GENETIC ALTERATIONS IN NON-SMALL CELL LUNG CARCINOMAS

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor in Philosophy by George Xinarianos.

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To my beloved parents

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George Xinarianos

Genetic alterations in non-small cell lung carcinoma

Lung cancer is responsible for more deaths than any other cancer. The Merseyside region in the Northwest England has got among the highest rates of lung cancer in the UK and across Europe. The poor prognosis associated with lung cancer urge for increased attention on early detection and intervention management. In this study, genetic alterations, in the form of loss of heterozygosity and microsatellite alterations were evaluated as a method of identifying individuals at high risk of developing lung cancer. Genetic alterations were detected in bronchial lavage specimens from lung cancer patients but also in patients with no clinical evidence of lung cancer. The latter raises the question whether microsatellite alterations are a genetic phenomenon solely associated with cancer or it may represent a feature of non-neoplastic diseases. The incidence and implications of telomerase in non-small cell lung carcinomas was also investigated. The results obtained in this study demonstrate that telomerase activity can be detected in the majority of lung cancer cases but not in normal lung. However, certain cases in former and/or moderate smokers may follow a telomerase independent pathway. No mutations were detected in the telomerase genes *hTR* and *hTERT*. Telomerase activity regulation was found to be $p21^{WAF1}$ and Rb independent. Telomerase was examined as a potential marker in bronchial lavage specimens from individuals undergoing diagnosis of lung cancer. Telomerase was found to be a specific marker for malignant lung disease and a potential complementary tool to cytology in the diagnosis of certain lung cancer cases. The expression levels of the DNA mismatch repair genes, *hMLH1* and *hMSH2* and their regulation was also studied in NSCLC. The findings in this study indicate that *hMLH1* and *hMSH2* gene inactivation is a common event in the development of NSCLC and their differential expression is histology type specific. LOH has been shown to be a major genetic event involved in hMLH1 silencing while a putative negative regulator of hMSH2 may be located at the locus 3p14. No mutations were detected in the promoter regions and the hot spot exons of these genes. Mutational and expression analysis of the *p53* gene was also performed. Both p53 mutations and overexpression were more frequent in squamous cell carcinomas than in lung adenocarcinomas. *p53* mutations were associated with abnormal Rb expression and p53 overexpression was associated with missense than null mutations. The results also suggest an interaction between p53 and MSH2. Although p53 is a transcriptional regulator of MSH2, MSH2 may act as a DNA damage signaler to p53. The results in this study also suggest an association between p53 and telomerase activity in NSCLC.

TABLE OF CONTENTS

Chapter Three: Telomerase Activity in the Molecular Pathogenesis of NSCLC

Chapter Four: Telomerase Activity in Bronchial Lavage

Abbreviations

AdenoCa:	Adenocarcinoma
BL:	Bronchial lavage
bp:	Base pairs
BSA:	Bovine serum albumin
CDK:	Cyclin dependent kinase
CDKI:	Cyclin dependent kinase inhibitor
COPD:	Chronic obstructive pulmonary diseases
CTC:	Cardiothoracic center
DNA:	Deoxyribonucleic acid
dNTP:	Deoxynucleotriphosphates
EDTA:	Ethylenediaminetetraacetic acid
EGF:	Epidermal growth factor
ESTs:	Expressed sequenced tags
FA:	Fibrosing alveolitis
GADD:	Growth arrest DNA damage
GTP:	Guanosine triphosphate
H:	Heterozygous
HA:	Heteroduplex analysis
HLA:	Human leukocyte antigen
HNPCC:	Hereditary non-polyposis colorectal carcinoma
HPV:	Human papillomavirus
H-ras:	Harvey-ras
kb:	Kilo bases

K-ras:	Kirsten-ras
LHC:	Left hilum consolidation
LLL:	Left lower lobe
LMB:	Left middle bronchus
LOH:	Loss of heterozygosity
LVF:	Left ventricular failure
MA:	Microsatellite alterations
MCI:	Myocardial Infraction
MD:	Mediastinal
MI:	Microsatellite instability
MMR:	Mismatch repair
ND:	Not determined
NED:	No evidence of disease
NF1:	Neurofibromatosis type 1
NMCS:	No malignant cells seen
N-ras:	Neuroblastoma-ras
NSCLC:	Non-small cell lung carcinoma
PCNA:	Proliferating cell nuclear antigen
PCR:	Polymerase chain reaction
Pk/yr:	Packs per year
R:	Right hilum
RA:	Rheumatoid arthritis
RER:	Replication errors
RFLP:	Restriction fragment length polymorphism
RLL:	Right lower lobe

RNA:	Ribonucleic acid
RT:	Room temperature
RUL:	Right upper lobe
SCCHN:	Small cell carcinoma of the head and neck
SCLC:	Small cell lung carcinoma
SDS:	Sodium dodecyl sulphate
SqCCL:	Squamous cell carcinoma of the lung
SSCP:	Single strand conformational polymorphism
TE:	Tris-EDTA
TRAP:	Telomeric repeat amplification protocol
UV:	Ultra-violet
WHO:	World health organization

Preface

Lung cancer is a major killer, accounting for more deaths than any other cancer worldwide. The Merseyside region in the Northwest England has got among the highest rates of lung cancer in the UK and across Europe. Multimodal strategies should be implemented in an attempt to improve the poor prognosis associated with the disease.

In this study, genetic alterations in the form of loss of heterozygosity (LOH) and microsatellite alterations (MA), were evaluated as a method of identifying individuals at high risk of developing lung cancer. Bronchial lavage (BL) specimens of individuals who were referred to an early lung cancer clinic in the Northwest of England with suspected lung cancer were studied for genetic alterations (LOH and MA). Genetic alterations were detected in patients with lung cancer, but also in patients with no cytological or radiological evidence of bronchial neoplasia. It was found that the prevalent type of alteration in specimens with cytological evidence of malignancy was LOH; in contrast, the individuals with no cytological evidence of malignancy showed a preponderance of MAs indicating that a substantial proportion of cells in the bronchial lavage from suspected lung cancer patients carry identifiable genetic alterations. However, the presence of genetic alterations in the bronchial lavage of individuals with no clinical evidence of lung cancer raised the question whether instability is a genetic phenomenon solely associated with cancer or it may represent a feature of non-neoplastic diseases.

Nevertheless, the results obtained in this study demonstrate that microsatellite PCR-based assays can be developed as tools for the earlier identification of genetic changes in cells exfoliating in the bronchus.

The incidence and implication of telomerase in the molecular pathogenesis of non-small cell lung carcinomas (NSCLC) was also investigated. Telomerase activity was detected in the majority of NSCLC cases examined but not in any of the matched normal lung tissues. Telomerase activity correlated with the T stage of the tumour, nodal metastasis and differentiation. Significant correlations were also found between the presence of telomerase activity and current smoking status at the time of diagnosis, daily tobacco consumption and total tobacco exposure (pack/years). Telomerase activity was not associated with the expression of $p21^{WAF1}$ and Rb. In addition, there was no association between telomerase activity and allelic imbalance on chromosome 3p. Mutations in the minimal functional region of the human telomerase RNA gene (*hTR*) and its promoter region were not detected. No mutations were detected in the promoter region of the human telomerase gene (*hTERT*) encoding for the catalytic subunit of human telomerase. These findings demonstrate that telomerase activity is a common genetic phenomenon in NSCLC cases but not in the normal lung. However, certain cases in former and/or moderate smokers may follow a telomerase independent pathway. These data also suggest that mutations in the in the minimal functional region of the *hTR* gene and its promoter region and the promoter region of the *hTERT* gene do not contribute in the regulation of telomerase

activity in NSCLC. In addition, these results indicate that regulation of telomerase activity in NSCLC is independent from two of the cell cycle regulators, $p21^{WAF1}$ and Rb.

Detection of telomerase activity was evaluated as a potential biomarker for the diagnosis of malignant lung disease. Telomerase activity was examined in BL samples from individuals undergoing diagnosis of lung cancer. Telomerase activity was detected in BL samples from lung cancer patients with a positive cytology report but also in BL sample from patients with non-malignant cells seen (NMCS). The final lung cancer diagnosis in the latter group was made by other means (surgery, biopsy, radiology). Telomerase activity was also detected in a BL specimen from an individual with cystic fibrosis who died 7 months after undergoing bronchoscopy. The results in this study indicate that telomerase is a specific marker for malignant lung disease and a potential complementary tool to cytology in the diagnosis of certain lung cancer cases. In addition, the findings of this study suggest that telomerase may be a marker of disease aggressiveness in certain non-neoplastic lung disorders.

The expression levels of MLH1 and MSH2 proteins in relation to LOH on chromosomes 3p and 2p, the mutational status of the promoters and certain hot spots in the genes were investigated in NSCLC. In adenocarcinomas, the reduction of hMSH2 expression was more frequently observed than that of hMLH1 while in squamous cell carcinoma of the lung (SqCCL), hMLH1

expression was more frequently reduced than hMSH2. Reduced expression of hMLH1 correlated with allelic imbalance on loci *D3S1289* and *D2S391*. It is of note that an inverse correlation was found between hMSH2 reduced expression and LOH at locus *D3S1300*. In addition, hMLH1 reduced expression was more frequently associated with heavy smoking, assessed by both daily tobacco uptake and total smoking exposure (pack-years). A correlation between hMLH1 reduced expression and nodal metastasis in SqCCL was also observed. No mutations were identified in the promoter regions and hot spot exons examined in these two genes. Differential expression of hMLH1 and hMSH2 was not associated with the expression of the cell cycle regulators Rb and $p21^{WAF1}$. These findings indicate that *hMLH1* and *hMSH2* gene inactivation is a common event in the development of NSCLC and allelic loss appears to be a major genetic event involved in *hMLH1* silencing. The results in this study also suggest that a putative negative regulator of the *hMSH2* gene may be located at the locus 3p14.

The status (mutations and expression) of p53 was analysed in NSCLC. p53 status data were analysed in relation to the expression of two of the cell cycle regulators, $p21^{WAF1}$ and pRB, LOH on chromosome 3p, differential expression of two of the DNA MMR genes, *hMLH1* and *hMSH2*. A possible role of p53 in the regulation of telomerase activity was also examined. Both p53 mutations and overexpression were more frequent in SqCCL than in lung adenocarcinomas. Intense p53 immunostaining strongly correlated with missense than null mutations. p53 mutations associated with abnormal Rb

expression. Interestingly, in NSCLC with wild type p53, p53 overexpression correlated with increased expression of MSH2. In addition, in SqCCL, p53 mutations correlated with reduced MSH2 expression. These data suggest of an interaction between p53 and MSH2. Although there is already evidence for p53 being a transcriptional regulator of MSH2, the results obtained in this study suggest that MSH2 may act as a DNA damage signaller to p53. p53 overexpression and total p53 aberrations (either mutation or overexpression or both) correlated with telomerase activity indicating a close association between p53 and telomerase activity. p53 mutations correlated with allelic imbalance at locus *D3S1266* while p53 overexpression correlated with allelic imbalance at loci *D3S1266*, *D3S1289*, *D3S1304*, indicating a possible interaction between gene(s) located on chromosome 3p and p53.

List of Figures

List of Tables

CHAPTER ONE

INTRODUCTION

1.1 Lung Cancer

The World Health Organisation (WHO) classification is accepted as the definite classification of lung cancer. Non-small cell lung carcinomas (NSCLC) was the term adopted by the WHO to group together adenocarcinoma, squamous cell carcinoma and large cell carcinoma. NSCLC accounts for approximately 75-80% of all lung cancers. The prognosis for patients with NSCLC is poor, with an overall 5-year survival rate of approximately 10% (Macdonald & Ford, 1997).

1.1.1 Incidence of Lung Cancer

Lung cancer is among the most commonly occurring malignancies in the world and is one of the few that continues to show an increasing incidence. In the United States, lung cancer is the leading cause of cancer death in men, and it has surpassed breast cancer as the leading cause of cancer death in women in the latter part of the 1980s (American Cancer Society, 1996).

Excluding this malignancy, most developed countries have shown declines in death rates from cancer in the past 20 years. During the same time period in countries such as the United States and Canada, the death rate from lung cancer increased more than three-fold but it has shown a recent decline. In developing countries, the death rate from lung cancer continues to accelerate. These changes appear to be affected significantly by the observed difference in

smoking habits and cigarette tar levels in developed and developing countries (Parkin, 1989). The incidence of lung cancer now exceeds 70 per 100,000 men in the United States. As we enter the 21st century, it is expected that the altered smoking habits of the past two decades and the decreased tar content of cigarettes consumed in most developed countries will lead to a decrease in lung cancer incidence (American Cancer Society, 1996).

Nevertheless, lung cancer is responsible for more deaths than any other cancer, accounting for almost 900,000 deaths per year worldwide (Parkin *et al,* 1999). It is the most common malignancy in males in the UK and is now the most frequent cancer in women in Liverpool. The highest incidence rates of lung cancer for both men and women are found in the Merseyside region of the Northwest England. In the Liverpool area the cumulative rate (0-74 years) in 1994-1995 was 11.6% for males and 7.2% for females compared with a national average of 7.3% for males and 3.0% for females in 1992 (Parkin, 1998).

Lung cancer is characterised by a very high mortality rate. In particular, approximately 10% of all patients (stage I-IV) who develop lung cancer survive 5 years. These mortality rates far exceed those of the acquired immunodeficiency syndrome epidemic. Unfortunately, this rate has been static in the past two decades, and it appears unlikely that in the near future marked improvements in survival rates will occur. However, it is anticipated that there

will be a decreased incidence of lung cancer in the next century, at least in developed countries, leading to a decrease in the total number of deaths per year attributed to this cancer (American Cancer Society, 1996).

1.1.2 Aetiology of Lung Cancer

It has been estimated that 80% of lung cancer deaths among men and 75% of lung cancer deaths among women are attributable to smoking. There is clear evidence for a dose-response relation for smoking and lung cancer. The risk of lung cancer increases with the number of cigarettes smoked, years of smoking duration, earlier age at onset of smoking, degree of inhalation, tar and nicotine content, and use of unfiltered cigarettes, and it decreases in proportion to the number of years after smoking cessation (Loeb *et al,* 1984; Garfinkel *et al,* 1988).

There is no apparent threshold in the dose-response relation between the degree of smoking and the incidence of lung cancer. Consequently, the potential of smoke in the environment of non-smokers to induce lung cancer has become an important issue. The smoke inhaled by non-smokers has a similar chemical composition to that inhaled by smokers but has higher N-nitrosamine levels and smaller size particles, which remain suspended in air and can more easily penetrate the bronchial tree (Hoffman *et al,* 1983).

Increases in lung cancer risk accompany exposure to carcinogens, such as asbestos, radon, bis-(chloromethyl)-ether, polycyclic aromatic hydrocarbons, chromium, nickel, and inorganic arsenic compounds (Fraumeni & Blott, 1982). The association with occupational exposure to these agents appears to be independent of cigarette smoking.

A number of other occupations are associated with an increased risk of lung cancer that appears to be dependent in part on high rates of smoking, which may interact with known and suspected carcinogens in the workplace. In addition, a number of occupations with a high smoking prevalence have increased cancer risk (Fraumeni & Blott, 1982).

The component fibers of asbestos, particularly crocidolite, are known carcinogens with a proven ability to cause lung cancer. Asbestos exposure has a dose-response effect, and a synergism between asbestos and smoking is evident (Kjuus *et al,* 1986; Seidman *et al,* 1986). Unlike other etiologic agents, it appears that even short-term exposure to asbestos can be a risk factor when the intensity of exposure is high (Talcott *et al,* 1989). It has been estimated that about 3% to 4% of all lung cancer cases are caused by asbestos (Omenn *et al,* 1986).

Radon is a naturally occurring chemically inert gas that is a decay product of uranium-238. Radon undergoes radioactive decay to short-lived secondary products, two of which emit heavily ionizing alpha particles. Inhaled alpha particles can deliver intense radiation to a depth of 41 to 71 micrometers in the epithelial lining of the airway. The carcinogenic effect results from non-lethal events in the nuclei. Radon has been recognised as a potentially important carcinogen because it is present in soil and rocks and seeps into homes and office buildings. When ventilation is restricted, it can accumulate in the atmosphere (Fabrikant, 1990).

Evidence is increasing that genetic factors can contribute to lung cancer risk because the metabolites of carcinogens cause the malignancy, and the pathways to create these metabolites are genetically determined (Heighway *et al,* 1986). Several approaches have been adopted to detect a genetic association: studies of familial clustering, studies of naturally occurring antigens, and studies of the metabolism of drugs. Studies of familial clustering are inconclusive and have been interpreted as showing a substantial effect or no effect at all (Law *et al,* 1987).

1.1.3 Pathology & Histology of Lung Cancer

Evidence is increasing that lung cancer is derived from a pluripotent stem cell that is capable of expressing a variety of phenotypes. This epithelial stem cell, in normal histogenesis, differentiates to those cells found in the

tracheobronchial tree, including pseudostratified reserved cells, ciliated goblet columnar cells, neuroendocrine cells, and type I and II pneumocytes seen lining the alveoli. Cells that are capable of division can express hyperplastic, metaplastic or neoplastic change (Linnoila, 1990).

It appears that squamous cell carcinoma and small cell carcinoma have a distinct dose-response relation, with increasing tobacco exposure producing increasing numbers of these histologic subtypes. Worldwide, however, adenocarcinoma appears to be increasing, especially in women, despite the fact that it does not have this significant dose-response relation with smoking. This increasing incidence of adenocarcinoma is especially seen in the United States and is less apparent in Europe and Japan (Gazdar & Minna, 1999).

Squamous cell carcinoma arises most frequently in proximal segmental bronchi and is associated with squamous metaplasia. In its earliest form known as carcinoma *in situ*, stratified squamous epithelium is replaced by malignant squamous cells, without invasion through the basement membrane. Because of the ability of these cells to exfoliate, this tumour can be detected by cytologic examination at its earliest stage. With further growth, the tumour invades the basement membrane and extends into the bronchial lumen, producing obstruction with resultant atelectasis or pneumonia (Hansen, 1991).

The squamous cell tumour is composed of sheets of epithelial cells, which may be well or poorly differentiated. Most well-differentiated tumours demonstrate keratin pearl. The more poorly differentiated tumours, if determined to be squamous cell carcinoma, have positive keratin staining. These tumours tend to be slow growing and it is estimated that up to 3 or 4 years are required from the development of *in situ* carcinoma to a clinically apparent tumour (Hansen, 1991).

Bronchoalveolar carcinomas form glands and produce mucin. Although they can be subdivided by light microscopy to the classic four types defined by the WHO classification, it appears that bronchoalveolar carcinoma is a distinct clinico-pathological entity (Clayton, 1988). This tumour appears to arise from type II pneumocytes, grows along alveolar septa by lepidic growth, and shows little if any desmoplastic or glandular change. These tumours are interesting in that they present in three different fashions: a solitary peripheral nodule, multifocal disease, or a rapidly progressive pneumonic form, which appears to spread from lobe to lobe, ultimately encompassing both lungs (Hansen, 1991).

Large cell carcinoma is the least common of all NSCLC tumours, accounting for about 15% of all lung cancers. Using immunohistochemical staining, electron microscopy, and the monoclonal antibodies, many tumours previously diagnosed as undifferentiated large cell carcinoma can now be classified more appropriately as poorly differentiated adenocarcinoma or

squamous cell carcinoma. For this reason, the incidence of this type of tumour continues to decrease (Hansen, 1995).

Adenocarcinomas arise in the periphery of the lung but they may also arise centrally in a bronchus. The diagnostic histopathological features of adenocarcinomas are gland formation and/or mucin production. Well-differentiated adenocarcinomas show abundant formation of glands or acini whereas moderately or poorly differentiated adenocarcinomas show relatively more solid areas of tumour and fewer glandular structures. In addition, the degree of cytological atypia is usually greater in the poorly differentiated adenocarcinomas (Hansen, 1995).

1.1.4 Molecular Biology & Genetics of Lung Cancer

Molecular analysis has demonstrated that lung cancer cells have accumulated a number of genetic lesions, predominantly in recessive oncogenes, with perhaps 10 or more such events required for the development of an overt lung cancer.

Proto-oncogenes that encode components of cell signalling pathways can become abnormally activated in lung cancer. Activation of ERBB1, which encodes the epidermal growth factor (EGF) receptor with tyrosine kinase activity, usually occurs by overexpression more commonly in NSCLC than in small cell lung carcinoma (SCLC), and may be related to tumour stage and

differentiation (Cerny *et al,* 1986). Another receptor, tyrosine kinase (p185), encoded by ERBB2 (HER2/neu) is highly expressed in more than one third of NSCLC, especially adenocarcinomas (Weiner *et al,* 1990). In addition, ERBB2 expression correlates with a shorter survival in lung adenocarcinomas and may also be a marker for intrinsic multi-drug resistance in NSCLC cell lines (Tsai *et al,* 1993).

Receptor tyrosine kinases act through several interacting molecules sending signals to the guanosine triphosphate (GTP)-binding RAS protein, which in turn transmits information to downstream effectors. The RAS gene family (K-ras, H-ras, and N-ras) can be activated by point mutations at codons 12, 13, or 61, and is mutated in approximately 20% to 30% of adenocarcinomas and 15% to 20% of all NSCLC cases but very rarely if ever in SCLC (Ritchardson & Johnson, 1993). It is of interest to note that K- ras mutations portend a poor prognosis in both early- and late-stage NSCLC cases and may prove to be clinically useful prognostic molecular markers (Slebos *et al,* 1990; Mitsudomi *et al,* 1991; Rasell *et al,* 1994). Mutations in the K- ras account for 90% of the RAS mutations found in lung adenocarcinomas while 85% of the K- ras mutations affect codon 12. It is of interest that 70% of K- ras mutations are G-T transversions with the substitution of the normal glycine (GGT) with either cysteine (TGT) or valine (GTT). Similar G-T transversions have also been found to affect the p53 gene in lung cancer. The later is attributed to DNA adducts caused by the polycyclic hydrocarbons and nitrosamines in tobacco smoke (Greenblatt *et al,* 1994).

A direct downstream effector of the RAS protein is the RAF1 oncoprotein. Despite the finding that one copy of RAF1, mapped at chromosome region 3p25, is frequently deleted, no mutations have been detected in human lung cancers. Moreover, molecules downstream of RAF in the signal transduction pathway have not been well studied yet and their involvement in lung carcinogenesis has to be elucidated (Sekido *et al,* 1998).

The signal transduction cascade results in the activation of nuclear oncoproteins such as those encoded by the MYC genes. Myc phosphoproteins control the transcription of other genes involved in growth regulation by forming heterodimers with the transcription-regulating proteins MAX (positive regulation) and MAD (negative regulation), possibly acting as a "master oncogene switch". The only member of the myc genes family, which is frequently activated in SCLC and NSCLC, is MYC. However, abnormalities of MYCN and MYCL usually only occur in SCLC. It has also been shown that MYC overexpression occurs in more than 50% of primary NSCLCs, and in a proportion of associated hyperplastic preneoplastic lesions, indicating that dysregulated MYC expression may be an early event in lung cancer development (Broers *et al,* 1993). In addition, there have also been reports of MYCL amplification with rearrangement in which MYCL fuses to the RLF gene causing a chimeric protein (Makela *et al,* 1991). Other oncogenes, such as ERBA, MYB, JUN, and FOS, have been implicated in lung cancer, although their biological importance has not as yet been fully elucidated (Sekido *et al,* 1998).

Cytogenetic and allelotyping studies have revealed deletions, suggesting the presence of underlying recessive oncogenes, at multiple chromosomal regions. Structural cytogenetic abnormalities have been identified in lung cancer suggestive of allele loss, including non-reciprocal translocations, deletions, and numeric abnormalities, whereas the presence of double minutes and homogeneously staining regions indicate gene amplification of oncogenes, such as for MYC. A landmark cytogenetic observation was the frequent deletion of chromosome region 3p14-23 in SCLC, and later extended to NSCLC (Sekido *et al,* 1998). Karyotyping has been supplemented by molecular allelotyping analysis of polymorphic DNA markers to identify loss of one gene copy, which indicates an underlying tumor suppressor gene. Reported regions frequently involved include 1p, 1q, 3p [multiple sites including 3p12-13, 3p14.2 (FHIT gene site), 3p21.3,and 3p25], 5q (APC, MCC loci), 8p, 9p21 (CDKN2), 11p13, 11p15, 13q14 (RB), 17p13 (p53), and 22q as well as other sites (Richardson & Johnson, 1993; Neville *et al,* 1995; Sekido *et al,* 1998).

1.1.5 Treatment of Lung Cancer

Surgery and radiotherapy have been used independently to obtain local control of the primary tumour and regional lymphatic drainage. Until recently, chemotherapy had been used in an attempt to prolong symptom-free life in patients with metastatic disease. In the past 20 years, however, combined-modality therapies have become much more prevalent and have spurred intensive investigation. All three modalities are now used as primary therapy

and, in combination, have been employed to improve disease-free intervals and ultimate survival (Hansen *et al,* 1995).

Until about 40 years ago, pneumonectomy was considered the surgical excision of choice in managing lung cancer. Presently, when complete excision can be obtained by lobectomy, this is the preferred resection with equal long-term success (Graham & Sedal, 1993).

Radiotherapy for the treatment of NSCLC has experienced significant changes in a short time with respect to the evolution of appropriate patient selection, radio-biologic principles, technical innovation, imaging, and the use and integration of chemotherapy and surgery. Factors such as quality of life during and after therapy, combined with the cost of treatment and management of treatment-related toxicity of radiotherapy, must be considered in the treatment decision in light of the current health care environment (Hansen, 1995).

Only about one third of patients diagnosed with NSCLC, present without clinical evidence of mediastinal nodal dissemination and are eligible for immediate surgical intervention. The remainder of patients referred for consideration of primary radiotherapeutic intervention include 10% to 15% with medically inoperable stage I or II disease, 30% to 40% with locally

advanced unresectable disease and for palliation, 40% to 50% with stage IV disease. Radiotherapy has now been integrated with surgery and chemotherapy in combined-modality treatment of potentially curable disease (Libenbaum & Green, 1994).

The use of chemotherapy in patients with NSCLC has been under investigation for several decades. In patients with advanced disease (stage IIIA or IIIB), traditional therapy has consisted of surgery and postoperative radiotherapy or radiotherapy alone for patients with unresectable disease. In these patients, chemotherapy is now used as a component of multimodality therapy. Therapy is given with curative intent, and it is hoped that the integration of chemotherapy will lead not only to an increased overall median survival time but also to an increase in the percentage of cured patients surviving for long periods of time (Ihde, 1992; Libenbaum & Green, 1994).

Strategies in this setting have included classic adjuvant chemotherapy in patients with fully resected disease and induction (or neoadjuvant) chemotherapy, whereby a specified number of chemotherapy cycles is administered before definitive local therapy with surgery, radiotherapy, or both. The simultaneous use of chemotherapy and radiotherapy has also been intensively investigated (Vokes, 1993)

It has been conclusively demonstrated that induction chemotherapy followed by radiotherapy prolongs the median survival time in patients with unresectable stage III disease when compared with radiotherapy alone (Sause *et al,* 1995). The use of induction chemotherapy in the surgical setting (stage IIIA) or with simultaneous radiotherapy remains investigational. Also, the use of adjuvant chemotherapy in patients with resected stage II or stage IIIA disease is considered experimental (Hansen, 1995; Sause *et al,* 1995).

Many patients with NSCLC, present with metastatic (stage IV) disease at initial diagnosis. There is no known curative therapy for these patients. The treatment goals are therefore broadly defined to maximize survival time and maintain acceptable quality of life (Hansen, 1995).

1.1.6 Early detection of Lung Cancer

Since most lung cancers are directly attributable to cigarette smoking the primary preventive strategy is to refrain from smoking. However, former smokers are still at high risk of developing lung cancer (Tockman *et al,* 1988; Fontana *et al,* 1991). Therefore secondary prevention such as a screening programme for these individuals is necessary. Early detection leads to early treatment and better chemoprevention, which in turn translates into higher cure rates.

Previous studies used screening by the means of x-ray and sputum cytology. Although these studies demonstrated that the number of diagnosed lung cancer cases is greater and that the stage distribution respectability and survival are more favourable for the screening group, they failed to give evidence of significant reduction in mortality from lung cancer in the group offered more extensive screening (Strauss *et al,* 1997; Miettinen, 1998; Petty, 1998). However, there were a number of limitations in those initial studies such as lack of an untested control group, inadequate compliance, and limited statistical analysis. Thus, it was concluded that screening for lung cancer saves lives.

Over the last 20 years, the pattern of disease has changed, conventional diagnostic techniques have improved and new early detection techniques have emerged. Molecular biomarkers in bronchial lavage and sputum cells may prove useful for early pre-clinical detection of lung cancer. In archive sputum specimens, mutations of K-ras and p53, and microsatellite alterations were identified as potential markers of malignancy (Mao *et al,* 1994; Somers *et al,* 1998; Sekido *et al,* 1998). In addition, detection of hetegeneous nuclear ribonucleoprotein (hnRNP) A2/B1 overexpression has shown promise for its inclusion in a panel of biomarkersfor early lung cancer detection (Tolkman *et al,* 1997; Fielding *et al,* 1999). Moreover, the use of low dose spiral CT scan may improve the outcome of the disease (Henschke *et al,* 1999). Radiation dose of low-dose spiral CT is about 10 times higher than that of plain chest radiography (Kaneko *et al,* 1996; Diederich *et al,* 1997). Lung cancer screening

with a mobile CT unit was started in 1996. A comparative study of the two screening methods observed that the rate of lung cancer detection by CT was 0.48%, while the detection rate of the same group before the introduction of CT was 0.03% (Sone *et al,* 1998). However, spiral CT is not as sensitive for small central cancers as it is for small peripheral cancers (Henschke *et al,* 1999). Therefore, a combination of methods should be used.

Another early detection technique is the use of fluorescent bronchoscopy instead of the standard white-light bronchoscopy. Squamous cell carcinoma of the bronchus arises after a series of progressive histological changes of the epithelium. Visualisation of early changes within the tracheobronchial tree may be difficult by conventional bronchoscopy because these early lesions are superficial and minute in size. Fluorescence broncoscopy is based on the differences in fluorescence intensity between normal tissue (green) and neoplastic (brown-brownish red depending on pathological grade). Preliminary studies have shown that the use of fluorescence bronchoscopy can improve the detection rate of pre-invasive bronchial lesions and could be clinically useful in detection and localisation of early lung cancer and in staging the extend of endobronchial spread of bronchial cancer (Yokomise *et al,* 1997; Lam *et al,* 1998).

1.2 Telomeres & Telomerase

1.2.1 Structure & Function of Telomeres

Telomeres are the DNA protein complexes found at the ends of linear chromosomes. They consist of specific proteins and simple repetitive DNA by the means of an array of tandem repeats. The evolutionary conservation and stringent sequence requirement of telomeric DNA adds to the importance of its sequence for telomere function. In addition, the repetitiveness of telomeric DNA may be functionally relevant as such DNA is packaged in chromatin in a characteristic manner (Rhodes & Giraldo, 1995).

Telomeric proteins comprise a class of structural proteins that bind sequence-specifically to telomeric DNA. The biological role of these proteins so far is to cap the ends of chromosomes confering stability. The later is achieved by protecting chromosomes from degradation and terminal fusion. Telomeres also serve as attachment points to the nuclear matrix, thus likely aiding in chromosome movement and organisation. In addition, they may alter transcription rate of nearby genes by a process referred to as telomere silencing. Further, telomeres are able to act as a buffer against the loss of terminal sequences inherent to the replication of linear DNA molecules by unidirectional RNA-primed DNA polymerases providing sites for *de novo* elongation of these molecules by the means of telomerase (Blackburn, 1991; Zakian, 1995).

Telomeric DNA usually consists of tandemly repeated sequences with a strand rich in guanosine (G). The G-rich strand always appears to be oriented in the 5' to 3' direction towards the end of the chromosome. Also, the length of telomeres varies widely between species (Allshire *et al,* 1989; Zakian, 1995). This length variation suggests differences in minimal functional sizes that may be related to number of telomeric proteins or of protein binding sites. However, it should be stressed that in most cases the precise length of telomeres remains still unknown (Zakian, 1989; Blackburn, 1991; Zakian, 1995).

1.2.2 Structure & Function of Telomerase

Telomerase is a ribonucleoprotein enzyme first discovered in *Tetrahymena* by Greider and Blackburn (1985) and afterwards in an immortal human tumour cell line by Morin (1989). Moreover, the RNA component of telomerase, cloned and sequenced from several ciliates, contains a short region that is referred to as the "template domain". This domain is complementary to the one or more repeats of the G-rich telomeric DNA of the species (Blackburn, 1992).

Furthermore, telomerase is a DNA polymerase that can be classified as a reverse transcriptase. The later is because its mechanism of action involves the copying of an RNA template into DNA. However, it is an unusual reverse transcriptase because unlike other reverse transcriptases, found in retroviruses

and prokaryotes, it is a ribonucleoprotein that contains its own RNA template as an integral part of the enzyme (Alberts *et al,* 1994; Feng *et al,* 1995).

According to our present understanding, the primary function of telomerase is the synthesis of telomeric DNA. This appears to be the prevalent pathway for maintenance of telomere length in the human germline and possibly in human stem cells. Telomerase may further serve in a repair capacity since naturally occurring chromosome breaks have been shown being capped by telomeric DNA and transfected short telomeres act as primers for telomerase-dependent elongation (Collins, 1996).

1.2.3 Human Telomerase

The candidate RNA component for human telomerase is 560 nucleotides long whereas its template region is complementary to approximately 1.5 repeats of the human telomere sequence. The later was identified by mutational analysis methods. In addition, the candidate human telomerase RNA component has no homology to any of the lower eukaryotic telomerase RNAs (Feng *et al,* 1995).

The identification of this clone paved the way for testing the function of human telomerase in human cancer cells and tissues using antisense or ribozyme technology. It has also been suggested that knockout experiments

could be planned to help elucidate the role of telomerase in germline and somatic tissue development, stem cell renewal, and hyperproliferative disorders. The later may also include cancer (Chong *et al,* 1995; Shay, 1995).

1.2.4 Telomeres and Cellular Senescence (AGING)

More than 30 years ago, Hayflick first described the limited replicative capacity of normal human fibroblasts in culture as a manifestation of cellular senescence (Hayflick, 1965; Shay, 1995). In this process, the majority of cells in a population fail to divide in response to a variety of normal growth stimuli after a characteristic number of divisions. However, as one would expect they do not die. It has been reported that they remain metabolically active characterised with an aberrant pattern of gene expression. Moreover, numerous other somatic cell types, including epithelial cells, endothelial cells, myoblasts, astrocytes, and lymphocytes, have shown evidence of a clock that limits their division capacity (Goldstein, 1990; Levy *et al,* 1992). This clock appears to utilise cell divisions as the unit of time rather than chronological or metabolic age. Therefore, it has been called a "mitotic clock". Although the maximum division capacity in culture of a human somatic cell population from a young, normal individual varies significantly from donor to donor and cell type to cell type, it typically falls in the range of 50-100. However, it has been shown that this limit decreases as a function of donor age. The later presumably reflects the replicative history of the cells *in vivo* (Goldstein, 1990; Vaziri *et al,* 1994).

1.2.5 Telomeres and the End Replication Problem

The inability of DNA polymerases to replicate the termini of linear molecules predicts loss of telomeric DNA sequences and shortening of telomeres with each round of semiconservative DNA synthesis. This is referred to as "end replication problem". In the absence of a mechanism to overcome this "end replication problem", organisms would fail to pass their complete genetic complement from generation to generation (Levy *et al,* 1992; Harley & Villeponteau, 1995). Hence, all species must possess at least a germ-line mechanism in order to prevent this type of incomplete replication of their genome.

Different organisms have evolved various methods to prevent net loss of DNA. In particular, the problem can be overcome by addition of terminal sequences by transposition, as in *Drosophila*, or by recombination as sometimes occurs in yeast. In addition, most of the eukaryotic species appear to acquire telomeric DNA by the *de novo* addition of nucleotides catalysed by telomerase (Zakian, 1989; Biessmann & Mason, 1992). In the absence of telomere maintenance, eukaryotic cells that would otherwise be immortal go through a number of doublings prior to cell cycle arrest and/or death (Singer & Gottschling, 1994). Further, the consequence of incomplete replication of terminal DNA fragments has been modelled and predicted to lead to a form of the binomial distribution of deletion events at each independent chromosome end.

1.2.6 The Telomere Hypothesis

Loss of telomeric DNA due to incomplete replication of chromosome ends was ascribed a potential role in the regulation of cell lifespan over 20 years ago. In particular, telomere shortening has been likened to a clock that monitors the number of somatic cell divisions and limit cell proliferation by signalling entry into senescence (Harley, 1991). It has been further proposed that cells with unlimited proliferative potential, such as unicellular organisms, reproductive cells and possibly tumour cells, would have a mechanism to compensate for the loss of DNA sequences at the chromosome termini. In addition, the identification of telomeric DNA sequences of vertebrates and the development of assays for the detection of the mammalian telomerase have given considerable evidence in support of this hypothesis in human cells (Biessmann & Mason, 1992).

Moreover, telomeres in human somatic cells from the adult differ in length and stability from those in germline and fetal cells. In sperm, telomeric DNA tracts are approximately 20 Kb long and are maintained at this length, or possibly even elongated, with increasing donor age. However, in somatic tissues, telomeres are significantly shorter and their size decreases as donor age increases. The later process is thought to be accelerated in individuals with premature-aging syndromes. Also, in cultured somatic cells, loss of telomeric DNA is dependent upon cell division while telomere length appears to be a good predictor of the residual proliferative capacity of cells both *in vivo* and *in vitro* (Biessmann & Mason, 1992; Holliday, 1996).

Although in human cells senescence is normally irreversible, *in vitro* transformation of cells in culture overrides the controls of cell proliferation. Transformed cells appear to acquire an extended lifespan terminating in a proliferative crisis during which cell death often predominates over cell division. However, this extended lifespan remains still finite. Also, the rare cells that escape crisis have unlimited proliferative capacity *in vitro*. Therefore they are considered to be immortal (Harley *et al,* 1994). Moreover, transformation does not directly activate or upregulate telomerase activity. The later suggests that transformed cells continue to lose telomeric DNA during cell division at the same rate as normal somatic cells. Consequently, they appear to have significantly shorter telomeres by the end of their extended lifespan than those of senescent cells (Greider, 1994). In addition, it has been reported that estimates of telomeric DNA length in cells at crisis suggest that telomeres may be absent on at least some chromosomes and their loss may be responsible for cell death. Nevertheless, the proposed relationships between telomeres and cell proliferative capacity argue that stabilisation or even elongation of critically short telomeres occurs upon cell immortalisation (Counter *et al,* 1992; Counter *et al,* 1994). It also correlates with the appearance of detectable telomerase activity.

Since methods for altering telomere length in normal cells are not available and the precise length of telomeres of senescent cells is unknown, the role of telomeres in growth arrest remains to be established. It should be stressed that many theories have been proposed (Holliday, 1996). However, a

particularly attractive one suggests that the Hayflick limit, also referred to as Mortality Phase I (M1), is simply a permanent cell cycle arrest due to a checkpoint mechanism recognising "damaged" DNA at the ends of one or more chromosomes. Such a mechanism presumably involves the tumour suppressor genes p53 and Rb in at least some cell types. The later is supported by the fact that viral oncoproteins and antisence oligonucleotides capable of decreasing p53 and pRb levels allow cells to bypass M1 (Bond *et al,* 1994; Shay *et al,* 1993). Although pRB is hypophosphorylated in non-dividing senescent cells, levels of p53 protein and mRNA do not seem to change significantly at senescence (Shay, 1995; Holliday, 1996). This emerges that p53 activity in senescent cells must be reviewed and determined more carefully.

1.2.7 Telomere Loss and Carcinogenesis

Molecular defects in the regulation of the cell cycle and loss of cell cycle checkpoints can be responsible, to a great degree, for the instability of tumour genomes. Also, environmental factors are a likely source of chromosomal lesions associated with tumourigenesis. In addition, the genotypic changes observed in a wide variety of human tumours may be due to malfunction of genes that normally maintain the integrity of the genome. The later are referred to as repair genes (Weinberg, 1989). A fourth factor that has recently come to the attention in the literature involves the status of telomeres in the chromosomes of cancer cells. It has been proposed that the programmed telomere shortening in human somatic cells and the resulting collapse in

telomere function may explain a significant portion of the genetic instability in tumours (Holliday, 1996; Shay, 1995).

Moreover, like telomeres in normal somatic cells, telomeres of primary human tumours appear to undergo progressive sequence loss at some stage in their development. This process is evident from the fact that tumour telomeres are generally shorter than the telomeres in neighbouring normal cells (Harley *et al,* 1994; Greider, 1994). However, comparison to the presumed precursor of the malignancy is important in order to evaluate changes in tumour telomeres. This is because possible differences in replicative history invalidate the use of other tissues as a control. Some investigators have also proposed that because of the large variation in telomere length in the human population the corresponding normal tissue should be from the same individual (Harley & Villeponteau, 1995).

The decline of tumour telomeres was initially observed in Wilm's tumour, breast, and colon carcinomas (de Lange *et al,* 1990; Hastie *et al,* 1990). However, more recently, telomere loss has been documented in most major human tumour types (Odagiri *et al,* 1994; Shirotani *et al,* 1994; Mehle *et al,* 1994; Ohyashiki *et al,* 1994). In the majority of the cases, the tumour telomeres have lost 3-5 Kb. If tumour telomeres shorten as fast as most normal somatic telomeres, loss of 3-5 Kb of telomeric DNA suggests that 30-100 population doublings have occurred during the clonal expansion of the

originally transformed cell. However, this replicative age is not unexpected. From the clinical point of view, most human tumours must go through at least 30 doublings to become sufficient large for detection. In addition, it should be stressed that growth period of a tumour may far exceed the number of divisions estimated from the tumour mass. The later is because of high rates of cell death tempering tumour expansion. These rates may sometimes be up to 80% of cell growth.

On the other hand, there are some large tumours with unexpectedly long telomeres. Moreover, it has been reported that some large (diameter > 5 cm) renal carcinomas appear to have lost only 1.5 Kb of telomeric DNA (Mehle *et al,* 1994). Similarly, long telomeres have been found in some cases of colon carcinomas that must have gone through at least 30 divisions, based on their size (Hastie *et al,* 1990). Two theories have been proposed in order to interpret these data. The first one suggests that these tumour telomeres initially declined but were restored at a later stage by telomerase. The second one proposes that there may be substantial intratumour variation in telomere length. Both theories conclude that telomere length is probably not a good indicator of the replicative history of a tumour. Therefore any relationship between telomere loss and tumour age cannot be established (Hastie *et al,* 1990; Mehle *et al,* 1994).

1.2.8 Loss of telomere function in tumours

Many primary and metastatic human tumours appear to have extremely short telomeres. Although it is not still possible to determine the length of the remaining telomeric repeats accurately, the average length of the remaining repeat arrays in some tumours is only 1-2 Kb per chromosome end. Because of the size heterogeneity of telomeric arrays, many ends must have considerably less copies of telomeric DNA. However, it is difficult to assess a possible functional breakdown in these tumour telomeres because the minimal *cis* - acting for human telomeres are not well defined (Zakian, 1995).

1.2.9 Telomerase activity and Carcinogenesis

Perhaps the best indication that cancer cells are affected by the status of their telomeres comes from the observation that telomerase activity is often increased in a wide variety of human tumours. In human cancer, high telomerase activity was first detected in ovarian carcinoma ascites. The later established a correlation between presence of the enzyme and metastatic or recurrent disease. Subsequent studies have confirmed the association between telomerase activity and cancer. Moreover, a survey of about 200 malignant samples from many different sites reported high telomerase activity in 90% of cancer tissues tested. Benign diseases and normal tissues, which were used as controls, appeared to be negative (Kim *et al,* 1994). The later clearly indicates that most often telomerase activation is associated with the transition to the malignant state of a tumour. However, some cases of telomerase negative

malignancies have been reported. These cases include chronic lymphocytic leukaemias and retinoblastoma. Furthermore, the vast majority of the cases of chronic lymphocytic leukaemia have reduced levels of telomerase activity relative to the positive tissue of origin. The later suggests a preponderance of telomerase negative malignant cells. Similarly, in the cases of retinoblastoma, over half of the examined samples appear to have no detectable enzyme activity (Counter *et al,* 1995). However, even in such cases telomerase may be eventually required for tumour expansion. It has been proposed that data available provide some evidence that telomerase is nearly ubiquitous in malignant tissues. The later also provides support for the requirement and role of the enzyme in the multistep process of carcinogenesis. Given the inherent heterogeneity of telomeres in any tissue and the available methods for their measurement, it is not surprising that the evidence relating the timing of telomerase activation to the presence of critically short telomeres is far less clear cut. However, there is support for the possibility that the two events may be concomitant. The later is based on three key observations. First, telomeres do shorten during tumourigenesis and a pattern of decreasing telomere length with increasing disease severity has been reported for some types of human tumours. Second, in nearly every type of cancer, with only a few exceptions, there are specimens with critically short telomeres. Third, longitudinal studies of late stage cancers have shown *in vivo* maintenance of short telomeres (Rhodes & Giraldo, 1995).

Tumours with long telomeres have also been described. Although there is no enough data for telomerase activity in these specimens, the widespread presence of the enzyme in malignant tissues implies that telomerase positive tumours with long telomeres should exist. Such cases have been reported in some types of human tumours, including lung cancers, lymphomas, and leukaemias. Contamination of tumour biopsies with normal cells, heterogeneity of the malignant cell population in terms of proliferative history, or elongation of short telomeres by a deregulated telomerase can all be plausible causes for this phenomenon. One more possibility is that telomerase may be reactivated independently of telomere length since stable telomeres of intermediate to long size have been detected in some cases (Kipling, 1995; Sharma *et al,* 1996). Nonetheless, tumours with very long telomeres may be the counterpart of recently described immortal human cells that maintain telomere length by telomerase independent mechanisms (Saltman *et al,* 1993).

Two genetic phenomena frequently observed in cancer genomes include loss of heterozygosity (LOH) and gene amplification. Both LOH and gene amplification could be attributed, at least in part, to deteriorating tumour telomeres. However, these observations need to be further corroborated and more is still to be known. Also, we need to know more about other genomic malformations, which are caused by deficient chromosome ends. In the near future, model systems should become available which will allow manipulation of telomere function in mammalian cells. The later is expected to give answers to some of the topics that appear still to be unclear. There is enough data

proving that telomerase activity appears to be elevated in a wide variety of human cancers. Its prevalence suggests that the increase in telomerase activity occurs at a pre-clinical stage in tumourigenesis. One possibility is that most tumours go through many more divisions than their accumulated mass suggests, depleting their telomeres at an early stage. They also require telomerase-mediated telomere restoration prior to their clinical appearance. Another possibility is that telomerase benefits budding tumours in ways we have not discovered yet. Moreover, telomere restoration may prove to be an eventual kind of side effect of a more acute demand for telomerase deregulation in the early stages of tumour development. Perhaps certain changes in telomerase can modify the cell cycle progression or may alter damaged DNA (Morin, 1996; Healy, 1995). Nevertheless, it seems to be crucial to know whether the enzyme is in other ways important to transformed cells.

One of the most important considerations appears to be the origin of telomerase positive cancer cells. One proposed hypothesis is that telomerase positive stem cells represent the progenitor cells for cancer. The fact that some early pre-malignant lesions are telomerase positive appears to be consistent with this opinion. However, for a pre-malignant lesion derived from a telomerase positive stem cell to be telomerase silent two assumptions should be made. The first one is that the overwhelming majority of the cells in the lesion represented more-differentiated telomerase negative progeny of the actual cell leading to cancer. The second one is that the population size of these precursor

cancer cells was large enough in order to permit sufficient secondary and tertiary mutations to occur, leading to cancer progression. The most widely accepted model for the origin of telomerase positive cancer cells argues that most adult tumours develop from telomerase negative precursors (Autexier & Greider, 1996; Villeponteau, 1996).

