allow tests to be done at a future date.

Should you not wish tissues to be removed, this will not affect your treatment in any way.

The research projects have been approved by the Research and Ethics Committee for North Glasgow Hospitals University Trust.

9.3 Abstracts and Presentations

Abstracts

Relationship between MLH1 expression and cisplatin sensitivity in small cell lung cancer.

S Davidson, J Plumb, G Strathdee, R Milroy, R Brown

British Journal of Cancer. 2001, Supplement 1. 85(1); P258

The Relationship between MLH1 expression and cisplatin sensitivity in small cell lung cancer.

S Davidson, J Plumb, G Strathdee, R Milroy, R Brown

Thorax Vol 56, Supplement III, December 01, iii68, P76

Presentations

The British Thoracic Society, Winter meeting 2001 (oral presentation)

The Relationship between MLH1 expression and cisplatin sensitivity in small cell lung cancer.

The British Cancer Research Meeting, July 2001 (poster presentation)

Relationship between MLH1 expression and cisplatin sensitivity in small cell lung cancer.

Plasma DNA Workshop, Manchester 2001

DNA mismatch repair and lung cancer