1.2.10 Clinical importance of telomerase

The apparent lack of telomerase in most somatic tissues and its presence in most malignant tissues render the enzyme a very prevalent tumour marker. The correlation between telomerase activity and the proliferative history of cells predicts that benign growth and pre-neoplastic disease should generally be telomerase negative. In addition, detection of the enzyme at these stages may signify disease progression and be of diagnostic value. On the other hand, lack of enzymatic activity in advanced neoplasias could predict a less aggressive course of disease or even spontaneous regression, making telomerase a prognostic marker. There have been numerous studies in order to assess telomerase as a diagnostic or prognostic marker in almost all types of human cancer (Shay & Gazdar, 1997). In addition, various forms of the telomeric repeat amplification protocol (TRAP) assay have been employed in many different types of clinical specimens to evaluate detection of telomerase activity as a diagnostic tool and compare it with other diagnostic methods currently used in the diagnosis in certain cancer cases (Califano *et al,* 1996; Muller *et al,* 1996; Kinoshita *et al,* 1997; Kyo *et al,* 1997; Shay & Gazdar, 1997; Yoshida *et al,* 1997; Aogi *et al,* 1998).

Since telomere reduction functions as a "mitotic clock", its occurrence points out cells that have undergone more divisions than surrounding cells. Whether or not telomerase has yet been activated, pronounced telomere reduction itself may indicate a relatively advanced stage of tumour cell evolution. Depending on tumour stage, it can reflect initial approach to M1, M1 bypass, or the period of genome destabilisation between M1 and M2. Tumours in which telomere length decreases with increasing tumour grade can be considered as candidates for studies of the correlation between telomerase activity and clinical grade. This is because of the role of telomere erosion in senescence and immortalisation (Sharma *et al,* 1996). Moreover, telomerase's functional association with M2 makes its presence a more robust indicator of cell immortality than are telomere lengths, whose relationship to clinical course and prognosis varies from cancer to cancer. Additionally, since cells with telomerase may stabilise rather than increase their telomere length, and telomeres may on occasion lengthen in the absence of telomerase, it is best not to rely on telomere length to indicate M2 escape. Although not all immortal cells express telomerase, and not all immortal cells are tumourigenic, the vast majority of metastatic cancers studied today have been found to be M2 immortal and express telomerase (Bacchetti & Counter, 1995; Kipling, 1995).

Since the genes for human telomerase RNA (hTRT) and protein moiety (hTERT) have been isolated and completely sequenced (Feng *et al,* 1995; Linger *et al,* 1997), they can provide probes for directly visualising the transcription of telomerase genes, and thus telomerase expression. Also, probes

for the gene encoding the telomerase protein moiety allow visualisation of messenger RNA for the telomerase protein by *in situ* hybridisation in tumour sections, mapping the distribution of telomerase-expressing cells within a solid tumour (Healy, 1995; Soder *et al,* 1997; Snijders *et al,* 1998; Soder *et al,* 1998). Furthermore, visualisation of transcription of the telomerase gene could make possible to trace the evolution of an immortal or metastatic clone out of a telomerase negative tumour cell population. It might also be especially effective where less malignant and more malignant cells inhabit adjacent tumour regions (Sharma *et al,* 1996).

Since telomerase activation appears to be a late event in tumour progression, therapies attacking telomerase are expected to affect cellular immortality and the metastatic phenotype. However, they will not kill tumour cells directly. Inhibition of telomerase activity in M2 immortalised cells would shove them back through the M2 portal, leaving them with the accumulated genetic damage of the M1-M2 interval and the entire preceding carcinogenic sequence. Such cells are expected to remain cancerous, but will also have a finite lifespan. In addition, the number of divisions in that lifespan will depend, in part, on the telomere length established while in the M2 bypassed state. This is because telomere reduction will begin again at the first mitosis after telomerase was inactivated (Healy, 1995).

Proposed anti-telomerase therapies could include pharmacological inhibition, transcriptional repression, or genetic intervention. The development of these telomerase inhibition approaches awaits the complete structural and functional characterisation of the human telomerase holoenzyme. Nevertheless, as mammalian telomerases begin to be isolated and studied structurally and functionally, comparison with eukaryotic telomerases should permit the development of specific inhibitors suitable for therapeutic induction of cancer-cell senescence.

The absence of telomerase activity in normal somatic mammalian cells probably reflects a gene that is turned off rather than an enzyme complexed to a diffusible inhibitor. This is because the mixing of telomerase negative cell extracts into telomerase positive ones does not seem to decrease assayable telomerase activity. It has also been proposed that telomeric heterochromatin silences genes near telomeres and the decrease in heterochromatin as telomere erosion occurs allow expression of these genes to begin. Thus, senescence could be mediated by position effects on the expression of regulatory genes. This mechanism could also mediate the expression of telomerase at M2 escape. When the mechanism responsible for repression of telomerase is identified, developmental therapeutic strategies to reinstate repression and induce M2 crisis in malignant cells may be able to be developed (Wright & Shay, 1992; Morin, 1996).

The recent isolation and sequencing of telomerase genes is expected to make genetic interventions possible. Antisense oligonucleotides to the mRNA or the enzymatic RNA could be used to prevent telomerase synthesis or inhibit enzymatic function. Introduction of a mutant telomerase gene could induce cellular senescence. The later will result in mutant telomeric repeats. Similar experiments carried out in yeast and *Tetrahymena* showed induction in cellular senescence (Lundblad *et al,* 1989, Yu *et al,* 1990). In addition, telomere binding proteins may represent other therapeutic targets.

1.3 The *p53* tumour suppressor gene

1.3.1 Structure of p53

The p53 gene, which has been mapped on the short arm of chromosome 17 (17p13.1), codes for a phosphoprotein made up of 393 amino acids and resides in the nucleus of the cell. In addition, the p53 gene consists of 11 exons but exon 1 is a non-coding exon. It was first discovered as a protein that could bind with the virally encoded large T antigen, responsible for the transformation of cells by simian virus 40. Antibodies raised against the large T antigen immunoprecipitated a protein of 53 kD, hence it was called p53 (Steele *et al,* 1998).

The central region of the open reading frame of p53, consisting of amino acids from about 100 to 300, contains the DNA-binding domain. This

proteolysis-resistant core is flanked by a C-terminal end, mediating oligomerization and an N-terminal end, containing a strong transcription activation signal (Vogelstein & Kinzler, 1994). The structure of p53 appears to be unique, consisting of a large beta-sandwich that acts as a scaffold for 3 loop-based elements. The sandwich is composed of 2 anti-parallel beta-sheets containing 4 and 5 beta-strands, respectively. The first loop binds to DNA within the major groove, the second loop binds to DNA within the minor groove, and the third loop packs against the second loop to stabilize it (Cho *et al,* 1994). In addition, it was pointed out that one of the most notable features of the structure is its correlation with the data on mutations. The residues most frequently mutated in cancers are all at or near the protein-DNA interface, and over two-thirds of the missense mutations are in 1 of the 3 DNA loops (Vogelstein & Kinzler, 1994).

The consensus binding site of p53 has been identified, appearing to have a striking internal symmetry and consisting of 2 copies of a 10-basepair motif separated by 0-13 bp. One copy of the motif was insufficient for binding, and subtle alterations of the motif, even when present in multiple copies, resulted in loss of affinity for p53 (El-Deiry *et al,* 1992). In addition, tetramerization of p53 occurs by interactions between the p53 monomers through a C-terminal domain comprising amino acid residues 325-356 (Pavletich *et al,* 1993). The crystal structure of this tetramerization domain at 1.7-angstrom resolution and the physical properties of the tetrameric interaction have also been described (Jeffrey *et al,* 1995).

The regulatory regions of the human p53 gene have been well characterized and studied. Two promoters have been identified; the first located 100 to 250 bp upstream of the non-coding first exon, and the second which is a stronger promoter, within the first intron (Reisman *et al,* 1988).

1.3.2 p53 in normal cells

Normal p53, also referred to as wild-type p53, suppresses outgrowth of genetically damaged, hence potentially neoplastic, cells in two distinct ways. The first one is by causing a pause in the cell cycle while the second one is by promoting exit from the cell cycle altogether. The latter process is also known as programmed cell death or apoptosis. This dichotomy is thought to allow an appropriate biological response to the two sequelae of DNA damage: Either genome integrity is restored by DNA repair, in which case cells can be released from transient cell cycle arrest, or when damage persists, cells can be permanently eliminated from the population by apoptosis. This function of p53 as "guardian of the genome" may extend to a role in initial monitoring and repair of DNA damage in addition to direct control of cell growth and death (Lane, 1992; Wang *et al,* 1995).

Suppression of cell transformation is mediated by specific binding of p53 tetrameres to DNA at its recognition motifs in the promoter of the wild-type p53-activated fragment (WAF1) gene (El-Deiry *et al,* 1993). WAF1 is also known as CIP1 or p21 gene, encoding for a universal inhibitor, known as

p21 or CDKI, of the cyclin-dependent kinases that govern cell cycle progression (Harper *et al,* 1993; Xiong *et al,* 1993). When levels of p21 inhibitor rise, the cyclin/CDK complexes it binds to can no longer phosphorylate members of the retinoblastoma (Rb) tumor suppressor protein family. Moreover, underphosphorylated Rb sequesters the E2F transcription factors required for producing the DNA synthesis machinery (Qin *et al,* 1995). Thus, the cell cycle is blocked prior to entering the S-phase of the cell cycle.

Regulation of this G1/S boundary is a critical checkpoint in the cell cycle and is potentially inhibited by p21. Cyclin/CDK inhibition is sufficient for growth suppression of cells (Chen *et al,* 1995), but the p21 inhibitor may also interfere with DNA synthesis directly by binding to proliferating cell nuclear antigen (PCNA), an essential factor in DNA replication (Waga *et al,* 1994). However, p21 levels can also be increased by a variety of other mechanisms independent of p53 transactivation (Parker *et al,* 1995).

A second gene under transcription control by p53 affecting cell cycle kinetics is GADD45 (growth arrest DNA damage) (Smith *et al,* 1994). GADD45 encodes a protein that, like p21, inhibits DNA synthesis by binding to PCNA. The MDM-2 protooncogene is also on the growing list of genes found to contain p53-binding consensus motifs and contributes to cell cycle control by a feedback loop to p53 itself (Chen *et al,* 1994). The MDM-2 protein binds to the transcription-promoting domain in the N-terminal of p53,

inhibiting this activity (Momand *et al,* 1992). Finally, p53 controls its own transcription. Other genes regulated by p53 do not affect growth response. However, they may modulate response(s) to other pathways contributing in the multistep process of carcinogenesis.

In response to DNA damage, p53 can trigger exit from the cell cycle and chromosomal disintegration by initiating an active enzymatic process of cell death, also referred to as apoptosis (Steller, 1995). The mechanism of p53-induced apoptosis is not well understood yet. However, the equilibrium of genes playing an important role in the process of apoptosis, like bax and bc1-2, may be shifted by p53 in favor of cell death (Steller *et al,* 1995). Furthermore, p53 increases levels of the apoptosis-promoting factor BAX, which has the p53 recognition motif in its promoter and represses levels of the apoptosis-blocking protein bc1-2 (Miyashita & Reed, 1995). However, mechanisms not involving transcriptional activation by p53 and pathways entirely independent of p53 have also been described (Steller *et al,* 1995). Nevertheless, the molecular pathways and gene families regulating cell death in differentiation and development are cell/tissue type specific raising the importance of p53 in clinical medicine.

Specific DNA binding of normal p53 protein is negatively regulated by its C- terminal domain, in which, sites for phosphorylation and tetramerization critical for high activity are located. The capacity for p53 to adopt different

conformations and hence assume different activity states is also influenced by its oxidation state and binding to zinc metal ions, potentially serving as sensors of oxidative and genotoxic stress (Hainaut & Milner, 1993). In addition, various cellular processes affect nuclear levels of active p53 protein. The latter includes protein and messenger RNA stability, cellular localization, and cytoplasmic sequestering (Maxwell & Roth, 1994).

When genetic damage such as DNA strand breaks occurs in a cell with normally functioning p53, levels of the active protein rise, and the cell cycle is arrested or the process of cell death is induced (Lee *et al,* 1995). It is not known what cellular signals decide between these alternatives or convert cell arrest to cell death. Nevertheless, cells deficient in functional p53 are genetically unstable and become permissive for opportunistic gene amplification or chromosome loss through DNA strand breakage and rejoining (Iskizaka *et al,* 1995). Increasing genetic disorder in the form of aneuploidy and genetic recombination events with loss of genetic material, known as loss of heterozygosity (LOH), can also accumulate in the cell population when unrepaired damage to nuclear macromolecules persists through the stages of cell division. The inability to delay cell division processes increases the probability that DNA damage will remain uncorrected during DNA replication, leading to neoplastic transformation and progression.

1.3.3 p53 in cancer cells

Between 30 and 70% of malignant tumours of almost every organ and histologic subtype have a point mutation in one of the two p53 gene copies and loss of the other allele (Greenblatt *et al,* 1994). In addition, loss of p53 function by other mechanisms may be important in some of the cancers that do not have p53 allele loss or mutation. Most mutations found in tumours are missense base substitutions in the p53 coding sequence that change a single amino acid in the core domain, which governs conformation and specific interactions with DNA. Sites where mutations are especially likely to occur (hot spots) cluster at points where the protein is in close proximity to DNA or makes direct contact when the tetramer binds to its recognition motif (Greenblatt *et al,* 1994).

One conformationally altered molecule of a p53 tetramer can disturb DNA binding, so that one aberrant allele can be sufficient to compromise tumor suppressor function (Hann & Lane, 1995). The latter, however, does not follow the classic "two-hit" model for inactivation of tumor suppressor genes. This model suggests that both parental copies have to be inactivated before growth suppressor activity in the cell is affected. Thus, the relative vulnerability of p53 as a site for genetic lesions in cancer development may rely partly on the large number of possible sites where minute damage will cause a major defect, and partly in the fact that damage to just one allele can already compromise suppressor activity and would be selected during growth. In addition, some p53 mutants may acquire new growth-stimulating potential.

The latter mutations have been characterized as oncogenic gain-of-function mutations Greenblatt *et al,* 1994; Hann & Lane, 1995).

p53 mutations represent clonal genetic alterations that provide these cells with a growth advantage over surrounding cells. Thus, p53 can be used as a marker to test the genetic relationship between separate clusters of neoplastic cells. In particular, studies in ovarian cancer have demonstrated identical p53 mutations in scattered peritoneal foci, providing convincing evidence that these foci are derived from a single progenitor cell (Mok *et al,* 1992). However, apparently independent primary tumors of the aerodigestive tract were found to harbor different p53 mutations (Chung *et al,* 1993). It should be stressed that there is emerging evidence indicating that p53 mutations usually arise later in progression. Therefore, an initial clone arising from an early genetic change may populate a large anatomical area and eventually give rise to individual lesions that have progressed through independent genetic alterations such as p53 mutation. Thus, the presence of identical p53 mutations is strong molecular proof for clonality, but distinct mutations in most tumor types are not sufficient to exclude the possibility of clonality (Greenblatt *et al,* 1994; Hann & Lane, 1995).

Since many but not all tumors contain p53 mutations several studies have been contacted to identify alternative mechanisms for inactivation of the p53 gene. An important endogenous pathway of p53 inactivation is by interaction with cellular protein MDM-2. Although mutations have not been

described in primary tumors, amplification of MDM2 appears to be the preferred mechanism for abrogating p53 function in some tumor types. In particular, in sarcomas, approximately one third of primary tumors contain amplification of MDM-2 associated with increased transcription and expression of the gene product but complete lack of p53 mutations (Leach *et al,* 1993). Amplification or overexpression of MDM-2 has also been shown in subsets of breast, lung, brain, and bladder cancers. In most of cases, p53 mutations are also exclusive, confirming the notion that these two events independently abrogate the p53 pathway.

The best-studied system for exogenous inactivation of p53 is the interaction of wild-type p53 with the E6 protein encoded by certain high risk types of human papillomavirus (HPV) (Galloway & McDougall, 1996). Several studies have demonstrated that the HPV E6 and E7 oncoproteins can cooperate to immortalize human keratinocytes (Hawley-Nelson *et al,* 1989; Steenbergen *et al,* 1996; Steenbergen *et al,* 1998). E6 has been shown to bind p53 and to mediate p53 degradation through the ubiquitin pathway (Scheffner *et al,* 1990; Scheffner *et al,*1993). Functional studies have also demonstrated that introduction of E6 into cells abrogates the p53-dependent G1 cell cycle arrest induced after exposure to DNA damage (Kessies *et al,* 1993). The great majority of cervical carcinomas are HPV positive and several studies have shown that p53 mutations are relatively rare in these tumours. The latter suggests mutually exclusive pathways for p53 inactivation. There are also certain cases of primary tumors that are both HPV positive and p53 mutation is

present (Howley, 1991). Although such cases are rare, they have led to the speculation that other pathways, like the E7 protein abrogating the Rb pathway, might be equally important factors for tumour progression. Furthermore, mutant p53 may still confer an additional growth advantage for affected cells. However, this question has yet to be addressed.

1.3.4 Mutagenesis of p53 in human tumours

Alteration or inactivation of p53 by mutation, or the interaction of p53 with oncogene products of DNA tumour viruses, can lead to cancer. These mutations seem to be the most common genetic change in human cancers. The effects of producing single copies of exogenous p53 genes, containing either point-mutated or wild-type versions of the P53 cDNA sequence, when introduced by infecting the cells with recombinant retroviruses, were initially studied in a human osteosarcoma cell line. Expression of wild-type p53 suppressed the neoplastic phenotype. In addition, using a 2-allele configuration, wild-type p53 was phenotypically dominant to mutated p53 (Chen *et al,* 1990).

The p53 tumour suppressor gene is found to be mutated and abundant in a wide variety of human tumours. p53 mutations were initially characterized in colon cancer where they invariably occurred between the pre-invasive (adenoma) and the invasive (carcinoma) stage (Baker & Preisinger, 1990). In addition, staining of primary colorectal carcinomas with antibodies specific for

p53, demonstrated overexpression of the protein in about 50% of cases. All benign adenomas included in the study were negative for p53 overexpression (Rodrigues *et al,* 1990). Further mutational analysis of p53 has revealed a similar progression in astrocytomas (low to high grade) (Sidransky *et al,* 1992), in squamous cell carcinoma of the head and neck (dysplasia/carcinoma *in situ* to invasive tumors) (Boyle *et al,* 1992), and in thyroid cancer (generally in poorly differentiated anaplastic carcinomas) and leukemias (Kastan *et al,* 1991; Wada *et al,* 1994). These research reports are based almost entirely on molecular analysis of the conserved portion of the p53 (exons 5-8), the region where most mutations occur. Many other studies have been conducted using immunohistochemical analysis to detect stabilization of mutant p53 protein as a marker of p53 mutation. However, many of these studies have been hampered by a lack of sequencing analysis to confirm the presence of these mutations.

Early reports on the mutational status of p53 in hepatocellular carcinomas from patients in high incidence areas revealed that 50% of the tumours had a point mutation at the third base position of codon 249 (exon 7). The great majority of these point mutations were G-to-T transversions (Hsu *et al,* 1991). The finding that no mutations were detected in exons 5, 6, 8, or the remainder of exon 7 was in contrast with previous reports in carcinomas and sarcomas of lung, colon, esophagus, and breast. The latter reports had demonstrated that p53 mutations are scattered over 4 of the 5 evolutionarily conserved domains (exons 5-8), which include codon 249. Later reports demonstrated that p53 mutations in hepatocellular carcinomas do not

exclusively occur at codon 249. The conflict was attributed to the high contamination of food with the mycotoxin aflatoxin B1 and high risk of Hepatitis B virus (HBV) in certain geographical regions (Bressac *et al,* 1991; Patel *et al,* 1992; Buetow *et al,* 1992; Greenblatt *et al,* 1994).

Rearrangements of the p53 gene were reported in 50% of osteogenic sarcomas in a relatively large study, which included sarcomas, leukemias, and lymphomas, (Masuda *et al,* 1987). In addition, the rearranged genes expressed levels of p53 protein that were elevated relative to other tumours. In osteosarcomas, homozygous deletion and lack of expression of p53 RNA or aberrant expression of p53 protein have also been detected. The fact that other, primary mutations have been defined in these tumours suggests that the change in p53 plays a progressional role (Mulligan *et al,* 1990).

Analysis of p53 mutational status in cases of astrocytic tumours identified mutations in glioblastomas and anaplastic astrocytomas, but in none of the more benign pilocytic astrocytomas (Schiffer *et al,* 1995). An earlier report had indicated that human glioblastomas in which p53 gene mutations were found had an earlier age of onset than did tumours without p53 gene mutations. Also, the average postoperative survival among patients with demonstrable p53 mutations was considerably longer than that of the group without such mutations (Chung *et al,* 1990).

The development of cervical cancers has been linked with infection by HPV. The virally encoded oncoproteins E6 and E7 form a complex with cell-encoded protein products of tumor suppressor genes p53 and RB. It is likely that these viral-host protein interactions result in loss of the negative growth control normally exerted by p53 and RB (Galloway & McDougall, 1996). There is evidence suggesting that HPV negative cervical cancers have point mutations in the p53 gene. The latter indicates that loss of wild-type p53 function is a critical event in the pathology of genital cancer, and that in the absence of HPV infection, p53 loss of function occurs via somatic mutation (Crook *et al,* 1992; Kaelbling *et al,* 1992). It has recently been suggested that p53 codon 72 polymorphism genotypes in association with HPV infection may confer a higher risk of developing cervical cancer. The latter has been demonstrated in p53 Arg/Arg women infected with HPV 16 350T variants (van Duin *et al,* 2000).

In certain breast carcinoma cases, an unusual pattern of p53 protein staining has been observed indicating that the protein may be limited to the cytoplasm and absent from the nucleus. Sequencing analysis revealed that p53 cDNAs derived from the samples with cytoplasmic staining revealed only wildtype p53. In contrast, cases showing nuclear p53 contained a variety of mutations. These observations have led to the suggestion that some breast cancers inactivate the p53 by sequestering the protein in the cytoplasm, away from its site of action in the cell nucleus. The latter phenomenon is referred to as nuclear exclusion (Moll *et al,* 1992).

p53 mutations can be found in about 55% of squamous cell carcinomas of the skin. Involvement of UV light in these p53 mutations is usually indicated by the presence of a CC-to-TT double-base change, which may be induced only by UV. Moreover, UV is implicated in such cancer cases by a UV-like occurrence of p53 mutations exclusively at dipyrimidine sites, including a high frequency of C to T substitutions (Brash *et al,* 1991, Dumaz *et al,* 1993). It has also been shown that p53 mutations induced by ultraviolet radiation can be found in more than 90% of actinic keratosis cases, which later progressed to squamous cell carcinomas of the skin. Actinic keratosis is considered a very early stage in the proposed progression model for squamous cell carcinomas of the skin (Ziegler *et al,* 1994).

The p53 tumour suppressor gene is commonly mutated in squamous cell carcinoma of the head and neck (SCCHN) and appears to be one of the molecular targets of tobacco-related carcinogens. Early studies demonstrated a positive correlation between immunohistochemical detection of p53 and a patient history of heavy smoking (Field *et al,* 1992). In addition, the majority of tumours from patients who had stopped smoking for more than five years prior to presentation immunostained for p53, suggesting that p53 gene alterations were an early event in the development of these cancers. It should be stressed that p53 positive staining is suggestive but not necessarily indicative of a p53 mutation. However, the association between p53 mutations and smoking, in SCCHN, has been confirmed in both carcinomas and pre-malignant lesions of the head and neck (Brennan *et al,* 1995; Lazarus *et al,*

1995). GC→AT transitions are the predominant type of mutations found in SCCHN from smokers. The p53 mutational profile of SCCHN cases is usually similar to that of non-small cell lung carcinoma cases of smokers. The latter is because of the chemical carcinogens found in tobacco smoke, which induce a specific pattern of mutations (Waridel *et al,* 1997). In addition, the incidence of p53 mutations among present and former smokers has been reported to be significantly higher than in non-smokers (Liloglou *et al,* 1997). It has also been reported that p53 mutations predominate in SCCHN cases with low genetic damage, as indicated by the fractional allelic loss (FAL) value (Liloglou *et al,* 1997). Therefore, an early initiating role for p53 has been suggested in the development of certain SCCHN cases.

Molecular analysis studies in the p53 gene have been performed in all types of human lung cancer and their findings indicated that the gene was frequently mutated or inactivated. The genetic abnormalities included gross changes such as homozygous deletions and abnormally sized mRNAs, along with a variety of point or small mutations (Carbone *et al,* 1994; Zheng *et al,* 1994). Moreover, mutations were localised in the p53 open reading frame conferring change of the amino acid sequence in a highly conserved region (exons 5-8). Very low or absent expression of p53 mRNA in lung cancer cell lines compared to normal lung was also seen (Takahashi *et al,* 1989). Another study suggested that p53 is the gene that most commonly undergoes mutation in lung cancer. Furthermore, it was found that several mutations of specific type (G-to-T transversions) were present resulting in missense changes in

amino acids highly conserved in evolution (Iggo *et al,* 1990). These specific mutations have been shown to occur more frequently in smokers than in non smokers with lung cancer and may be the result of specific carcinogenic agents present in tobacco smoke, like benzo[a]pyrene. However, there are reports demonstrating a prevalence of GC-AT transitions instead of GC-TA transversions as expected from a smoking population.. These mutations have been detected preferentially at non-CpG sites suggesting that different environmental carcinogens, apart from those found in the tobacco smoke, may be involved in the pathogenesis of certain lung cancer cases (Liloglou *et al,* 1997b; De Anta *et al,* 1997).

Although p53 mutations can usually be detected in more than 30% of human cancers, haematologic malignancies show a low percentage of p53 mutations (Felix *et al,* 1992). The latter suggests that p53 mutations are not a primary event responsible for cancers of the blood. p53 mutations have not been found in retinoblastoma tumours, despite the frequency with which such alterations can be found in the clinically associated tumour, osteosarcoma. However, retinoblastoma tumours are closely associated with molecular abnormalities in the Rb tumour suppressor gene (Walker *et al,* 1999).

1.3.5 Clinical importance of p53

Tumours progress through a series of genetic changes during their development. Elucidating the timing of critical genetic changes in different

types of cancer contributes significantly to the development of molecular progression models. These progression models can then be used to target early genetic changes, potentially useful for early detection strategies, and those that occur later between the pre-invasive and the invasive, potentially useful for prognostic assays. Moreover, early changes may be particularly attractive as therapeutic targets for intervention. p53 mutations are a common genetic event in almost all human cancers, therefore the timing of their occurrence in different tumour types is of great importance.

In some types of human cancer p53 status has been compared and correlated with overall survival. In particular, it has been shown that p53 overexpression or 17p LOH is associated with a statistically significant decrease in survival in colon cancer (Kern *et al,* 1989; Zeng *et al,* 1994). However, studies in non-small cell lung cancer and head and neck cancer, have shown a growing discrepancy regarding the difference in survival based on p53 status. This has been reported to depend on the method employed in each study in order to assess p53 mutagenicity (Sidransky & Hollstein, 1996). Therefore, controlled studies utilizing a combination of methods in order to analyse the mutational status of p53 are required to settle the issue in most tumour types.

The identification of clonal genetic alterations is an emerging and powerful tool for the detection of human malignancy. Because p53 mutations are so ubiquitous, they are excellent candidate markers for molecular studies.

Although p53 mutations occur often between the preinvasive and the invasive state, many of these lesions are still small, and detection of p53 mutations can be used for detection of pre-clinical cancers. Initial studies have indicated the feasibility of this approach demonstrating the presence of rare p53 mutations in the urine of patients with bladder cancer (Sidransky *et al,* 1991). Thus, morphologic analysis of cytologic samples could be greatly augmented by the use of molecular techniques to detect possible clonal p53 mutations. Other reports have demonstrated the detection of p53 mutations in sputum samples from patients who went on to develop clinical lung cancer. In all cases, the primary tumors that were resected contained the identical p53 mutation identified in the sputum (Mao *et al,* 1994).

p53 mutations can also be used as markers of tumor spread. In the case of head and neck cancer, p53 mutations were detected in the primary tumor. In the cases where a p53 mutation was present, further investigation identified rare cells carrying the same p53 mutations in apparently normal surgical margins and lymph nodes. The latter demonstrated that rare neoplastic cells were often left behind after apparently complete surgical resection. Many of these patients would have been significantly upstaged if lymph node analysis based on molecular analysis had been included. In many of these cases, the positive molecular margin predicted the precise location of tumor recurrence. This type of approach may also be useful for many types of tumors where p53 mutations are common (Brennan *et al,* 1995).

A humoral immune response to mutant p53 has been seen in all types of cancers tested, with the possible exception of gliomas. Current estimates of antibody production in cancer patients range from 5–40%, based on simple ELISA tests (Angelopoulou *et al,* 1994; Rainov *et al,* 1995). It is not clear why only certain patients are able to mount an immune response to mutant p53. Possible explanations for this selective anti-p53 response include loss of tolerance due to accumulation of the more stable mutant forms and increased immunogenicity due to conformational tertiary changes induced by specific mutations (Lubin *et al,* 1993). A prerequisite for antibody synthesis in most malignancies appears to be a missense mutation. However, the latter is not sufficient to determine whether or not antibody will be present. Certain p53 mutations in combination with specific class-I and -II HLA antigens involved in processing and presenting the p53 oncoproteins may determine whether an immune response will occur (Wiedenfeld *et al,* 1994). Instances where p53 antibodies in asymptomatic individuals have heralded the development of clinical cancer months to years later suggest that serum screening of high-risk groups may be an appropriate addition to early detection screening strategies (Lubin *et al,* 1995). Moreover, antibody titers drop sharply after therapy, which implies that monitoring the efficacy of a therapy or the presence of an occult recurrence with anti-p53 titers may be possible.

The role of p53 in response to DNA damage induced by radiation or chemotherapy has prompted significant interest in alternative strategies for therapy of human cancers. An important hypothesis has emerged proposing

that primary tumours with p53 mutation may not recognize DNA damage and thus may not induce the normal apoptotic pathway for self-destruction. It would follow that most human tumors with abrogated p53 pathways would be relatively resistant to most therapeutic agents. This in turn would explain the severe resistance of most epithelial tumours to commonly used agents (Lowe *et al,* 1993). p53 may also induce suppression of angiogenesis, potentially critical for the early neo-vasculization seen in primary tumours. Mutant p53 is unable to suppress this process in gliomas, and p53 may also directly affect transcription of thrombospondin-1, an angiogenesis inhibitor (Dameron *et al,* 1994).

Strategies to circumvent this critical apoptotic decision point in p53 mutant tumours might provide therapeutic advantages. However, it is already clear that p53 induction of apoptosis in response to DNA damage varies according to cell type. Some studies on epithelial cells have shown little difference in response to radiation-induced change regardless of p53 status. However, hematologic malignancies appear to depend on this critical recognition by wild-type p53 for apoptosis. This notion is strengthened by resistance to therapeutic agents in hematologic neoplasms with p53 mutation. Thus, the role of p53 in the apoptotic pathway, the presence of different genetic changes in different tumor types, and the growth factors in which the neoplastic cell resides may all be critical in determining response to these agents (Hartwell & Kastan, 1994).

1.4 DNA Mismatch Repair

One of the most exciting recent advances in the molecular biology of cancer is the discovery that mismatch repair (MMR) defects are carcinogenic. As DNA repair systems are designed to maintain the integrity of the genome and a fundamental feature of cancer is genomic instability, it is perhaps not so surprising that defects in these systems are carcinogenic (Eshleman & Markowitz, 1996). The later has been well demonstrated by the excision repair defects in *Xeroderma pigmentosum* (Sancar, 1994).

MMR was described first in bacteria, later in yeast and finally in higher eukaryotes. One of its roles in bacteria is to recognise and repair mistakes made by the DNA polymerases during replication. This provides the bacterial genome with a level of protection against mutation. It also guards the genome by preventing recombination between non-homologous regions of DNA (Modrich, 1991).

1.4.1 Bacterial Mismatch Repair Pathway

The bacterial system determines which base(s) is incorrect by cueing on the fact that the error-containing nascent DNA strand is transiently unmethylated. Although the system requires 10 independent components, three critical ones have been identified. These are MutS, MutL and MutH which have been named after their corresponding bacterial mutator strains. Moreover,

MutS is the molecule that recognises and binds to the mispair or loop. Afterwards, MutL and MutH form a complex that scans the duplex for the nearest hemimethylated site. MutH then nicks on the unmethylated strand and an exonuclease excises the nascent strand from the nick back past the mismatch. This patch is resynthesised and finally ligated (Umar *et al,* 1994).

1.4.2 Human Mismatch Repair Pathway

Comparison of the human MMR system to the bacterial system reveals extensive similarities. Both systems have been shown to provide the genome with a 100-1000-fold level of protection against mutations arising during DNA replication. Also, both direct repair to the newly replicated DNA strand, require multiple components and can excise the nascent strand in either direction to the mismatch. However, a major difference between the two systems is that the human system has multiple homologues for each bacterial component. In particular, homologues of MutS include hMSH2 (human MutS hologue 2), GTBP2 (G-T mismatch binding protein) and hMSH3. Although it has been demonstrated that the proteins encoded by hMSH2 and GTBP2 bind the mismatch as a heteroduplex, it is not yet fully understood how hMSH3 participates in the pathway. In addition, multiple human homologues of MutL have been identified. These include hMLH1 (human MutL homologue 1), PMS1 (post-meiotic segregation 1) and PMS2. The proteins coded by hMLH1 and PMS2 bind as a heteroduplex after the initial binding to the mismatch by the MutS heterodimer (Schaaper, 1993; Fishel & Wilson, 1997).

1.4.3 The Replication Error (RER) Phenotype and Carcinogenesis

The first indication that defective MMR is an important factor in carcinogenesis was the discovery of the replication error (RER) phenotype of microsatellite instability in sporadic and inherited colon cancer. Microsatellites are normally stable repetitive sequences where the repeating fragment is between one to six bases long. It is their repeating nature that makes microsatellites particularly prone to slippage during replication. The later results in the formation of a small loop in either the template or nascent DNA strand. Despite these replicative mistakes which occur in all cells, microsatellites are normally stable in length because of the efficiency of the MMR system. However, a defect in the MMR system leads to the RER phenotype of microsatellite instability. In addition, it has been shown that RER cancer cells exhibit an increased mutation rate in endogenous expressed genes. The later is referred to as mutator phenotype (Aaltonen *et al,* 1993; Thibodeau *et al,* 1993; Eshleman & Markowitz, 1996).

Ionov et al (1993) provided evidence for the mutator phenotype hypothesis as a molecular mechanism in carcinogenesis. This involves a mutation in a DNA replication or repair gene which results in decreased efficiency of the MMR system. They estimated that cells from tumours with a mutator phenotype can carry more than 100,000 mutations in microsatellite repeat sequences. They concluded that these mutations reflect a previously undescribed form of carcinogenesis in the colon mediated by a mutation in a DNA replication factor resulting in reduced fidelity for replication or repair. In

addition, it has been demonstrated that the mutation rate of (CA)n repeats in RER(+) tumour cells is at least 100-fold that in RER(-) tumour cells and affects extrachromosomal as well as endogenous genomic sequences (Parsons *et al,* 1993). Moreover, using *in vitro* assays, it has been showen that the mutability of RER(+) cells is associated with a profound defect in strand-specific mismatch repair. This deficiency was observed with microsatellite heteroduplexes as well as with heteroduplexes containing single base-base mismatches and affected an early step in the repair pathway. Thus, a true mutator phenotype exists in a subset of human tumours (Parsons *et al,* 1993).

The subset of sporadic colorectal tumours and most tumours developing in hereditary nonpolyposis colorectal cancer (HNPCC) patients, containing alterations in microsatellite sequences, are thought to manifest replication errors and are referred to as RER(+). Moreover, RER has been well demonstrated in a wide variety of human tumours and linked with mutations in DNA repair genes that participate in the human mismatch repair pathway (Shibata *et al,* 1994; Bodmer *et al,* 1994; Risinger *et al,* 1995; Merlo *et al,* 1994; Orth *et al,* 1994; Wooster *et al,* 1994; Burks *et al,* 1994). The later includes hMLH1, hMSH2, hPMS1, and hPMS2. It is also of note that the mutator phenotype results to an increased mutation rate in a receptor for Tumour Growth Factor beta (TGF-β). It has been reported that TGF-β functions as a tumour suppressor gene in colon cancers that appear to be RER positive. TGF-β is a gene at risk because its coding region contains a base

polyadenine repeat. Frameshift mutations have been reported in over 90% of RER positive colon cancers (Markowitz *et al,* 1995; Parsons *et al,* 1995).

1.4.4 hMLH1 gene

1.4.4.1 Mapping of the MLH1 gene

Using RFLPs and microsatellite markers for linkage analysis in 3 hereditary nonpolyposis colon cancer (HNPCC) families, Lindblom et al (1993) demonstrated linkage to 3p23-p21. After human homologs of the mutS gene of bacteria and yeast were found to have mutations responsible for HNPCC, a survey of a large database of expressed sequenced tags (ESTs) derived from random cDNA clones revealed 3 additional human MMR genes, all related to the bacterial mutL gene. One of these genes (MLH1) was mapped to 3p21.3 by fluorescence *in situ* hybridisation (FISH). The other 2 genes had a slightly greater similarity to the yeast mutL homolog PMS1 and were therefore denoted PMS1 and PMS2, respectively (Papadopoulos *et al,* 1994). Another study by Bronner and co-workers (1994) also implicated the human MutL homolog, MLH1, in the form of HNPCC that maps to 3p. They mapped the MLH1 gene to the same region, 3p23-p21.3, by FISH. Using 19 dinucleotide markers and haplotype analysis in 2 families Tannergard et al (1994) also localized the gene specifically to 3p23-p21.3.

1.4.4.2 Structure of the MLH1 gene

It has been shown that the human MLH1 gene consists of 19 coding exons spanning approximately 100 kb, and that exons 1 to 7 contain a region that is highly conserved in the MLH1 and PMS1 genes of yeast (Han *et al,* 1995). Genuardi et al (1998) characterized the normal alternative splicing of the MLH1 gene and reported a number of splice variants that exist in various tissue types. They observed splice variants lacking exons 6/9, 9, 9/10, 9/10/11, 10/11, 12, 16, and 17.

1.4.4.3 The MLH1 gene in Hereditary Non Polyposis Colorectal Cancer (HNPCC)

Mutations in the MLH1 gene were detected in 7 Finnish HNPCC kindreds (Papadopoulos *et al,* 1994). It is of note that all affected individuals exhibited a heterozygous deletion of codons 578 to 632. Codons 578 to 632 were found to constitute a single exon that encodes several highly conserved amino acids found at identical positions in yeast MLH1 (Papadopoulos *et al,* 1994). In other 3p-linked families, a 4-nucleotide deletion, a 4 nucleotide insertion were also detected (Papadopoulos *et al,* 1994). Furthermore, a missense mutation in affected individuals in 1 chromosome 3 linked HNPCC family has been reported (Bronner *et al,* 1994). Another study also found germline mutations in 8 (24%) of 34 unrelated cancer patients from HNPCC pedigrees (Han *et al,* 1995). A study which evaluated tumours from 74 HNPCC kindreds for genomic instability, characteristic of a mismatch repair deficiency,

found such instability in 68 (92%) of the kindreds. Further investigation demonstrated that in 48 of the kindreds with instability, 15 (31%) harboured mutations in the MLH1 gene (Liu *et al,* 1996). A study of unrelated HNPCC families reported that a cluster of MLH1 mutations were found in the region encompassing exons 15 and 16, which accounts for 50% of all the independent MLH1 mutations described (Wijnen *et al,* 1996).

Maliaka et al (1996) identified 6 different new mutations in the MLH1 gene in Russian and Moldavian HNPCC families. Three of these mutations occurred in CpG dinucleotides. Analysis of a compilation of published mutations including the new data suggested to the authors that CpG dinucleotides within the coding regions of the MLH1 gene are hotspots for single basepair substitutions.

It has been demonstrated that loss of heterozygosity of markers within or adjacent to the MLH1 gene on 3p occurs non-randomly in tumours from members of families in which the disease phenotype co-segregates with MLH1. In every informative case, the loss affected the wild-type allele. These results suggested that DNA MMR genes resemble tumour suppressor genes in that 2 hits are required to cause a phenotypic effect (Hemminki *et al,* 1994).

1.4.4.4 The MLH1 gene in sporadic cancers

It has been reported that hypermethylation of the 5-prime CpG island of the MLH1 gene is found in most sporadic primary colorectal cancers with microsatellite instability (MSI) and that this methylation was often, but not invariably, associated with loss of MLH1 protein expression. Such methylation also occurred, but was less prominent, in MSI-negative tumours, as well as in MSI-positive tumours with known mutations of a mismatch repair gene. Hypermethylation of colorectal cancer cell lines with MSI also was frequently observed, and in such cases, reversal of the methylation with 5-aza-2-prime-deoxycytidine not only resulted in re-expression of MLH1 protein, but also in restoration of the mismatch repair capacity in MMR-deficient cell lines. The results suggested that MSI in sporadic colorectal cancer often results from epigenetic inactivation of MLH1 in association with DNA methylation (Herman *et al,* 1998).

Sasaki et al (1996) studied 43 tumours and corresponding normal tissues from 23 Japanese patients with multiple primary cancers. They found no germline mutations of the MLH1 gene and detected only 2 somatic missense mutations among the 43 tumors examined. These 2 tumours had each shown increased replication error (RER+) at more than 1 of the 5 microsatellite loci examined.

Approximately 20% of endometrial cancers exhibit MSI. Although the frequency of MSI is higher in endometrial cancers than in any other common malignancy, the genetic basis of MSI in these tumours is not clearly understood. A recent study investigated the role that methylation of the MLH1 DNA mismatch repair gene plays in the genesis of MSI in a large series of sporadic endometrial cancers. The MLH1 promoter was found to be methylated in 41 of 53 (77%) MSI-positive cancers investigated. In MSI-negative tumours, on the other hand, there was evidence for limited methylation (1 of 11 tumours studied). Immunohistochemical investigation of a subset of the tumours revealed that methylation of the MLH1 promoter in MSI-positive tumours was associated with loss of MLH1 expression. Immunohistochemistry proved that 2 MSI-positive tumours lacking MLH1 methylation failed to express the MSH2 mismatch repair gene. Both of these cancers came from women who had family and medical histories suggestive of inherited cancer susceptibility. These observations suggested that epigenetic changes in the MLH1 locus account for MSI in most cases of sporadic endometrial cancers and provide additional evidence that the MSH2 gene may contribute substantially to inherited forms of endometrial cancer (Simpkins *et al,* 1999).

Two recent studies demonstrated that homozygosity for MLH1 mutations results in a mutator phenotype characterized by leukemia and/or lymphoma associated with type I neurofibromatosis (NF1). It is of note that these individuals developed hematologic malignancy at a very early age. This

observation indicated that MMR deficiency is compatible with human development, but may lead to mutations during embryogenesis. These observations suggested that the NF1 gene may be a preferential target for such alterations (Ricciardone *et al,* 1999; Wang *et al,* 1999).

1.4.5 hMSH2 gene

1.4.5.1 Mapping of the MSH2 gene

In studies of 2 large kindreds, many individuals who had colon cancer with or without endometrial cancer were found to show linkage to an anonymous microsatellite marker on chromosome 2, specific at locus *D2S123*, which suggested that a gene is in the region of 2p16-p15 (Peltomaki *et al,* 1993; Aaltonen *et al,* 1993). Fishel et al. (1993) cloned and characterized a human MutS homolog, MSH2, and demonstrated that the gene maps to 2p22-p21 by studying of a mapping panel of somatic cell hybrid DNAs using PCR. A study by Leach and collegues (1993) used chromosome microdissection to obtain highly polymorphic markers from 2p16. These and other markers were ordered in a panel of somatic cell hybrids and used to define a 0.8-Mb interval containing the locus linked to HNPCC. Candidate genes were mapped with respect to this locus, and one gene was found to lie within the 0.8-Mb interval. This gene was homologous to a prokaryotic gene, MutS, which participates in mismatch repair (Leach *et al,* 1993).

1.4.5.2 Structure of the MSH2 gene

Kolodner et al (1994) reported that the genomic MSH2 locus covers approximately 73 kb and contains 16 exons. The highest homology was to the yeast Msh-2 gene in the helix-turn-helix domain, perhaps responsible for MutS binding to DNA. The yeast and human Msh-2 proteins were 77% identical between codons 615 and 788. There were 10 other blocks of similar amino acids distributed throughout the length of the 2 proteins (Fishel *et al,* 1993). The existence of alternative splicing in the MSH2 gene has also been reported. Coupled RT-PCR of various tissue samples from normal individuals and HNPCC patients identified MSH2 gene products lacking exons 5, 13, 2-7, and 2-8 (Genuardi *et al,* 1998).

1.4.5.3 The MSH2 gene in HNPCC

Aaltonen et al (1993) reported no LOH for the *D2S123* or other chromosome 2 markers in either familial cases of HNPCC. They demonstrated, however, that most of these cancers had widespread alteration in short repeated DNA sequences, (CA)n dinucleotide repeat fragments, suggesting that numerous replication errors had occurred in the sequences during tumour development. It was proposed that their findings reflect the existence on chromosome 2 of a gene, which is neither an oncogene nor a tumour suppressor gene but rather a gene leading to genomic instability. Furthermore, studies succeeded in identifying specific germline mutations in each of the 2 kindreds that originally established linkage to chromosome 2 (Leach *et al,* 1993). It has also been demonstrated that cancer susceptibility arises either due to the inheritance of a frameshift mutation or a nonsense mutation in the MSH2

gene (Hall *et al,* 1994). Novel pathogenic germline mutations resulting in stop codons, either directly or through frameshifts have also been reported. In addition, MSH2 mutations were found in 21% of the specimens examined but a correlation between the site of the individual mutations and the spectrum of tumour types was not established (Wijnen *et al,* 1995). A finding of note by Pensotti and collegues (1997) was that the Italian kindreds with demonstrated MSH2 mutations had a mean age of colorectal cancer onset of 43 years versus an average age of 53 years for the families without mutations (Pensotti *et al,* 1997). In addition, a high level of microsatellite instability (16 of 18 cases) from individuals with MSH2 mutations and infrequently (1 of 21 cases) in colorectal cancers from individuals without detectable mutations has been reported. Families with germline mutations had individuals affected at younger ages and with multiple tumours (Bapat *et al,* 1999).

Seven new pathogenic mutations in the MSH2 gene were identified in Russian and Moldavian HNPCC families. Three of these mutations occurred in CpG dinucleotides and led to a premature stop codon, splicing defect, or an amino acid substitution in evolutionarily conserved residues. Analysis of a compilation of published mutations including the new data suggested to the authors that CpG dinucleotides within the coding regions of the MSH2 gene are hotspots for single base-pair substitutions. These authors also demonstrated that the latter holds true for the MLH1 gene as well (Maliaka *et al,* 1996).

1.4.5.4 The MSH2 gene in sporadic cancers

MSH2 gene aberrations have been reported in 13% of sporadic colorectal cancers. It is of note that identical abnormalities were also found in HNPCC cases. Both sporadic and HNPCC cases examined shared biologic properties, such as location on the right side of the colon and preservation of diploidy or near-diploidy (Aaltonen *et al,* 1993). Another study by Thibodeau and co-workers examined sporadic colorectal tumour DNA for somatic instability at (CA)n repeats. Differences between tumour and normal DNA were detected in 25 of the 90 tumours studied. The instability appeared as either a substantial change in repeat length (often heterogeneous in nature) or a minor change (typically 2 bp). There was a significant correlation between microsatellite instability and location of the tumour in the proximal colon (right colon) and with increased patient survival. Instability was also correlated with mutations in the MSH2 gene (Thibodeau *et al,* 1993). Another study also found that 12% of sporadic colorectal carcinomas carry somatic deletions in poly-(dA/dT) sequences and other simple repeats which are associated with mutations in the MSH2 gene (Ionov *et al,* 1993).

A study by Orth and collegues found that 50% of ovarian tumour cell lines examined were genetically unstable at most microsatellite loci analyzed. They identified the source of the genetic instability as a point mutation in the MSH2 gene. The patient, whose tissue was used to establish one of the cell lines, was a 38-year-old heterozygote for this mutation and her normal tissue carried both mutant and wild-type alleles of the MSH2 gene. However, the

wild-type allele was lost at some point early during tumourigenesis so that DNA isolated either from the patients ovarian tumour or from the cell line carried only the mutant MSH2 allele. The genetic instability observed in the tumour and cell line DNA, together with the germline mutation in a mismatch-repair gene, suggested that MSH2 is involved in the onset and/or progression in a subset of sporadic ovarian cancer (Orth *et al,* 1994).

Nevertheless, the microsatellite DNA instability that is associated with alteration in the MSH2 gene in HNPCC and several forms of sporadic cancer is thought to arise from defective repair of DNA replication errors that create insertion-deletion loop-type (IDL) mismatched nucleotides. It has been demonstrated that purified MSH2 protein efficiently and specifically binds DNA containing IDL mismatches of up to 14 nucleotides. The above findings supported a direct role for MSH2 in mutation avoidance and microsatellite stability in human cells (Fishel *et al,* 1994).

The aim of this study was to evaluate and assess potential biomarkers, like genetic alterations and telomerase activity, for the identification of individuals at high risk of developing the disease. The potential role of DNA MMR genes, hMLH1 and hMSH2, in the molecular pathogenesis of NSCLC and their regulation and relation to the major cell cycle regulators; p53, $p21^{WAF1}$ and Rb, was also examined. The patient population recruited in this study was from the Merseyside region, Northwest England, which appears to have some of the highest incidence rates of lung cancer in the UK and across Europe.

CHAPTER TWO

LOSS OF HETEROZYGOSITY AND MICROSATELLITE ALTERATIONS IN BRONCHIAL LAVAGE

Abstract

DNAs from the bronchial lavage of 90 individuals who were referred to an early lung cancer clinic in the Northwest of England with suspected lung cancer were studied using 12 microsatellite markers. Genetic alterations were detected in 15 of 43 (35%) patients with lung cancer, but also in 11 of 47 (23%) patients with no cytological or radiological evidence of bronchial neoplasia. No significant differences were found between the referring symptoms in any of the second group of individuals with and without genetic alterations. No correlation was found between smoking exposure and loss of heterozygosity (LOH)/microsatellite alterations (MAs) in the microsatellite markers. On comparing LOH with MAs based on cytology review, it was found that the prevalent type of alteration in specimens with cytological evidence of malignancy was LOH; in contrast, the individuals with no cytological evidence of malignancy showed a preponderance of MAs ($p = 0.01$). These data indicate that a substantial proportion of cells in the bronchial lavage from suspected lung cancer patients carry identifiable genetic alterations. However, the presence of genetic alterations in the bronchial lavage of individuals with no clinical evidence of lung cancer, raises the question whether instability is a phenomenon solely associated with cancer or it may represent a feature of non-neoplastic diseases. The results obtained in this study suggest that microsatellite PCR-based assays can be developed as tools for the earlier identification of genetic changes in cells exfoliating in the bronchus.

2.1 Introduction

Genomic instability is considered to be a hallmark of cancer. It has been proposed as a novel mechanism in the multistep process of carcinogenesis (Lengauer *et al,* 1998; Loeb, 1998; Perucho, 1996). Genomic instability in cells is found in many forms of genetic alterations. Allelic imbalance or LOH mainly represents chromosomal instability (Lengauer *et al,* 1998) whereas microsatellite instability (MI), also found in the literature as replication errors (RER) or MAs, is most often attributed to DNA repair machinery errors. In this study, the term "genetic alterations" will be used to include both LOH and MA phenomena.

The comparative analysis of microsatellite loci in tumour and normal counterpart tissue has become the most widely used method to determine such genetic alterations. Genomic instability is a common phenomenon in lung cancer (Field *et al,* 1996; Merlo *et al,* 1994; Miozzo *et al,* 1996; Neville *et al,* 1996; Shridhar *et al,* 1994) and, in some reports, has been associated with prognosis (Mitsudori *et al,* 1996; Pifarre *et al,* 1997). In a previous study from our group, 42 of 45 (93%) of NSCLC specimens were found to carry LOH or MAs in at least one of the 92 markers examined (Field *et al,* 1996). Furthermore, genetic alterations have been detected in the plasma (Chen *et al,* 1996), bronchial mucosa and sputum specimens of lung cancer patients (Miozzo *et al,* 1996; Mao *et al,* 1994a,b). It is of note that genetic alterations were detected in specimens presenting with minimal atypia (Miozzo *et al,* 1996) and moreover, that LOH and MAs have also been demonstrated in bronchial specimens from chronic smokers

who do not have lung cancer (Mao *et al,* 1997; Wistuba *et al,* 1997). These findings suggest that these genetic alterations precede morphological transformation of the cells.

In this study, DNA from 90 BL specimens was assayed for LOH and MAs to examine whether genetic alterations can be identified in BL from individuals with no cytological or radiological evidence of lung cancer.

2.2 Materials & Methods

2.2.1 Patient Selection and Clinical Samples

90 BL and control blood samples were collected from individuals with suspected lung cancer who have been referred to the Cardiothoracic Centre (CTC) over a twelve-month period. The criteria for referral to the CTC included: (a) unresolved chest infection; (b) abnormal chest X ray; (c) cough (> 4 weeks); (d) non-specific weight loss; (e) stridor; (f) persistent (>3 weeks) hoarse voice and (g) other suspicious features that would prompt referral to the lung cancer clinic. Each patient underwent a full clinical workup for lung cancer including a chest X-ray, spirometry and bronchoscopy. BL specimens were obtained from all of these patients; the choice of site was based on bronchoscopic findings within the large airways, into which, approximately 50 ml of saline were introduced via the bronchoscope and then aspirated.

The selection of patients was undertaken on the basis of an adequate cytology preparation and blood sample availability. The age of the patients selected ranged between 38 and 89 (mean 65). Thirty-three of the individuals were females and 57 were males. Smoking data were available for 85 individuals (74 smokers, 11 non-smokers). The total smoking exposure was calculated as follows:

Pack-years = years smoked × pack/day

A differential cell count was undertaken for all BL samples that were reported as "no malignant cells seen" (NMCS) and the lung cancer patients with genomic instability. The epithelial cells present varied between samples (20-90%).

2.2.2 DNA extraction

2.2.2.1 Bronchial Lavage Specimens

One ml of BL was transferred into 1.5 ml tubes and centrifuged for 5 min, 10,000 rpm, at room temperature (RT). The resulting pellet was resuspended in 400 mM Tris-HCl pH 7.0, 150mM NaCl, 60 mM EDTA, 1% SDS, 100μg/ml Proteinase K and incubated at 42°C for 12-15 hours in an orbital shaker. Deproteinization included the addition of 150 μl 5M Sodium Perchlorate and 500 μl Chloroform. After mixing and microcentrifugation for 2 min at 14,000 rpm, RT, the aqueous phase was transferred into a fresh tube and DNA was precipitated by the addition of an equal volume of isopropanol. After incubation at -20°C for 12-14 h, DNA was recovered with microcentrifugation (14,000 rpm) for 15 min at 4°C, washed with 70% ice-cold ethanol and resuspended in 50 μl of sterile distilled H_2O.

2.2.2.2 Blood Samples

Three ml of blood were washed three times with 10mM Tris-HCl pH 8.0, 320 mM Sucrose, 1% Triton X-100, 5 mM $MgCl_2$ to remove red blood cells. Each washing step was followed by centrifugation at 3,000 rpm for 5 min, RT. Lysis of the resulting white blood cells was similar to that of BL. Deproteinization was carried out by the addition of 500 µl Phenol/Chloroform. After mixing and microcentrifugation (14,000rpm, RT) for 2 min the aqueous phase was transferred into a fresh tube and DNA was precipitated by the addition of an equal volume of isopropanol. DNA was recovered with a sterile loop, washed with 500 µl ice-cold 70% ethanol and resuspended in 200 µl of sterile distilled H_2O.

2.2.3 PCR Amplification of Microsatellite Loci

The selection of microsatellite markers was based on the previous work of the lung cancer group (Deaprtment of Clinical Dental Sciences, University of Liverpool) (Field *et al,* 1995; Field *et al,* 1996; Neville *et al,* 1995; Neville *et al.* 1996) and other workers' published results (Merlo *et al,* 1994; Miozzo *et al,* 1996; Schridhar *et al,* 1994). 12 markers were chosen: *ACTBP*, *D14S50*, *D3S1215*, *D3S1339*, *D3S1351*, *D4S194*, *D4S392*, *FGA*, *D6S271*, *D9S286*, *TCRD* and *TP53*. These markers have been previously shown to exhibit a high frequency of genetic alterations in lung cancer.

Oligonucleotide primers purchased from Research Genetics (Huntsville, USA). The reaction mixture contained 16 mM $(NH_4)_2SO_4$, 67 mM Tris-HCl pH 8.8, 0.1% Tween-20, 200 mM dNTPs, 0.1 mM of each primer, 2 mM $MgCl_2$ and 0.5 U BIOPRO polymerase (BIOLINE, UK). The amplification parameters were: initial denaturation for 2 min followed by 28-30 cycles at 94°C for 30 sec, 55-58°C for 30 sec and 72°C for 30 sec, and final extension step at 72 °C for 5 min.

PCR products were analysed by electrophoresis on 10% non-denaturing polyacrylamide gels and visualised by silver staining (10 min washing in fixation buffer consisting of 10% absolute ethanol and 0. 5% acetic acid. Gels are then stained with 0.1% $AgNO_3$ for 20 min and developed in developer solution consisting of 1.5% NaOH and 0.15% formaldehyde followed by a final fixation step in 0.75% Na_2CO_3 for 5 min. All steps were carried out on a shaker plate).

MA was judged from the presence of an allele (band) with different electrophoretic mobility. LOH was judged visually on the basis of a ≥40% relative reduction of one allele intensity. MA was judged from the mobility shift of one of the alleles or presence of an additional allele on the gel. Positive samples were scored only when the LOH/MA results was confirmed at least twice in separate PCR reactions.

2.2.4 Sensitivity and MA detection

To assess the sensitivity of LOH and MA of our method we prepared DNA samples of various tumour (T) to normal (N) ratios from tumour specimens with known LOH and MAs.

2.2.5 Statistical analysis

Fisher's exact test was employed to analyse the data.

2.3 Results

2.3.1 Sensitivity for LOH and MA detection

The sensitivity analysis showed that LOH may be detected in 1:10 dilution (T/N), while MA was detected in 1:16 dilution (Figure 2.3.1.1).

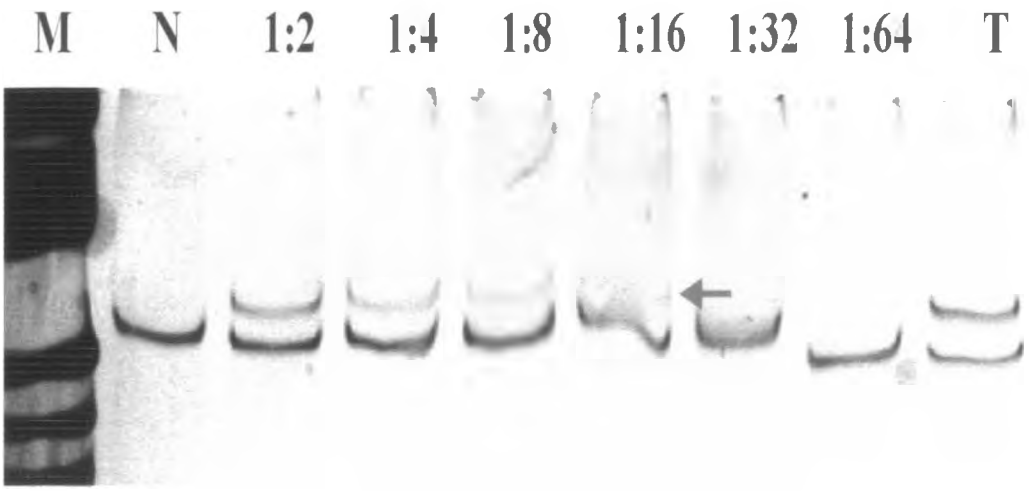

Figure 2.3.1.1. Sensitivity assay for MA detection. DNA from tumours with known MA were spiked with DNA from normal tissue in a range of ratios.

2.3.2 Genetic alterations in lung cancer patients

The clinical findings (cytology, radiology, biopsy) revealed 43 patients with lung cancer, of which 18 individuals were reported with malignant cells present in the cytological specimen; 25 individuals were reported as NMSC but did have a radiological and/or histological diagnosis of lung cancer. The remaining 47 individuals had no clinical evidence of lung cancer (Figure 2.3.2.1).

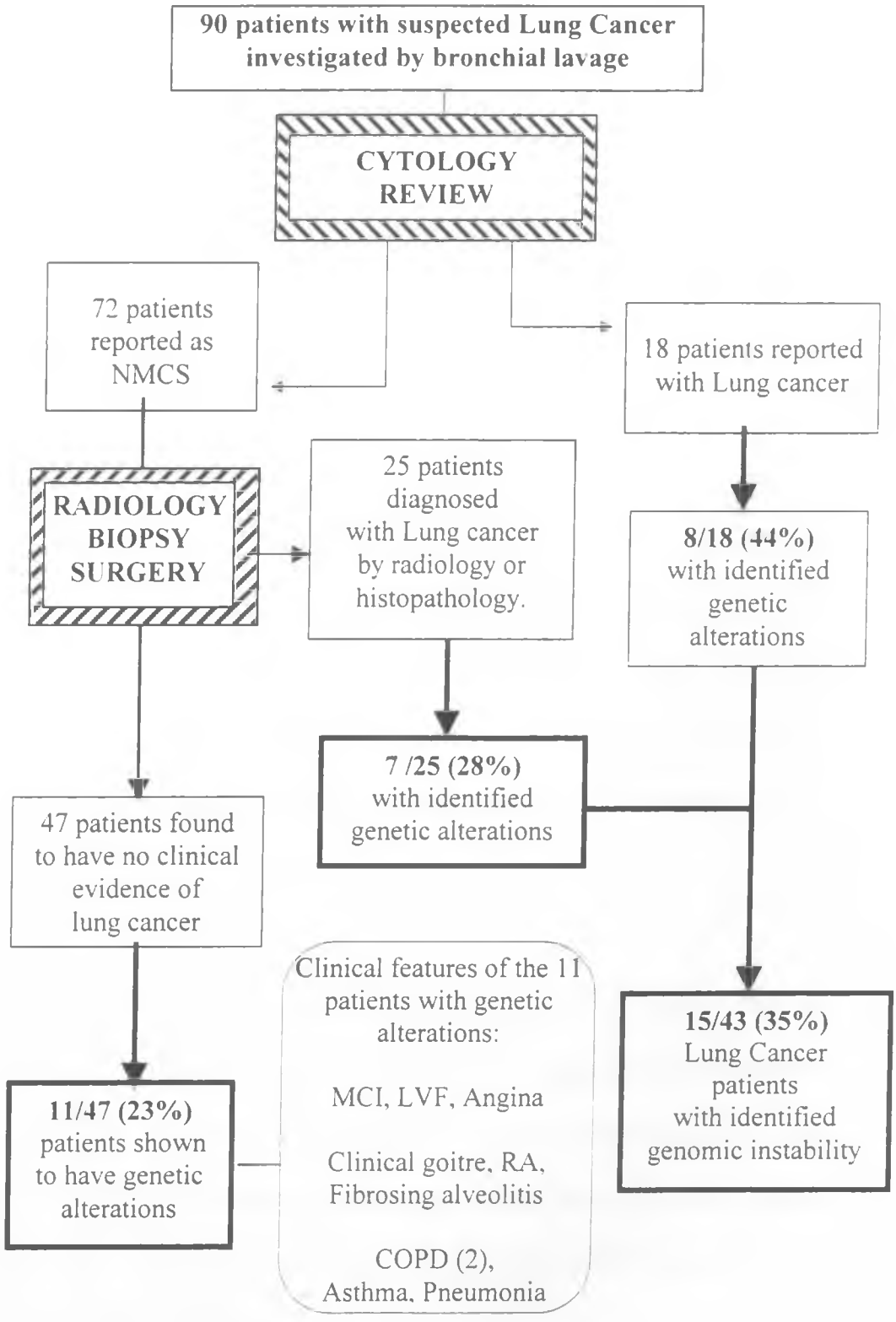

Figure 2.3.2.1. Flow diagram of the 90 patients undergoing a clinical workup for lung cancer in this study. This demonstrates genomic instability in the bronchial lavage specimens from individuals with lung cancer and also in the individuals with no cytological or radiological evidence of lung cancer.

Genetic alterations were identified in 8 of 18 (44%) of the positive cytology cases. All of the eight individuals demonstrated LOH, and three specimens had LOH and or MAs in more than one marker (Table 2.3.2.1). The remaining 10 of 18 carcinomas of the positive cytology group with no evidence of genetic alterations included 9 squamous cell carcinomas and 1 adenocarcinoma.

Seven of the 25 (28%) individuals, who were diagnosed with bronchial carcinoma but had no malignant cells in their BL, exhibited genetic alterations (Table 2.3.2.1). The remaining 18 carcinomas in this group with no genetic alterations, were composed of 9 squamous cell carcinomas, one adenocarcinoma and 8 individuals without histological diagnosis (diagnosed radiographically).

The following abbreviations were used in table 2.3.2.1. pk/yr: pack per year, NED: no evidence of disease, SCLC: small cell lung carcinoma, AdenoCa: adenocarcinoma, RA: rheumatoid arthritis, FA: fibrosing alveolitis, RUL: right upper lobe, LLL: left lower lobe, RLL: right lower lobe, LMB: left middle bronchus, LHC: left hilum consolidation, R: right hilum, MD: mediastinal, H: heterozygous, ND: not determened

Table 2.3.2.1. Clinical details of patients with genetic alterations detected in their BL specimen

Sample	Cytology	Radiology	Biopsy	Surgery	Final Diagnosis	Smoking (pk/yr)[a]	ACTBP2	D14250	D321215	D321339	D321351	D42194	D42392	D62271	D92286	FGA	TCRD	TP53
EDC034	AdenoCa	Mass LLL	-	-	AdenoCa	60	H	**MA**		**L**	**L**		H	H		H		H
BL192	NSCLC	Mass RUL	-	AdenoCa	AdenoCa	25	H		H		H		H	H				**L**
BL145	SCLC	Mass LMB	SCLC	-	SCLC	123	H	H			H	**L**	H	H		**L**	H	H
BL176	SCLC	Mass RUL	SCLC	-	SCLC	98	H		H	**L**	H	H		H		H		**L**
BL112	SqCCL	Mass R Hilum	SqCCL	-	SqCCL	220	H	H	H	H	H		H	H		H	**L**	H
BL075	SqCCL	Mass R Hilum	SqCCL	-	SqCCL	78	H	H	H			H	H	H	**L**	H	H	H
BL154	SqCCL	Mass RLL	SqCCL	-	SqCCL	135	H	H			**L**	H	H	H	H	H	H	H
BL115	SqCCL	Collapse LLL	SqCCL	-	SqCCL	118	H	H		H	H	H	**L**	H			H	**L**
BL125	nmcs	Collapse RUL	-	-	2ndary AdenoCa	ND	H	H		H		H	H			**MA**	H	H
BL187	nmcs	Mass RUL	-	-	Metastatic melanoma	0				H				H		H		**MA**
BL152	nmcs	Shadow RUL	-	AdenoCa	AdenoCa	76	H	H		H	H	**L**	H	H		H	H	H
BL212	nmcs	Mass LLL	-	AdenoCa	AdenoCa	37	H	H	H				H	H		H		**L**
BL114	nmcs	Mass RUL	-	AdenoCa	AdenoCa	34	**MA**		H	**MA**	H	H	H	H		H		H
BL081	nmcs	Mass RUL	-	-	Lung Ca	40		**MA**	H		H		H	H		H	H	
BL181	nmcs	Shadow RUL	-	SqCCL	SqCCL	120		H	H	H	H	H		H		**MA**		H
BL119	nmcs	normal	-	-	Angina	38			H		H	H	H	H	**MA**	H		
EDC067	nmcs	clear	-	-	Asthma	40	H	H		H			**L**	H		**MA**		H
BL084	nmcs	Shadow LLL	inflammation	-	FA	0	H			H	H	H	H	H	H	**MA**	**L**	H
BL111	nmcs	Atelectasis	-	-	COPD	ND		**MA**	H	H	H		H	H				H
BL090	nmcs	normal	-	-	COPD	48	H	H	H		H		H	H	**L**	H	H	H
BL126	nmcs	MD shadow	-	-	Clinical Goitre	45	H	H	H	H				H		**MA**		**MA**
BL067	nmcs	LHC	-	-	LVF	59	**L**			H	**MA**		H			H		
BL085	nmcs	Pleural effusion LLL	inflammation	-	MI	160	H	H	**L**	H	H	H		H	H	H		H
BL180	nmcs	normal	-	-	NED	0	H		H	H	H	H	**MA**	H	H	H	H	H
EDC029	nmcs	consolidation	-	-	Pneumonia	32	H	H		H	H		H	H		**MA**	H	H
BL086	nmcs	clear	-	-	RA	0	H	**MA**	H	H	H			H	H	H	H	H

In 9 of the cases with lung cancer, tumour tissue was also available. Three of the nine tumours demonstrated genetic alterations. Of these, 2 (BL114, BL176) had the same aberration in BL (Figure 3.3.2.2b,c), whereas in patient BL154, the aberration was not detectable in the BL specimen, although it was present in the tumour (Figure 2.3.2.2a). Examining the relationship between genetic alterations and confirmed cytology/ histopathology, it was found that LOH/MAs tend more frequent among the BL of patients with adenocarcinomas (6/8) than squamous carcinomas (5/25; $p = 0.069$).

On comparing LOH with MAs based on the cytology review, it was found that the prevalent type of alteration in specimens with cytological evidence of malignancy is LOH (Table 2.3.2.1). In contrast, the individuals with negative cytology show a preponderance of MAs (Fisher's exact, $p = 0.01$). To examine this, the number of LOH/MA findings was compared with the differential cell count in the cytology specimens from patients with no clinical evidence of cancer by any diagnostic method. In the BL specimens with >50% epithelial cells had 3 with LOH and 5 with MAs, while those cells with < 50% epithelial cells in the BL had 2 with LOH and 4 with MAs. No significant difference was found between LOH/MA frequency and the epithelial cell content of the BL specimen.

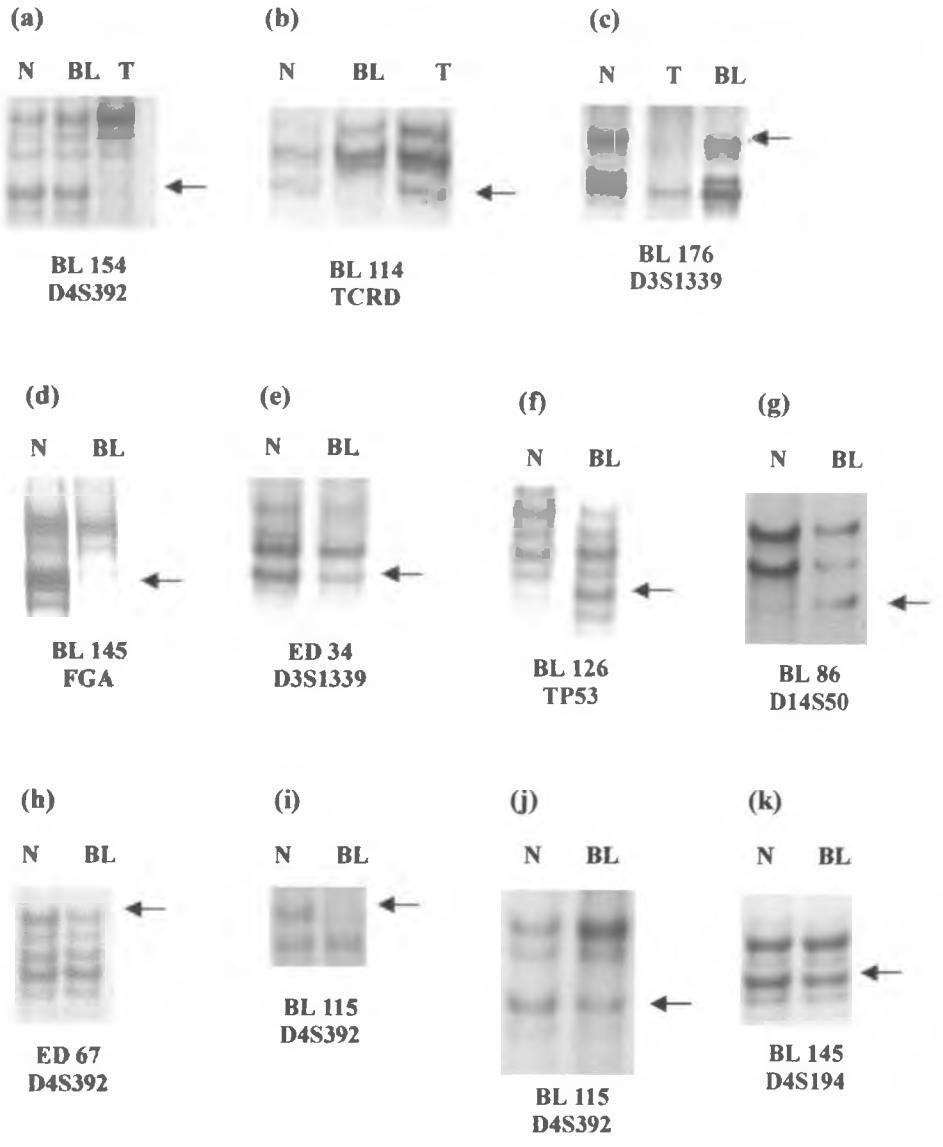

Figure 2.3.2.2. Genomic instability in BL specimens from individuals with lung cancer and also from individuals with no clinical evidence of lung cancer. (a, b & c): analysis of genomic instability in blood (N), BL and tumour specimens (T). LOH is seen in the tumour tissue but not in the BL (a); LOH is seen in both BL and T specimens (b and c). Also, cases d,e,h,i,j and k show LOH in the BL specimen while f and g show MA (band shift). It is of note that in some cases (d, i) loss of one allele is almost complete indicating a large proportion of cancer cells in the BL specimen while in others (c, h, j, k) the loss is incomplete indicating a higher proportion of normal cells.

2.3.3 Genetic alterations in the BL of individuals with no clinical evidence of lung cancer

Eleven of 47 (23%) of the individuals had no cytological or radiological evidence of lung cancer but did demonstrate genomic alterations. Nine individuals had evidence of LOH/MA and two individuals were found to have LOH alone (Table 2.3.2.1). The statistical analysis in the group of individuals with no clinical evidence of lung cancer revealed an association (6 of 9) between genomic alterations and individuals with rheumatoid arthritis, clinical goitre, cardiac problems and fibrosing alveolitis compared with those (4 of 31) with common chest disease (e.g. asthma, COPD or chest infections; $p = 0.003$). No significant differences were found between the referring symptoms in the individuals with and without genetic alterations. On examination of the prescribed drugs to these 47 patients over the past five years, no association was found between genetic alterations and any specific drugs. Cytology review of these 47 individuals, showed nine with squamous metaplasia of regular type of whom 4 had genetic alterations, but this trend was not found to be significant (Fishers' exact test, $p = 0.09$).

The statistical analysis on either the total number of individuals or separate groups of lung cancer and non-lung cancer individuals did not produce any significant correlation between genetic alterations of the markers examined with age or gender. Similar was the case with the smoking history or tobacco exposure in patients with lung cancer because, as only two of the 42 individuals

with a complete smoking data, were non-smokers (one patient with squamous cell carcinoma of the lung and metastatic melanoma; Table 2.3.2.2).

Table 2.3.2.2. Genomic instability and smoking history of the lung cancer patients investigated in this study.

Smoking Status	LOH	LOH+MA	MA	No evidence of genetic alterations
Smokers				
Lung Cancer	9	1	3	27
Non-cancer[a]	2	2	3	27
Nonsmokers				
Lung Cancer			1	1
Non-cancer		1	2	6
No smoking data				
Lung Cancer			1	
Noncancer			1	3

[a] No clinical lung cancer has been observed in these patients on follow-up to date (31/12/1999).

In the group of 47 patients with no clinical evidence of lung cancer, smoking data were available for 43 individuals (34 smokers, 9 non-smokers). Seven of the smokers (2 with LOH only, 3 with MAs only and 2 with LOH/MA) and 3 of the non-smokers (1 with LOH/MAs and 2 with MAs only) demonstrated genetic alterations. The clinical diagnosis for these three patients was rheumatoid arthritis, fibrosing alveolitis and one individual with no evidence of any disease.

2.4 Discussion

90 BL specimens and blood samples from individuals with suspected lung cancer were analysed with twelve microsatellite markers that were found to exhibit the most frequent MAs and LOH in previous publications in NSCLC and SCCHN (Field *et al,* 1995; Field *et al,* 1996; Merlo *et al,* 1994; Miozzo *et al,* 1996; Neville *et al,* 1995; Neville *et al,* 1996; Shridhar *et al,* 1994). Genetic alterations (LOH and MAs) were detected in the BL of 35% lung cancer patients. It is of particular interest that genetic alterations were detected in BL specimens from patients who were reported as NMCS on cytological examination. This finding indicates that a proportion of the cells in the BL carried genetic aberrations but presented with no morphological evidence of malignancy. However, an alternative source of LOH/MAs may be naked tumour DNA in the BL sample. This may also explain the fact that LOH/MAs were detected in samples with a cytology report indicating possible target epithelial cells in very low abundance, lower than the sensitivity it was demonstrated for this assay (1:10 for LOH and 1:16 for MA). Naked DNA released in body fluids is also considered to be a source of genetic alterations detected in the plasma of lung cancer patients (Chen *et al,* 1996). It should be noted that the sensitivity of MA detection in this study is in agreement with that of Foucault et al (1996), who reported detection of the less abundant allele when it represents 6% of the total DNA. Genetic alterations have also been detected in body fluids, such as urine (Steiner *et al,* 1997), plasma (Chen *et al,* 1996) and sputum (Miozzo *et al,* 1996) and may, therefore, be considered as an attractive candidate for the development of strategies for the early diagnosis of cancer, alongside other molecular methods that require no invasive techniques.

In an allelotype study of NSCLC undertaken with 92 markers, it has been shown that 93% of the samples exhibited LOH/MA in at least one marker (Neville *et al,* 1996). Thus, it may be argued that the majority of NSCLC cases contain such genetic damage because it may be considered to be a fundamental feature of carcinogenesis. The detection of LOH/MA in 35% of lung cancer patients in this study can, therefore, be considered as an underestimate and a larger number of markers are, therefore, required to increase the detection efficiency. In addition, a possible underestimation of the LOH/MA frequency may be due to the visual identification. MAs are easier to detect, because they are represented by an additional band, whereas LOH demands quantitation of the relative density of the two alleles. The detection method used (i.e. silver staining of the PCR products analysed on polyacrylamide gel), has been widely used for the detection of genetic alterations. However, it is clear that there are certain limitations to its applicability on clinical specimens, such as BL, with a possible low number of target cells. In the future, biomagnetic separation for epithelial cell enrichment may be a solution to this problem. In addition, the use of fluorescent chemistry will increase the sensitivity of the assays because the quantitation of band intensities would become far more accurate. The latter is supported by recent research findings of Liloglou and colleagues who have established a robust experimental platform with increased sensitivity and specificity for the detection of genetic alterations in lung tumours and BL specimens using high throughput fluorescent microsatellite analysis (Liloglou *et al,* 2000, Liloglou *et al,* submitted).

It is of note that 23% of the patients referred for investigation with no cytological or radiological evidence of lung cancer do have genetic instability in the same microsatellite markers as found in patients with proven neoplasia. These patients, however, cannot be considered as a "control group", based on their referral criteria and are still under long-term follow-up. The results obtained in this study are in agreement with recent reports demonstrating genetic alterations in biopsy specimens from current and former smokers who do not have lung cancer (Mao *et al,* 1997; Wistuba *et al,* 1997) and MI in cytological material from patients with COPD (Spandidos *et al,* 1996). In this group of 47 patients, genetic alterations were more common in rheumatoid arthritis, goiter, fibrosing alveolitis and cardiac problems such as myocardial infraction (MCI), left ventricular failure (LVF) and angina than in common chest disease. Thus, this may imply that genetic alterations occur in disease processes such as cardiac and autoimmune conditions and thereby, suggests a possible genetic link between certain neoplastic and non-neoplastic diseases. Moreover, LOH/MA in this group may just represent molecular damage due to tobacco exposure in smokers or some other carcinogens in non-smokers. Such genetic changes have been previously reported in smokers in a range of histological types including normal epithelium (Mao *et al,* 1997; Wistuba *et al,* 1997). The individuals included in this study are currently being followed-up in the context of the Liverpool Lung Project (Field *et al,* 1999) and it remains to be shown whether those with genetic alterations will develop lung cancer at a future date.

In this study, no statistical correlation was found between genomic instability and age or gender. Because there were only 11 non-smokers (2 in the lung cancer group and 9 in the non-cancer group) in this study, significant comparisons between genetic alterations and smoking history cannot be produced. LOH tends to be the predominant genetic alteration found among smokers and MA is more frequent in non-smokers, although not significantly (Fishers' exact test, p= 0,19). However, among patients with no malignancy, non-smokers did not demonstrate a significant difference in the frequency of total genetic alterations (LOH+MA). This is in contrast to the findings of Mao et al (1997) and Wistuba et al (1997). One hypothesis for this observation may lie in the argument that this population in the Northwest of England may be exposed to additional carcinogens. This hypothesis is consistent with previous observations on NSCLC and SCCHN patients in this region, who demonstrated a p53 mutational profile with prevalence of GC to AT transitions, which is not typical of a smoking population (Liloglou *et al,* 1997a,b).

A trend was observed for LOH/MA to be found more frequently in BL patients with adenocarcinomas (6 of 8) than with SqCCL (5 of 25). The reason for this is unclear, mainly because adenocarcinomas are usually associated with peripheral airways whereas SqCCL is usually associated with large-airway disease and is more efficiently sampled by BL. Furthermore, the prevalent type of genetic alteration found in specimens with cytological evidence of malignancy was LOH, whereas individuals with NMCS at cytology mainly demonstrated MA. This finding implies that LOH (i.e. chromosomal imbalance) may be

associated with neoplastic cell transformation, and thus, altered cytological appearance of the cell, whereas MA may not be associated with identifiable morphological changes. MA may, therefore, represent genetic changes of subclones arising from a cell population with high genetic diversity. It may be argued that this difference may be due to the fact that MA is more easily detected than LOH in samples with low number of target cells, such as the cytological specimens that have been reported as NMCS. However, these results indicate that there was no bias in the detection of MA in samples with < 50% epithelial cells. It was also investigated whether this bias was due to the different histological composition of the NMCS group (prevalence of adenocarcinomas) compared with the cytology-positive group (prevalence of SqCCL). However, the absence of a significant difference in the type of genetic alteration seen in adenocarcinomas (4 with LOH, 2 with MA) and SqCCL (4 with LOH,1 with MA) does not support this argument.

In this study, it has been demonstrated that genetic alterations can be detected in the BL. In time, additional markers and advances in technology may increase the present success rate. Additional studies and long-term follow-up are required to clarify the value of such genetic alterations as a tool for the identification of individuals with a high risk of developing the disease.

This chapter has been published. J.K.Field, T. Liloglou, G. Xinarianos, W. Prime, P. Fielding, M.J. Walshaw, L. Turnbull. Genetic alterations in bronchial lavage as a potential marker for individuals with a high risk of developing lung cancer. *Cancer Research* 59: 2690-2695, 1999.

CHAPTER THREE

TELOMERASE ACTIVITY IN THE MOLECULAR PATHOGENESIS OF NON-SMALL CELL LUNG CARCINOMAS

Abstract

The TRAP assay was used to examine 108 NSCLC and their adjacent normal tissue. Telomerase activity was detected in 93 of 108 (86%) NSCLC examined but not in any of the matched normal lung tissues. Telomerase activity correlated with the T stage of the tumour ($p=0.008$), nodal metastasis ($p<0.05$) and differentiation ($p=0.04$). Significant correlations were also found between the presence of telomerase activity and current smoking status at the time of diagnosis ($p=0.01$), daily tobacco consumption ($p<0.05$) and total tobacco exposure (pack/years) ($p<0.05$). Telomerase activity was not associated with the expression of $p21^{WAF1}$ and Rb. In addition, there was no association between telomerase activity and allelic imbalance on chromosome 3p. Mutations in the minimal functional region of the human telomerase RNA gene (*hTR*) and its promoter region were not detected. No mutations were detected in the promoter region of the human telomerase gene (*hTERT*) encoding for the catalytic subunit of human telomerase. Our findings demonstrate that telomerase activity is a common genetic phenomenon in NSCLC cases but not in the normal lung. However, certain cases in former and/or moderate smokers may follow a telomerase independent pathway. These data also suggest that mutations in the in the minimal functional region of the *hTR* gene and its promoter region and the promoter region of the *hTERT* gene do not contribute in the regulation of telomerase activity in NSCLC. In addition, our results indicate that telomerase activity in NSCLC is independent from two of the cell cycle regulators, $p21^{WAF1}$ and Rb.

3.1 Introduction

Telomeres are specific structures consisting of short tandem repeat sequences at the ends of chromosomes. Their major role is to help maintain chromosome stability. The maintenance of telomeres is considered to be essential for the progression of cells through a normal, mortal life span (Zakian, 1995). Telomerase is a ribonucleoprotein enzyme which replaces chromosome ends lost during successive cycles of cell division (Morin, 1989). Telomerase activity has been detected in almost all human cancer types and in all human tumour cell lines tested. Telomerase activity has also been detected in some somatic tissues, including hematopoietic cells and some stem cells (Bacchetti & Counter, 1995; Shay & Bacchetti, 1997; Wright *et al*, 1996).

Early reports on telomerase in lung cancer indicated that enzyme activity is present in most of such cases, ranging from 78-96% in NSCLC while it was present in all of the small cell carcinomas tested (Albanell *et al*, 1997; Hiyama *et al*, 1995; Yashima *et al*, 1997). In one of the reports, similar proportions of telomerase positive cases were detected in the two different patient populations, American and Japanese, included in the study (Hiyama *et al*, 1995). It has also been demonstrated that dysregulation of telomerase is an early event in the multistage pathogenesis of bronchogenic lung carcinomas (Yashima *et al*, 1997).

The TRAP assay was employed, in a radioactive or non-radioactive form, to detect telomerase activity in NSCLC samples and matching normal lung tissues in a patient population from the Merseyside region of the Northwest England. In addition, in order to investigate further the regulation of telomerase activity in NSCLC, the minimal functional region of the *hTR* gene and its promoter region and the promoter region of the *hTERT* gene were examined for mutations. Telomerase activity data were further comparative analysed with allelic imbalance on chromosome 3p and the expression levels of two of the cell cycle regulators, $p21^{WAF1}$ and Rb.

3.2 Materials & Methods

3.2.1 Cell Lines

The human non-small cell lung cancer cell lines Calu3, H1155, HTB58, and A549, and human colon adenocarcinoma HCT15 and LoVo cell lines were used as positive controls for telomerase activity. The human lung fibroblast cell line LL47 was used as a negative control. All cell lines were obtained from the American Type Culture Collection (USA) and grown according to the conditions recommended by the supplier. Cell pellets were collected after trypsinisation [addition of 5ml trypsin solution (Sigma, UK), incubation at 37°C for 3 min, addition of growth medium to neutralise the trypsin and transfer to 20ml universal tubes] followed by centrifugation at 3,000 rpm for 5 min and frozen at -80°C until preparation of telomerase extracts.

3.2.2 Patients and Tissue Samples

Primary lung tumour and corresponding normal tissue samples were obtained from 108 patients, 46 males and 62 females, who underwent surgery in the CTC of Broadgreen University Hospital, Liverpool (Merseyside, UK). The age of the patients selected ranged between 45 and 95 (mean 66).

Current smoking status data were available for 89 individuals; 47 current smokers, 11 recently stopped smokers (1-4 years prior to presentation), 25

former smokers (≥5 years prior to presentation) and 6 non-smokers. Complete data for calculating the total smoking exposure was available for 60 smokers. Total smoking exposure is expressed in pack-years:

$$pack\text{-}years = \text{years smoked} \times \text{packs/day}.$$

The patients' pack years ranged from 20 to 165 (mean = 75).

All specimens (n=108; 35 adenocarcinomas, 52 squamous cell carcinomas, 21 other NSCLC) were microdissected to ensure content of at least 80% tumour cells and immediately snap-frozen in liquid nitrogen and stored at -80°C prior to preparation of protein extracts.

Each frozen tissue sample was first washed in 500 µl ice-cold buffer (10 mM HEPES-KOH [pH 7.5], 1.5 mM $MgCl_2$, 10 mM KCl and 1 mM dithiothreitol), then homogenised in 200 µl of ice-cold CHAPS lysis buffer (0.5% CHAPS (3-[(3-cholamidopropyl)-dimethyl-ammonio]-1-propane-sulphonate), 10 mM Tris -HCl pH 7.5, 1 mM $MgCl_2$, 1 mM egtazic acid (EGTA), 5 mM beta-mercaptoethanol, 0.1 mM phenylmethylsulphonyl fluoride (PMSF) and 10% glycerol). Homogenisation was performed in 1.5 ml microfuge tubes using disposable pestles rotated by a motor. Frozen pelleted cultured cells were also lysed in 200 µl of ice-cold CHAPS lysis buffer.

Lysates were incubated on ice for 30 min followed by centrifugation at 14,000 rpm for 20 min at 4°C. Resulting supernatants were transferred into fresh tubes and snap frozen in liquid nitrogen and stored at -80°C until they were subjected to the TRAP assay. A protein concentration for each extract was determined using the BSA protein assay kit following the protocol recommended from the supplier (Pierce Chemical Co, UK).

3.2.3 TRAP Assay

The TRAP assay was performed as previously described with a few modifications (Kim *et al*, 1994; Wright *et al*, 1995). Six μg total protein of a cell line or lung tissue were added to TRAP reaction mix, containing 50 mM of each dNTP, 100 ng of TS primer, 2.5 U *BIOPRO* polymerase (Bioline, UK), and 5 μl 10X buffer (50 mM KCl, 10 mM Tris-HCl pH 8.8, 0.1% Triton X-100, 1.5 mM $MgCl_2$), and allowed to react at room temperature for 30-60 minutes. The reaction mixture was then heated at 90°C for 90 sec to inactivate telomerase and 100 ng of CX primer were added to the reaction. Samples were subjected to 31 (radioactive TRAP) or 37 (non-radioactive TRAP) cycles of amplification using the following parameters: 94°C for 30 sec, 50°C for 30 sec and 72°C for 45 sec. For radioactive TRAP, 1-2 μCi per reaction [μ-^{32}P]-dCTP, 3000 Ci/mmole, were added.

Specificity controls were performed by pre-treatment of protein extracts with 0.5 μg RNase A (Sigma, UK) for 30 min at 37°C or heat inactivation at 95°C for 5 min before addition to the TRAP assay. Blank control samples containing CHAPS lysis buffer were also run.

TRAP products were run on 10% non-denaturing polyacrylamide gels for 1000-1,100 Vh and the 6 bp DNA ladder was visualised by silver staining (10 min washing in fixation buffer consisting of 10% absolute ethanol and 0.5% acetic acid. Gels are then stained in 0.1% $AgNO_3$ solution for 20 min and developed in developer solution consisting of 1.5% NaOH and 0.15% formaldehyde followed by a final fixation step in 7.5% Na_2CO_3 solution for 5 min. All steps were carried out on a shaker plate.) or autoradiography after a 1-2 days exposure at -80°C.

A sample was scored as telomerase positive when an RNase A and/or heat sensitive 6 bp DNA ladder was observed. All experiments were carried out in duplicate. All tumour and normal negative samples were also examined by the radioactive form of the TRAP assay.

3.2.4 PCR Controls for Telomerase Inhibitors

PCR for exon 10 of the p53 gene using intronic primers was used to check for the presence of contaminating genomic DNA in tumour extracts. In

addition, tumour extracts from negative samples were mixed with extracts from telomerase positive specimens and subjected to the TRAP assay to assess any inhibitory action on the assay.

3.2.5 DNA Extraction and PCR Amplification for hTR, hTR and hTERT Promoter Regions

Frozen tumour tissue specimens were available from 108 individuals. Five 10 μm sections of each sample were microdissected to ensure presence of more than 80% tumour cells. Sections were lysed in 400 mM Tris-HCl pH 8.0, 150 mM NaCl, 60 mM EDTA, 1% SDS, 100 μg/ml Proteinase K and incubated at 42°C for 16 hours in an orbital shaker. Deproteinization included extraction with an equal volume of phenol/chloroform and chloroform. Each step was followed by centrifugation at 14,000 rpm for 5 min (RT) and the aqueous phases were transferred into fresh tubes. DNA was precipitated by the addition of an equal volume of isopropanol and mixing by inversion. DNA was spooled onto sterile microbiology loops, washed with 500 μl 70% ethanol and resuspended in 200 μl TE (10mM Tris-HCl, pH 7.0 and 1mM EDTA). Working stocks were prepared by 5-fold dilution in sterile distilled H_2O.

PCR reactions were performed in a 25 μl reaction volume and contained 100 ng of genomic DNA, 200 μM of each dNTP, 6 pM of each primer, 0.6U *BIOPRO* polymerase (Bioline, UK) and 2.5 μl 10X buffer (50mM

KCl, 10 mM Tris-HCl pH 8.8, 0.1% Triton X-100, 1.5 mM $MgCl_2$. Samples were subjected to PCR amplification using the following primers and amplification parameters:

hTR: 5' TCT TCG CGG TGG CAG TGG GT 3'

5' GGG AGG GGT GGT GGC CAT TT 3'

Initial denaturation at 95 °C for 3 min followed by 37 cycles of: denaturation at 94 °C for 45 sec, annealing at 61 °C for 35 sec and extension at 72 °C for 35 sec (PCR amplification product: 281 bp).

hTR promoter: 5' ACGCCTTTCTTTCCTAATAA 3'

5' GCTTTGCGTCTTTACTTCC 3'

Initial denaturation at 95 °C for 3 min followed by 37 cycles of: denaturation at 94 °C for 45 sec, annealing at 53 °C for 35 sec and extension at 72 °C for 40 sec (PCR amplification product: 300 bp).

*hTR*T promoter: 5' CGCCTCCGTCCTCCCCTTCA 3'

5' GCTGCCTGAAACTCGCGCCG 3'

Initial denaturation at 95 °C for 3 min followed by 37 cycles of: denaturation at 94 °C for 45 sec, annealing at 64 oC for 40 sec and extension at 72 °C for 40 sec (PCR amplification product: 400 bp). Five μl of the amplification products

were analysed on a 2% agarose gel, stained with ethidium bromide (5μg/ml) and visualised on a UV transilluminator.

3.2.6 hTR, hTR and hTERT Promoters Single Strand Conformation Polymorphism (SSCP) and Heteroduplex Analysis (HA)

Single strand conformation polymorphism (SSCP) and heteroduplex analysis (HA) were primarily used to screen for mutations in the minimal functional region of *hTR*, *hTR* and *hTER*T promoter regions.

For SSCP analysis, 2-4 μl of the PCR product were mixed with 10 μl of denaturing solution consisted of 80% formamide, 100 mM NaOH, 1 mM EDTA, 0.1% Bromophenol Blue, 0.1% Xylene Cyanol FF. Samples were then heated at 95°C for 3 min, chilled on ice and loaded onto 8-10% native polyacrylamide gels, containing 5-10% glycerol. Gels were run at 15°C for 2,500-3,500 Volt hours and silver stained after electrophoresis.

HA was performed as follows: 2-5 μl of the PCR product were denatured at 95 °C for 5 min and allowed to cool down slowly at RT for 1 hour. Samples were then analysed on 8% native polyacrylamide gels and run for 1,600-1,800 Volt hours. Gels were silver stained after electrophoresis.

3.2.7 Sequencing

PCR products were used for subsequent sequencing after purification with QIAquick PCR Purification Kit following the procedure recommended by the supplier (Qiagen,Ltd, West Sussex, UK). Sequencing was performed using the ABI PRISM® BigDye™ Terminator Cycle Sequencing kit and analysis on a 377 sequencer (PE Biosystems, Warrington, UK) using the supplier's protocol. The primers used for PCR amplification were also used for sequencing but only one primer (either forward or reverse) was used in each sequencing reaction.

3.2.8 Statistical analysis

Fisher's exact test was used to analyse the molecular and clinicopathological data. T-test was performed to investigate the relation of molecular data to age and pack-year parameters. Analysis was performed using the SPSS software.

3.3 Results

3.3.1 Telomerase Activity in NSCLC

The TRAP assay was optimised by running serial dilutions (6 μg, 0.6 μg and 0.06 μg) of extracts of positive control cell lines (Figure 3.3.1.1A and B). Positive telomerase activity in a tumour sample was determined by the presence of a 6-bp ladder, which was sensitive to heat inactivation and/or RNase A treatment of the protein extract.

Telomerase activity was detected in 93 of 108 (86%) NSCLC examined. In particular, enzyme activity was present in 30 of 35 (86%) adenocarcinomas, 48 of 52 (92%) squamous cell carcinomas and 15 of 21 (76%) other NSCLC cases (Table 3.3.1.1).

Table 3.3.1.1. Telomerase activity in NSCLC

Lung Tumour	n	Telomerase (+)	%
Adenocarcinoma	35	30	86
SqCCL	52	48	92
Other NSCLC	21	15	76
TOTAL	**108**	**93**	**86**

Representative examples of the detection of telomerase activity in NSCLC are shown in Figure 3.3.1.1C. None of 108 normal matching lung tissues were found to be telomerase positive (Figure 3.3.1.1D).

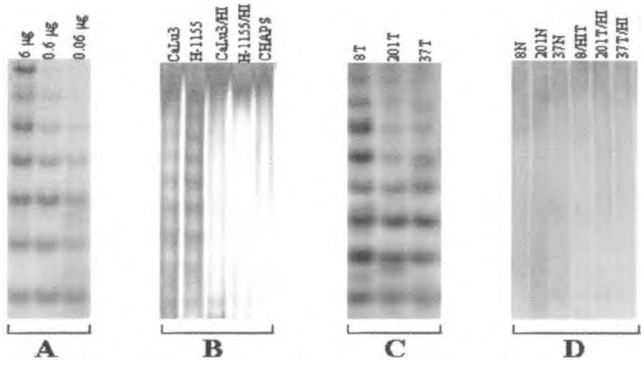

Figure 3.3.1.1 Telomerase activity in NSCLC using a non-radioactive (silver staining) TRAP assay. **A)** LoVo (colon adenocarcinoma) cell line extracts assayed at 3 concentrations (6 µg, 0.6 µg, 0.06 µg) to establish the sensitivity of the assay. **B)** Telomerase positive lung cancer cell lines CaLu3 and H-1155, and negative control for the reaction (CHAPS lysis buffer). Lanes marked HI indicate heat-inactivated samples of the indicated cancer cell lines. **C)** Telomerase positive NSCLC tumour extracts with different levels of enzyme activity. **D)** Telomerase negative samples marked "N" are corresponding normal lung tissue from the indicated telomerase-positive specimens. Lanes marked HI are heat-inactivated samples of the indicated tumour cell extracts.

Tumour and normal samples found to be telomerase negative, were investigated further for the presence of TRAP assay inhibitors and genomic DNA. Our results revealed that genomic DNA was not present in any of the samples examined indicating absence of false positive results. Moreover, telomerase activity was retained when these samples were mixed with extracts from telomerase positive tumours.

No differences in the results gained were observed when the radioactive and the non-radioactive forms of the TRAP assay were compared.

Telomerase activity was more frequently detected in T2-4 stage tumours (85 of 95) than in T1 stage tumours (6 of 11) ($p=0.008$). Telomerase activity was also more frequently detected in tumours with nodal metastasis (35 of 37) than in tumours with no nodal metastasis (56 of 69) ($p<0.05$). Telomerase activity was associated with moderate/poor differentiation ($p=0.04$). Telomerase activity data in relation to pathological parameters are shown in Table 3.3.1.2.

No association was found between telomerase activity and histological diagnosis, age and gender of the patient.

Table 3.3.1.2. Telomerase activity and pathological parameters of the NSCLC studied.

Pathological parameter	n	Telomerase (+)	Fisher's exact (p value)
T status			
T1	11	6	p=0.008
T2-4	14	9	
Nodal metastasis			
Positive nodes	37	35	p<0.05
Negative nodes	69	56	
Differentiation			
Well differentiated	16	11	p=0.04
Moderate/Poor differentiated	47	43	

Telomerase activity was more frequently detected in current smokers (45 of 47) than former smokers (19 of 25) (stopped smokers for at least 5 years prior to presentation) (Fisher's exact p=0.01). The patients analysed in this study were divided into two groups, current smokers and former smokers (smokers who had stopped for at least 5 years). Individuals who had given up smoking for 1-4 years were not included in either of the groups. However, if they were also included in the analysis the result was still statistically significant (Fisher's

exact p=0.01). Telomerase activity was associated with daily tobacco consumption (Fisher's exact $p<0.05$). In particular, telomerase activity was more frequently detected in heavy smokers (53 of 57) (smokers who smoked more than 20 cigarettes per day) than in moderate smokers (20 of 26) (smokers who smoked less than 20 cigarettes per day). Telomerase activity was also found to correlate with overall smoking exposure (pack years) [tel (-) mean 78.7 +/- 4.8 (std error), tel (+) mean 60.1 +/- 7.2 (std error), T-test, independent samples-2 tailed, $p<0.05$]. Telomerase activity data in relation to smoking are shown in Table 3.3.1.3.

Table 3.3.1.3. Telomerase activity in relation to smoking status and tobacco consumption in NSCLC.

Smoking	n	Telomerase (+)	Fisher's exact (p value)
Smoking status			
Current smokers	47	45	p=0.01
Former smokers[1]	25	19	
Former smokers[2]	36	28	p=0.01
Non-smokers	6	4	
Tobacco consumption			
Heavy smokers (>20 cigarettes/day)	57	53	p<0.05
Moderate smokers (<20 cigarettes/day)	26	20	

[1] Only stopped smokers for at least 5 years are included

[2] Stopped smokers for 1-4 year have also been included

3.3.2 Mutational Analysis of *hTR*, *hTR* and *hTERT* Promoter Regions

No mutations were detected in the *hTR* (Figure 3.3.2.1a) and *hTERT* (Figure 3.3.2.1b) promoter regions as well as in the minimal functional region of the *hTR* gene (Figure 3.3.2.2)

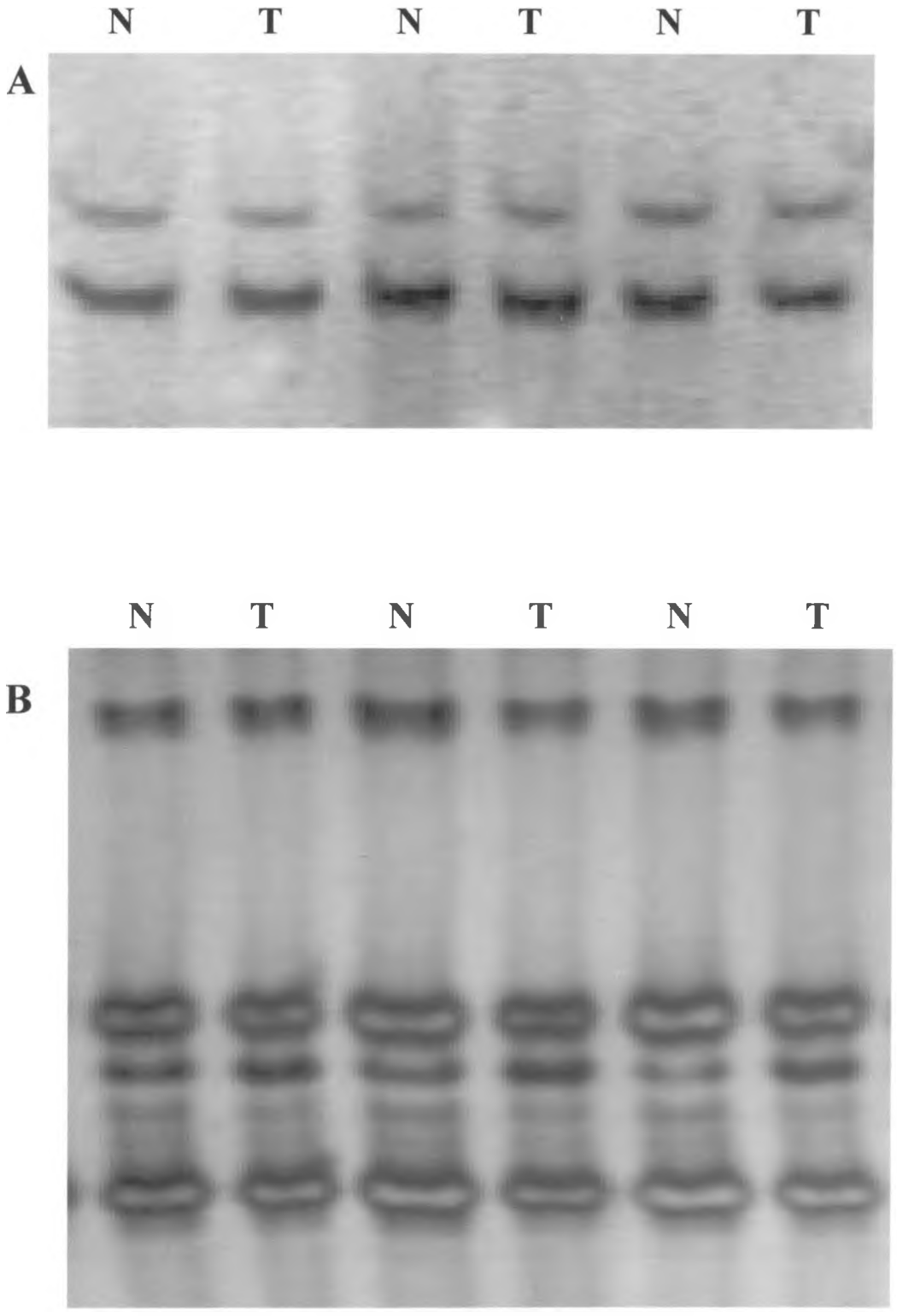

Figure 3.3.2.1. Representative examples of SSCP analysis of hTR (A) and hTERT (B) promoters in NSCLC. T: DNA from lung tumour tissue, N: DNA from corresponding normal lung tissue.

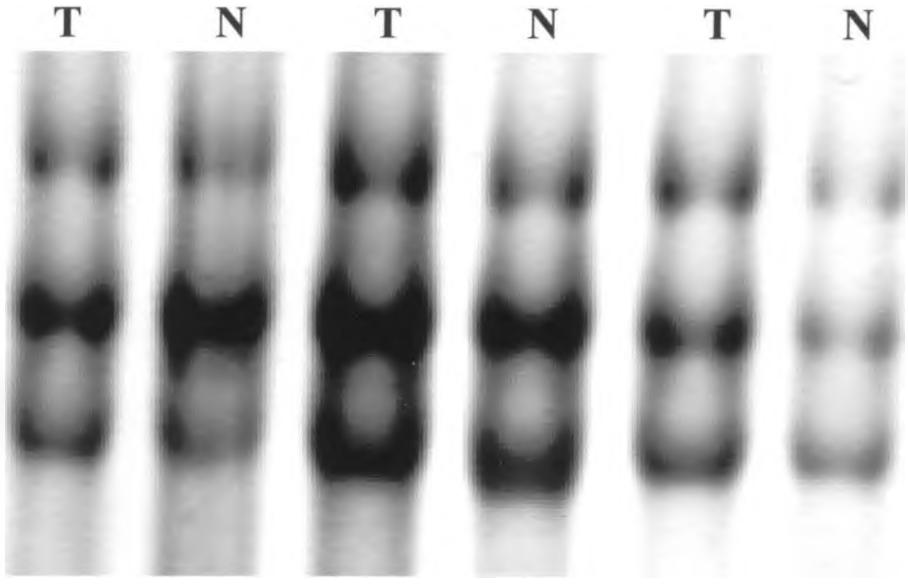

Figure 3.3.2.2. Representative examples of SSCP analysis of hTR gene in NSCLC. T: DNA from lung tumour tissue, N: DNA from corresponding normal lung tissue.

3.3.3 Telomerase Activity in Relation to Rb and $p21^{WAF1}$ Expression

Rb and $p21^{WAF1}$ expression data were available from previous unpublished studies of the Molecular Oncology Unit in the Roy Castle International Centre for Lung Cancer Research. Telomerase activity was detected in 40 of 48 (83%) specimens with normal and 45 of 51 (88%) specimens with abnormal $p21^{WAF1}$ expression levels. In addition, telomerase activity was detected in 59 of 67 (88%) specimens with normal and 21 of 27 (78%) specimens with abnormal Rb expression levels (Table 3.3.3.1).

Table 3.3.3.1. Telomerase activity in relation to $p21^{WAF1}$ and Rb expression in NSCLC investigated in this study.

Telomerase	$p21^{WAF1}$ expression		Rb expression	
	Normal	**Abnormal**	**Normal**	**Abnormal**
Positive	40	45	59	21
Negative	8	6	8	6

3.3.4 Telomerase Activity in Relation to Allelic Imbalance on Chromosome 3p

Allelic imbalance data for four microsatellite markers located on chromosome 3p (*D3S1289, D3S1266, D3S1300 and D3S1304*) were available for 83 individuals from a previous study of ours (Liloglou *et al*, 2000). The comparative analysis of telomerase activity and the allelic imbalance data was based on 4 markers on chromosome 3p in 83 individuals and is presented in Table 3.3.4.1. No association was found between telomerase activity and allelic imbalance at *D3S1289*, *D3S1266*, *D3S1300* and *D3S1304* loci.

Table 3.3.4.1 Telomerase activity in relation to allelic imbalance on chromosome 3p in NSCLC. Only informative cases (heterozygous and allelic imbalance) have been included.

	Telomerase	
Cytogenetic locus	**positive**	**negative**
D3S1266		
H	16	4
AI	36	3
D3S1300		
H	14	3
AI	36	4
D3S1289		
H	18	3
AI	36	4
D3S1304		
H	18	1
AI	28	1

H: Heterozygous AI: Allelic Imbalance

3.4 Discussion

Telomerase activity has been detected in a range of human cancers (Hiyama *et al*, 1996; Huang *et al*, 1998; Muller *et al*, 1996, Ohyashiki *et al*, 1996; Reynolds *et al*, 1997). This is the first study to examine telomerase activity in NSCLC in a European population. In particular, the incidence of telomerase activity was investigated, in NSCLC from a patient population in the Northwest England, Merseyside region, which appears to have some of the highest incidence rates of lung cancer in the UK (Parkin, 1998). Telomerase activity was detected in 86% of the tumour but it was completely absent in the normal adjacent tissues. This finding is in agreement with previous reports, which examined Japanese and American populations (Albanell *et al*, 1997; Hiyama *et al*, 1995; Lee *et al*, 1998). Although telomerase activity was detected in the vast majority of the samples examined, there is, however, a distinct group of NSCLC tumours, which exhibits undetectable levels of telomerase activity. Therefore, it may be argued that these telomerase negative tumours may overcome the telomere shortening using an alternative pathway (Dionne & Wellinger, 1996; Lundbland & Blackburn, 1993; Murname *et al*, 1994).

The TRAP assay, as performed in this study, may be considered to be semi-quantitative because equal amounts (6 μg) of protein from each specimen were used in the assay and equal volumes of each reaction mix were analysed (45 μl). Variable levels of telomerase activity were observed in tumour

specimens, ranging from no activity to high activity, as judged by the varying intensity and number of bands in the TRAP ladder. Other studies (Albanell *et al*, 1997; Hiyama *et al*, 1995; Yashima *et al*, 1997) have also reported variable levels of telomerase activity in lung tumour specimens. It has been suggested that tumours with low or undetectable telomerase activity may contain primarily mortal cells, while tumours with high levels of telomerase are likely to contain mainly immortal cells (Hiyama *et al*, 1995). The tumour samples in the current study were microdissected to ensure that tumour extracts came from at least 80% cancer cells. However, it is still possible that the variation of telomerase activity could be attributed to an admixture of normal and cancer cells or the presence of different telomerase levels within individual tumour subpopulations. Further studies, including long-term patient follow-up, are required to define the prognostic significance, if any, of varying levels of telomerase activity in lung tumours.

Telomerase activity was associated with the T stage and nodal metastasis of the tumours examined. This is consistent with the findings of other studies that have reported high telomerase activity in NSCLC with high tumour proliferation rates and advanced pathological stage (Albanell *et al*, 1997). Therefore, it may be argued that telomerase may be a marker of disease progression and aggressiveness in NSCLC. Telomerase activity was also found to correlate with moderate/poor differentiation. The latter suggests that telomerase activity may be affected by the degree of differentiation of the

neoplastic cells. No correlation was found between telomerase activity and histopathological diagnosis, age and gender of the patient.

It is of interest that telomerase activity was not detected in any of the 108 adjacent normal lung tissues. This is in contrast to a previous report, which demonstrated weak telomerase activity in approximately 23% of normal peripheral lung tissues from corresponding lung tumours (Yashima *et al*, 1997). The authors of the study proposed that the presence of regenerating stem cells or damage to the epithelial cells due to exposure in chemical carcinogens found in tobacco could have led to these observations. Other studies have demonstrated either a very small incidence (approximately 4%) of weak or no detectable telomerase activity in normal lung tissues (Albanell *et al*, 1997; Hiyama *et al*, 1995; Lee *et al*, 1998). Our p53 PCR experiments indicated that it is highly unlikely TRAP assay inhibitors and genomic DNA to be the reasons for the absence of detectable telomerase activity in this subset of NSCLC studied, as well as in the adjacent normal tissues.

Smoking status data were available for 89 of the specimens examined. Since there were only 6 non-smokers and 83 current or former smokers in this series of samples, a comparison between telomerase and smokers versus non-smokers could not be made. However, telomerase activity was more frequently detected in current than in former smokers ($p=0.01$). This is in contrast to the findings of Yashima and co-workers (1997) who demonstrated

no difference in telomerase activity between former and current smokers with lung tumours. Our observations may indicate that certain NSCLC cases from former smokers, in this population in the Northwest England, may undergo a telomerase independent initiating pathway.

It is of note that telomerase activity was associated with a history of heavy smoking (>20 cigarettes/day) compared with moderate smoking (<20 cigarettes/day) ($p<0.05$). In addition, telomerase activity was found to correlate with smoking exposure (packyears) ($p<0.05$).

Telomerase activity was also investigated in relation to the expression levels of two of the cell cycle regulators, $p21^{WAF1}$ and Rb. There was no association between telomerase activity and the expression levels of these proteins. This suggests that telomerase activity regulation is $p21^{WAF1}$ and Rb independent in this set of NSCLC examined. This is in agreement with a previous study in breast cancer cases which reported a similar lack of association between Rb expression and telomerase activity (Landberg *et al,* 1997).

It is of note that no mutations were detected in the minimal functional and promoter regions of the *hTR* gene which encodes for the RNA component of human telomerase. Also, no mutations were detected in the promoter region

of the *hTERT* gene which encodes for the catalytic subunit of human telomerase. This demonstrates that mutations in these regions do not contribute to the activation or deregulation of telomerase in NSCLC.

Telomerase activity was not associated with allelic imbalance on chromosome 3p. This is in contrast to the findings of previous studies which have demonstrated that telomerase activity may be regulated by gene(s) located on chromosome 3p (Cuthbert *et al,* 1999; Mehle *et al,* 1998; Steenbergen *et al,* 1996). The lack of association observed in this study may be primarily due to the fact that only a subset of the tumours examined for telomerase activity has been analysed for allelic imbalance on chromosome 3p. In addition, the high percentage of telomerase (86%) results in just a few telomerase negative cases, which in combination with the exclusion of homologous samples makes statistical analysis difficult due to the small numbers. Further studies are required to elucidate the relationship between telomerase activation and allelic imbalance on chromosome 3p.

In this study, it has been demonstrated that telomerase activity is present in the vast majority of NSCLC and correlated with smoking status at the time of presentation and heavy smoking history. In addition, telomerase activity in the tumours correlated with T stage, nodal metastasis and differentiation. These data suggest that telomerase activity is implicated in the molecular pathogenesis of NSCLC and a subset of telomerase negative

tumours from former smokers may represent a discrete population with a distinct telomerase-independent initiating pathway. Further study of telomerase activity as a diagnostic/prognostic marker is required. In addition, *in situ* assays for detection of the telomerase RNA (*hTR*) and catalytic protein (*hTER*T) components (Kolquist *et al*, 1998; Meyerson *et al*, 1997; Snijders *et al*, 1998; Soder *et al*, 1997; Soder *et al*, 1998) may improve our understanding of telomerase regulation and its molecular initiating pathways.

Part of this chapter has been published. G. Xinarianos, F.M. Scott, T. Liloglou, W. Prime, J. Callaghan, J.R. Gosney, J.K.Field. Telomerase activity in non-small cell lung carcinomas correlates with smoking status. *International Journal of Oncology* 15: 961-965, 1999.

CHAPTER FOUR

TELOMERASE ACTIVITY IN BRONCHIAL LAVAGE SPECIMENS

Abstract

The TRAP assay was used to examine telomerase activity in BL samples from individuals undergoing diagnosis of lung cancer. Telomerase activity was detected in 17 of 36 (47%) samples examined. In particular, 16 of 23 (70%) BL specimens obtained from lung cancer patients showed detectable telomerase activity, while only 1 of 13 (8%) specimens obtained from patients without lung cancer demonstrated activity (p=0.00038). Moreover, 9 of 10 (90%) BL specimens, which were cytologically positive for lung cancer were also positive for telomerase activity, while 7 of 13 (54%) cytologically negative BL specimens for lung cancer showed detectable telomerase activity. Detection of telomerase activity combined with cytology were able to identify 17 of 23 (74%) lung cancer cases whereas cytology alone identified 10 of 23 (43%) such cases (p=0.035). These results indicate that telomerase is a specific marker for malignant lung disease and a potential complementary tool to cytology in the diagnosis of certain lung cancer cases.

4.1 Introduction

Telomerase is a ribonucleoprotein enzyme which replaces chromosome ends lost during successive cycles of cell division (Morin, 1989). Telomerase activity has been detected in almost all types of human cancer, including lung cancer (Xinarianos *et al,* 1999, Albanell *et al,* 1997; Bacchetti & Counter, 1995; Hiyama *et al,* 1995; Shay & Bacchetti, 1997). Previous studies in lung cancer patients indicated that telomerase activity is present in most of such cases, ranging from 78-96% in NSCLC while it was present in all of the small cell carcinomas tested (Xinarianos *et al,* 1999, Albanell *et al.* 1997; Hiyama *et al,* 1995; Lee *et al,* 1998). Moreover, a recent study demonstrated that dysregulation of telomerase is an early event in the multistage pathogenesis of bronchogenic lung carcinomas (Yashima *et al,* 1997). There have been numerous studies in order to evaluate telomerase as a diagnostic marker in various types of human cancer (Cunningham *et al,* 1998; Shay & Gazdar, 1997).

In this study, the TRAP assay was used to detect telomerase activity in BL specimens in order to establish conditions to assess telomerase as a potential diagnostic marker for malignant lung disease.

4.2 Materials & Methods

4.2.1 Cell Lines

The human non-small cell lung cancer cell lines Calu3, H1155, HTB58, were used as positive controls for telomerase activity. All cell lines were purchased from the American Type Culture Collection and grown according to the conditions recommended by the supplier. Cell pellets were collected after trypsinisation and frozen at -80°C until preparation of telomerase extracts.

4.2.2 Patients and Bronchial Lavage Specimens

BL specimens were collected from 36 individuals with suspected lung cancer who have been referred to the CTC in Liverpool. The criteria for referral to the CTC included; unresolved chest infection, abnormal chest X ray, cough (>4 weeks), non-specific weight loss, stridor, persistent (>3 weeks) hoarse voice and other suspicious features which would prompt referral to the lung cancer clinic. The choice of site was based on bronchoscopic findings within the large airways and approximately 20 ml of saline was introduced via the bronchoscope and then aspirated. Two to four hundred µl of each BL fluid were frozen at -80°C for later preparation of protein extracts. Diagnoses of the patients from which the specimens were collected included: 20 NSCLC (5 adenocarcinomas, 1 adenosquamous carcinoma, 11 squamous cell carcinomas, 2 non-small cell lung carcinomas, 1 lung carcinoid), 3 small cell

carcinomas, and 13 patients who were found to have no clinical evidence of lung cancer.

4.2.3 TRAP Assay

Each BL sample was first pelleted by centrifugation at 14,000 rpm for 5 min at RT, then lysed in 100 µl of ice-cold CHAPS lysis buffer as previously described (Kim *et al,* 1994, Chapter 3 in this thesis). Resulting supernatants were snap frozen in liquid nitrogen and stored at 80°C until they were subjected to the TRAP assay. A protein concentration for each extract was determined using the BSA protein assay kit (Pierce Chemical Co, UK). The TRAP assay was performed as previously described with a few modifications (Kim *et al,* 1994; Wright *et al,* 1995). Samples were subjected to 31 or 37 cycles of PCR amplification. Specificity controls were performed by pre-treatment of protein extracts from BL with 0.5 µg RNase A (Sigma, UK) for 30 min at 37°C or heat inactivation at 95°C for 5 min before addition to the TRAP assay. Blank control samples containing CHAPS lysis buffer were also run. TRAP products were run on 10% non-denaturing polyacrylamide gels for 1000-1,100 Vh and the 6 bp DNA ladder was visualised by silver staining (10 min washing in fixation buffer consisting of 10% absolute ethanol and 0. 5% acetic acid. Gels are then stained with 0.1% $AgNO_3$ for 20 min and developed in developer solution consisting of 1.5% NaOH and 0.15% formaldehyde followed by a final fixation step in 0.75% Na_2CO_3 for 5 min. All steps were carried out on a shaker plate.) or autoradiography after 1-7 days exposure at

-80°C. A BL sample was scored as telomerase positive when an RNase A and/or heat sensitive 6 bp DNA ladder was observed. All experiments were carried out in duplicate.

4.2.4 PCR Controls for Telomerase Inhibitors

The presence of *Taq* polymerase inhibitors in the BL extracts was examined by mixing genomic DNA derived from cultured cells and protein extracts derived from the BL specimens followed by PCR amplification of either exon 1 of *K-ras* or exon 10 of *p53*. Samples which failed to amplify in the PCR reaction were subjected to two further centrifugations at 14,000 rpm for 20 min at 4°C. In each step, supernatants were transferred into fresh tubes. Then, samples were further analysed for the presence of inhibitors. When all samples had demonstrated that no inhibitors were present, they were then examined for genomic DNA contamination. An equal volume to the one subjected to the TRAP assay from each BL specimen was used in the PCR reaction for either *K-ras* or *p53*. When all samples had demonstrated that no inhibitors and genomic DNA were present they were finally subjected to the TRAP assay.

4.2.5 Statistical Analysis

Results were analysed using Fisher's exact test.

4.3 Results

Protein extracts for telomerase activity were prepared from BL specimens from subjects undergoing diagnosis of lung cancer or other inflammatory lung diseases. In the TRAP assay, each BL sample was run at a series of dilutions. A specimen was determined to be positive if a distinct 6 bp ladder was seen in two separate assays. Examples of telomerase activity detected in BL specimen extracts are shown in Figure 4.3.1.

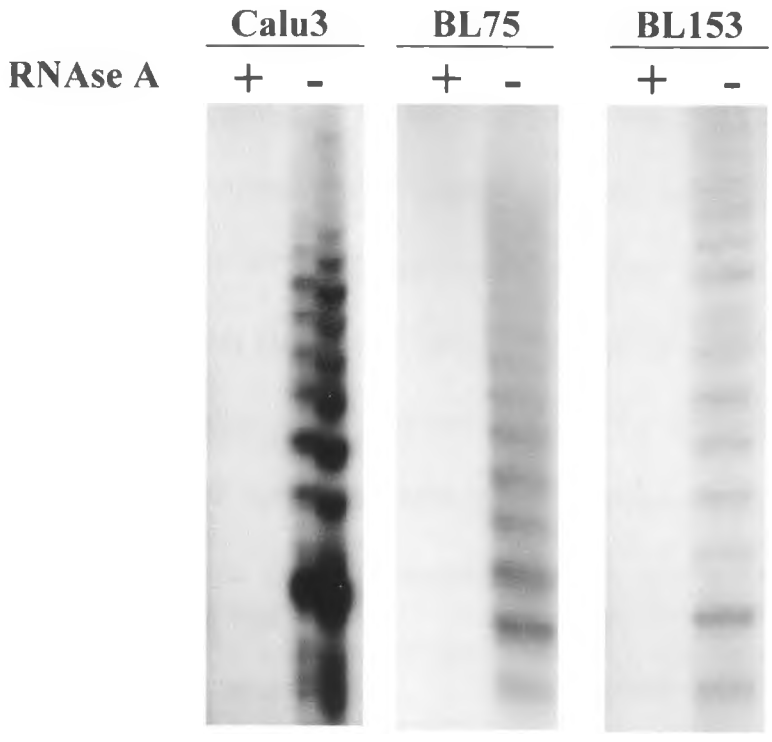

Figure 4.3.1. Detection of telomerase activity in bronchial lavage specimens by TRAP assay. Lanes marked "+" are RNase A digestion controls for each sample. BL specimens 75 and 153 show abolishment of the TRAP assay product ladder after digestion with RNase A, indicating product is due to telomerase activity in the sample. Lane marked Calu3 is a positive control TRAP reaction using a cell extract from a lung cancer cell line.

Fifteen of the 36 BL specimen extracts exhibited inhibition of the PCR reactions for K-ras exon 1 or p53 exon 10. These specimens were subjected to two further centrifugations at 14,000 rpm for 20 min at 4°C. Further analysis revealed that no inhibitors were present. Overall, telomerase activity was detected in 17 of 36 (47%) BL specimens examined. In particular, 16 of 23 (70%) specimens from lung cancer patients and one of 13 (8%) BL specimens from patients with other conditions were positive for telomerase (p=0.00038). Nine of 10 (90%) BL specimens with positive cytology for lung cancer were positive for telomerase. One telomerase positive specimen was from a patient with cystic fibrosis. In addition, telomerase activity was detected in 7 of 13 (54%) BL specimens from patients with lung cancer with a negative cytology report. The results of this study are summarised in Table 4.3.1.

Table 4.3.1. Telomerase activity in BL specimens

Patient Diagnoses	n	Telomerase (+)	%
Lung Cancer	23	16	70
Cytology positive[a]	10	9	90
Cytology negative[b]	13	7	54
No Clinical Evidence of Lung Cancer	13	1	8
Total	**36**	**17**	**47**

[a]Cytology positive: malignant cells seen

[b]Cytology negative: no malignant cells seen

When detection of telomerase activity was combined with cytology we could identify 17 of 23 (74%) lung cancer cases while cytology alone could only identify 10 of 23 (43%) such cases (p=0.035). Statistical analysis of the 36 specimens showed no significant correlation of telomerase activity in BL specimens with current smoking status, tobacco consumption, age and sex of the patient. Telomerase activity was detected more frequently in patients with squamous cell carcinomas than adenocarcinomas, 10 of 11 (91%) and 2 of 5 (40%) respectively. However, this was not found to be statistically significant (p=0.06).

4.4 Discussion

Telomerase activity has been measured in a number of clinical specimen types that are relevant to early diagnosis of a range of cancers (Aogi *et al,* 1998; Califano *et al,* 1996; Kinoshita *et al,* 1997; Kyo *et al,* 1997; Muller *et al,* 1996; Yoshida *et al,* 1997). Each type of specimen provides individual challenges. In order to develop new diagnostic tools for lung cancer, it is necessary to establish conditions for the measurement of potential new biomarkers in sputum and bronchial lavage specimens (Field *et al,* 1998). It has been previously demonstrated that genetic alterations in the form of microsatellite instability and/or loss of heterozygosity may be detected in BL specimens from lung cancer patients and individuals at high risk of developing lung cancer (Field *et al,* 1999).

In this study, the TRAP assay was employed to detect telomerase activity in 36 BL specimens from individuals with suspected lung cancer. It is of particular interest to note that telomerase activity was detected in BL specimens from individuals who had a negative cytology report but the patient's lung cancer was diagnosed by other methods (radiology, biopsy or surgery). The latter indicates that a proportion of the cells in the BL had telomerase activity although the specimen had no cytological evidence of malignancy. This is also in agreement with previous published reports (Arai *et al,* 1998; Yahata *et al,* 1998).

In a paper by Yahata and co-workers, (1998) 16 of 22 (73%) BL specimens from lung cancer patients, assayed by an extract-based fluorescent TRAP assay, were positive for telomerase. Another study reported telomerase activity by TRAP in 29 of 37 (78%) BL specimens from cancer patients, including 8, which were negative by cytology (Arai *et al,* 1998). In the data presented here, measurable telomerase activity was found in 16 of 23 (70%) BL specimens from patients with a final diagnosis of lung cancer. It is of note that in this study only 200 to 400 μl of the total volume of each BL specimen was used to prepare an extract for the TRAP assay compared to larger volumes (1-10 ml) used in previous studies (Arai *et al,* 1998; Hiyama *et al,* 1998; Yahata *et al,* 1998).

In a previously published report telomerase activity was detected in 4 bronchoalveolar lavage samples from patients with non-cancerous lung diseases. Two of the telomerase-positive samples were from patients who died of aggressive pulmonary inflammation within a month after the specimen was taken (Hiyama *et al,* 1998). In this study, telomerase activity was detected in a sample from a patient with cystic fibrosis who died from respiratory failure seven months after presentation. Thus, it may be argued that increased telomerase activity in bronchial specimens may be a marker of aggressiveness in non-malignant lung disease as well as neoplasia. Since telomerase activity was detected in 70% of lung cancer cases and only in 8% of cases with a final diagnosis of benign lung disease, it may be argued that telomerase is a highly specific marker for malignant lung disease.

On examining the combined results of telomerase activity and cytology, 74% of lung cancer cases were identified, whereas with cytology alone 43% of the lung cancer patients were identified. However, it should also be stressed that detection of telomerase activity could identify 70% of lung cancer cases. It has been demonstrated that telomerase activity may be detected in most of lung cancer cases, ranging from 78-96% in surgically excised tumour tissues (Albanell *et al,* 1997; Hiyama *et al,* 1995; Lee *et al,* 1998; Xinarianos *et al,* 1999). Nevertheless, detection of telomerase activity in clinical specimens such as BL or sputum appears to be a promising complementary method to cytology for the diagnosis of certain lung cancer cases.

A trend has been observed for telomerase activity to be detected more frequently in BL specimens from patients with SqCCL (10 of 11) than with adenocarcinomas (2 of 5). This may be explained by the fact that adenocarcinomas are usually associated with peripheral airways while SqCCL are usually associated with large airways. Consequently, the latter are more efficiently sampled by bronchoscopy and BL.

In this study, it was demonstrated that telomerase activity is a specific molecular marker for the diagnosis of certain lung cancer cases and could serve as a complementary diagnostic method to cytology. In addition, telomerase may have a role as a marker of aggressiveness in non-neoplastic inflammatory lung diseases. However, further studies of telomerase activity as a diagnostic/

prognostic marker are required. Moreover, *in situ* assays for detection of the telomerase RNA (hTR) and catalytic protein (hTRT) components may need to be applied to reduce the number of false negative results found in clinical specimens such as BL or sputum (Snijders *et al,* 1998; Soder *et al,* 1997; Soder *et al,* 1998).

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CHAPTER FIVE

MOLECULAR INVESTIGATION OF THE MISMATCH REPAIR GENES hMLH1 AND hMSH2 IN NON-SMALL CELL LUNG CARCINOMAS

Abstract

In this study, the expression levels of hMLH1 and hMSH2 proteins in relation to LOH on chromosomes 3p and 2p, the mutational status of the promoters and certain hot spots in the genes were investigated in 150 NSCLC. It has been demonstrated that 88 of 150 (59%) tumour specimens had reduced expression levels of the hMLH1 while 85 of 147 (58%) specimens had reduced expression levels of the hMSH2 protein. Reduced expression levels of both proteins were observed in 51 of 150 (34%) specimens. In adenocarcinomas, the reduction of hMSH2 expression was more frequently observed than that of hMLH1 ($p<0.003$) while in SqCCL, hMLH1 expression was more frequently reduced than hMSH2 ($p<0.006$). Reduced expression of hMLH1 correlated with allelic imbalance on loci *D3S1289* ($p<0.0002$) and *D2S391* ($p<0.05$). It is of note that an inverse correlation was found between hMSH2 reduced expression and LOH at locus *D3S1300* ($p=0.016$). In addition, hMLH1 reduced expression was more frequently associated with heavy smokers, assessed by daily tobacco uptake ($p=0.018$) and total smoking exposure (pack-years) ($p<0.05$). In addition, a correlation between hMLH1 reduced expression and nodal metastasis in SqCCL was observed ($p=0.015$). No mutations were identified in the promoters or exons examined in these two genes. Differential expression of hMLH1 and hMSH2 was not associated with the expression of the cell cycle regulators Rb and $p21^{WAF1}$. These findings indicate that *hMLH1* and *hMSH2* gene inactivation is a common event in the development of NSCLC and allelic loss appears to be a major genetic event involved in

hMLH1 silencing. In addition, it is suggested that a putative negative regulator of *hMSH2* gene may be located at the locus 3p14.

5.1 Introduction

hMLH1 and *hMSH2* are two of the genes known to be implicated in the DNA MMR system. Inactivation of the MMR machinery has been closely associated with a mutator phenotype, which is a hallmark of almost all human cancers (Loeb, 1998). Molecular defects in one or both of the genes account for a significant proportion of hereditary non-polyposis colorectal carcinomas (HNPCC) and a small proportion of sporadic colorectal cancer cases (Peltomaki & de la Chapelle, 1997; Rhyu, 1996). Inactivation of DNA mismatch repair genes occurs in two steps following the same pattern as in tumour suppressor genes (Kolodner, 1996). Previous studies have demonstrated that loss of heterozygosity (LOH) at the DNA MMR loci is a frequent genetic event in human cancers, including lung cancer (Benachenhou *et al,* 1998a,b; Benachenhou *et al,* 1999; MacDonald *et al,* 1998; Wieland *et al,* 1996). It has also been shown that methylation of the promoter region of the *hMLH1* gene leads to lack of expression of the encoding protein (Fleisher *et al,* 1999; Kane *et al,* 1997; Simpkins *et al,* 1999). However, *hMSH2* promoter methylation has not been demonstrated in tumours lacking expression of the relative protein (Cunningham *et al,* 1998; Esteller *et al,* 1998; Herman *et al,* 1998). Recent reports have indicated that reduced expression levels of the DNA MMR genes may be implicated in the pathogenesis of certain human cancers and may predict disease free survival following primary chemotherapy (Curia *et al,* 1999; Mackay *et al,* 1999; Shin *et al,* 1998; Soliman *et al,* 1998; Thibodeau *et al,* 1996; Wei *et al,* 1997; Wei *et al,* 1998). A possible role of

hMLH1 and hMSH2 overexpression in the induction of apoptosis has been also suggested (Zhang *et al,* 1999).

Multiple molecular defects have been identified to play a role in the molecular pathogenesis of lung tumours, including alterations in oncogenes and tumour suppressor genes (Field *et al,* 1998; Sekido *et al,* 1998). Mutations in the p53 and K-ras genes as well as allelic losses and deletions at chromosomes 3p and 9p appear to be among the most commonly found genetic defects in carcinomas of the lung (Liloglou *et al,* 1997; Neville *et al,* 1995a,b; Neville *et al,* 1996; Sekido *et al,* 1998). Although the role of the hMLH1 and hMSH2 genes in the molecular pathogenesis of HNPCC and sporadic colorectal carcinoma has been well studied, little is known about the involvement of these genes in lung cancer.

In this study, the expression levels of hMLH1 and hMSH2 proteins were investigated in relation to allelic imbalance at chromosomes 2p and 3p in NSCLC. The mutational status of the promoter regions and the most frequently mutated exons reported of the hMLH1 and hMSH2 genes were also examined. In addition, the expression levels of hMLH1 and hMSH2 proteins were investigated in relation to the expression levels of the cell cycle regulators Rb and $p21^{WAF1}$.

5.2 Materials & Methods

5.2.1 Patients

Lung tumour tissue samples were obtained from 150 patients, 59 males and 91 females, who were operated in the CTC of Broadgreen University Hospital, Liverpool (Merseyside, UK). The age of the patients ranged between 41 and 95 years (median=65). The histology of the specimens included in this investigation was: 49 adenocarcinomas, 85 squamous cell carcinomas, 8 adenosquamous, 6 large cell carcinomas and 2 unclassified non-small cell lung carcinomas. Smoking history (daily consumption, current status) was available for 111 individuals; 54 current smokers, 16 recently stopped smokers (1-4 years prior to presentation), 35 former smokers (≥5 years prior to presentation) and 6 non-smokers. However, complete data for calculating the total smoking exposure was available for only 65 smokers. Total smoking exposure is expressed in pack-years:

$$\text{pack-years} = \text{years smoked} \times \text{packs/day}$$

In this study the patients' pack years ranged from 17 to 165 (median = 69).

5.2.2 Immunohistochemical Detection of hMLH1 and hMSH2 Protein Expression.

Protein expression was demonstrated immunohistochemically by a modified avidin–biotin complex method. Formalin fixed paraffin process tissues were sectioned at 4μm thickness, mounted on APES coated slides and dried at 37°C overnight. Sections were deparaffinised in xylene for 5 min and rehydrated in a series of graded alcohols (100%, 90%, 75%) to tap water, 5 min in each one. Heat mediated antigen retrieval was required to expose the epitopes and was performed by microwaving the sections on full power in 0.01M citrate buffer (pH 6.0) for 15 mins in a 800W microwave oven. The sections were left to stand for 15mins to cool and then rinsed for 5 mins in running tap water. Endogenous peroxidase activity was blocked by 1.5% hydrogen peroxide in methanol for 10 mins. Sections were incubated in the primary antibody buffer (5% goat serum in PBS) for 20mins. Monoclonal antibodies against hMLH1 and hMSH2 (Serotec UK) were diluted 1:10 and 1:20 respectively in the primary antibody buffer and incubated for 1 hour at room temperature. The primary antibodies were visualised with Dako LSAB 2 Peroxidase kit (DAKO, UK). The secondary and tertiary reagents were incubated for 30 mins each and rinsed in between each stage with 0.05M TBS (pH 7.6). The signal was developed with diaminobenzidine (Merck, UK) and hydrogen peroxide. The sections were counterstained with haematoxylin. Normal mouse IgG replaced the primary antibody as a negative control. The frequency of the nuclear staining was scored on a scale from (-) to (+++) [as absent (-), weak (+), moderate (++) and strong (+++)] without the knowledge

of clinical, pathological, 3p allelic imbalance status, Rb and $p21^{WAF1}$ expression data.

5.2.3 DNA Extraction

Paired tumour-normal frozen tissue specimens were available from 85 individuals. Tumour frozen tissue specimens were also available for 35 individuals with no corresponding normal tissue. Five 10 μm sections of each sample were microdissected to ensure presence of more than 80% tumour cells. Sections were lysed in 400 mM Tris-HCl pH 8.0, 150 mM NaCl, 60 mM EDTA, 1% SDS, 100 μg/ml Proteinase K and incubated at 42°C for 16 hours in an orbital shaker. Deproteinization included extraction with an equal volume of phenol/chloroform and chloroform. Each step was followed by centrifugation at 14,000 rpm for 5 min (RT) and the aqueous phases were transferred into fresh tubes. DNA was precipitated by the addition of an equal volume of isopropanol and mixing by inversion. DNA was spooled onto sterile microbiology loops, washed with 500 μl 70% ethanol and resuspended in 200 μl TE (10mM Tris-HCl, pH 7.0 and 1mM EDTA). Working stocks were prepared by 5-fold dilution in sterile distilled H_2O.

5.2.4 Allelic Imbalance Analysis

Four markers located proximal and distal to hMLH1 gene (*D3S1289, D3S1266, D3S1300* and *D3S1304*) and one marker proximal to hMSH2 gene

(*D2S391*) were available for 85 individuals from a previous study of the Molecular Oncology Unit in the Roy Castle International Centre for Lung Cancer Research (Liloglou *et al,* 2000). An additional marker (*D2S2259*) also located on 2p16 was examined in this study and added in the existing database. All fluorescent microsatellite markers were selected from the Linkage Mapping Set V2.0 (PE-Applied Biosystems, Warrington, UK). The reaction mixture contained 1×GeneAmp Buffer II, 350 μM dNTPs, 2.66 mM $MgCl_2$ and 0.35 U AmpliTaq Gold™ polymerase (PE-Applied Biosystems, Warrington, UK).

Amplification parameters were the following: Initial denaturation for 12 min; 30 cycles consisted of 94°C for 30 sec, 55°C for 30 sec and 72°C for 30 sec; final extension step at 72 °C for 20 min. PCR products were diluted 2.5 fold in ddH_2O and mixed with loading buffer: size marker ROX-350 (PE-Applied Biosystems, Warrington, UK), dextran blue, formamide (1:1:5). After denaturation at 95°C for 5 min samples were analysed on an ABI-PRISM 377 automatic sequencer (PE-Applied Biosystems, Warrington, UK). Results were analysed using the Genescan™ and Genotyper™ software (PE-Applied Biosystems, Warrington, UK).

5.2.5 Mutational Analysis

Mutational analysis was performed on 120 samples. Screening of *hMLH1* promoter region and exons 9, 13 and 16 and *hMSH2* promoter region

and exons 5, 7 and 8 was performed by PCR followed by single strand conformational polymorphism (SSCP) and heteroduplex analysis (HA). The primers used for *hMLH1* (exons 9, 13 and 16) and *hMSH2* (exons 5, 7 and 8) and PCR amplification parameters have been previously described (Wijnen *et al,* 1996). The primers used for the amplification of the promoter regions of *hMLH1* and *hMSH2* are as follows:

hMLH1 promoter: 5' AGGCTCCACCACCAAATAAC 3' (sense)

5' CGCTGTCCGCTCTTCCTATT 3' (antisense)

(PCR amplification product 311 bp).

hMSH2 promoter: 5' CCTTGCATACACCCCACCCA 3' (sense)

5' GCGACCCCACACCCACTAA 3' (antisense)

(PCR amplification product 287 bp).

PCR reactions were performed in a 25 μl reaction volume and contained 100 ng of genomic DNA, 200 μM of each dNTP, 8 pM of each primer, 0.6U *BIOPRO* polymerase (Bioline, UK) and 2.5 μl 10X buffer (50mM KCl, 10 mM Tris-HCl pH 8.8, 0.1% Triton X-100, 1.5 mM $MgCl_2$. Samples were subjected to 37 cycles of amplification. Five μl of the amplification products were analysed on a 2% agarose gel, stained with ethidium bromide (5μg/ml) and visualised on a UV transilluminator.

For SSCP analysis, 2-4 μl of the PCR product were mixed with 10 μl of denaturing solution consisted of 80% formamide, 100 mM NaOH, 1 mM EDTA, 0.1% Bromophenol Blue, 0.1% Xylene Cyanol FF. Samples were then heated at 95°C for 3 min, chilled on ice and loaded onto 8-10% native polyacrylamide gels, containing 5-10% glycerol. Gels were run at 15°C for 2,500-3,500 Volt hours and silver stained after electrophoresis. HA was performed as follows: 2-5 μl of the PCR product were denatured at 95 °C for 5 min and allowed to cool down slowly at RT for 1 hour. Samples were then analysed on 8% native polyacrylamide gels and run for 1,600-1,800 Volt hours. Gels were silver stained after electrophoresis.

5.2.6 Sequencing

PCR products were used for subsequent sequencing after purification with QIAquick PCR Purification Kit following the procedure recommended by the supplier (Qiagen,Ltd, West Sussex, UK). Sequencing was performed using the ABI PRISM® BigDye™ Terminator Cycle Sequencing kit and analysis on a 377 sequencer (PE Biosystems, Warrington, UK) using the supplier's protocol. The forward or reverse primer used for PCR amplification of each fragment was also used in sequencing reactions.

5.2.7 Statistical Analysis

Fisher's exact test was used to analyse the molecular and clinicopathological data. Analysis was performed using the SPSS software.

5.3 Results

5.3.1 hMLH1 and hMSH2 Expression in Lung Tumours

Fifteen normal lung tissues, adjacent to tumours examined in this study were investigated for the expression of hMLH1 and hMSH2. In all cases normal bronchial epithelium adjacent to tumour demonstrated strong staining (+++) for both proteins (Figure 5.3.1.1A and B). Hence, tumours demonstrating strong (+++) immunoreactivity were classified as "normal expression" while tumours demonstrating absent, weak and moderate immunoreactivity were classified as "reduced expression".

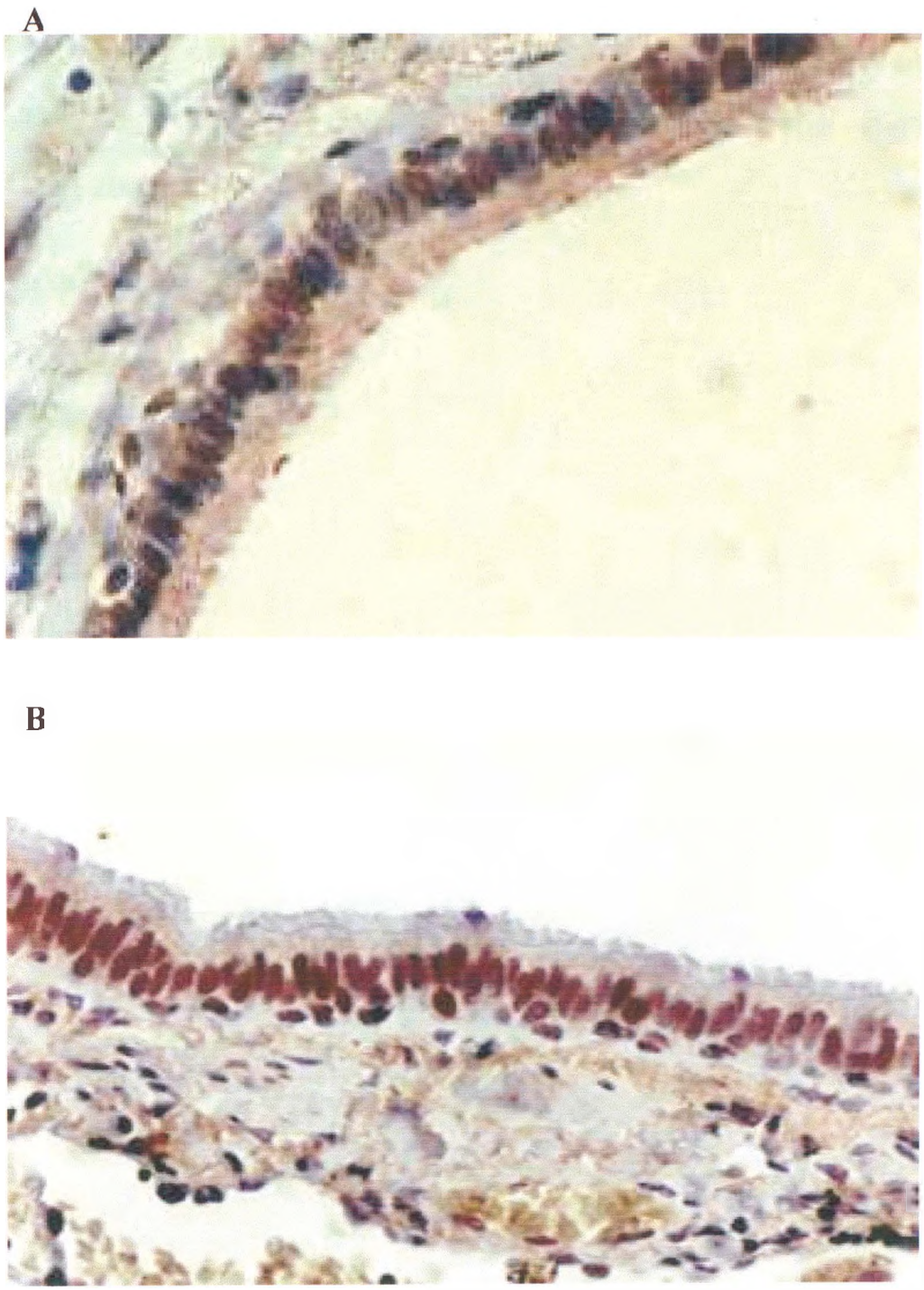
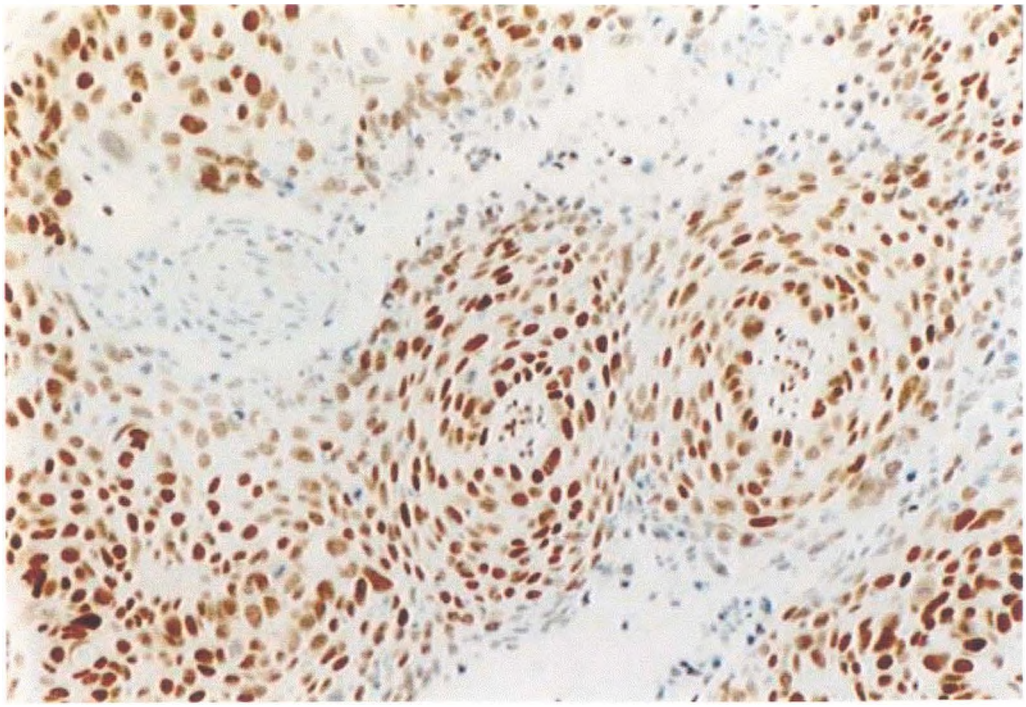


Figure 5.3.1.1. Representative examples of hMLH1 (A) and hMSH2 (B) expression in normal lung adjacent to tumour.

hMLH1 expression was examined in 150 NSCLC tissues. Sixty-two (41%) were found to have intense staining (+++) (Figure 5.3.1.2A) and 88 (59%) showed reduced (absent, weak or moderate) staining (-, + or ++) (Figure 5.3.1.2B-D).

A



B

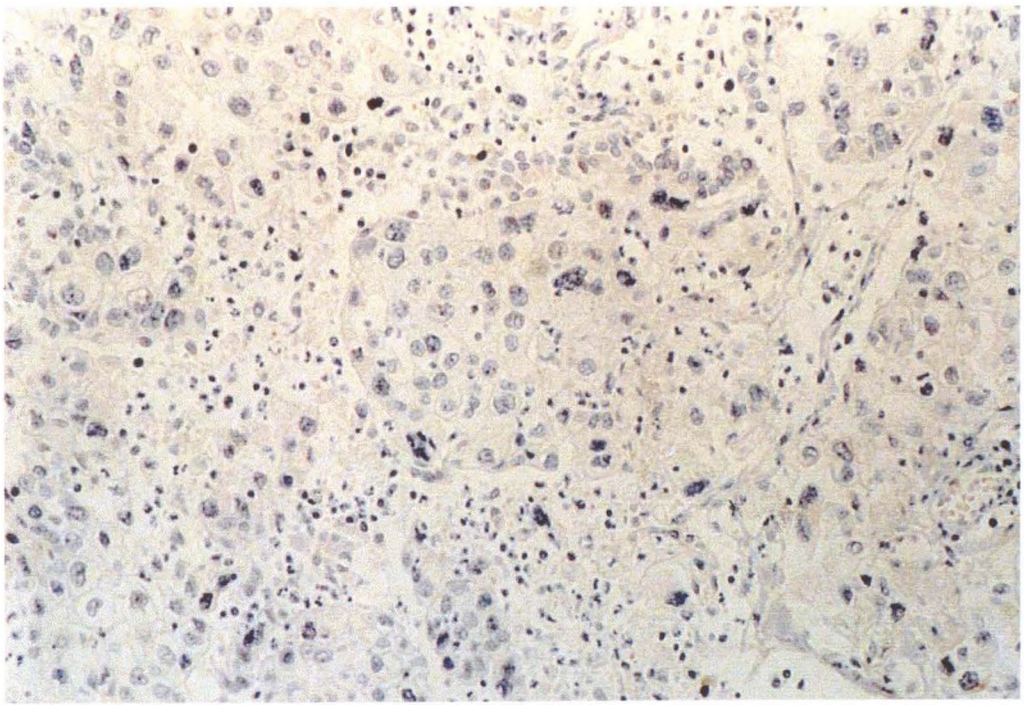
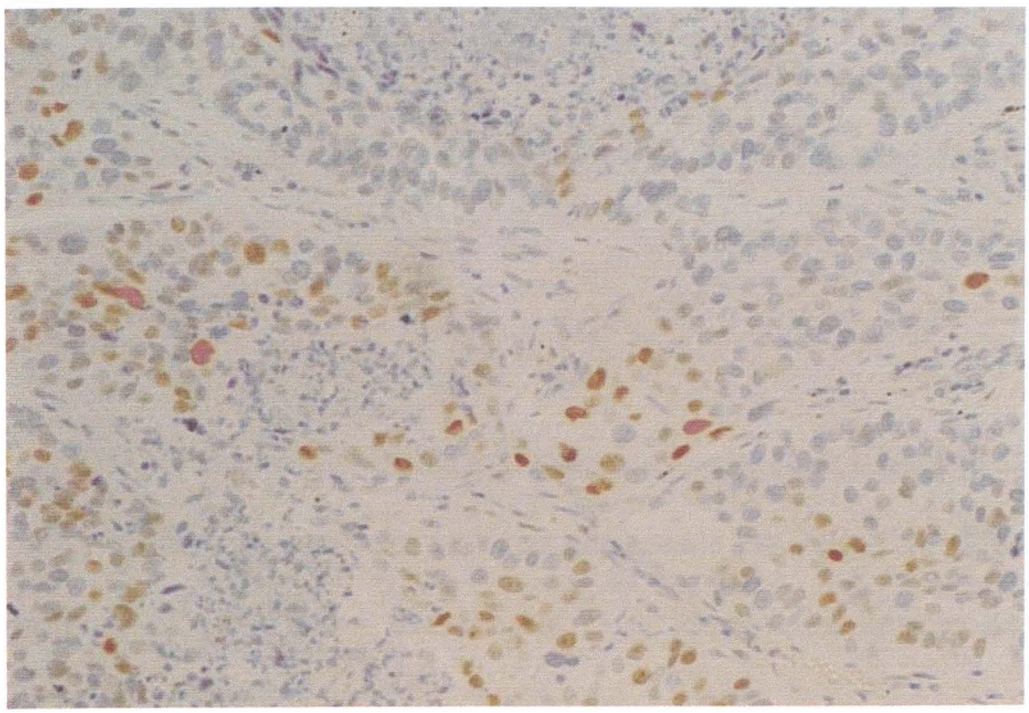


Figure 5.3.1.2A-B. Representative examples of strong (A) and absent (B) hMLH1 expression in NSCLC

C



D

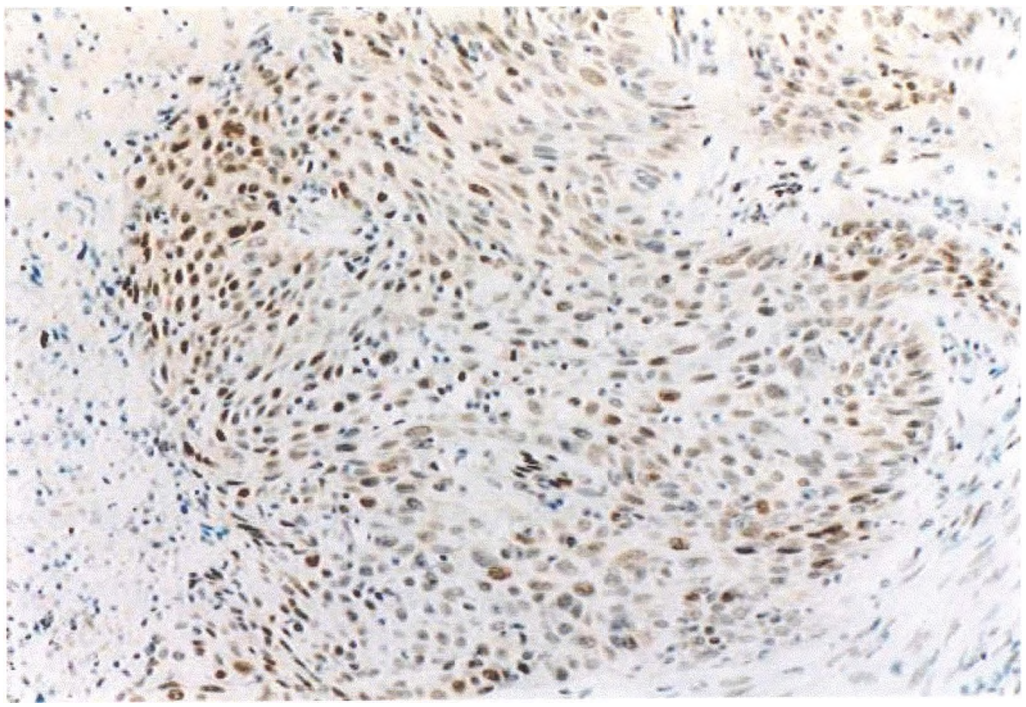


Figure 5.3.1.2C-D. Representative examples of weak (C) and moderate (D) hMLH1 expression in lung carcinomas.

Of the specimens with reduced expression, 8 showed absence (-) of hMLH1 expression whereas 22 showed weak (+) and 58 showed moderate (++) expression (Table 5.3.1.1). On examining the NSCLC subtypes; 26/49 (53%) adenocarcinomas and 56/85 (66%) SqCCL showed reduced hMLH1 expression. (Table 5.3.1.1).

It is of note that hMLH1 reduced expression was more frequently found in heavy smokers (>1 packs per day) than in moderate smokers (≤ 1 pack per day). Forty-six of 71 heavy smokers and 13 of 32 moderate smokers had reduced hMLH1 expression (Fisher's exact test, $p=0.018$) (Table 5.3.1.2). In addition, hMLH1 reduced expression was more frequently found among patients with total smoking exposure higher than the median (69 pack-years) ($p<0.05$) (Table 5.3.1.2). No association was found between the non-smoker/former/current smoker status and hMLH1 expression levels. A correlation between hMLH1 reduced expression and nodal metastasis was found in SqCCL ($p=0.015$). In particular, hMLH1 reduced expression was found in 29 of 51 (57%) SqCCL specimens with negative nodes and in 24 of 29 (83%) SqCCL with positive nodes. No significant associations were found between hMLH1 expression and other clinico-pathological parameters (age, gender, differentiation and T stage).

Table 5.3.1.1. Levels of expression of hMLH1 and hMSH2 genes in NSCLC detected by immunohistochemistry.

Histology	hMLH1 expression				hMSH2 expression			
	Reduced			Normal	Reduced			Normal
	-	+	++	+++	-	+	++	+++
AdenoCa	2	6	18		1	20	19	
		26		**23**		**40**		**9**
SqCCL	5	14	37		7	6	24	
		56		**29**		**37**		**45**
Other NSCLC	1	2	3		0	2	6	
		6		**10**		**8**		**8**
Total NSCLC		**88**		**62**		**85**		**62**

(-): absence of expression, (+): weak expression, (++): moderate expression, (+++): strong expression.

Table 5.3.1.2. Expression levels of hMLH1 and hMSH2 proteins in lung tumors in relation to the patients' smoking exposure

Gene	Expression level	Smoking status				Daily tobacco consumption		Total tobacco exposure[1]	
		Non smokers	Current	Former ≥5 years	Former 1-4 years	≤1 pack/day	>1 packs/day	<69 pk-ys	≥69 pk-ys
								19	
hMLH1	normal	3	24	16	4	19	25	22	15
	reduced	3	30	19	12	13	46	10	18
hMSH2	normal	2	24	13	6	11	31	13	19
	reduced	4	29	22	9	21	38	15	17

[1]The patients have been grouped according to their pack-year data based on the mean pack-year value found in this study (69 pack-years)

hMSH2 expression was examined in 147 NSCLC tissues. No tissue was available for 3 other samples that had already been examined for hMLH1. Sixty-two (42%) were found to have strong expression (+++) (Figure 5.3.1.3A) and 85 (58%) showed reduced (absent, weak or moderate) expression (-, + or ++) (Figure 5.3.1.3B-D). Nine of 49 adenocarcinomas, (18%) demonstrated hMSH2 strong expression while reduced expression was observed in 40 (82%). Forty-five of 82 (55%) SqCCL showed strong expression whereas 37 (45%) showed reduced expression (Table 5.3.1.1). No significant associations were identified between hMSH2 expression and T stage, nodal metastasis, differentiation, smoking status, age or gender of the patient.

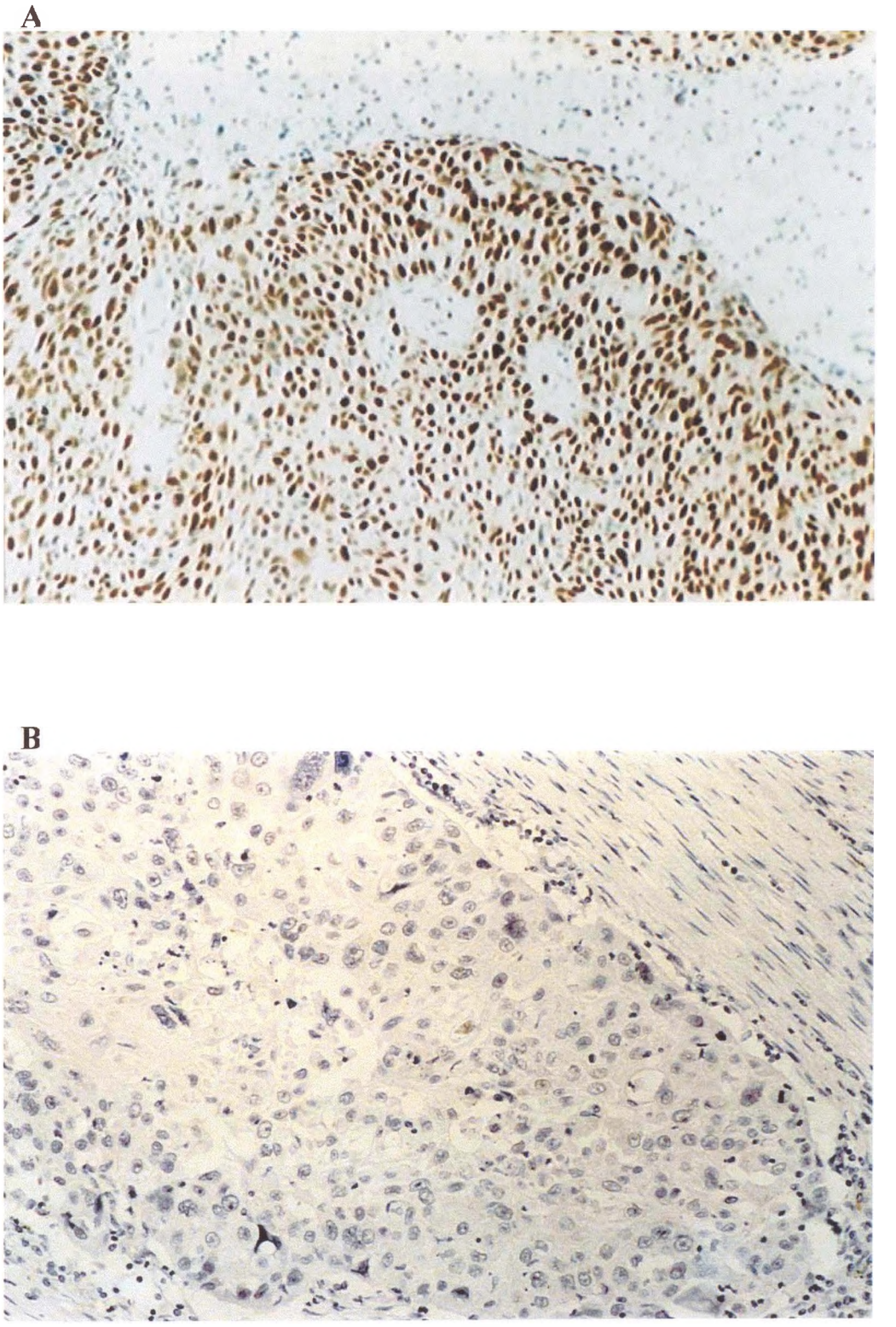


Figure 5.3.1.3A-B. Representative examples of strong (A) and absent (B) hMSH2 expression in NSCLC.

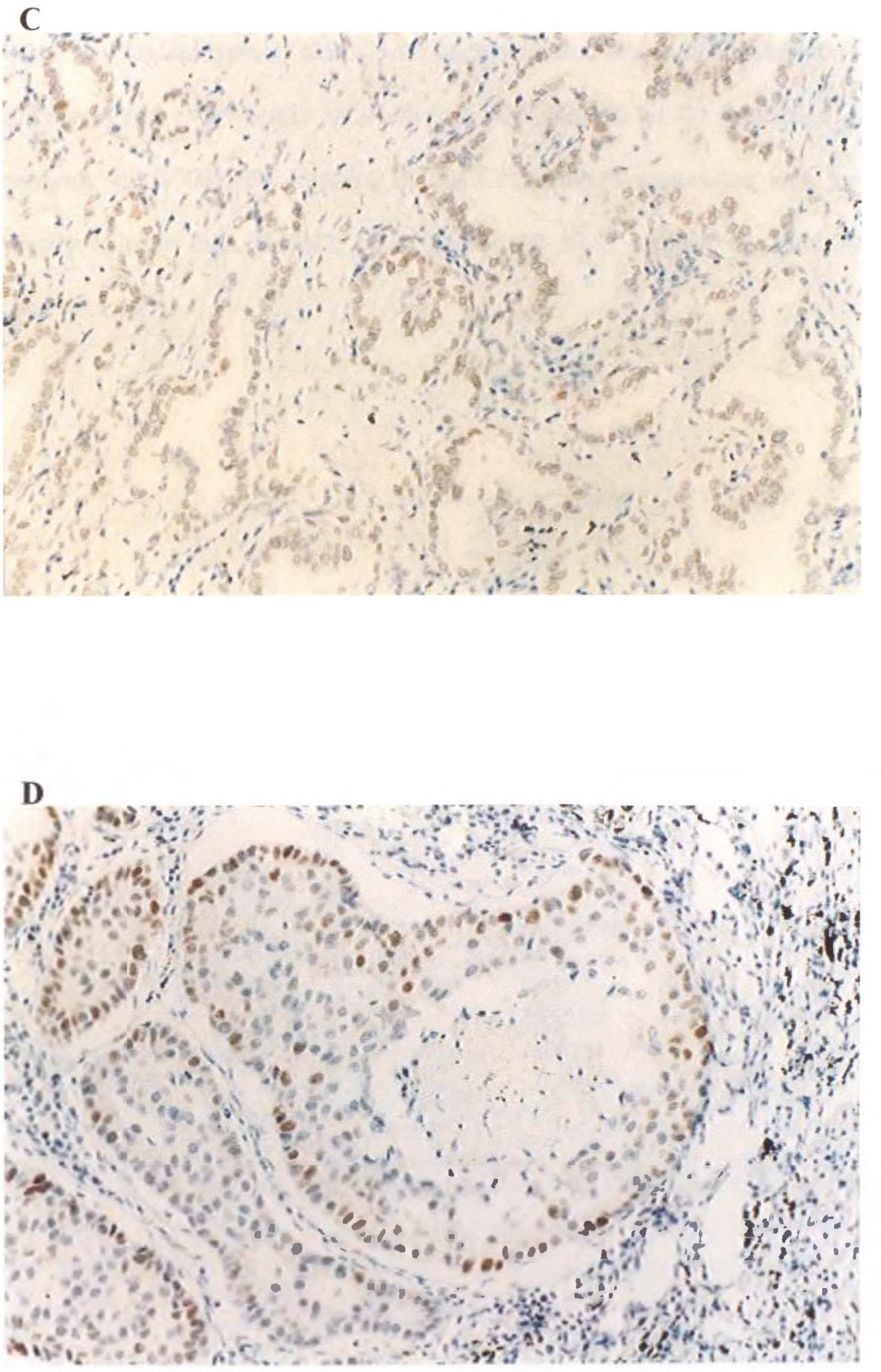


Figure 5.3.1.3C-D. Representative examples of weak (C) and moderate (D) hMSH2 expression and in NSCLC.

The comparative analysis of expression levels of hMSH2 and hMLH1 in different histological types (Table 5.3.1.1) demonstrated that in adenocarcinomas, hMSH2 was more frequently reduced than hMLH1, 40 of 49 and 26 of 49 respectively ($p<0.003$). In contrast, in SqCCL, hMLH1 expression was more frequently reduced than hMSH2, 56 of 85 and 37 of 82 respectively ($p<0.006$) (Figure 5.3.1.4).

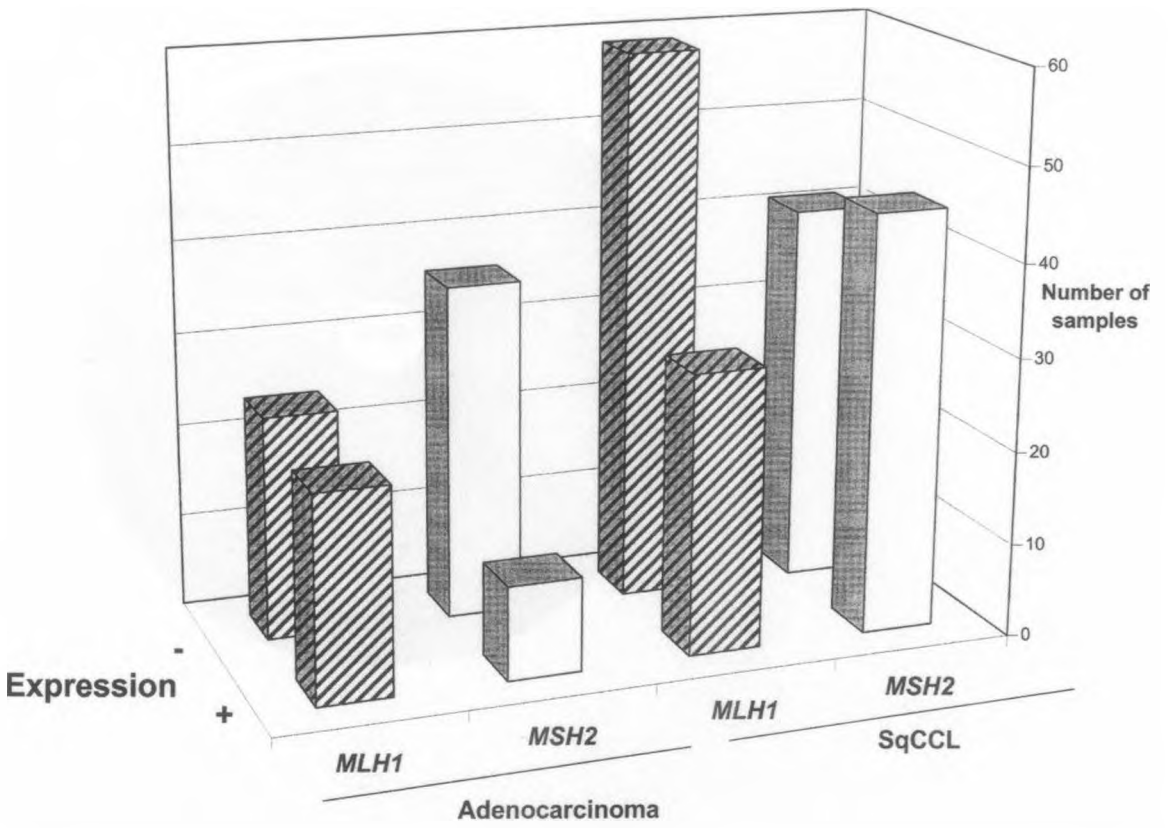


Figure 5.3.1.4. Histological type specific reduced expression of hMLH1 and hMSH2 in lung adenocarcinomas and squamous cell lung carcinomas. hMSH2 expression was reduced more than hMLH1 in lung adenocarcinomas while hMLH1 expression was reduced more than hMSH2 in squamous cell carcinomas of the lung (SqCCL).

Simultaneous reduced expression of both hMLH1 and hMSH2 was found in 51 of 150 (34%) samples examined (Figure 5.3.1.5). Samples with reduced expression of both MMR proteins, compared to samples with reduced expression of only one of the examined proteins, did not show any associations with the clinical and pathological parameters examined.

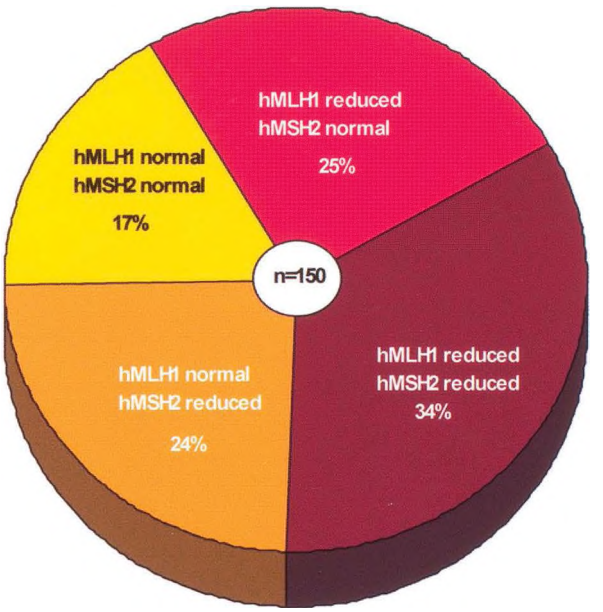


Figure 5.3.1.5. hMLH1/hMSH2 expression in NSCLC patients investigated in this study.

5.3.2 Mutational Analysis of *hMLH1* and *hMSH2* Promoter Regions and Hotspot Exons

Mutational analysis using SSCP and HA was performed on the promoter regions of *hMLH1* and *hMSH2* genes in 120 samples and in exons shown to have mutational hot spots in HNPCC and sporadic colorectal carcinomas (exons 9, 13 and 16 of *hMLH1* and exons 5, 7 and 8 of *hMSH2*) (Figures 5.3.2.1A-B and 5.3.2.2A-B respectively). No mutations or polymorphisms were detected in any of the examined regions.

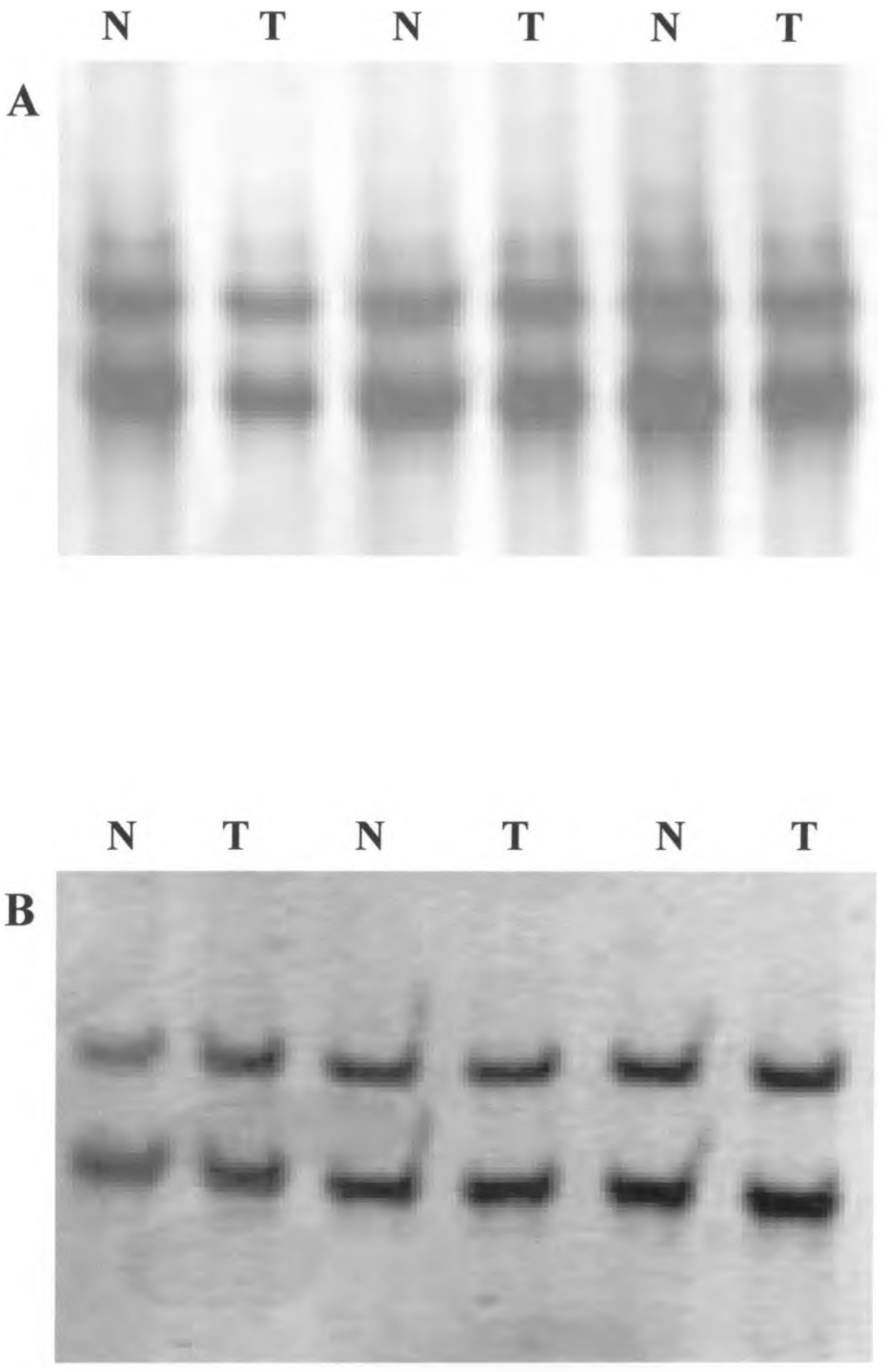


Figure 5.3.2.1. Representative examples of SSCP analysis of hMLH1 (A) and hMSH2 (B) promoters in NSCLC. T: DNA from lung tumour tissue, N: DNA from corresponding normal lung tissue.

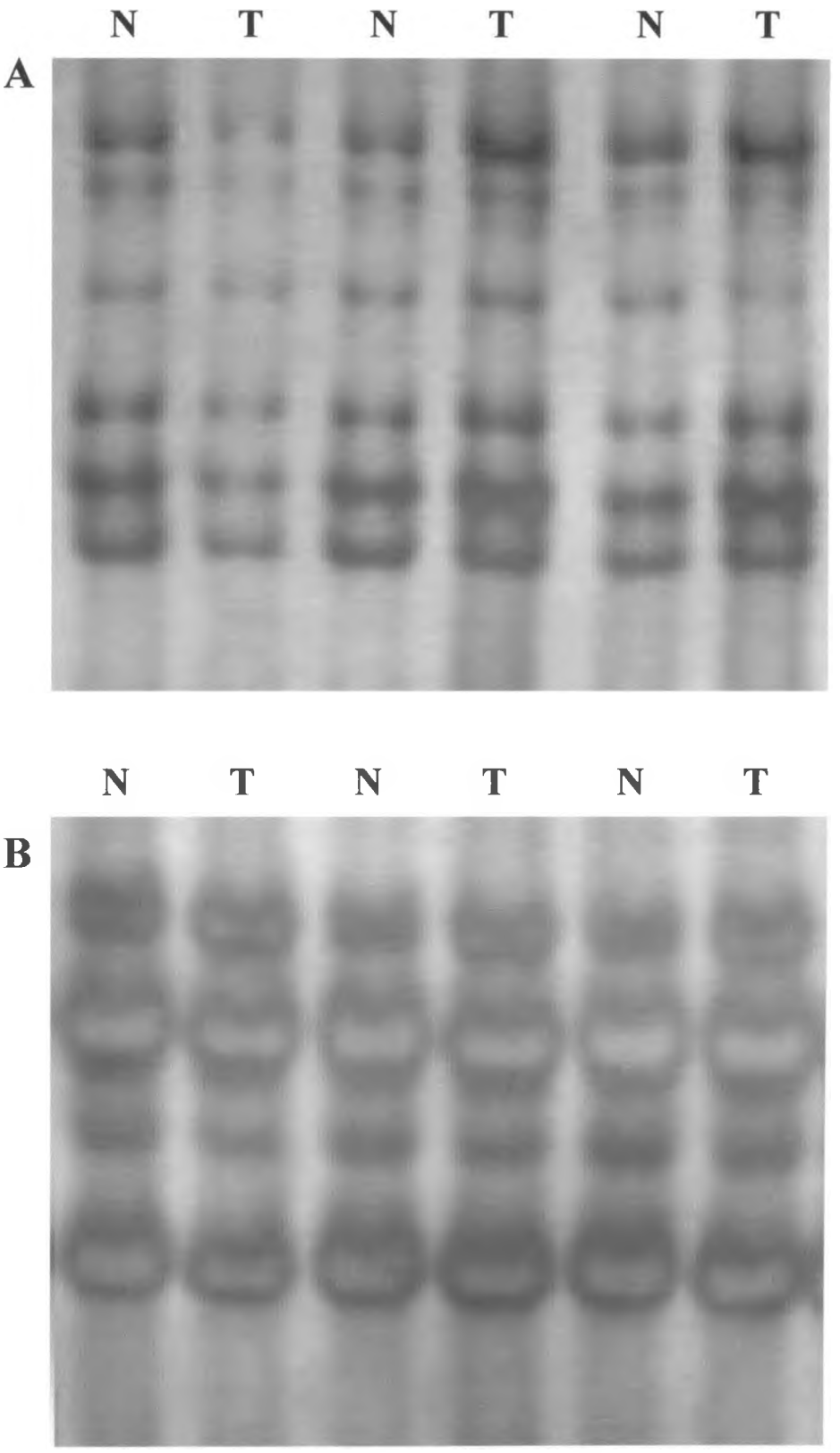


Figure 5.3.2.2. Representative examples of SSCP analysis of hMLH1 exon 16 (A) and hMSH2 exon 8 (B). T: DNA from lung tumour tissue, N: DNA from corresponding normal lung tissue.

We have also undertaken automated sequencing analysis for both of these genes in 20 randomly selected samples, in order to check possible SSCP false negatives, however, no mutations were found.

5.3.3 hMLH1 and hMSH2 Expression in Relation with Allelic Imbalance at Chromosomes 2p and 3p

Eighty-five of the 150 samples examined in the current study were previously investigated for allelic imbalance using fluorescent microsatellite markers and analysis on a 377 ABI PRISM automatic sequencer (Liloglou *et al.* 2000). In this study, an additional marker on 2p16 (*D2S2259*) was also examined. Allelic imbalance for the latter was identified in 26 of 60 (43%) informative cases (Figure 5.3.3.1).

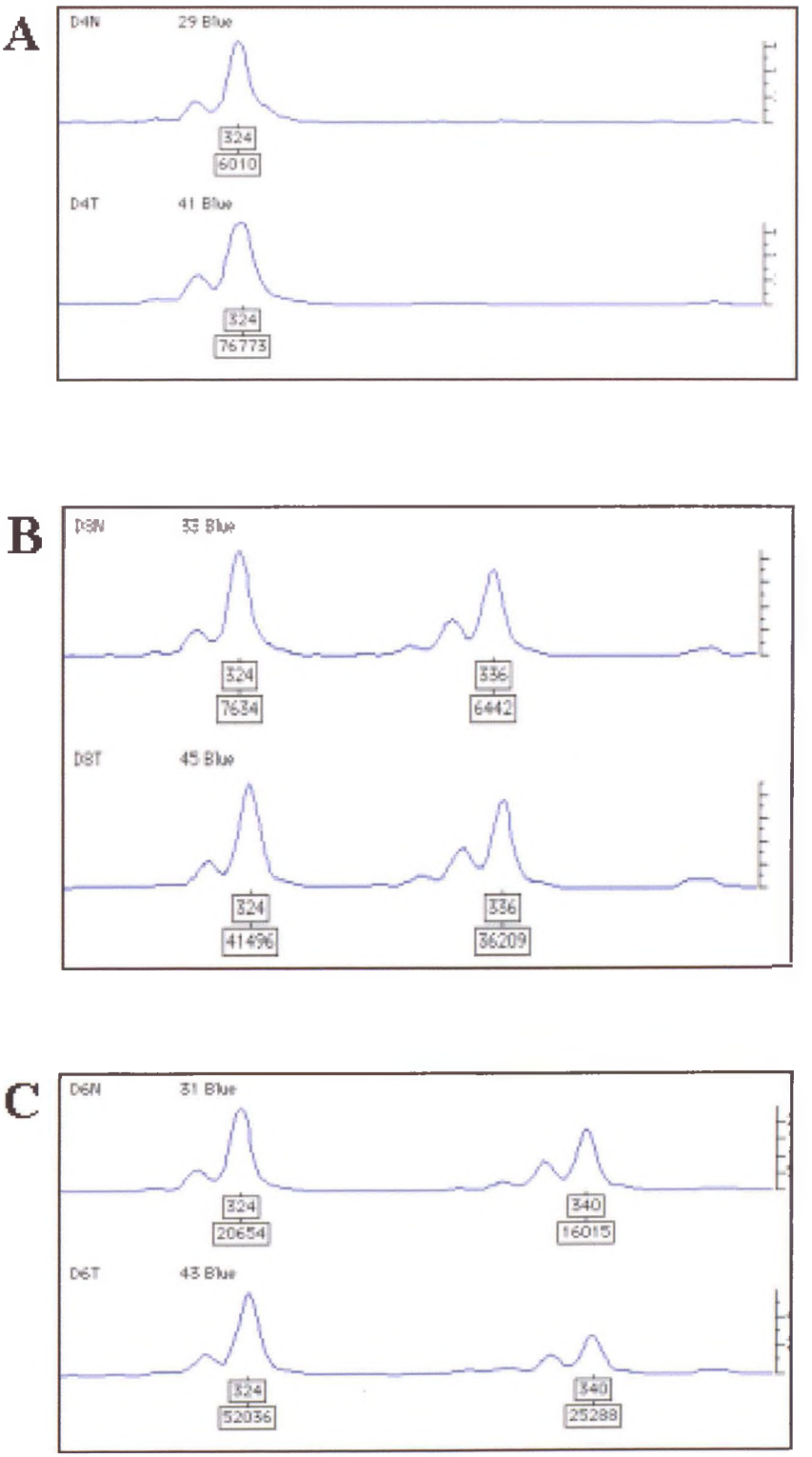


Figure 5.3.3.1. Representative example of allelic imbalance analysis at the D2S122 locus. A: Homozygous, B: Heterozygous, C: Allelic Imbalance

The comparative expression analysis of hMLH1 and hMSH2 and the allelic imbalance data was based on 4 markers on chromosome 3p in 85 individuals (Table 5.3.3.1). The results showed that hMLH1 reduced expression correlated with allelic imbalance at the *D3S1289* locus on 3p21 (p=0.00019). No such correlation was found with the loci; *D3S1304* (3p26) (p=0.14); *D3S1266* (3p24) (p=0.1) and *D3S1300* (3p14) (p=0.57). However, an inverse correlation was found between allelic imbalance at locus *D3S1300* (p=0.016) and expression of hMSH2 protein (Table 5.3.3.1). In addition, a trend was observed between a higher expression level of the hMSH2 protein and allelic imbalance at loci *D3S1266* (p=0.063) and *D3S1289* (p=0.059), but not with locus *D3S1304* (p= 0.55).

No association was found between hMSH2 expression and allelic imbalance at the *D2S391* (p=0.28) and *D2S2259* (p=0.24) loci. However, a correlation was found between hMLH1 reduced expression and allelic imbalance at the *D2S391* (p=0.048) but not at the *D2S2259* locus (p=0.14).

Table 5.3.3.1. Expression levels of hMLH1 and hMSH2 proteins in lung tumors in relation to allelic imbalance on chromosomes 3p and 2p. Only informative cases (heterozygous status in normal) were included.

Gene	Expression level	D3S1304		D3S1266		D3S1289		D3S1300		D2S391		D2S2259	
		H	L	H	L	H	L	H	L	H	L	H	L
hMLH1	normal	11	11	13	16	18	13	8	18	24	11	19	10
	reduced	8	18	7	21	3	24	9	19	13	16	15	16
hMSH2	normal	11	15	8	24	9	25	6	26	23	14	18	17
	reduced	8	12	12	13	12	12	11	11	14	13	16	9

H: heterozygous, L: loss of heterozygosity

5.3.4 hMLH1 and hMSH2 expression in relation with expression of Rb and $p21^{WAF1}$

Rb and $p21^{WAF1}$ expression data were available from previous unpublished studies of the Molecular Oncology Unit in the Roy Castle International Centre for Lung Cancer Research.

Reduced expression of MLH1 was detected in 13 of 56 (50%) specimens with abnormal Rb expression and 43 of 78 (55%) specimens with normal Rb expression. In addition, reduced MSH2 expression was detected in 14 of 26 (54%) specimens with abnormal Rb expression and 35 of 78 (45%) specimens with normal Rb expression (Table 5.3.4.1).

Reduced MLH1 expression was detected in 25 of 52 (48%) specimens with abnormal $p21^{WAF1}$ expression and 31 of 50 (62%) of specimens with normal $p21^{WAF1}$ expression. In addition, reduced MSH2 expression was detected in 22 of 53 (42%) specimens with abnormal $p21^{WAF1}$ expression and 25 of 50 (50%) specimens with normal $p21^{WAF1}$ expression (Table 5.3.4.1).

No statistically significant correlations were found between Rb and $p21^{WAF1}$ expression levels and simultaneous reduced expression of both MLH1 and MSH2.

Table 5.3.4.1. MLH1 and MSH2 expression in relation to the expression of $p21^{WAF1}$ and Rb in NSCLC

		MLH1 expression			MSH2 expression		
		Normal	**Reduced**	**Fisher's test p value**	**Normal**	**Reduced**	**Fisher' test p val**
p21 expression	abnormal	27	25	0.11	31	22	0.37
	normal	19	31		25	25	
Rb expression	abnormal	13	13	0.41	12	14	0.28
	normal	35	43		43	35	

5.4 Discussion

The *hMLH1* and *hMSH2* DNA mismatch repair genes are known to be implicated in human carcinogenesis through a mutator phenotype. Indeed, familial and sporadic colon cancers are the most well studied models. However, the information of the status of these two genes in lung cancer is limited. In this study, the expression of the *hMLH1* and *hMSH2* DNA mismatch repair genes were investigated in NSCLC lesions. The immunohistochemical analysis demonstrated that 59% of the examined tumours had reduced expression of hMLH1 and 58% had reduced expression of hMSH2, while 34% demonstrated reduction of expression in both of these genes. It is of note that 82% of all examined lung tumours showed reduced expression of at least one of the two investigated genes. These results suggest a critical role for these DNA MMR genes in lung carcinogenesis.

It is of note that the reduction of expression of these two genes is associated with the histological subtypes; in adenocarcinomas hMSH2 expression was more frequently reduced than that of hMLH1 while the converse was observed in SqCCL. As both genes are considered to be inactivated in a two-hit model (Kolodner, 1996), the relationship between MMR gene expression levels and allelic imbalance on 3p and 2p chromosome arms, the locations of these genes, was investigated. The results indicated that reduced hMLH1 expression correlated with allelic imbalance at the *D3S1289* (3p21) locus. This suggests that loss of one allele of the *hMLH1* gene may be one of the major genetic events

involved in its inactivation. This may explain the finding that hMLH1 expression is more frequently reduced in SqCCL than in adenocarcinomas, as the former have demonstrated a greater incidence of LOH on chromosome 3p (Liloglou *et al,* 2000; Neville *et al,* 1996; Tsuchiya *et al,* 1992). Hypermethylation of the *hMLH1* promoter has been demonstrated in human tumours (Cunningham *et al,* 1998; Fleisher *et al,* 1999; Herman *et al,* 1998; Kane *et al,* 1997; Simpkins *et al,* 1999) and this most likely also contributes to changes in the gene's expression. The mutational analysis of the *hMLH1* promoter region and the hot spot exons did not reveal any mutations, which is in agreement with previous reports (Benachenhou *et al,* 1998b; Hatta *et al,* 1997) and suggests that mutations are unlikely to be a major cause of *hMLH1* inactivation in lung carcinogenesis. A correlation was found between the reduced expression of hMLH1 and allelic imbalance at the *D2S391* (2p16) locus, which may suggest that hMLH1 expression regulatory gene(s) are located in this region but further studies are required to clarify this aspect.

It is of particular note that an inverse relationship between allelic imbalance at the *D3S1300* locus and hMSH2 expression was identified where hMSH2 reduced expression was more prevalent in samples retaining heterozygosity at this locus. This may imply the presence of a negative hMSH2 regulatory gene on 3p, suggestive of negative feedback mechanism; however, further studies are required to elucidate the nature of this relationship. This inverse correlation provides a possible explanation for the lower incidence of reduced hMSH2 expression in SqCCL compared to adenocarcinomas. This is possibly due

to the relatively higher incidence of LOH on 3p found in SqCCL (Liloglou *et al,* 2000; Neville *et al,* 1996; Tsuchiya *et al,* 1992).

No association was found between hMSH2 expression and allelic imbalance at the *D2S391* and *D2S2259* (2p16) loci, which suggests that allelic imbalance at these loci is not the main event contributing to the reduction of hMSH2 expression in NSCLC. Our results indicated no mutations in the promoter and the hot spot exons of *hMSH2*, which is in agreement with previous reports (Anbazhagan *et al,* 1999; Gotoh *et al,* 1999; Hatta *et al,* 1997). Furthermore, no hypermethylation of *hMSH2* promoter has been demonstrated in certain human tumours (Cunningham *et al,* 1998; Esteller *et al,* 1998; Herman *et al,* 1998), thus, inactivation of the *hMSH2* gene may rely on alternative mechanisms involving changes in its upstream regulatory genes. Recent reports have revealed p53-binding sites on the *hMSH2* promoter (Scherer *et al,* 1996) and a possible hMSH2 expression regulatory role of p53 in leukaemias (Zhu *et al,* 1999).

hMLH1 reduced expression correlated with both higher daily tobacco uptake and total tobacco exposure (pack-years) indicating that tobacco carcinogens are implicated in *hMLH1* inactivation and moreover, that they may have an additive effect. The lack of association with the current/former smoking status argues that smoking related MMR inactivation may be irreversible and it is in agreement with the fact that smoking cessation does not significantly reduce risk for lung cancer development in chronic smokers to the base line of non-

smokers. Moreover, hMLH1 expression levels did not appear to differ between smokers and non-smokers. Although only 6 non-smokers were included in this study, and a "passive smoker" status is difficult to assess, the above finding indicates that carcinogens apart from those found in tobacco may also affect hMLH1 expression. Reduced expression of hMLH1 in SqCCL correlated with nodal metastasis, suggesting that the *hMLH1* gene may contribute to a more aggressive tumour phenotype in this histological subtype. Thus, hMLH1 expression may be a useful molecular marker for a clinician, suggesting towards a more radical treatment.

The comparative analysis of hMLH1 and hMSH2 expression in the tumours in this study did not reveal any associations between the expression of these two genes. Furthermore, tumour specimens with combined reduced expression of both hMLH1 and hMSH2 proteins did not correlate with any clinical and pathological parameters. Thus, no complementary role of these two proteins was demonstrated. The frequencies of these two genes reduced expression are different in SqCCL and adenocarcinomas and this may be due to the different incidence of LOH on chromosome 3p in these tumour subtypes. Also, the results in this study demonstrated that smoking affects hMLH1 but not hMSH2, while hMLH1 but not hMSH2 correlates with nodal metastasis in SqCCL. All the above findings support distinct roles for the two genes in lung carcinogenesis. The results suggest that at least some of the environmental and endogenous factors involved in their inactivation pathway are different. Moreover, the comparative analysis of hMLH1 and hMSH2 differential expression with the

expression levels of Rb and $p21^{WAF1}$ demonstrated that the regulation of these MMR proteins is independent of the expression levels of two of the major cell cycle regulators. Nevertheless, further investigations are required to elucidate the complete pathways of inactivation of these two genes in NSCLC and reveal additional factors implicated in their regulation.

Part of this chapter has been published. G. Xinarianos, T. Liloglou, W. Prime, P. Maloney, J. Callaghan, P. Fielding, J. R. Gosney, J.K.Field. hMLH1 and hMSH2 expression correlates with allelic imbalance on chromosome 3p in non-small cell lung carcinomas. *Cancer Research* 60: 4216-4221, 2000.

CHAPTER SIX

MOLECULAR ANALYSIS OF THE p53 STATUS IN NON-SMALL CELL LUNG CARCINOMAS

Abstract

The status (mutations and expression) of the p53 gene was examined in 108 NSCLC. The relation of p53 to the differential expression of two of the DNA MMR proteins, MLH1 and MSH2, was also investigated. p53 status was comparatively analysed in relation to the expression of two of the cell cycle regulators, $p21^{WAF1}$ and Rb, and allelic imbalance on chromosome 3p. A possible association between p53 and telomerase activity was also investigated. p53 overexpression was demonstrated in 64% while p53 mutations were detected in 43% of the samples examined. Both p53 mutations and overexpression were more frequent in SqCCL (57% and 73% respectively) than in lung adenocarcinomas (22% and 50% respectively) (p=0.0008 and p=0.02 respectively). Intense p53 immunostaining strongly correlated with missense (90%) rather than null (36%) mutations (p=0.0004). p53 mutations were more frequent in samples with abnormal Rb expression (37%) than those with normal Rb expression (17%) (p=0.015). Interestingly, in NSCLC with wild type p53, increased expression of MSH2 correlated with p53 intense staining (p=0.018). In addition, in SqCCL, p53 mutations correlated with reduced MSH2 expression (p=0.019). These data suggest an interaction between p53 and MSH2. Although there is already evidence for p53 being a transcriptional regulator of MSH2, these findings suggest that MSH2 may act as a DNA damage signaller to p53. p53 overexpression and p53 aberrations (mutation, overexpression or both) correlated with telomerase activity, p=0.01 and p=0.004 respectively, suggesting a close association between p53 damage and activation of telomerase in NSCLC. p53 mutations correlated with allelic

imbalance at locus *D3S1266* (p=0.04) while p53 overexpression correlated with allelic imbalance at loci *D3S1266* (p=0.02), *D3S1289* (p=0.04) and *D3S1304* (p=0.02), indicating a possible interaction between gene(s) located on chromosome 3p and p53.

6.1 Introduction

The p53 gene codes for a 53 KDa nuclear phosphoprotein which holds a key position in the complex network controlling genome stability , cell cycle, and apoptosis (Steele *et al,* 1998). p53 acts as a tumour-suppressor gene by arresting cells with DNA damage in the Go/G1 phase providing adequate time for the cell's DNA repair mechanism to function and, if unsuccessful, leads cells to apoptotic death (Oren 1999). Wild-type p53 transactivates $p21^{WAF1}$ which is a CDK inhibitor, preventing phosphorylation of pRb by the Cyclin/CDK complexes and consequently sequestration from E2F and progression into the S phase (Steele *et al,* 1998, Oren 1999).

The p53 gene has been found to be inactivated in a large range of human tumors (Holstein *et al,* 1991, Greenblatt *et al,* 1994, Holstein *et al,* 1996, Beroud and Soussi, 1998, Bennet *et al,* 1999). This inactivation can arise by means of mutations in the *p53* gene or binding of the p53 protein by cellular (MDM2) or viral (AdE1b, HBx, HPV E6 etc) proteins. In non-small cell lung carcinomas (NSCLC), p53 mutations have been previously reported and are considered among the most frequent genetic events contributing in the molecular pathogenesis of such neoplasms (Mitsudomi *et al,* 1992, Carbone *et al,* 1994, Liloglou *et al,* 1997a). The majority of the resulting p53 mutant proteins due to missense mutations are resistant to degradation and therefore have prolonged half-lives. The latter allows detection of mutant proteins by immunohistochemical staining (Steele *et al,* 1998). Previous studies have reported the increased p53

staining in human cancers, including lung cancer (Field *et al,* 1991, Field *et al,* 1992, Harris and Holstein, 1993, Bodner *et al,* 1992, Hartman *et al,* 1995, Casey *et al,* 1996). A possible role of p53 expression as a prognostic marker in lung cancer has also been suggested (Papadakis *et al,* 1992, Brambilla *et al,* 1993, Carbone *et al,* 1994). Although missense mutations in the p53 gene have been frequently correlated with p53 overexpression, null mutations are often not detected by immunohistochemical staining (Bodner *et al,* 1992, Hartman *et al,* 1995, Casey *et al,* 1996, Hashimoto *et al,* 1999). The majority of the p53 mutations described, have been detected within exons 5-8, which encodes 88% of the DNA binding domain of the protein. However, there is considerable evidence suggesting that p53 mutations and their biological effects may have been underestimated since the whole coding region (exons 2-11) of the p53 gene has not been extensively investigated (Hartman *et al,* 1995, Casey *et al,* 1996, Walker *et al,* 1999). It has been demonstrated that p53 mutations outside of exons 5-8 account for about 20% of the total number of mutations (Casey *et al,* 1996, Brattstrom *et al,* 1998, Fujita *et al,* 1999, Tomizawa *et al,* 1999).

p53 levels increase in response to DNA damage (Kastan et al 1991, Cox and Lane, 1995) and this is mainly accomplished through post-translational modifications (Lakin & Jackson, 1999). The DNA-PK, ATM and ATR genes are among the known signallers of DNA damage to p53. p53 can bind *in vitro* to the promoter region elements of the h*MSH2* (Scherer et al 1996) and synergies with c-Jun in regulating MHS2 expression (Scherer *et al,* 2000). Further support for a MSH2 expression regulatory role of p53 comes

from an association between p53 mutations and MSH2 downregulation in adult acute leukaemia cases (Zhu *et al,* 1999).

p53 is believed to play a central role in maintaining genomic stability (Marx, 1994). Telomerase adds telomeric repeat sequences to the ends of chromosomes in order to compensate for the losses that occur with each round of DNA replication (Harley & Villeponteau, 1995). It has been proposed that one of the main functions of p53 may be to detect telomere erosion and subsequently signal growth control pathways (Wynford-Thomas *et al*, 1995). An association between telomerase activity and p53 overexpression has been demonstrated in breast and lung tumours (Roos *et al*, 1998; Wu *et al*, 1999).

In this study, the status (expression and mutations) of the p53 tumour suppressor gene was investigated in NSCLC cases from a population in Northwest England (Merseyside, UK). The possible relations of p53 with two of the DNA MMR genes, *hMLH1* and *hMSH2*, two of the cell cycle regulators, Rb and $p21^{WAF1}$ and allelic imbalance on chromosome 3p were examined. The possible association between p53 and telomerase in NSCLC was also investigated.

6.2 Materials and Methods

6.2.1 Patients and Tissue Samples

Lung tumour tissue samples were obtained from 108 patients, 41 males and 67 females, who were operated in the CTC of Broadgreen University Hospital, Liverpool (Merseyside, UK). The age of the patients ranged between 41 and 95 years (median=63). The histology of the specimens included in this investigation was: 36 adenocarcinomas, 58 squamous cell carcinomas, 7 adenosquamous, 5 large cell carcinomas and 2 unclassified non-small cell lung carcinomas. Smoking history (daily consumption, current status) was available for 108 individuals; 58 current smokers, 12 recently stopped smokers (1-4 years prior to presentation), 30 former smokers (≥5 years prior to presentation) and 8 non-smokers. Complete data for calculating the total smoking exposure was available for 98 smokers. Total smoking exposure is expressed in pack-years:

$$\text{pack-years} = \text{years smoked} \times \text{packs/day}$$

The patients' pack years ranged from 16 to 177 (median = 77).

6.2.2 DNA Extraction and PCR Amplification for p53

Frozen tumour tissue specimens were available from 108 individuals. Five 10 μm sections of each sample were microdissected to ensure presence of

more than 80% tumour cells. Sections were lysed in 400 mM Tris-HCl pH 8.0, 150 mM NaCl, 60 mM EDTA, 1% SDS, 100 μg/ml Proteinase K and incubated at 42°C for 16 hours in an orbital shaker. Deproteinization included extraction with an equal volume of phenol/chloroform and chloroform. Each step was followed by centrifugation at 14,000 rpm for 5 min (RT) and the aqueous phases were transferred into fresh tubes. DNA was precipitated by the addition of an equal volume of isopropanol and mixing by inversion. DNA was spooled onto sterile microbiology loops, washed with 500 μl 70% ethanol and resuspended in 200 μl TE (10mM Tris-HCl, pH 7.0 and 1mM EDTA). Working stocks were prepared by 5-fold dilution in sterile distilled H_2O.

Twenty five μl PCR reactions were performed containing 100 ng of genomic DNA, 200 μM of each dNTP, 50mM KCl, 10 mM Tris-HCl pH 8.8, 0.1% Triton X-100, 1.5 mM $MgCl_2$, 6 pM of each primer and 0.6U *BIOPRO* polymerase (Bioline, UK) Samples were subjected to PCR amplification using the following parameters: Initial denaturation at 95 °C for 3 min followed by 37 cycles of denaturation at 94 °C for 40 sec, annealing at 57-66 °C for 30 sec and extension at 72 °C for 30 sec, followed by a final extension step at 72 °C for 5 min. Oligonucleotide primers, annealing temperatures and PCR amplification product sizes are shown in Table 6.2.2.1. Five μl of the amplification products were analysed on a 2% agarose gel, stained with ethidium bromide (5μg/ml) and visualised on a UV transilluminator.

Table 6.2.2.1. Primers used for p53 PCR amplification in this study.

Exon(s)	Primer Sequence (5'-3')	Annealing (^{0}C)	PCR product (bp)
2/3	F: GCG TCT CAT GCT GGA TCC CC	65	307
	R: CCT CCA GGT CCC AGC CCA AC		
4	F: TGA GGA CCT GGT CCT CTG AC		
	R: AGA GGA ATC CCA AAG TTC CA	57	294
5	F: TGT GCC CTG ACT TTC AAC TC		
	R: TCA GTG AGG AAT CAG AGG CC	57	297
6	F: GGC CTC TGA TTC CTC ACT GA		
	R: GCC ACT GAC AAC CAC CCT TA	66	192
7	F: CTC ATC TTG GGC CTG TGT TA		
	R: GAA ATC GGT AAG AGG TGG GC	57	228
8	F: GGG AGT AGA TGG AGC CTG GT		
	R: ACT GCA CCC TTG GTC TCC TC	66	272
9	R: TAT CAC CTT TCC TTG CCT CT		
	F: ACG GCA TTT TGA GTG TTA GA	57	163
10	F: CCC CCT CCT CTG TTG CTG CA		
	R: GGA AGG CAG GGG AGT AGG GC	66	172
11	F: TGT CAT CTC TCC TCC CTG CT		
	R: AGT GGG GAA CAA GAA GTG GA	60	141

6.2.3 p53 SSCP and Heteroduplex Analysis

Single strand conformation polymorphism (SSCP) and heteroduplex analysis (HA) were primarily used to screen for p53 mutations.

For SSCP analysis, 2-4 µl of the PCR product were mixed with 10 µl of denaturing solution consisted of 80% formamide, 100 mM NaOH, 1 mM EDTA, 0.1% Bromophenol Blue, 0.1% Xylene Cyanol FF. Samples were then heated at 95°C for 3 min, chilled on ice and loaded onto 8-10% native polyacrylamide gels, containing 5-10% glycerol. Gels were run at 15°C for 2,500-3,500 Volt hours and silver stained after electrophoresis.

HA was performed as follows: 2-5 µl of the PCR product were denatured at 95 °C for 5 min and allowed to cool down slowly. Samples were then analysed on 8% native polyacrylamide gels and run for 1,600-1,800 Volt hours. Gels were silver stained after electrophoresis.

All specimens which demonstrated altered SSCP or HA electrophoretic patterns were subjected to sequencing analysis.

6.2.4 Sequencing

PCR products were used for subsequent sequencing after purification with QIAquick PCR Purification Kit following the procedure recommended by the supplier (Qiagen Ltd, West Sussex, UK). Sequencing was performed using the ABI PRISM® BigDye™ Terminator Cycle Sequencing kit and analysis on a 377 sequencer (PE Biosystems, Warrington, UK) using the supplier's protocol. The primers used for PCR amplification of each exon were also used for sequencing but only one primer (either forward or reverse) was used in each sequencing reaction.

6.2.5 Immunohistochemical Analysis of p53 Protein

Protein expression was demonstrated immunohistochemically by a modified avidin–biotin complex method. Formalin fixed paraffin process tissues were sectioned at 4μm thickness, mounted on APES coated slides and dried at 37°C overnight. Sections were deparaffinised in xylene for 5 min and rehydrated in a series of graded alcohols (100%, 90%, 75%) to tap water, 5 min in each one. Heat mediated antigen retrieval was required to expose the epitopes and was performed by microwaving the sections on full power in 0.01M citrate buffer (pH 6.0) for 15 mins in a 800W microwave oven. The sections were left to stand for 15mins to cool and then rinsed for 5 mins in running tap water. Endogenous peroxidase activity was blocked by 1.5% hydrogen peroxide in methanol for 10 min. Sections were incubated in the

primary antibody buffer (5% goat serum in PBS) for 20mins. The monoclonal antibody against p53 (Serotec UK) was diluted 1:40 in the primary antibody buffer and incubated for 1 hour at room temperature. The primary antibody was visualised with Dako LSAB 2 Peroxidase kit (DAKO, UK). The secondary and tertiary reagents were incubated for 30 mins each and rinsed in between each stage with 0.05M TBS (pH 7.6). The signal was developed with diaminobenzidine (Merck, UK) and hydrogen peroxide. The sections were counterstained with haematoxylin. Normal mouse IgG replaced the primary antibody as a negative control. The frequency of the nuclear staining was scored on a scale from (-) to (+++) [as absent (-), weak (+), moderate (++) and strong (+++)] without the knowledge of clinical, pathological and molecular data. The evaluation of staining intensity was performed as follows: absent and weak staining was scored as normal while moderate and strong staining was scored as abnormal.

6.2.6 Statistical Analysis

Fisher's exact and Chi square (Pearson's correlation) tests were employed to comparatively analyse the molecular and clinicopathological data tables. T-test was performed to investigate the relation of molecular data to continuous values such as age and pack-year parameters. Analysis was performed using the SPSS 10.0 software for windows.

6.3 Results

6.3.1 Mutational Analysis of the p53 Gene in NSCLC

One hundred and eight DNAs from NSCLC cases were screened by SSCP and HA for the presence of mutations within exons 2-11 of the p53, which cover the entire coding region of the gene. Representative examples of SSCP and HA screening are shown in figures 6.3.1.1 and 6.3.1.2 respectively.

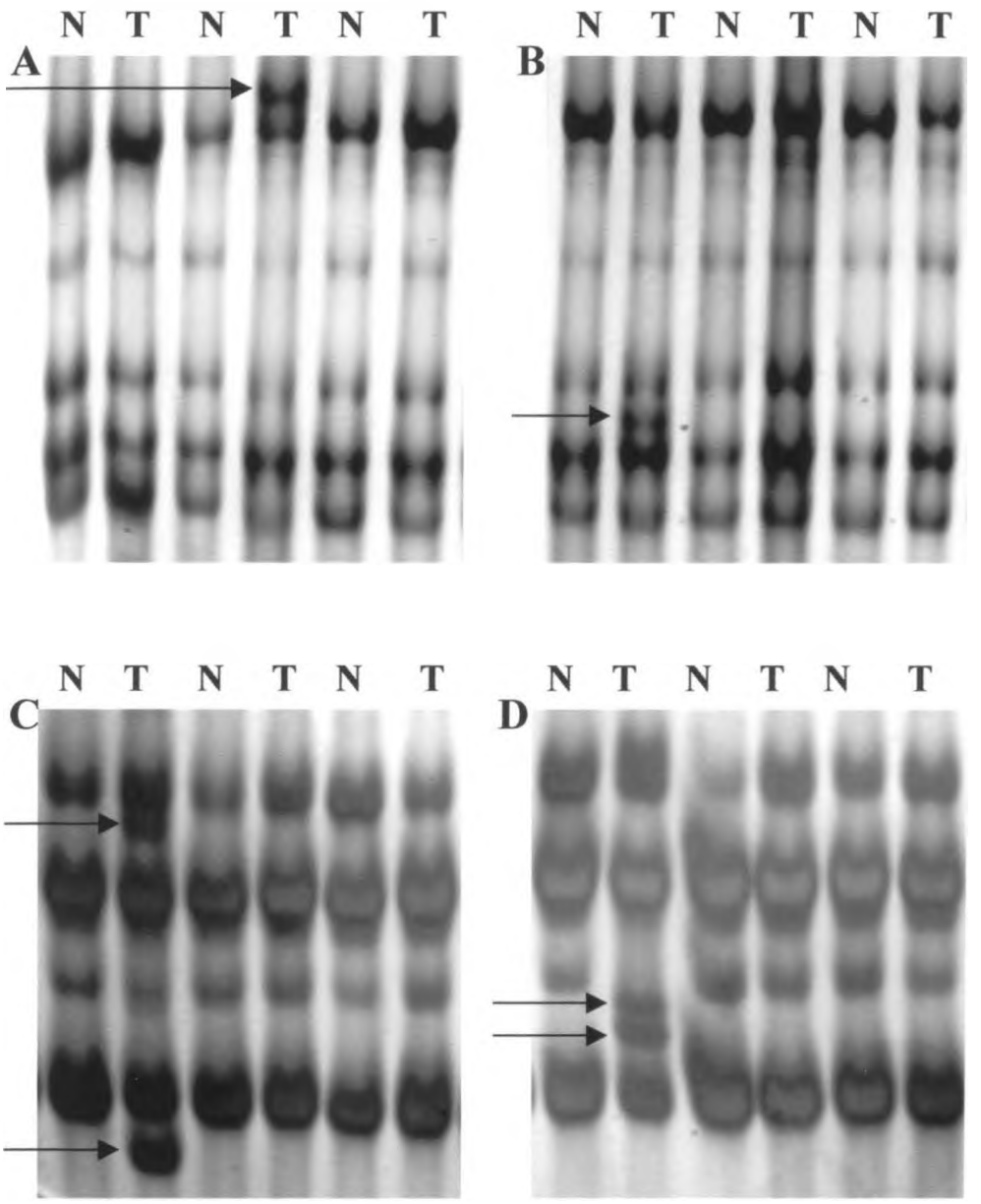


Figure 6.3.1.1. Representative examples of mutational screening in exon 5 (A), exon 6 (B), exon 9 (C) and exon 10 (D) by SSCP analysis. Bands with mobility shifts are indicated by arrows.

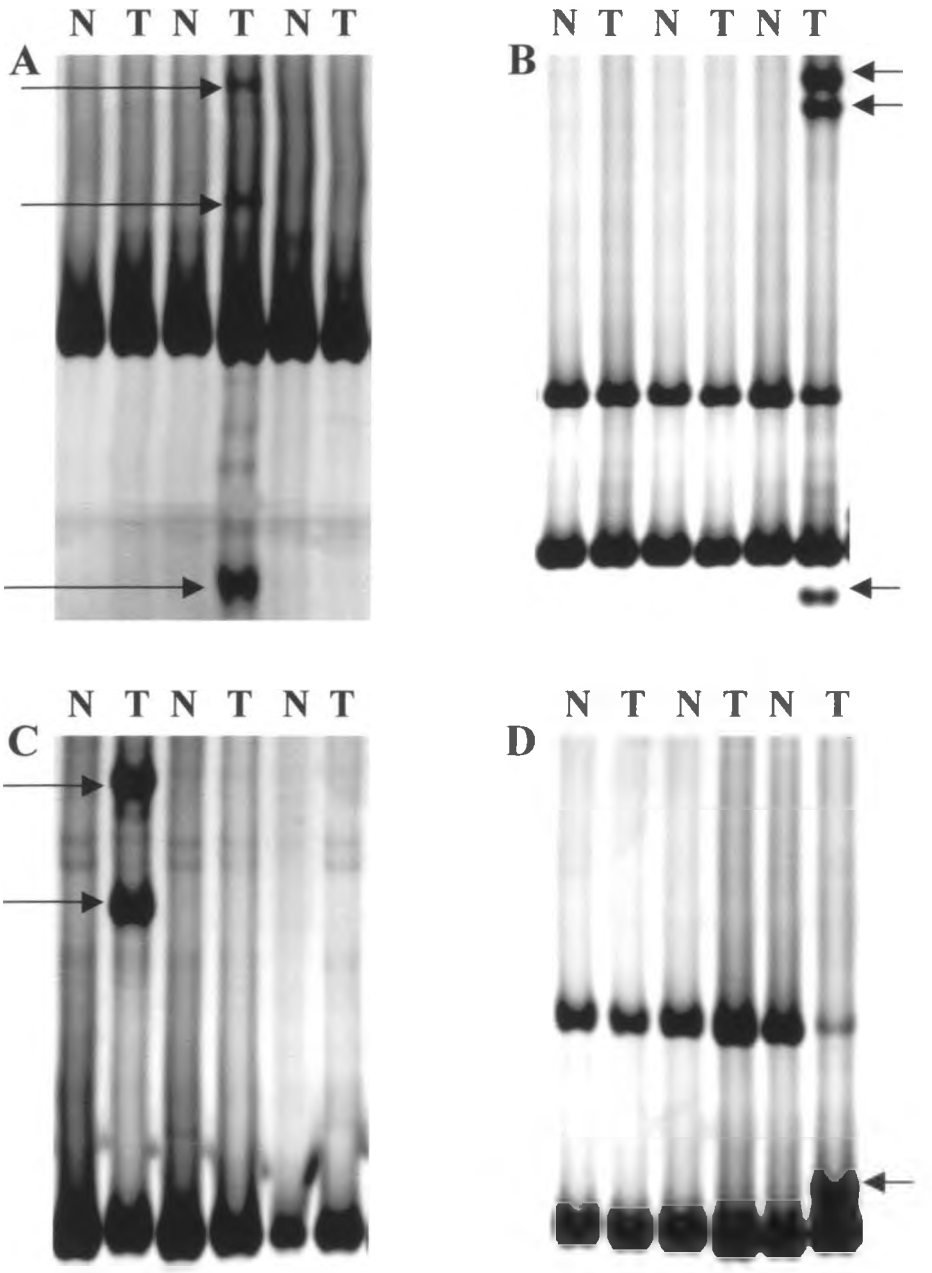


Figure 6.3.1.2. Representative examples of mutational screening in exon 5 (A), exon 6 (B), exon 7 (C) and exon 8 (D) by HA analysis. Bands with mobility shifts are indicated by arrows.

Forty seven mutations were detected in 46 of the 108 (43%) samples examined (Table 6.3.1.1). Representative examples of mutations detected in this investigation are shown in Figure 6.3.1.3. In order to validate the sensitivity of SSCP and HA primary screening results, 15 of the SSCP/HA negative tumour samples were chosen at random and sequenced for all the exons and no further mutations were revealed.

Of the 46 tumours with mutations, 36 (78%) were found to harbour the mutation within the DNA binding domain of p53 (exons 5-8) while 10 (22%) mutations were detected in the exons outside of 5-8. Sequencing analysis demonstrated 33 missense, 9 nonsense, 3 frameshift and 2 mutations affecting splicing. In particular, the mutational profile found was 4 deletions, 1 insertion and 42 base substitutions (Table 6.3.1.1). The base substitutions consisted of 22 transitions (13 GC→AT and 9 AT→GC) and 20 transversions (13 GC→TA and 7 AT→TA). Four of 13 GC→AT transitions occurred at CpG dinucleotides. All the mutations were found to be somatic since no mutations were detected in the corresponding normal tissues.

Table 6.3.1.1. p53 mutations in NSCLC detected in this study.

No	Diagnosis	exon (codon)	codon change	aa change	p53 IHC	p21 IHC	Rb IHC	pk/ys	T status	N status	Age
L145	SqCCL	10 (337)	CGC-CCC	Arg-Pro	abnormal	normal	normal	56	2	0	77
L151	SqCCL	10(364)	GCT-CCT	Ala-Pro	abnormal	normal	abnormal	55	2	0	70
L127	SqCCL	4(58)	CCA-ACA	Pro-Thr	normal	abnormal	normal	40	2	2	72
L052	LCCL	5 (158)	g del	Frameshift	normal	normal	abnormal	52	2	1	67
L003	SqCCL	5(136)	CAA-TAA	Gln-Stop	normal	normal	normal	104	2	2	67
L041	SqCCL	5(158)	CGC-CCC	Arg-Pro	abnormal	abnormal	abnormal	51	2	0	65
L086	AdenoCa	5(159)	CGC-CTG	Arg-Leu	abnormal	abnormal	normal	123	2	0	67
L055	SqCCL	5(163)	TAC-TGC	Tyr-Cys	abnormal	normal	abnormal	70	2	0	68
L179	SqCCL	5(163) 6(188)	TAC-TGC TCT-TTT	Tyr-Cys Ser-Phe	abnormal	abnormal	abnormal	35	3	2	57
L112	SqCCL	5(165)	CAG-TAG	Gln-Stop	normal	abnormal	abnormal	NA	2	0	74

L172	SqCCL	5(179)	CAT-CGT	His-Arg	abnormal	abnormal	normal	94	2	1	66
L129	SqCCL	5(181)	CGC-CCC	Arg-Pro	abnormal	normal	abnormal	NA	2	1	74
L143	AdenoCa	5(184)	GAT-AAT	His-Asn	abnormal	normal	abnormal	35	2	1	56
L159	AdenoCa	6 (218)	GAA-TAA	Glu-Stop	normal	normal	normal	32	2	0	48
L152	SqCCL	6(188)	TCT-TTT	Ser-Phe	abnormal	abnormal	normal	35	2	0	56
L014	SqCCL	6(195)	ATC-TTC	Ile-Phe	abnormal	abnormal	normal	110	2	0	57
L093	SqCCL	6(204)	GAG-TAG	Glu-Stop	abnormal	abnormal	normal	174	2	0	76
L124	AdenoCa	6(216)	GTG-ATG	Val-Met	abnormal	abnormal	normal	94	2	0	65
L173	SqCCL	6(218	GTG-GAG	Val-Glu	abnormal	abnormal	normal	NA	2	0	63
L021	AdenoSq	7(229-235)	19 bp del	Truncation	normal	normal	normal	39	2	0	59
L087	AdenoCa	7(239)	AAC-GAC	Asn-Asp	abnormal	normal	normal	34	2	0	72
L035	SqCCL	7(243)	g del	Frameshift	normal	normal	normal	60	2	1	48
L034	AdenoCa	7(245)	GGC-TGC	Gly-Cys	abnormal	abnormal	normal	112	2	0	73

L027	SqCCL	7(248)	CGG-TGG	Arg-Trp	abnormal	abnormal	normal	147	2	1	57
L025	SqCCL	7(248)	CGG-CAG	Arg-Gln	abnormal	normal	normal	90	3	1	69
L061	LCCL	7(249)	AGG-TGG	Arg-Trp	abnormal	normal	normal	140	2	1	73
L028	LCCL	7(258)	GAA-AAA	Glu-Lys	abnormal	normal	abnormal	108	2	0	72
L141	SqCCL	7(259)	GAC-GTC	Glu-Val	abnormal	abnormal	normal	NA	2	0	71
L080	SqCCL	8(261)	ATG-TTG	Met-Leu	abnormal	normal	abnormal	74	3	1	70
L163	SqCCL	8(266)	GGA-GTA	Gly-Val	ND	ND	ND	72	2	0	54
L043	AdenoCa	8(273)	CGT-CAT	Arg-His	abnormal	normal	normal	98	2	0	65
L049	AdenoCa	8(273)	CGT-CTT	Arg-Leu	abnormal	normal	normal	156	4	1	59
L024	SqCCL	8(275)	TGT-TTT	Cys-Phe	abnormal	normal	normal	123	2	0	67
L057	SqCCL	8(277)	TGT-TTT	Cys-Phe	abnormal	normal	normal	42	2	0	69
L146	SqCCL	8(278)	c del	Frameshift	abnormal	normal	abnormal	58	2	0	79
L107	SqCCL	8(282)	CGG-TGG	Arg-Trp	abnormal	normal	normal	NA	2	2	64

L092	SqCCL	8(285)	GAG-GTG	Glu-Val	abnormal	abnormal	abnormal	112	2	0	73
L044	SqCCL	8(294)	GAG-TAG	Glu-Stop	normal	normal	abnormal	92	2	0	64
L019	AdenoSq	8(297)	3bp ins	His-GlnSer	normal	normal	normal	20	2	0	65
L161	SqCCL	9(307)	GCA-TCA	Ala-Ser	normal	abnormal	abnormal	61	2	0	73
L048	SqCCL	9(319)	AAG-TAG	Lys-Stop	abnormal	abnormal	abnormal	92	2	0	68
L164	SqCCL	9(325)	GGA-TGA	Gly-Stop	normal	normal	normal	0	2	1	64
L012	SqCCL	9(326)	GAA-TAA	Glu-Stop	abnormal	abnormal	ND	68	2	0	68
L144	SqCCL	9(331)	CAG-TAG	Gln-Stop	abnormal	normal	abnormal	62	2	0	61
L154	SqCCL	intron 2	G-A	splicing	ND	abnormal	normal	93	2	2	72
L007	SqCCL	intron 4	G-C	splicing	normal	abnormal	abnormal	70	2	2	55

AdenoCa: Adenocarcinoma, SqCCL: Squamous Cell Carcinoma of the Lung, AdenoSq: Adenosquamous, LCCL: Large Cell Carcinoma of the Lung, ND: Not Done, NA: Not Available, aa change: amino acid change, IHC: Immunohistochemistry.

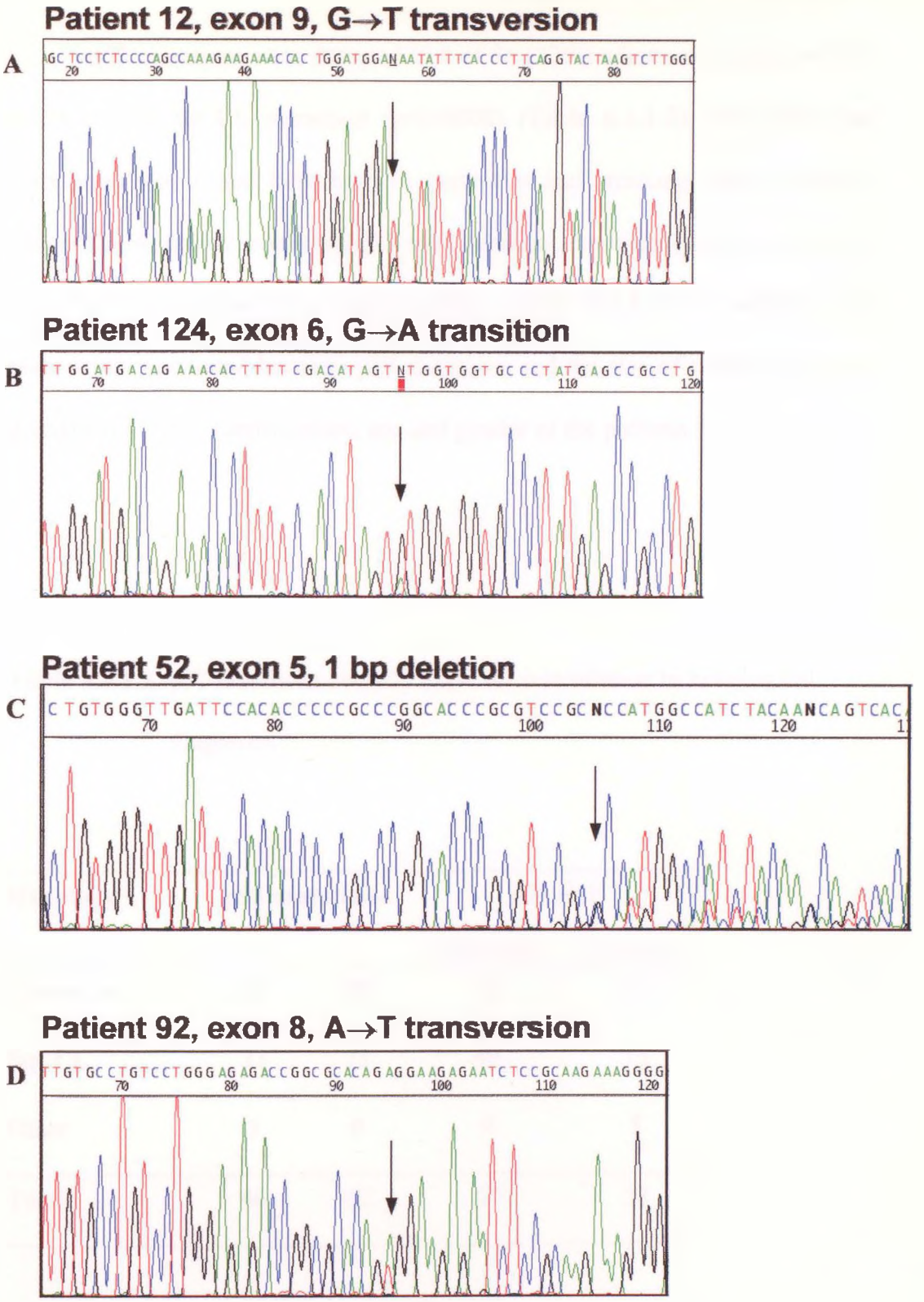


Figure 6.3.1.3. Representative sequencing examples of p53 mutations detected in NSCLC. **A:** Sequence analysis of exon 5 from patient no. 12 showing a G-T transversion. **B:** Sequence analysis of exon 6 from patient no. 124 showing a G-A transition. **C:** Sequence analysis of exon 5 from patient no. 52 showing a deletion.**D:** Sequence analysis of exon 8 from patient no. 12 showing an A-T transversion.

p53 mutations were detected in 8 of 36 (22%) adenocarcinomas and 33 of 58 (57%) SqCCL examined (p=0.0008) (Table 6.3.1.2). No significant correlation was found between p53 mutations and smoking status (current, former smokers and non-smokers) total tobacco exposure (pack- years) and daily tobacco consumption (cigarettes/day, Table 6.3.1.3). In addition, no association was found between p53 mutations and the size of tumor and nodal metastasis (TNM classification), age and gender of the patients.

Table 6.3.1.2. p53 mutations and p53 expression in relation to histological diagnosis.

Histology	**p53 mutations**		**p53 IHC**	
	+	-	**Abnormal**	**Normal**
AdenoCa	8	28	18	18
SqCCL	33	25	40	15
Other	5	9	9	5
Total	46	62	67	38

AdenoCa: Adenocarcinoma, SqCCL: Squamous Cell Carcinoma of the Lung, IHC: Immunohistochemistry. +: mutant p53, -: wild type p53.

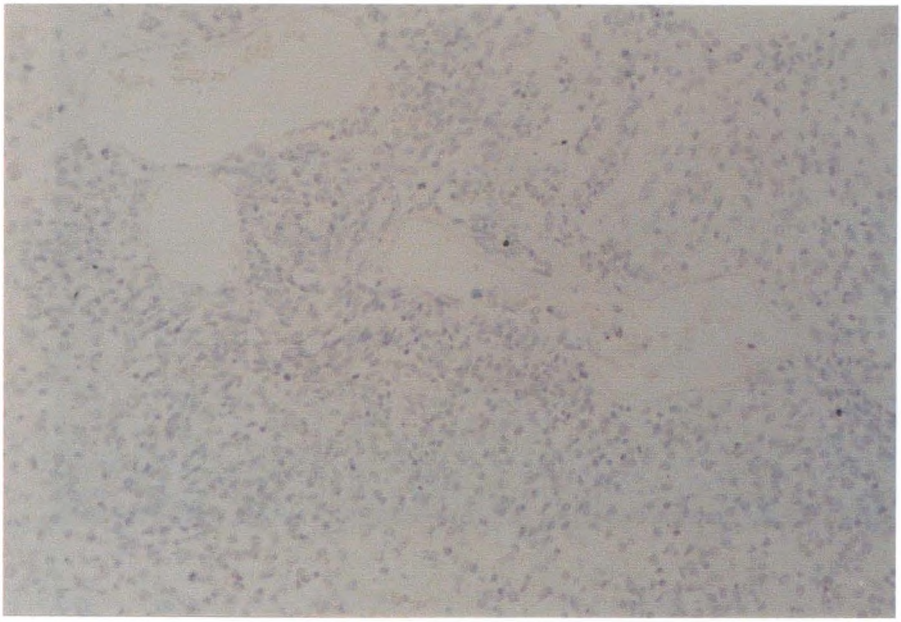
Table 6.3.1.3. p53 mutations and p53 expression in relation to Rb, $p21^{WAF1}$, MLH1 and MSH2 differential expression and telomerase activity in NSCLC.

		p53 mutations		**p53 expression**	
		mutant	**wild type**	**Normal**	**Abnormal**
MLH1 expression	normal	22	26	38	28
	reduced	22	35	18	19
MSH2 expression	normal	22	35	16	39
	reduced	22	27	21	27
$p21^{WAF1}$expression	abnormal	20	32	22	30
	normal	25	27	15	36
Rb expression	abnormal	17	10	10	17
	normal	29	50	28	49
Telomerase activity	positive	41	49	29	59
	negative	3	12	10	5

6.3.2 Expression of p53 in NSCLC

The expression levels of the p53 gene were examined by IHC in 105 NSCLC cases. p53 positive immunostaining was demonstrated in 67 of 105 (64%) cases examined. In particular, p53 immunopositivity was detected in 18 of 36 (50%) adenocarcinomas and 40 of 55 (73 %) SqCCL (p=0.02) (Table 6.3.1.2). Representative examples of p53 IHC detection are shown in Figure 6.3.2.1A and B. p53 positive staining did not correlate with the overall presence of mutations (p=0.08), however, it correlated with the type of mutation; p53 staining was more frequent in specimens harbouring missense (27 of 30) than null (5 of 14) mutations (p=0.0004). No significant correlation was found between p53 overexpression and smoking history (Table 6.3.1.3), TNM classification, age and gender of the patient.

A



B

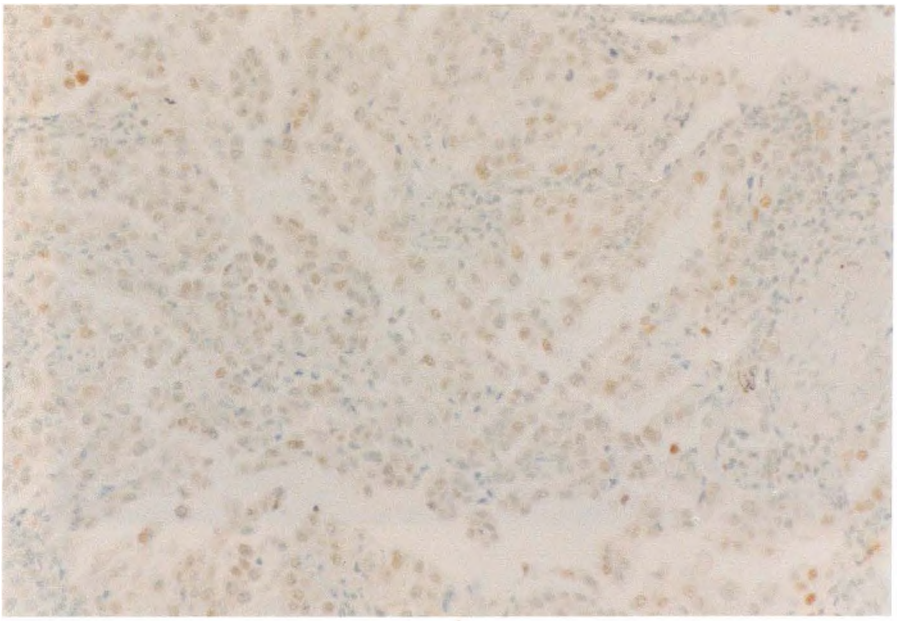
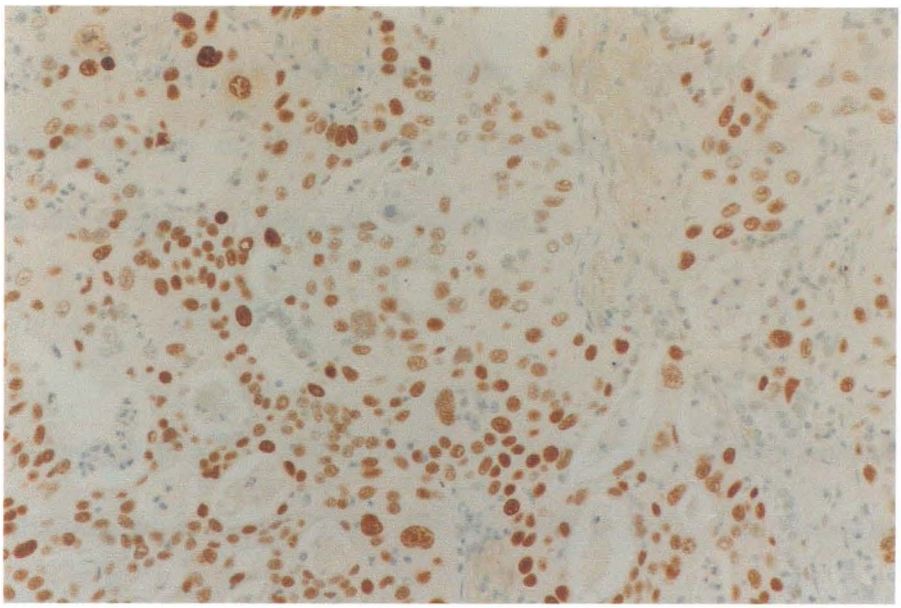


Figure 6.3.2.1A. Representative examples of absent (A) and weak (B) p53 expression in NSCLC

C



D

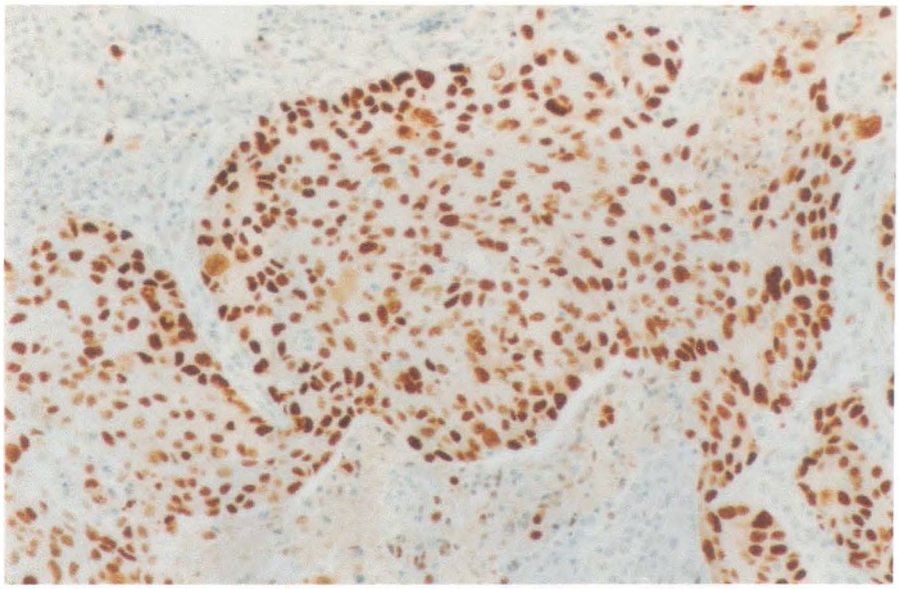


Figure 6.3.2.1B. Representative examples of moderate (C) and strong (D) p53 expression in NSCLC

6.3.3 p53 in relation to Rb and $p21^{WAF1}$ expression

$p21^{WAF1}$ and Rb expression data were available from previous unpublished studies of the Molecular Oncology Unit in the Roy Castle International Centre for Lung Cancer Research.

Although $p21^{WAF1}$ expression was not found to correlate overall with p53 mutations, when the p53 DNA binding domain (exons 5-8) was examined, mutations in exons 5-6 were the most related to p21 overexpression (10/16) in comparison to exons 7-8 (4/19), (p=0.015) (Figure 6.3.3.1).

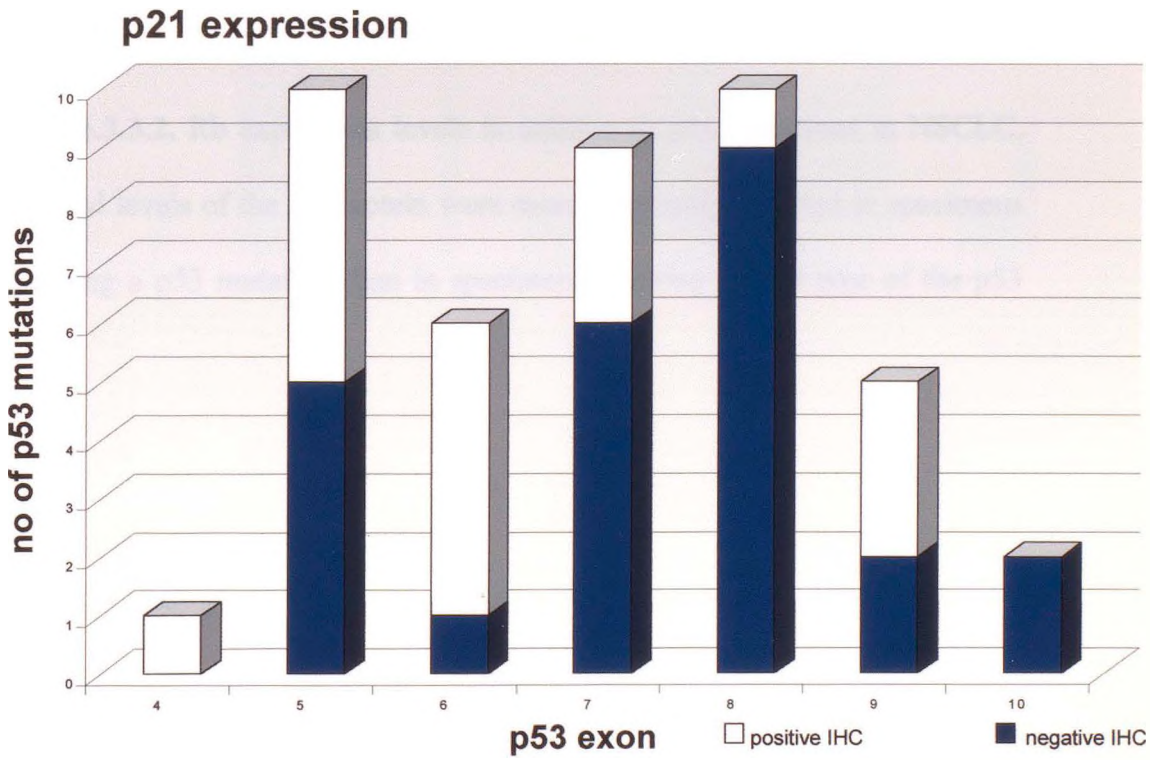


Figure 6.3.3.1. Differential impact of p53 mutations in NSCLC on the expression levels of p21 in relation to the mutation position.

Abnormal levels of the Rb protein were detected in 17 of 46 (37 %) specimens harbouring a p53 mutation and 10 of 60 (17 %) carrying a wild type of the p53 gene (p=0.015) (Figure 6.3.3.2).

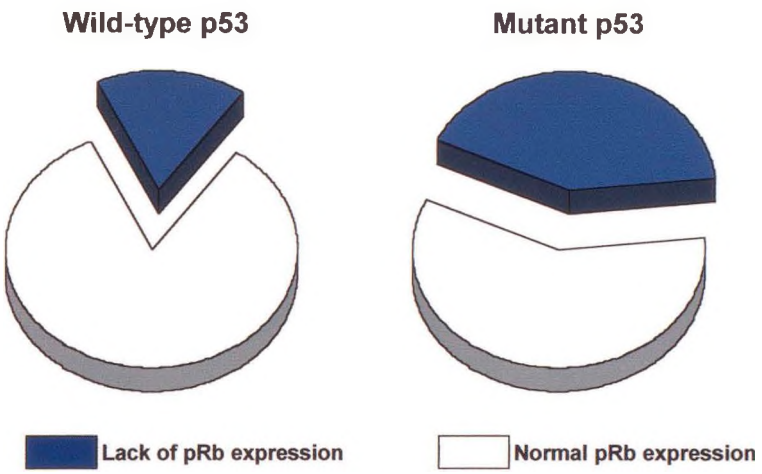


Figure 6.3.3.2. Rb expression levels in relation to p53 mutations in NSCLC. Abnormal levels of the Rb protein were more frequently detected in specimens harbouring a p53 mutation than in specimens carrying a wild type of the p53 gene.

Moreover, among mutations within the p53 DNA binding domain, mutations in exons 5 and 8 appeared to associate with abnormal expression of pRb (11/20) in contrast to exons 6 and 7 (1/15) (p= 0.003) (Figure 6.3.3.3).

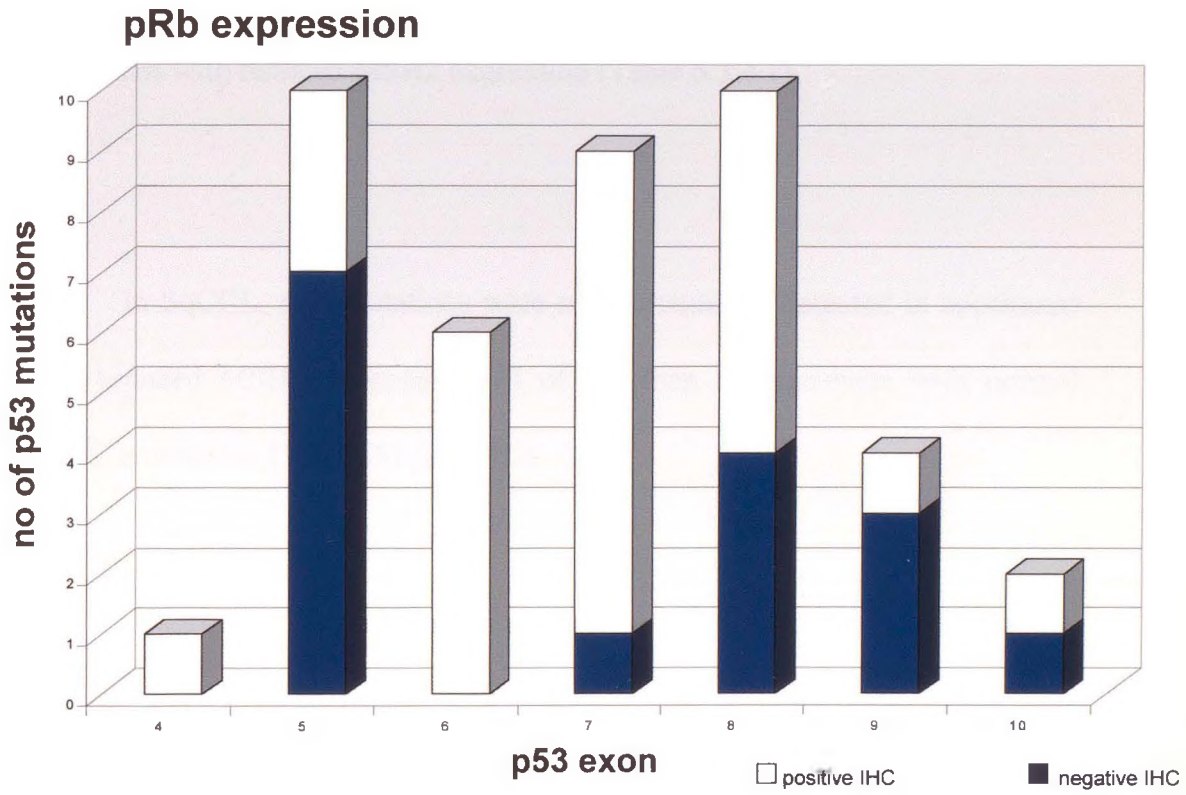


Figure 6.3.3.3. Differential impact of p53 mutations in NSCLC on the expression levels of pRb in relation to the mutation position.

6.3.4 p53 in Relation to MLH1 and MSH2 Differential Expression in NSCLC

Overall, p53 mutations were detected in 22 of 48 (46%) specimens with normal expression of MLH1 and 22 of 57 (39%) specimens with reduced expression levels of MLH1. In addition, p53 mutations were detected in 22 of 57 (39%) specimens with normal MSH2 expression and 22 of 49 (45%) specimens with reduced MSH2 expression (Table 6.3.4.1).

In SqCCL, p53 mutations were more frequently detected in specimens with reduced MSH2 expression (14 of 18) than in specimens with normal MSH2 expression (17 of 38) (p=0.019).

Table 6.3.3.1. p53 mutations, p53, p21 and Rb expression in relation to the expression of MLH1 and MSH2 in NSCLC.

		MLH1 expression			**MSH2 expression**		
		Normal	Reduced	Fisher's test p value	Normal	Reduced	Fisher's test p value
p53 mutations	mutant	22	22	0.29	22	22	0.32
	wild type	26	35		35	27	
p53 expression	abnormal	28	38	0.25	39	27	0.09
	normal	19	18		16	21	
p21 expression	abnormal	27	25	0.11	31	22	0.37
	normal	19	31		25	25	
Rb expression	abnormal	13	13	0.41	12	14	0.28
	normal	35	43		43	35	

p53 intense staining was demonstrated in 38 of 56 (68%) specimens with reduced MLH1 expression and 28 of 47 (60%) specimens with normal MLH1 expression. In addition, 27 of 48 (56%) specimens with reduced MSH2 expression and 39 of 55 (71%) specimens with normal MSH2 expression were found with intense p53 staining (Table 6.3.4.1). This trend (p=0.09) became a

significant correlation when wild type p53 specimens were analysed separately, excluding thus samples with p53 staining due to DNA mutation. In this case, p53 intense staining was found in 11/27 samples with reduced MSH2 and in 24/35 samples with normal MSH2 expression (p=0.018) (Figure 6.3.4.1).

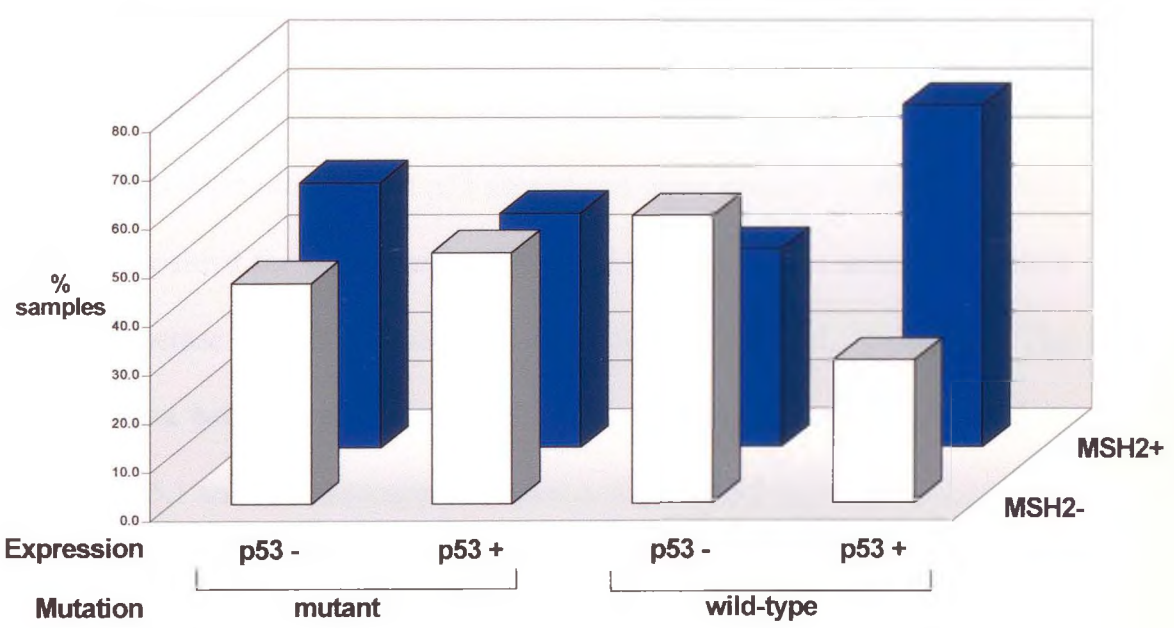


Figure 6.3.4.1. MSH2 expression relative to p53 mutation and expression status. While strong MSH2 expression has no apparent effect on p53 expression in samples with mutant p53, it appears to increase p53 staining in samples carrying wild type alleles.

6.3.5 p53 in Relation to Telomerase Activity

Telomerase activity data were available from a previous study (Xinarianos *et al*, 1999; Chapter 3 in this thesis). p53 mutations were detected in 41 of 90 (46%) samples positive for telomerase activity and 3 of 15 (20%) samples negative for telomerase activity (Pearson's Chi Square-2 sided, p=0.06). p53 overexpression was demonstrated in 59 of 88 (67%) of specimens positive for telomerase activity and 5 of 15 (33%) of specimens negative for telomerase activity (Pearson's Chi Square-2 sided, p=0.01). When bringing together samples with p53 aberrations, either mutation or overexpression or both, it was demonstrated that p53 aberrations were detected in 69 of 88 (78%) samples positive for telomerase activity and 6 of 15 (40%) samples negative for telomerase activity (Pearson's Chi Square-2 sided, p=0.002). The associations between p53 status and telomerase activity are summarised in Table 6.3.5.1 and schematically presented in Figure 6.3.5.1.

Table 6.3.5.1. p53 status (mutations, expression, total aberrations) in relation to telomerase activity in NSCLC

		Telomerase activity		
		Positive	**Negative**	**Pearson's Chi Square (2-sided) test p value**
p53 mutations	mutant	41	3	0.06
	wild type	49	12	
p53 expression	abnormal	59	5	0.01
	normal	29	10	
p53 aberration	aberrant[1]	69	6	0.002
	normal[2]	19	9	

1: This category includes specimens with wild type DNA and abnormal expression, mutant DNA and normal staining and mutant DNA and abnormal staining.

2: This category includes specimens with wild type DNA and normal staining.

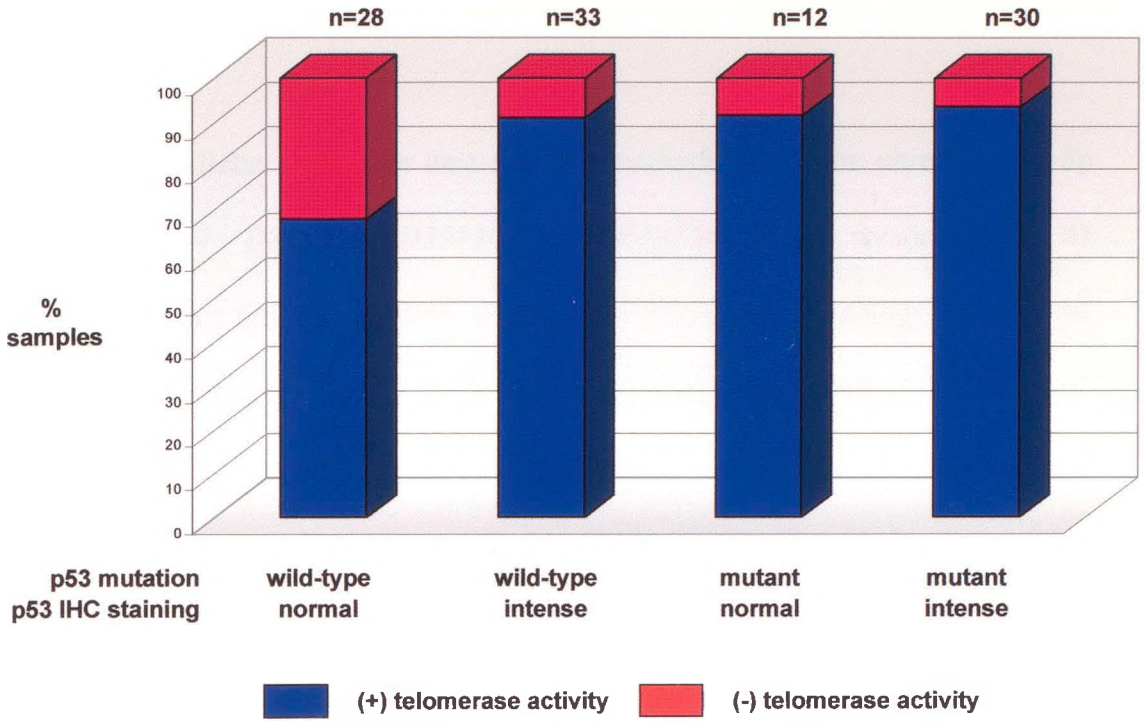


Figure 6.3.5.1. p53 status (mutation, overexpression and total aberrations) in relation to telomerase activity in NSCLC.

6.3.6 p53 in Relation to Allelic Imbalance on Chromosome 3p

Allelic imbalance data for four markers located on chromosome 3p (*D3S1289, D3S1266, D3S1300* and *D3S1304*) were available for 85 individuals from a previous study of the Molecular Oncology Unit in the Roy Castle International Centre for Lung Cancer Research (Liloglou *et al,* 2000). The comparative analysis of p53 status and the allelic imbalance data on chromosome 3p is summarised in Table 6.3.6.1. The results showed that p53 mutations correlated with allelic imbalance at the *D3S1266* (3p24) locus (p=0.04). No such correlation was found with the loci; *D3S1304* (3p26) (p=0.1); *D3S1289* (3p21) (p=0.1) and *D3S1300* (3p14) (p=0.2). p53 expression correlated with allelic imbalance at the loci; *D3S1266* (p=0.02), *D3S1304* (p=0.02) and *D3S1289* (p=0.04). No such correlation was found with the locus and *D3S1300* (p=0.5).

Table 6.3.6.1. p53 mutations and expression in relation to allelic imbalance on chromosome 3p in NSCLC

		D3S1304		D3S1266		D3S1289		D3S1300	
		H	L	H	L	H	L	H	L
p53 mutations	wild type	16	15	15	21	16	21	14	24
	mutant	8	16	5	22	8	22	6	18
p53 expression	normal	13	8	11	12	13	13	7	14
	abnormal	10	22	8	30	10	29	12	27

6.4 Discussion

In the present study, the status of p53 (mutations and expression) was examined in relation to two other major cell cycle regulation controllers such as the Rb and $p21^{WAF1}$ genes as well as two of the DNA MMR genes, *hMLH1* and *hMSH2*. In addition, p53 status was examined in relation to telomerase activity. For mutational analysis, exons 2-11, which cover the whole coding region of p53, were examined. Mutational screening in this study was performed by both SSCP and HA in order to reduce false negatives. p53 mutations were found in 43% of the samples. This is a higher frequency to that reported in a previous study of the same research group examining exons 4-9 in 45 lung tumours (Liloglou *et al,* 1997a). The dual screening, the study of exons outside 5-8 and new primer pairs designed for amplification of shorter fragments size are the most likely reasons for this increased efficiency in mutation detection. It is of note that 22% of mutations were located outside exons 5-8, which code for 88% of the DNA binding domain of p53. This is in agreement with Tomizawa et al 1999, Casey et al 1996 and Brattstrom et al 1998 who reported 19%, 17% and 20% of such p53 mutations outside exons 5-8 in lung tumors respectively. Similar frequencies have been reported in breast and ovarian tumors (Hartmann *et al,* 1995, Casey et al 1996) while a higher frequency (33%) of such "outside" mutations was found in head and neck tumors (Kropveld *et al,* 1999). It is also of note that in this study no mutations were found in exons 2, 3 and 11. This, being in agreement with previous reports (Tomizawa et al 1999, Casey et al 1996 and Brattstrom et al 1998), indicates that exons 4-10 may comprise a minimal region of required

mutational analysis to cover the vast majority of p53 mutations in lung cancer. This is also supported by the presence of only 2 mutations outside exons 4-10 in the p53 mutation database (Béroud and Soussi, 1998) although it must be acknowledged that the majority of references for this database have not covered exons outside 5-8.

Only 8 patients from those with available smoking history in this study were non smokers. This did not allow one to draw statistically significant conclusions concerning the mutation frequency in relation to smoking. However, no difference was found between current and former smokers which is in agreement with previous observations in patients with upper respiratory tract tumours (Liloglou *et al,* 1997a and 1997b) and indicates that p53 mutations may be acquired during the patients' early smoking history.

The mutational profile was found to include similar frequencies of GC→AT transitions and GC→TA transversions. The lack of GC→TA transversion preponderance that is usually reported for smoking populations gives further support to the previous suggestion for additional environmental carcinogens in the area and/or predisposition factors that may also play a role in lung carcinogenesis (Liloglou *et al,* 1997a). Similar lack of preponderance of GC→TA transversion has been reported in patients from Gdansk, Poland which is also an industrialised area with analogous climate. (de Anta *et al,* 1997).

p53 positive staining correlated with missense mutations as null and frameshift mutations frequently result in a truncated protein that can not be detected immunohistochemically. This suggests that immunohistochemical detection of p53 is not adequate by itself for identification of p53 abnormalities. However, 56.5% of samples with no detected p53 sequence mutations, showed positive immunostaining. In this study, 75% of the examined samples showed p53 aberrations and 46% were mutations. Consequently, both mutational and IHC analysis are required to identify the whole spectrum of p53 aberrations and this is in agreement with Casey *et al,* 1996.

A lack of association was observed between $p21^{WAF1}$ expression and p53 aberrations and this is additional evidence to previous studies (Brambilla et al 1998, Bennet et al. 1998, Marchetti *et al,* 1996) supporting a dual, p53-dependent and p53-independent, regulation of $p21^{WAF1}$ expression. Lack of Rb expression correlated with p53 mutations (p=0.015) (Figure 4). This is the first study to report such a relationship in NSCLC. However, additional studies have to be undertaken in order to elucidate the nature of this relationship. No association was found between p53 staining and Rb expression and this is in agreement with previous studies (Geradts *et al,* 1999). Interestingly, when examining the DNA binding domain (exons 5-8), a differential effect of p53 mutations on the expression levels of $p21^{WAF1}$ and pRb was observed, depending on the location of the mutated nucleotide. Thus, mutations in exons 5 and 6 are mainly associated with $p21^{WAF1}$ overexpression while exons 5 and

8 were mostly associated with lack of pRb expression. This is possibly an example of specific p53 regions being involved in different functions of the protein.

Comparative analysis between p53 status and differential expression of the DNA MMR genes, *hMLH1* and *hMSH2*, revealed that MSH2 expression correlated (p= 0.018) with p53 staining in samples with wild type p53, while a trend (p=0.09) occurred for the overall examined population. Two hypotheses can be generated from this result. The first hypothesis is that p53 overexpression results in MSH2 upregulation as it is a transcriptional activator of it (Scherer et al 1996, Scherer et al 2000). This is also supported by the correlation of p53 mutations with reduced MSH2 expression in SqCCL as well as a similar association shown in adult acute leukaemia (Zhu *et al,* 1999). However, this might suggest that intense IHC staining of p53 indicates stabilisation but not necessarily complete inactivation, since stabilised p53 can transactivate MSH2. However, further investigations are required to support this argument. The second hypothesis is that MSH2 may act as a DNA damage signaller to p53, similarly to ATM and DNA PK, thus binding and stabilising the protein. Therefore, high levels of MSH2 expression result in p53 stabilisation and correlate with positive p53 immunophenotype in samples carrying wild type p53. However, in samples carrying a mutant p53, stabilisation of the protein due to the mutation masks the effect of MSH2. Taking both hypotheses together, therefore, it may be speculated that a possible

feedback loop exists between p53 and MSH2, however, additional functional studies are required to confirm and reveal the full nature of this relationship.

Analysis of the p53 status data in comparison with telomerase activity data showed that p53 overexpression was associated with telomerase activity. In particular, p53 overexpression was more frequently detected in telomerase positive samples than in telomerase negative samples. However, a similar association was not observed between p53 mutations and telomerase activity. Although there was a preponderance of p53 mutations to be more frequent in telomerase positive than in telomerase negative samples, this was not statistically significant ($p=0.06$). Thus, the association of p53 mutations with telomerase activity is masked by non-mutational inactivation events (e.g. MDM-2 binding). It is of note that total p53 aberrations, mutation, overexpression or both, were also correlated with telomerase activity. A study by Roos and colleagues has reported that telomerase activity correlates with p53 overexpression in breast cancer. However, a similar association was not found between p53 mutations and telomerase activity (Roos *et al,* 1998). It should be stressed that the study by Roos and colleagues examined the p53 mutational status only by SSCP screening in exons 5-8, therefore the p53 mutational data was inadequate to provide further evidence for a possible association between p53 mutations and telomerase activity. A similar association between p53 overexpression and telomerase activity has previously been demonstrated in NSCLC (Wu *et al,* 1999). However, the latter study did not examine the mutational status of the p53 gene. The data presented in this

study and previously published reports have clearly demonstrated that both immunohistochemical and mutational analysis are required in order to examine the whole spectrum of p53 aberrations in human tumours (Casey *et al,* 1996). This relationship between p53 and telomerase activity gives further support to the hypothesis that wild type p53 may form part of a mechanism which detects either the loss of telomeres directly or the structural consequences of telomere erosion and consequently signals growth arrest in G_1 phase of the cell cycle. At this point, only cells that lack functional p53 will be able to pass this barrier. Ultimately, the cells which will pass this barrier will have destabilising effect of further telomere erosion and will have to re-activate telomerase (Wynford-Thomas *et al*, 1995).

p53 mutations correlated with allelic imbalance at locus *D3S1266* while p53 overexpression correlated with allelic imbalance at loci *D3S1266*, *D3S1289*, *D3S1304*. Deletion of one copy of the short arm of chromosome 3p is considered to be among the most frequent genetic events in lung cancers, indicating that putative tumour suppressor gene(s) implicated in lung tumourigenesis is located on this region (Neville *et* al, 1996; Todd *et* al, 1997). The data presented in this study suggests a possible interaction between gene(s) located on chromosome 3p and p53 inactivation.

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CHAPTER SEVEN

DISCUSSION AND CONCLUSION

Lung cancer remains the leading cause of death from cancer worldwide and is associated with high mortality and morbidity rates. The poor prognosis of lung cancer is related to the progression from a localised primary to a disseminated metastatic disease. In addition, with the current diagnostic technology by the time lung cancer reaches a clinically detectable point, the disease is already in a late stage and closely from reaching a lethal tumour burden. Thus, lung cancer is a disease for which screening approaches are appealing and an important goal for lung cancer management is to develop improved techniques of identifying the disease at its pre-clinical stages. Nevertheless, there is now a clear need for early diagnostic methods in lung cancer, which will most likely involve multiple biomarkers to screen individuals with a high risk of developing lung cancer. Therefore, there is a continuous need for research and development in the area of biomarkers as well as establishing the genetic processes and phenomena underlying the molecular pathogenesis of lung cancer.

The aim of this study was to evaluate and assess potential biomarkers, like genetic alterations and telomerase activity, for the identification of individuals at high risk of developing the disease. The potential role of DNA MMR genes, hMLH1 and hMSH2, in the molecular pathogenesis of NSCLC and their regulation and relation to the major cell cycle regulators; p53, $p21^{WAF1}$ and Rb, was also examined. The patient population recruited in this study was from the Merseyside region.

Northwest England, which appears to have some of the highest incidence rates of lung cancer in the UK and across Europe.

Previous studies have demonstrated that the majority of NSCLC cases exhibit genetic damage in the form of genetic alterations (LOH and MAs). In addition, genetic alterations are considered to be a fundamental feature of carcinogenesis. Therefore, it was of interest to evaluate and assess such genetic alterations in BL specimens from individuals with suspected lung cancer. In this study, such alterations were detected in the BL of 35% of lung cancer patients. However, it is of particular interest that genetic alterations were detected in BL specimens from patients who were reported as NMCS on cytological examination indicating that a proportion of the cells in the BL carried genetic aberrations but presented with no morphological evidence of malignancy.

A group (23%) of the patients referred for investigation with no cytological or radiological evidence of lung cancer do have genetic instability in the same microsatellite markers as found in patients with proven neoplasia. In this group of patients, genetic alterations were more common in individuals with rheumatoid arthritis, goiter, fibrosing alveolitis and cardiac problems (MI, LVF, angina) than in common chest disease. These findings may imply that genetic alterations occur in disease processes such as cardiac and autoimmune conditions suggesting a possible genetic

link between certain neoplastic and non-neoplastic diseases. Another possible explanation could be that genetic alterations in this group may just be indicators of molecular damage to the bronchial epithelium due to tobacco exposure in smokers or some other carcinogens in non-smokers. In addition, a "passive smoking" effect should not be excluded for the non-smokers but it is very difficult to assess at this stage. However, it should be stressed that these patients cannot be considered as a "control group". based on their referral criteria and long-term follow-up is needed. The individuals included in this study are currently being followed-up in the context of the Liverpool Lung Project and it remains to be shown whether those with genetic alterations will develop lung cancer at a future date. The latter could not be included as part of this thesis given the 4 year time limitation for its completion.

Nevertheless, genetic alterations detected in body fluids, such as BL, may be considered as an attractive candidate for the development of strategies for the early diagnosis of cancer, alongside other molecular methods that require no invasive techniques. In time, additional markers and advances in technology may increase the present success rate. Additional studies and long-term follow-up are required to clarify further the value of such genetic alterations as a tool for the identification of individuals with a high risk of developing lung cancer.

There has been an urge for the identification, evaluation and assessment of molecular markers that could serve as diagnostic tools for neoplastic disease. Telomerase activity has been detected at high rates in almost all types of human cancer. This makes detection of telomerase activity a potential marker for the diagnosis of neoplasia. Therefore, it was of interest to examine the incidence of telomerase activity in NSCLC and study further its potential use as a diagnostic marker for malignant lung disease. In this study, telomerase activity was detected in 86% of the tumour but it was completely absent in the normal adjacent tissues. Although telomerase activity was detected in the vast majority of the samples examined, there is, however, a distinct group of NSCLC tumours, which exhibits undetectable levels of telomerase activity. Therefore, it may be argued that these telomerase negative tumours may overcome the telomere shortening using an alternative pathway.

Telomerase activity was associated with the T stage and nodal metastasis of the tumours examined. This indicates that telomerase may be a marker of disease progression and aggressiveness in NSCLC. Telomerase activity was also found to correlate with moderate/poor differentiation. The latter suggests that telomerase activity may be affected by the degree of differentiation of the neoplastic cells. Telomerase activity was also associated with current smoking status at the time of diagnosis, with a history of heavy smoking and overall tobacco exposure. In particular, telomerase activity was more frequently detected in current than in former

smokers and in heavy than in moderate smokers (daily tobacco consumption and tobacco exposure) indicating that certain NSCLC cases from former and/or moderate smokers may undergo a telomerase independent initiating pathway. These findings also suggest a possible role for telomerase as a marker of extensive damage to the bronchial epithelium due to the carcinogens found in tobacco.

Telomerase activity regulation was further investigated in relation to the expression levels of two of the cell cycle regulators, $p21^{WAF1}$ and Rb. No association between telomerase activity and the expression levels of these proteins suggesting that telomerase activity regulation is $p21^{WAF1}$ and Rb independent in this set of NSCLC examined. In addition, no mutations were detected in the minimal functional and promoter regions of the *hTR* gene which encodes for the RNA component of human telomerase. Also, no mutations were detected in the promoter region of the *hTERT* gene which encodes for the catalytic subunit of human telomerase demonstrating that mutations in these regions do not contribute in the activation or deregulation of telomerase in NSCLC. Comparative analysis of telomerase activity with allelic imbalance on chromosome 3p did not produce evidence that putative gene(s) located on this chromosome regulate telomerase activity. However, this is in contrast to the findings of recent studies which have demonstrated that telomerase activity may be regulated by gene(s) located on chromosome 3p. The lack of association observed in this study may be primarily due to the fact that only a subset of the tumours examined for

telomerase activity has been analysed for allelic imbalance on chromosome 3p. Further studies are required to elucidate the relationship between telomerase activation and allelic imbalance on chromosome 3p.

Nevertheless, telomerase is implicated in the molecular pathogenesis of NSCLC. However, a specific subset of telomerase negative tumours may represent a discrete population with a distinct telomerase-independent initiating pathway. Further detailed studies are required to improve our understanding of telomerase regulation and its molecular initiating pathways.

Telomerase activity has been detected in a number of clinical specimen types that are relevant to early diagnosis of a range of human cancers. In this study, conditions were established for the detection of telomerase activity in BL specimens from individuals with suspected lung cancer. Telomerase activity was detected in 70% of BL specimens from patients with a final diagnosis of lung cancer. However, it is of particular interest to note that telomerase activity was detected in BL specimens from patients who had a negative cytology report but the patient's lung cancer was diagnosed by other methods (radiology, biopsy or surgery). The latter indicates that a proportion of the cells in the BL had telomerase activity although the specimen had no cytological evidence of malignancy. Moreover, analysis of the combined results of telomerase activity and

cytology demonstrated that a two-method approach identified 74% of lung cancer cases whereas with cytology alone only 43% of the lung cancer patients were identified. Thus, detection of telomerase activity in clinical specimens such as BL appears to be a promising complementary method to cytology for the diagnosis of certain lung cancer cases.

Telomerase activity was also detected in the BL specimen from a patient with cystic fibrosis who died from respiratory failure seven months after presentation. The latter supported further the findings of a previous study which reported that telomerase activity can be detected in BL specimens from patients with non-cancerous inflammatory lung disorders who died of disease aggressiveness within a short period after the specimen was taken Therefore it may be argued that detection of telomerase activity in BL specimens may be a marker of aggressiveness in non-malignant lung disease as well as neoplasia.

Nevertheless, it has been demonstrated that telomerase activity is a specific molecular marker for the diagnosis of certain lung cancer cases and could serve as a complementary diagnostic method to cytology. In addition, telomerase may have a role as a marker of aggressiveness in non-neoplastic inflammatory lung diseases. This urges for further studies of telomerase activity as a diagnostic/prognostic marker in a large cohort of patients with close monitoring of outcome.

DNA mismatch repair genes have been implicated in human carcinogenesis through a mutator phenotype. Although this family of genes has been extensively studied in certain types of human cancer, like familial and sporadic colon cancers, these genes have been barely studied in lung cancer. In this study, it has been demonstrated that 59% of the examined NSCLC had reduced expression of *hMLH1* and 58% had reduced expression of *hMSH2*, while 34% demonstrated reduction of expression in both of these genes. It is of particular interest that 82% of all NSCLC examined showed reduced expression of at least one of the two investigated genes. These results suggest a critical role for these DNA MMR genes in lung carcinogenesis. The differential expression of these two genes was associated with the histological subtypes. In particular, in adenocarcinomas *hMSH2* expression was more frequently reduced than that of *hMLH1* while the converse was observed in SqCCL. In addition, reduced expression of *hMLH1* in SqCCL correlated with nodal metastasis, suggesting that the *hMLH1* gene may contribute to a more aggressive tumour phenotype in this histological subtype and prompting for its use as a useful molecular marker towards a more radical management of these patients. *hMLH1* reduced expression correlated with both higher daily tobacco uptake and total tobacco exposure (pack-years) indicating that tobacco carcinogens are implicated in *hMLH1* inactivation and moreover, that they may have an additive effect.

Further investigation of the genes' possible inactivation mechanisms indicated that reduced hMLH1 expression correlated with allelic imbalance at the *D3S1289* (3p21) locus suggesting that loss of one allele of the *hMLH1* gene may be one of the major genetic events involved in its inactivation in NSCLC. In addition, mutational analysis of the *hMLH1* promoter region and the hot spot exons did not reveal any mutations suggesting that mutations are unlikely to be a major cause of *hMLH1* inactivation in NSCLC. It has also been demonstrated that differential expression of *hMLH1* may be due to allelic imbalance at the *D2S391* (2p16) locus, suggesting that *hMLH1* expression regulatory gene(s) are located in this region. In addition, an inverse relationship between allelic imbalance at the *D3S1300* locus and *hMSH2* expression was identified implying the presence of a negative *hMSH2* regulatory gene on chromosome 3p and suggestive of a negative feedback mechanism. However, further studies employing more microsatellite markers on chromosomes 3p and 2p are required to elucidate the nature of this relationship.

hMSH2 differential expression was not found to be associated with allelic imbalance at the *D2S391* and *D2S2259* (2p16) loci. The latter suggests that allelic imbalance at these loci is not the main event contributing to the differential expression of *hMSH2* in NSCLC. In addition, there was no evidence of mutations in the promoter and the hot spot exons of *hMSH2*. It is of note that previous studies have reported no hypermethylation of *hMSH2* promoter. Nevertheless, inactivation of the

hMSH2 gene may be due to alternative mechanisms involving changes in its upstream regulatory genes.

Moreover, the comparative analysis of *hMLH1* and *hMSH2* differential expression in the NSCLC cases included in this study did not reveal any associations with the expression levels of Rb and $p21^{WAF1}$ demonstrating that the regulation of these MMR proteins is independent of the expression levels of two of the major cell cycle regulators. Furthermore, tumour specimens with combined reduced expression of both *hMLH1* and *hMSH2* proteins did not correlate with any clinical and pathological parameters demonstrating a non-complementary role of these two proteins.

Nevertheless, it has been demonstrated that *hMLH1* and *hMSH2* genes have distinct roles in the molecular pathogenesis of NSCLC. The findings of this study suggest that at least some of the environmental and endogenous factors involved in their inactivation pathway are different. Further investigations are required to elucidate the complete pathways of inactivation of these two genes in NSCLC and reveal additional factors implicated in their regulation.

The *p53* gene is one of the most well characterised and studied tumour suppressor genes. Its implication in smoking related cancers, like

lung cancer, has been well established. However, the vast majority of the studies have only examined mutations within the DNA binding domain (exons 5-8) or expression of the protein. In this study, the status of p53 (mutations and expression) was investigated, examining the whole coding region (exons 2-11). p53 mutations were found in 43% of the NSCLC studied. It has been demonstrated that 22% of the mutations were located outside exons 5-8, providing further support to other studies stating similar frequencies in other types of human cancer. Thus, it may be argued that the frequency of p53 mutations in human cancers has been underestimated since the great majority of the studies have examined only the DNA binding domain, consisting of exons 5-8. However, no mutations were found in exons 2, 3 and 11 giving further support to previous reports and indicating that exons 4-10 may comprise a minimal region of required mutational analysis to cover the vast majority of p53 mutational spectrum in lung cancer.

Since there were only 8 non smokers among the patients studied significant conclusions concerning the mutation frequency in relation to smoking cannot be made. However, no difference was found between current and former smokers, indicating that p53 mutations may be an early molecular event in the molecular pathogenesis of NSCLC.

There was lack of GC→TA transversion preponderance that is usually reported for smoking populations giving further support to previous suggestions for additional environmental carcinogens and/or predisposition factors in the area (Merseyside, UK) that may play a significant role in lung carcinogenesis.

p53 positive staining, suggestive but not absolutely indicative of a p53 mutant status, was detected in 75% of the NSCLC examined. It has been demonstrated that missense mutations correlated with p53 positive staining, further supporting that null and frameshift mutations frequently result in a truncated protein that cannot be detected immunohistochemically. This also suggests that immunohistochemical detection of p53 is not adequate by itself for identification of p53 abnormalities. However, 56.5% of samples with no detected p53 sequence mutations, showed positive immunostaining. Consequently, both mutational and immunohistochemical analysis are required to identify the whole spectrum of p53 aberrations.

There was no association between p53 aberrations and $p21^{WAF1}$ expression providing further support to previous studies suggesting a dual, p53-dependent and p53-independent, regulation of $p21^{WAF1}$ expression. It has also been demonstrated that abnormal Rb expression correlated with p53 mutations whereas there was no association between p53 staining and Rb expression providing further support to previous studies with similar

findings. Interestingly, when examining the DNA binding domain (exons 5-8), a differential effect of p53 mutations on the expression levels of $p21^{WAF1}$ and pRb was observed, depending on the location of the mutated nucleotide. Thus, specific p53 regions may be involved in different functions of the protein.

Comparative analysis revealed that MSH2 expression correlated with p53 staining in samples with wild type p53, while a trend occurred for the overall examined population. One hypothesis is that p53 overexpression results in MSH2 upregulation as it is a transcriptional activator of it. This is also supported by the correlation of p53 mutations with reduced MSH2 expression in SqCCL as well as a similar association shown in other studies. However, this might suggest that intense immunohistochemical staining of p53 indicates stabilisation but not necessarily complete inactivation, since stabilised p53 can transactivate MSH2. Another hypothesis is that MSH2 may act as a DNA damage signaller to p53, similarly to ATM and DNA PK, thus binding and stabilising the protein. Therefore, high levels of MSH2 expression result in p53 stabilisation and correlate with positive p53 immunophenotype in samples carrying wild type p53. However, in samples carrying a mutant p53, stabilisation of the protein due to the mutation masks the effect of MSH2. Nevertheless, it may be speculated that a possible feedback loop exists between p53 and MSH2. However, additional functional studies are required to confirm and reveal the full nature of this relationship. p53 overexpression and total p53 aberrations (either mutation

or overexpression or both) correlated with telomerase indicating a close association between p53 and telomerase activity. Further studies are required to elucidate the nature of this relationship. p53 mutations and p53 overexpression correlated with allelic imbalance on chromosome 3p. The latter suggests that putative tumour suppressor gene(s) located on chromosome 3p may be associated with p53 in the molecular pathogenesis of certain NSCLC cases.

The challenging and hard task of eradicating lung cancer should involve a multi-modal approach. Prevention strategies should include a number of components, such as public awareness and education as well as public health policies on national and international levels. Characterisation, evaluation and assessment of biomarkers should be implemented towards the identification of individuals at high risk of developing lung cancer and its diagnosis in the pre-clinical stages of the disease. The latter is also going to provide the rationale for the identification of new therapeutic targets and assist the development of chemoprevention strategies. Integration of these strategies in lung cancer studies will pave the way to a brighter future and make sure that lung cancer patients will never walk alone!

CHAPTER EIGHT

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APPENDICES

APPENDIX I

Publications generated from this thesis

G. Xinarianos, T. Liloglou, W. Prime, G. Sourvinos, A. Karachristos, P. Maloney, J.R. Gosney, D.A. Spandidos, J.K. Field: Molecular alterations in the cell cycle regulators p53, Rb and p21 in relation to the expression of the DNA mismatch repair proteins MLH1 and MSH2 in non-small cell lung carcinomas. *Submitted.*

G. Xinarianos, T. Liloglou, W. Prime, P. Maloney, P. Fielding, J. Callaghan, J.K. Field: hMLH1 and hMSH2 expression correlates with allelic imbalance on chromosome 3p in non-small cell lung carcinomas. *Cancer Research* (2000) 60: pp 4216-422.

G. Xinarianos, F. M. Scott, T. Liloglou, W. Prime, M. Walshaw, L. Turnbull, J. K. Field: Evaluation of telomerase activity in bronchial lavage as a potential early and diagnostic marker for malignant lung disease. *Lung Cancer* (2000) 28: pp 37-42.

G. Xinarianos, F. M. Scott, T. Liloglou, W. Prime, J. Callaghan, J. R. Gosney, J. K. Field,: Telomerase activity in non-small cell lung carcinomas correlates with smoking status. *International Journal of Oncology* (1999) 15: pp 961-965.

J. K. Field, T. Liloglou, **G. Xinarianos,** W. Prime, M. Walshaw, L. Turnbull: Genomic instability in bronchial lavage as a potential marker for individuals with a high risk of developing lung cancer. *Cancer Research* (1999) 59: pp 2690-2695.

Manuscript in preparation:

G. Xinarianos, T. Liloglou, J. Callaghan, P. Maloney, J. K. Field: Telomerase activity interactions with cell cycle regulators in non-small cell lung carcinomas.

Chapters in books:

G. Xinarianos, T. Liloglou, J. K. Field: Detection of telomerase activity in lung tumours and bronchial lavage specimens. In Lung Cancer: Methods and Protocols. Methods in Molecular Medicine. Barbara Driscoll (ed), Humana Press Inc., New Jersey, USA. *Forthcoming.*

T. Liloglou, **G. Xinarianos,** J. K. Field: Detection of allelic imbalance in lung tumours and bronchial lavage specimens. In Lung Cancer: Methods and Protocols. Methods in Molecular Medicine. Barbara Driscoll (ed), Humana Press Inc., New Jersey, USA. *Forthcoming.*

APPENDIX II

Conference presentations generated from this thesis

T. Liloglou, **G. Xinarianos,** P. Maloney, J. Callaghan, P. Fielding, J. R. Gosney, J. K. Field: hMLH1 and hMSH2 expression in non-small cell lung carcinomas correlates with allelic imbalance on chromosome 3p. 91st Annual Meeting of the American Association for Cancer Research (AACR), San Francisco, CA, USA, April 2000.

G. Xinarianos, T. Liloglou, Jill Callaghan, P. Maloney, J. K. Field: Molecular investigation into the role of telomerase and its components in the pathogenesis of non-small cell lung carcinomas. Annual Meeting of the British Society of Human Genetics (BSHG). York, UK, September 1999.

J. K. Field, T. Liloglou, **G. Xinarianos,** L. Turnbull, M. Walshaw: Genomic instability in bronchial lavage specimens from individuals with no evidence of lung cancer: An early detection marker? ECCO 10, The European Cancer Conference, Vienna, Austria, September 1999.

G. Xinarianos, T. Liloglou, W. Prime, P. Maloney, P. Fielding, J. Callaghan, J.K. Field. Expression of hMLH1 and hMSH2 and allelic imbalance on chromosomes 3p21 and 2p16 in non-small cell lung carcinomas. Annual Meeting of the British Association for cancer Research (BACR). Edinburgh, UK, July 1999.

G. Xinarianos, T. Liloglou, J. Nunn, P. Maloney, G. Sourvinos, A. Karachristos, D.A. Spandidos, J.K. Field. Molecular analysis of p53 in current and former smokers with carcinomas of the upper and lower respiratory tract. 90th Annual Meeting of the American Association for Cancer Research (AACR), Philadelphia, PA, USA, April 1999.

G. Xinarianos, F. M. Scott, T. Liloglou, W. Prime, Jill Callaghan, J. R. Gosney , L. Turnbull, J. K. Field: Telomerase activity in non-small cell lung carcinomas. Swiss Institute for Experimental Cancer Research Conference: Cancer and the Cell Cycle, Lausanne, Switzerland, January 1999.

G. Xinarianos, F. M. Scott, W. Prime, M. Walshaw, L. Turnbull and J. K. Field: Detection of telomerase activity in lung tumours and bronchoalveolar lavage. Annual Meeting of the British Association for cancer Research (BACR). Dublin, Ireland, June 1998.

J. K. Field, T. Liloglou, **G. Xinarianos**, W. Prime, L. Turnbull, M. Walshaw: Genomic instability in bronchial lavage specimens from individuals with no evidence of lung cancer: An early detection marker? 89th Annual Meeting of the American Association for Cancer Research (AACR), New Orleans, LA, USA, April 1998.

F. M. Scott, **G. Xinarianos,** W. Prime, M. Walshaw, L. Turnbull and J. K. Field: Development of telomerase activity for early detection of lung cancer: studies in human bronchoalveolar lavage fluid. 89th Annual Meeting of the American Association for Cancer Research (AACR). New Orleans, LA, USA, April 1998.

G. Xinarianos, F. M. Scott, W. Prime, M. Walshaw, L. Turnbull and J. K. Field: Telomerase activity in lung cancer and human bronchoalveolar lavage fluid. British Association for Cancer Research (BACR) Winter Meeting, London, UK, November 1997.

T. Liloglou, **G. Xinarianos,** W. Prime M. Walshaw, L. Turnbull and J. K. Field: Microsatellite instability and loss of heterozygocity in bronchial lavage specimens. British Association for Cancer Research (BACR), London, UK, November 1997.

J. K. Field, T. Liloglou, **G. Xinarianos,** H. Ross, W. Prime, F. Scott, J. R. Gosney, L. Turnbull, R. J Donnelly: Microsatellite instability in non-small-cell lung cancer and bronchial lavage specimens. 88th Annual Meeting of the American Association for Cancer Research (AACR), San Diego, CA, USA, 1997.

APPENDIX III

[CANCER RESEARCH 59, 2690–2695, June 1, 1999]

Genetic Alterations in Bronchial Lavage as a Potential Marker for Individuals with a High Risk of Developing Lung Cancer[1]

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ABSTRACT

Using 12 microsatellite markers, we have studied DNAs from the bronchial lavage of 90 individuals who were referred to an early-lung-cancer clinic in the Northwest of England with suspected lung cancer. Genetic alterations were detected in 15 (35%) of 43 patients with lung cancer but also in 11 (23%) of 47 patients with no cytological or radiological evidence of bronchial neoplasia. No significant differences were found between the referring symptoms in any of the second group of individuals with and without genetic alterations. No correlation was found between smoking exposure and loss of heterozygosity (LOH)/microsatellite alterations (MAs) in the microsatellite markers. On comparing LOH with MAs based on cytology review, we found that the prevalent type of alteration in specimens with cytological evidence of malignancy was LOH; in contrast, the individuals with no cytological evidence of malignancy showed a preponderance of MAs ($P = 0.01$). Our results indicate that a substantial proportion of cells in the bronchial lavage from suspected lung cancer patients carry identifiable genetic alterations. However, the presence of genetic alterations in the bronchial lavage of individuals with no clinical evidence of lung cancer raises the question whether instability is a phenomenon solely associated with cancer or represents a feature of nonneoplastic diseases. Our results suggest that microsatellite PCR-based assays can be developed as tools for the earlier identification of genetic changes in cells exfoliating in the bronchus.

INTRODUCTION

Lung cancer is responsible for more deaths than any other cancer, accounting for 785,000 deaths per year world-wide. It is the most common malignancy in males in the United Kingdom, and the highest incidence rates of lung cancer for both men and women are found in the Merseyside region of the Northwest of England. Current clinical techniques are not capable, in the majority of lung cancer cases, to detect tumors before they have already progressed beyond effective treatment; consequently, there is a high mortality rate with a five-year overall survival rate of 5% (1). Thus, increased attention on earlier detection and intervention management is imperative.

Genomic instability is considered to be a hallmark of cancer. It has been proposed as a novel mechanism in the multistep process of carcinogenesis (2–4). Genomic instability in cells is found in many forms of genetic alterations. Allelic imbalance or LOH[3] mainly represents chromosomal instability (4) whereas MI, also found in the literature as replication errors (RER) or MAs, is most often attributed to DNA repair machinery errors. In this study, we will use the term "genetic alterations" to cover both LOH and MA phenomena.

The comparative analysis of microsatellite loci in tumor and normal counterpart tissue has become the most widely used method to determine such genetic alterations. Genomic instability is a common phenomenon in lung cancer (5–9) and, in some reports, has been associated with prognosis (10, 11). In a previous study from our group (8), 42 (93%) of 45 of NSCLC specimens were found to carry LOH or MAs in at least one of the 92 markers examined. Furthermore, genetic alterations have been detected in the plasma (12), bronchial mucosa, and sputum specimens of lung cancer patients (6, 13, 14). It is of note that genetic alterations were detected in specimens presenting with minimal atypia (6) and, moreover, that LOH and MAs have also been demonstrated in bronchial specimens from chronic smokers who do not have lung cancer (15, 16). These findings suggest that these genetic alterations precede morphological transformation of the cells. In this study, we have assayed DNA from 90 BL specimens for LOH and MAs, and we pose the question as to whether genetic alterations can be identified in BL from individuals with no cytological or radiological evidence of lung cancer. To date this question has not been answered.

MATERIALS AND METHODS

Patient Selection and Clinical Samples

We have collected 90 BL and control blood samples from individuals with suspected lung cancer who have been referred to the Cardiothoracic Center in Liverpool over a twelve-month period. The criteria for referral to the Cardiothoracic Center included: (*a*) unresolved chest infection; (*b*) abnormal chest X-ray; (*c*) cough (>4 weeks); (*d*) nonspecific weight loss; (*e*) stridor; (*f*) persistent (>3 weeks) hoarse voice; and (*g*) other suspicious features that would prompt referral to the lung cancer clinic. Each patient underwent a full clinical workup for lung cancer including a chest X-ray, spirometry, and bronchoscopy. BL specimens were obtained from all of these patients: the choice of site was based on bronchoscopic findings within the large airways, into which ~50 ml of saline was introduced via the bronchoscope and then aspirated.

The selection of patients was undertaken on the basis of an adequate cytology preparation and blood sample availability. The age of the patients selected ranged between 38 and 89 (average, 65). Thirty-three of the individuals were females, and 57 were males. Smoking data were available for 85 individuals (74 smokers, 11 nonsmokers). The total smoking exposure was calculated as follows:

$$\text{Pack-years} = (\text{age at presentation} - \text{age started} - \text{years stopped}) \times \frac{\text{pack}}{\text{day}}$$

A differential cell count was undertaken for all of the BL samples that were reported as NMCS and for the lung cancer patients with genomic instability. The epithelial cells present varied between samples (20–90%).

DNA Extraction

BL. BL (1 ml) was transferred into 1.5-ml tubes and centrifuged for 5 min, 10,000 rpm, at room temperature. The resulting pellet was resuspended in 400

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[3] The abbreviations used are: LOH, loss of heterozygosity; NSCLC, non-small cell lung cancer; MA, microsatellite alterations; NMCS, no malignant cells seen; COPD, chronic obstruction pulmonary disease; SqCCL, squamous cell carcinoma of the lung; MI, microsatellite instability; LVF, left ventricular failure; SCCHN, squamous cell carcinoma of the head and neck; MCI, myocardial infarct; BL, bronchial lavage.

mM Tris-HCl (pH 7.0), 150 mM NaCl, 60 mM EDTA, 1% SDS, and 100 μg/ml proteinase K and incubated at 42°C for 12–15 h in an orbital shaker. Deproteinization included the addition of 150 μl of 5 M sodium perchlorate and 500 μl of chloroform. After mixing and microcentrifugation for 2 min, the aqueous phase was transferred to a fresh tube, and DNA was precipitated by the addition of an equal volume of isopropanol. After incubation at −20°C for 12–14 h, DNA was recovered with microcentrifugation for 15 min at 4°C, washed with 70% ice-cold ethanol, and resuspended in 50 μl of sterile distilled H_2O.

Blood. Blood (3 ml) was washed three times with 10 mM Tris-HCl (pH 8.0), 320 mM sucrose, 1% Triton X-100, and 5 mM $MgCl_2$ to remove red cells. Lysis of the resulting WBCs was similar to that of BL. Deproteinization was carried out by the addition of 500 μl of phenol/chloroform. After mixing and microcentrifugation for 2 min, the aqueous phase was transferred to a fresh tube, and DNA was precipitated by the addition of an equal volume of isopropanol. DNA was recovered with a sterile loop, washed with ice-cold 70% ethanol, and resuspended in 200 μl of sterile distilled H_2O.

PCR Amplification of Microsatellite Loci

The selection of microsatellite markers was based on the previous work of our group (7, 8, 17, 18) and other workers' published results (5, 6, 9). We chose 12 markers: ACTBP, D14S50, D3S1215, D3S1339, D3S1351, D4S194, D4S392, FGA, D6S271, D9S286, TCRD, and TP53. These markers have been previously shown to exhibit a high frequency of instability in lung cancer.

Oligonucleotide primers purchased from Research Genetics (Huntsville, AL). The reaction mixture contained 16 mM $(NH_4)_2SO_4$, 67 mM Tris-HCl (pH 8.8), 0.1% Tween 20, 200 mM dNTPs, 0.1 mM of each primer, 2 mM $MgCl_2$ and 0.5 units BIOPRO polymerase (BIOLINE, United Kingdom). The amplification parameters were: initial denaturation for 2 min, followed by 28–30 cycles at 94°C for 30 s, 55–58°C for 30 s, and 72°C for 30 s, and final extension step at 72°C for 5 min. PCR products were analyzed by electrophoresis on 10% nondenaturing polyacrylamide gels and stained with silver. MA was judged from the presence of an allele (band) with different electrophoretic mobility. LOH was judged visually on the basis of a ≥40% relative reduction of one allele intensity. MA was judged from the mobility shift of one of the alleles or the presence of an additional allele on the gel. Positive samples were scored only when the LOH/MA results were confirmed at least twice in separate PCR reactions.

Sensitivity and MA Detection

To assess the sensitivity of LOH and MA of our method, we prepared DNA samples of various tumor:normal (*T*:*N*) ratios from tumor specimens with known LOH and MAs. The analysis showed that LOH may be detected in 1:10 dilution (*T*:*N*), and MA was detected in 1:16 dilution (Fig. 1).

Statistical Analysis

Fisher's exact test was used to analyze the data.

RESULTS

Genetic Alterations in Lung Cancer Patients. The clinical findings (cytology, radiology, biopsy) revealed 43 patients with lung cancer, of which 18 individuals were reported with malignant cells present in the cytological specimen; 25 individuals were reported as NMCS but did have a radiological and/or histological diagnosis of lung cancer. The remaining 47 individuals had no clinical evidence of lung cancer (Fig. 2).

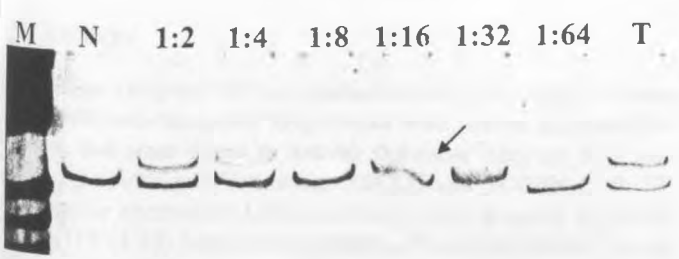

Fig. 1. Sensitivity assay for MA detection. DNA from a tumor with known MA was spiked with DNA from normal tissue in a range of ratios.

Genetic alterations were identified in 8 (44%) of 18 of the positive cytology cases (Fig. 2). All of the eight individuals demonstrated LOH, and three specimens had LOH and/or MAs in more than one marker (Table 1). The remaining 10 of 18 carcinomas of the positive cytology group with no evidence of genetic alterations included 9 squamous cell carcinomas and 1 adenocarcinoma.

Seven (28%) of the 25 individuals who were diagnosed with bronchial carcinoma but had no malignant cells in their BL exhibited genetic alterations (Table 1). The remaining 18 carcinomas in this group with no genetic alterations were composed of 9 squamous cell carcinomas, 1 adenocarcinoma, and 8 individuals without histological diagnosis (diagnosed radiographically). No association was found between the radiological findings and genetic alterations.

In nine of the cases with lung cancer, tumor tissue was also available. Three of nine tumors demonstrated genetic alterations. Of these, two (BL114 and BL176) had the same aberration in the BL (Fig. 3*b* and *c*), whereas in patient BL154, the aberration was not detectable in the BL specimen, although it was present in the tumor (Fig. 3*a*). We have examined the relationship between genetic alterations and confirmed cytology/histopathology and found that LOH/MAs tend to be more frequent among the BL of patients with adenocarcinomas (6 of 8) than squamous carcinomas (5 of 25; $P = 0.069$).

On comparing LOH with MAs based on the cytology review, we found that the prevalent type of alteration in specimens with cytological evidence of malignancy is LOH (Table 1). In contrast, the individuals with negative cytology show a preponderance of MAs (Fisher's exact test, $P = 0.01$). To examine this, we compared the number of LOH/MA findings against the differential cell count in the cytology specimens from patients with no clinical evidence of cancer by any diagnostic method. In the BL specimens with >50% epithelial cells had three with LOH and five with MAs, whereas those samples with <50% epithelial cells in the BL had two with LOH and four with MAs. No significant difference was found between LOH/MA frequency and the epithelial cell content of the BL specimen.

Genetic Alterations in the BL of Individuals with No Clinical Evidence of Lung Cancer. Eleven (23%) of 47 of the individuals had no cytological or radiological evidence of lung cancer but did demonstrate genomic alterations. Nine individuals had evidence of MA/LOH, and two individuals were found to have LOH alone (Table 1). The statistical analysis in the group of individuals with no clinical evidence of lung cancer revealed an association (6 of 9) between genomic alterations and individuals with rheumatoid arthritis, clinical goiter, cardiac problems, and fibrosing alveolitis compared with those (4 of 31) with common chest disease (*e.g.*, asthma, COPD, or chest infections; $P = 0.003$). No significant differences were found between the referring symptoms in the individuals with and without genetic alterations. On examination of the prescribed drugs to these 47 patients over the past 5 years, no association was found between genetic alterations and any specific drugs. Cytology review of these 47 individuals showed 9 with squamous metaplasia of regular type, of whom 4 had genetic alterations, but this trend was not found to be significant (Fisher's exact test, $P = 0.09$).

The statistical analysis on either the total number of individuals or the separate groups of lung-cancer and non-lung-cancer individuals did not produce any significant correlation between genetic alterations of the markers examined with age or gender. Similar was the case with the smoking history or tobacco exposure in patients with lung cancer because, of 42 with complete smoking data, we had only two nonsmokers (one patient with squamous cell carcinoma of the lung and

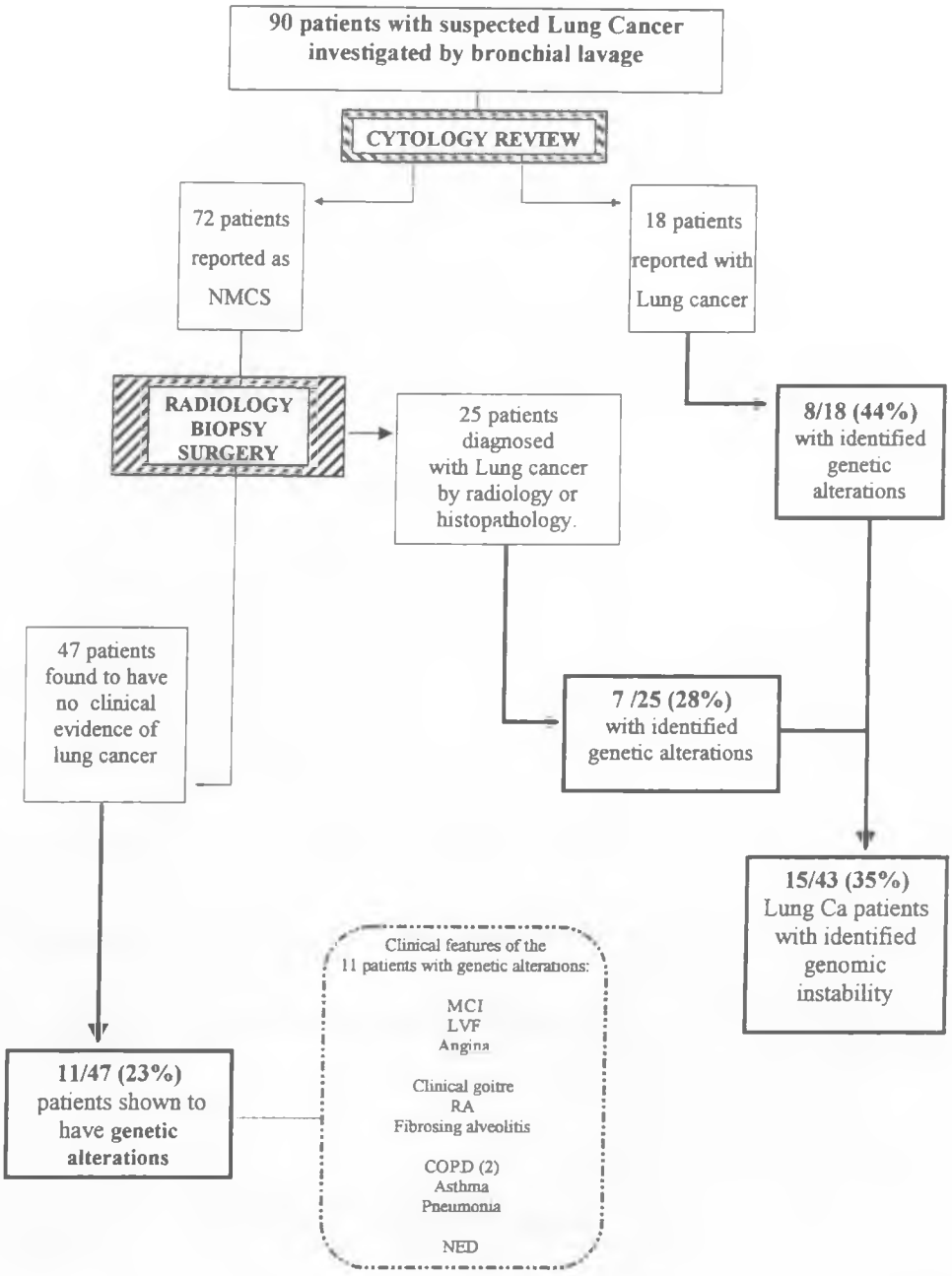

Fig. 2. Flow diagram of the 90 patients undergoing a clinical workup for lung cancer in this study. This demonstrates genetic alterations in the BL specimens from individuals with lung cancer and also in the individuals with no cytological or radiological evidence of lung cancer. *RA*, rheumatoid arthritis; *NED*, no evidence of disease.

metastatic melanoma; Table 2). In the group of 47 patients with no clinical evidence of lung cancer, smoking data were available for 43 individuals (34 smokers, 9 nonsmokers). Seven of the smokers (two with LOH only, three with MAs only, and two with LOH/MAs) and three of the nonsmokers (one with LOH/MAs and two with MAs only) demonstrated genetic alterations. The clinical diagnosis for these three patients was rheumatoid arthritis, fibrosing alveolitis, and one individual with no evidence of any disease.

DISCUSSION

We have analyzed 90 BL specimens and blood samples from individuals with suspected lung cancer with twelve microsatellite markers that were found to exhibit the most frequent MAs and LOH in previous publications in NSCLC and SCCHN (5–9, 17, 18). Genetic alterations (LOH and MAs) were detected in the BL of 35% (15 of 43) lung cancer patients. It is of particular interest that genetic alterations were detected in BL specimens from patients who were reported as NMCS on cytological examination. This finding indicates that a proportion of the cells in the BL carried genetic aberrations but presented with no morphological evidence of malignancy. However, an alternative source of LOH/MAs may be naked tumor DNA in the BL sample. This may also explain the fact that LOH/MAs were detected in samples with a cytology report indicating possible target epithelial cells in very low abundance, lower than the sensitivity we have demonstrated for this assay (1:10 for LOH and 1:16 for MAs; Fig. 1). Naked DNA released in body fluids is also suggested to be a source of genetic alterations detected in the plasma of lung cancer patients (12). It should be noted that the sensitivity of MA detection in our experiments is in agreement with that of Foucault *et al.* (19), who reported detection of the less abundant allele when it represents 6% of the total DNA. Genetic alterations have also been detected in body fluids such as urine (20), plasma (12), and sputum (6) and may, therefore, be considered as an attractive candidate for the development of strategies for the early diagnosis of cancer, alongside other molecular methods that require no invasive techniques.

Table 1 *Clinical details of patients with genetic alterations detected in their BL specimen*

Sample	Cytology	Radiology	Biopsy	Surgery	Final diagnosis	Smoking (pk-yr)[a]	ACTBP2	D14250	D321215	D321339	D321351	D42194	D42392	D62271	D92286	FGA	TCRD	TP53
EDC034	AdenoCa	Mass LLL			AdenoCa	60	H	MA		L	L		H	H		H		H
BL192	NSCLC	Mass RUL		AdenoCa	AdenoCa	25	H		H		H		H	H				L
BL145	SCLC	Mass LMB	SCLC		SCLC	123	H	H			H	L	H	H		L	H	H
BL176	SCLC	Mass RUL	SCLC		SCLC	98	H		H	L	H	H		H		H		L
BL112	SqCCL	Mass R hilum	SqCCL		SqCCL	220	H	H	H	H	H		H	H		H	L	H
BL075	SqCCL	Mass R hilum	SqCCL		SqCCL	78	H	H	H			H	H	H	L	H	H	H
BL154	SqCCL	Mass RLL	SqCCL		SqCCL	135	H	H			L	H	H	H	H	H	H	H
BL115	SqCCL	Collapse LLL	SqCCL		SqCCL	118	H	H		H	H	H	L	H			H	L
BL125	*nmcs*	Collapse RUL			Secondary AdenoCa	ND	H	H		H		H	H			MA	H	H
BL187	*nmcs*	Mass RUL			Metastatic melanoma	0				H				H		H		MA
BL152	*nmcs*	Shadow RUL		AdenoCa	AdenoCa	76	H	H		H	H	L	H	H		H	H	H
BL212	*nmcs*	Mass LLL		AdenoCa	AdenoCa	37	H	H	H				H	H		H		L
BL114	*nmcs*	Mass RUL		AdenoCa	AdenoCa	34	MA		H	MA	H	H	H	H		H		H
BL081	*nmcs*	Mass RUL			Lung Cancer	40		MA	H		H		H	H		H	H	
BL181	*nmcs*	Shadow RUL		SqCCL	SqCCL	120		H	H	H	H	H		H		MA		H
BL119	*nmcs*	Normal			Angina	38			H		H	H	H	H	MA	H		
EDC067	*nmcs*	Clear			Asthma	40	H	H		H			L	H		MA		H
BL084	*nmcs*	Shadow LLL	Inflammation		FA	0	H			H	H	H	H	H	H	MA	L	H
BL111	*nmcs*	Atelectasis			COPD	ND		MA	H	H	H		H	H				H
BL090	*nmcs*	Normal			COPD	48	H	H	H		H		H	H	L	H	H	H
BL126	*nmcs*	MD shadow			Clinical goiter	45	H	H	H	H				H		MA		MA
BL067	*nmcs*	LHC			LVF	59	L			H	MA		H			H		
BL085	*nmcs*	Leural effusion LLL	Inflammation		MCI	160	H	H	L	H	H	H		H	H	H		H
BL180	*nmcs*	Normal			NED	0	H		H	H	H	H	MA	H	H	H	H	H
EDC-29	*nmcs*	Consolidation			Pneumonia	32	H	H		H	H		H	H		MA	H	H
BL086	*nmcs*	Clear			RA	0	H	MA	H	H	H			H	H	H	H	H

[a] pk-yr, pack-year; NED, no evidence of disease; SCLC, small cell lung carcinoma; AdenoCa, adenocarcinoma; RA, rheumatoid arthritis; FA, fibrosing alveolitis; RUL, right upper lobe; LLL, left lower lobe; LMB, left middle bronchus; LHC, left hilum consolidation; MD, mediastinal; H, heterozygous; ND, not determined; R, Hilum, right helum.

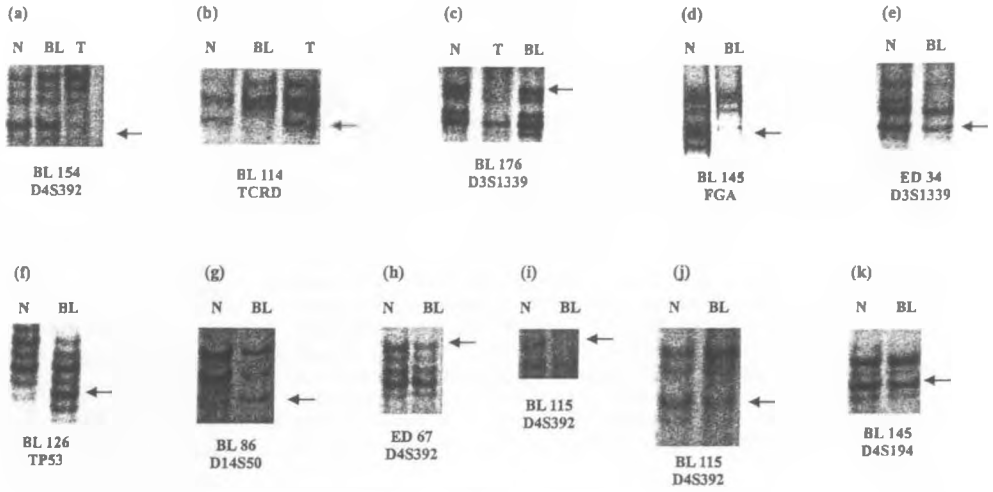

Fig. 3. Genomic instability in BL specimens from individuals with lung cancer and also from individuals with no clinical evidence of lung cancer. *a*, *b*, and *c*, analysis of genetic alterations in blood (*N*), BL, and tumor specimens (*T*). LOH is seen in the tumor tissue but not in the BL (*a*); LOH is seen in both BL and tumor specimens (*b* and *c*). Also, cases *d*, *e*, *h*, *i*, *j*, and *k* show LOH in the BL specimen, and *f* and *g* show MA (*bandshift*). It is of note that in some cases (*d* and *i*), the loss of one allele is almost complete, which indicates a large proportion of cancer cells in the BL specimen, whereas in others (*c*, *h*, *j*, and *k*), the loss is incomplete, which indicates a higher proportion of normal cells.

In an allelotype study of NSCLC undertaken with 92 markers, we have showed that 93% of the samples exhibited LOH/MA in at least one marker (7). Thus, it may be argued that the majority of NSCLC cases contain such genetic damage because it may be considered to be a fundamental feature of carcinogenesis. The detection of LOH/MA in 35% of lung cancer patients in this study can, therefore, be considered as an underestimate, and a larger number of markers are, therefore, required to increase the detection efficiency. In addition, a possible underestimation of the LOH/MA frequency may be due to the visual identification. MAs are easier to detect because they are represented by an additional band, whereas LOH demands quantitation of the relative density of the two alleles. The detection method that we used (*i.e.*, silver staining of the PCR products analyzed on polyacrylamide gel), has been widely used for the detection of genomic instability. However, it is clear that there are certain limitations to its applicability on clinical specimens, such as BL, with a possible low number of target cells. In the future, biomagnetic separation for epithelial cell enrichment may be a solution to this problem. In addition, the use of fluorescent chemistry will increase the sensitivity of the assays because the quantitation of band intensities would become far more accurate.

It is of note that 23% (11 of 47) of the patients referred for investigation with no cytological or radiological evidence of lung cancer do have genetic alterations in the same microsatellite markers as found in patients with proven neoplasia. These patients, however, cannot be considered as a "control group," based on their referral criteria and are still under long-term follow-up. Our results are in agreement with recent reports demonstrating genetic alterations in biopsy specimens from current and former smokers who do not have lung cancer (15, 16) and MI in cytological material from patients with COPD (21). In this group of 47 patients, genetic alterations were more common in rheumatoid arthritis, goiter, fibrosing alveolitis, and cardiac problems (MCI, LVF, and angina) than in common chest disease. Thus, this may imply that genetic alterations occur in disease processes such as cardiac and autoimmune conditions and, thereby, suggests a possible genetic link between certain neoplastic and nonneoplastic diseases. Moreover, LOH/MA in this group may just represent molecular damage due to tobacco exposure in smokers or some other carcinogens in nonsmokers. Such genetic changes have been previously reported in smokers in a range of histological types including normal epithelium (15, 16). The individuals included in this study are currently being followed up in the context of the Liverpool Lung Project (22), and it remains to be shown whether those with genetic alterations will develop lung cancer at a future date.

Table 2 *Genomic instability and smoking history of the lung cancer patients investigated in this study*

Smoking status	LOH	LOH + MA	MA	No evidence of genetic alterations
Smokers				
Lung cancer	9	1	3	27
Noncancer[a]	2	2	3	27
Nonsmokers				
Lung cancer			1	1
Noncancer		1	2	6
No smoking data				
Lung cancer			1	
Noncancer			1	3

[a] No clinical lung cancer has been observed in these patients on follow-up to date.

In this study, no statistical correlation was found between genomic instability and age or gender. Because there were only 11 nonsmokers (2 in the lung cancer group and 9 in the noncancer group) in this study, we are unable to produce significant comparisons between genetic alterations and smoking history. LOH tends to be the predominant genetic alteration found among smokers, and MA is more frequent in nonsmokers, although not significantly (Fisher's exact test, $P = 0.19$; Table 2). This might reflect the exposure to different carcinogens between smokers and nonsmokers. However, among patients with no malignancy, nonsmokers did not demonstrate a significant difference in the frequency of total genetic alterations (LOH + MA; Table 2). This is in contrast to the findings of Mao *et al.* (15) and Wistuba *et al.* (16). One hypothesis for this observation may lie in the argument that this population in the Northwest of England may be exposed to additional carcinogens. This hypothesis is consistent with our previous observations on NSCLC and SCCHN patients in this region, who demonstrated a *p53* mutational profile with prevalence of GC→AT transitions, which is not typical of a smoking population (23, 24).

We have observed a trend for LOH/MA to be found more frequently in BL patients with adenocarcinomas (6 of 8) than with SqCCL (5 of 25). The reason for this is unclear, mainly because adenocarcinomas are usually associated with peripheral airways whereas SqCCL is usually associated with large-airway disease and is more efficiently sampled by BL. Furthermore, the prevalent type of genetic alteration found in specimens with cytological evidence of malignancy was LOH (Table 1), whereas individuals with NMCS at cytology mainly demonstrated MA. This finding implies that LOH (*i.e.*, chromosomal imbalance) may be associated with neoplastic cell transformation and, thus, altered cytological appearance of the cell, whereas MA may not be associated with identifiable morphological changes. MA may, therefore, represent genetic changes of subclones arising from a cell population with high genetic diversity. It may be argued that this difference may be due to the fact that MA is more

easily detected than LOH in samples with low numbers of target cells, such as the cytological specimens that have been reported as NMCS. However, our results indicate that there was no bias in the detection of MA in samples with <50% epithelial cells. We also investigated whether this bias was due to the different histological composition of the NMCS group (prevalence of adenocarcinomas) compared with the cytology-positive group (prevalence of SqCCL; Table 1). However, the absence of a significant difference in the type of genetic alteration seen in adenocarcinomas (four with LOH, two with MA) and SqCCL (four with LOH, one with MA) does not support this argument.

In this study, we have demonstrated that genetic alterations can be detected in the BL. In time, additional markers and advances in technology may increase the present success rate. Additional studies and long-term follow-up are required to clarify the value of such genetic alterations as a tool for the identification of individuals with a high risk of developing the disease.

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Telomerase activity in non-small cell lung carcinomas correlates with smoking status

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Abstract. Human telomerase is a ribonucleoprotein DNA polymerase which maintains the telomeric region of human chromosomes and has been detected in all types of human cancer tested. We used the telomeric repeat amplification protocol (TRAP) assay to examine 71 non-small cell lung carcinomas (NSCLC) and their adjacent normal tissue. Telomerase activity was detected in 61 (86%) of the 71 NSCLC examined but not in any of the matched normal lung tissues. A significant correlation was found between the presence of telomerase activity and current smoking status at the time of diagnosis (p=0.0076). In addition, a trend was found between telomerase activity and smoking exposure (p=0.06). Our findings demonstrate that telomerase activity is a common phenomenon in NSCLC cases but not in the normal lung. However, certain cases in former smokers may follow a telomerase independent pathway.

Introduction

Telomeres are specific structures consisting of short tandem repeat sequences at the ends of chromosomes. Their major role is to help maintain chromosome stability. The maintenance of telomeres is considered to be essential for the progression of cells through a normal, mortal life span (1). Telomerase is a ribonucleoprotein enzyme which replaces chromosome ends lost during successive cycles of cell division (2). Telomerase activity has been detected in almost all human cancer types and in all human tumour cell lines tested. Telomerase activity has also been detected in some somatic tissues, including hematopoietic cells and some stem cells (3-5).

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Key words: telomerase activity, TRAP assay, non-small cell lung carcinoma

Lung cancer is responsible for more deaths than any other cancer, accounting for 900,000 deaths per year worldwide (6). It is the most common malignancy in males in the UK and is now the most frequent cancer in women in Liverpool. The highest incidence rates of lung cancer for both men and women are found in the Merseyside region of Northwest England. In the Liverpool area the cumulative rate (0-74 years) in 1994-1995 was 11.6% for males and 7.2% for females compared with a national average of 7.3% for males and 3.0% for females in 1992 (7-9).

Early reports on telomerase in lung cancer indicated that enzyme activity is present in most of such cases, ranging from 78-96% in NSCLC while it was present in all of the small cell carcinomas tested (10-12). In one of the reports, similar proportions of telomerase positive cases were detected in the two different patient populations, American and Japanese, included in the study (10). It has also been demonstrated that dysregulation of telomerase is an early event in the multistage pathogenesis of bronchogenic lung carcinomas (12).

We used the TRAP assay, in radioactive and non-radioactive forms, to detect telomerase activity in NSCLC samples and matching normal lung tissues in a patient population from the Merseyside region of Northwest England.

Materials and methods

Cell lines. The human non-small cell lung cancer cell lines Calu3, H1155, HTB58, and A549, and human colon adenocarcinoma HCT15 and LoVo cell lines were used as positive controls for telomerase activity, and the human lung fibroblast cell line LL47 as a negative control. All cell lines were obtained from the American Type Culture Collection and grown according to the conditions recommended by the supplier. Cell pellets were collected and frozen at -80°C until preparation of telomerase extracts.

Patients and tissue samples. Primary lung tumour and corresponding normal tissue samples were obtained from 71 patients, 42 males and 29 females, who underwent surgery in the Cardiothoracic Centre of Broadgreen Hospital, Liverpool (Merseyside, UK). The age of the patients selected ranged between 45 and 77 years (average 62 years). Smoking

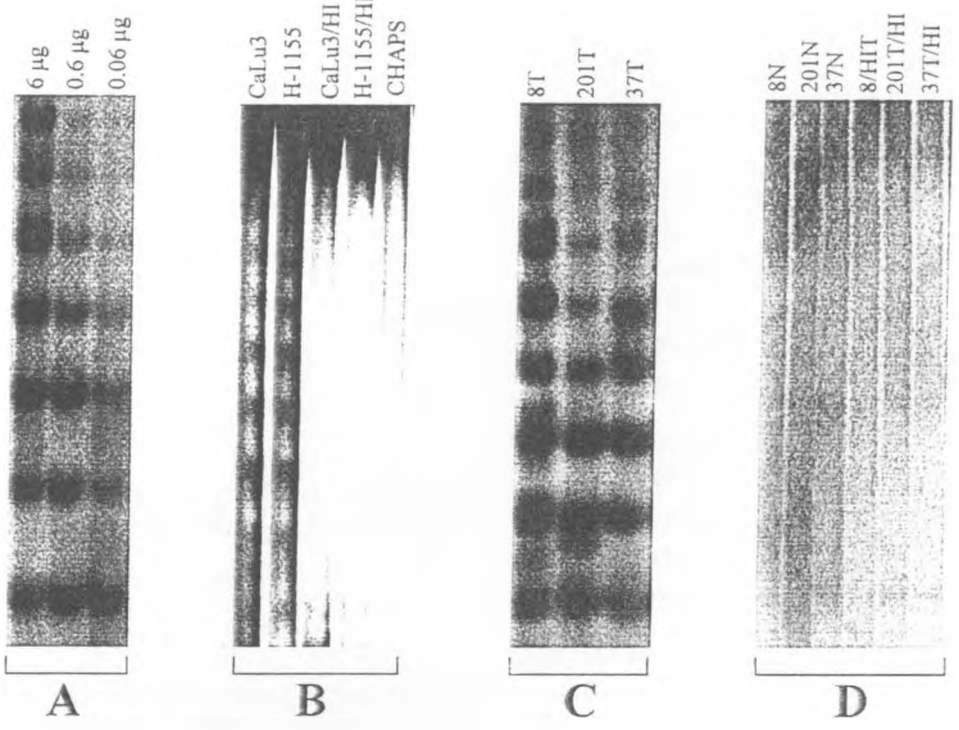

Figure 1. Telomerase activity in NSCLC using a non-radioactive (silver staining) TRAP assay. A. LoVo (colon adenocarcinoma) cell line extracts assayed at 3 concentrations (6 µg, 0.6 µg, 0.06 µg) to establish the sensitivity of the assay. B. Telomerase positive lung cancer cell lines CaLu3 and H-1155, and negative control for the reaction (CHAPS lysis buffer). Lanes marked HI indicate heat-inactivated samples of the indicated cancer cell lines. C. Telomerase positive NSCLC tumour extracts with different levels of enzyme activity. D. Telomerase negative samples marked 'N' are corresponding normal lung tissue from the indicated telomerase-positive specimens. Lanes marked HI are heat-inactivated samples of the indicated tumour cell extracts.

data were available for 55 individuals (52 current or former smokers, 3 non-smokers). The total smoking exposure was calculated in pack-years = [Years smoked] x [packs/day]. All specimens (n=71; 32 adenocarcinomas, 32 squamous cell carcinomas, 7 other NSCLC) were microdissected to ensure content of at least 70% tumour cells and immediately snap-frozen in liquid nitrogen and stored at -80°C prior to preparation of protein extracts.

TRAP assay. Each frozen tissue sample was first washed in ice-cold buffer (10 mM HEPES-KOH [pH 7.5], 1.5 mM $MgCl_2$, 10 mM KCl and 1 mM dithiothreitol), then homogenised in 200 µl of ice-cold CHAPS lysis buffer (0.5% CHAPS (3-[(3-cholamidopropyl)-dimethyl-ammonio]-1-propanesulphonate), 10 mM Tris-HCl, pH 7.5, 1 mM $MgCl_2$, 1 mM EGTA (egtazic acid), 5 mM ß-mercaptoethanol, 0.1 mM phenylmethylsulphonyl fluoride (PMSF) and 10% glycerol). Homogenisation was performed in 1.5 ml microfuge tubes using disposable pestles rotated by a motor. Lysates were incubated on ice for 30 min followed by centrifugation at 14,000 rpm for 20 min at 4°C. Resulting supernatants were snap-frozen in liquid nitrogen and stored at -80°C until they were subjected to the TRAP assay. A protein concentration for each extract was determined using the BCA protein assay kit (Pierce Chemical Co., UK). The TRAP assay was performed as previously described with a few modifications (13,14). Six µg total protein of a cell line or lung tissue were added to TRAP reaction mix, containing 50 mM of each dNTP, 100 ng of TS primer, 2.5 U BIOPRO polymerase (Bioline, UK), and 5 µl 10X buffer (50 mM KCl, 10 mM Tris-HCl, pH 8.8, 0.1% Triton X-100, 1.5 mM $MgCl_2$), and allowed to react at room temperature for 30-60 min. The reaction mixture was then heated at 90°C for 90 sec to inactivate telomerase and 100 ng of CX primer was added to the reaction. Samples were subjected to 31 (radioactive TRAP) or 37 (non-radioactive TRAP) cycles of amplification using the following parameters: 94°C for 30 sec, 50°C for 30 sec and 72°C for 45 sec. For radioactive TRAP, 1-2 µCi per reaction [α-^{32}P]dCTP, 3000 Ci/mmole, were added. Specificity controls were performed by pre-treatment of protein extracts with 0.5 µg RNase A for 30 min at 37°C or heat inactivation at 95°C for 5 min before addition to the TRAP assay. Blank control samples containing CHAPS lysis buffer were also run. TRAP products were run on 10% non-denaturing polyacrylamide gels for 1000-1,100 Vh and the 6-bp DNA ladder was visualised by silver staining or autoradiography after a 1-2 day exposure at -80°C. We scored a sample as telomerase positive when an RNase A and/or heat sensitive 6-bp DNA ladder was observed. All experiments were carried out in duplicate. All tumour and normal negative samples were also examined by the radioactive form of the TRAP assay.

PCR controls for telomerase inhibitors. PCR for exon 10 of the p53 gene using intronic primers was used to check for the presence of contaminating genomic DNA in tumour extracts (Xinarianos *et al*, manuscript in preparation). In addition, tumour extracts from negative samples were mixed with extracts from telomerase positive specimens and subjected to the TRAP assay to assess any inhibitory action on the assay.

Table I. Telomerase activity in NSCLC.

Lung tumour	n	Telomerase positive	%
Adenocarcinoma	32	26	81
SqCCL	32	29	91
Other NSCLC	7	6	86
Total	71	61	86

Statistical analysis. Results were analysed using Fisher's exact test. Values of $p<0.05$ were considered to be statistically significant.

Results

The TRAP assay was optimised by running serial dilutions (6 µg, 0.6 µg and 0.06 µg) of extracts of positive control cell lines (Fig. 1A and B). Positive telomerase activity in a tumour sample was determined by the presence of a 6-bp ladder which was sensitive to heat inactivation and/or RNase A treatment of the protein extract. Seventy-one NSCLC samples and their corresponding normal lung tissue were investigated for telomerase activity (Table I). Overall, telomerase activity was detected in 61 (86%) of 71 specimens examined. Enzyme activity was present in 26 (81%) of 32 adenocarcinomas, 29 (91%) of 32 squamous cell carcinomas and 6 (86%) of 7 other NSCLC cases (Fig. 1C). None of 71 normal matching lung tissues were found to be telomerase positive (Fig. 1D).

Tumour and normal samples which were found to be telomerase negative were investigated further for the presence of TRAP assay inhibitors and genomic DNA. Our results revealed that genomic DNA was not present in any of the samples examined indicating absence of false positive results. Moreover, telomerase activity was retained when these samples were mixed with extracts from telomerase positive tumours.

Data obtained for telomerase activity were examined in relation to clinical and pathological parameters. No significant correlation was found between the presence or absence of telomerase activity and tumour size or stage (TNM classification), differentiation, histology, alcohol consumption and gender of the patient. However, telomerase activity was more frequently detected in current smokers (30 of 31) than former smokers (9 of 14) (stopped for at least 5 years prior to presentation) (Fisher's exact test p=0.0079). The patients analysed in this study were divided into two groups, current smokers and former smokers (smokers who had stopped for at least 5 years). Individuals who had given up smoking for 1-4 years were not included. However, if they had been included in the analysis the result would still have been statistically significant (p=0.013). A trend (p=0.06) was found between telomerase activity and overall smoking exposure. Telomerase activity was more frequently detected in heavy smokers (>100 pack-years) than in light smokers (<40 pack-years). Also, a trend was found for an inverse correlation between telomerase positivity and the age of the patient (p=0.08) (Table II).

Table II. Telomerase activity and smoking status, tobacco exposure, and age of the NSCLC studied.

Characteristic	n	Telomerase (+)	Fisher's exact test
Smoking status			
Current smokers	31	30	p=0.0079
Former smokers[a]	14	9	
Former smokers[b]	21	15	p=0.013
Non-smokers	3	2	
Tobacco exposure			
Heavy smokers (>100 pack-years)	9	9	p=0.06
Light smokers (<40 pack-years)	7	4	
Age (years) (median 62)			
>62	47	38	p=0.08
<62	24	23	

[a]Only non-smokers for at least 5 years are included. [b]Non-smokers for 1-4 years have also been included.

Discussion

Telomerase activity has been detected in a range of human cancers (15-19). This is the first study to examine telomerase activity in NSCLC in a European population. In particular, we investigated the incidence of telomerase activity in NSCLC from a patient population in the Northwest England, Merseyside region, which appears to have some of the highest incidence rates of lung cancer in the UK (20). Telomerase activity was detected in 86% of the tumours but it was completely absent in the normal adjacent tissues. Our finding is in agreement with previous reports which examined Japanese and American populations (10,11,24). Although telomerase activity was detected in the vast majority of the samples examined, there is a distinct group of NSCLC tumours which exhibits undetectable levels of telomerase activity. Therefore, it may be argued that these telomerase negative tumours may overcome the telomere shortening using an alternative pathway (21-23).

The TRAP assay, as performed in this study, may be considered to be semi-quantitative because equal amounts (6 µg) of protein from each specimen were used in the assay and equal volumes of each reaction mix were analysed (45 µl). We have observed variable levels of telomerase activity in tumour specimens, ranging from no activity to high activity,

as judged by the varying intensity and number of bands in the TRAP ladder (Fig. 1). Other studies (10-12) have also reported variable levels of telomerase activity in lung tumour specimens. It has been suggested that tumours with low or undetectable telomerase activity may contain primarily mortal cells, while tumours with high levels of telomerase are likely to contain mainly immortal cells (10). High telomerase activity has been frequently detected in NSCLC with high tumour proliferation rates and advanced pathological stage (11). The tumour samples in the current study were micro-dissected to ensure that tumour extracts derived from 70-90% of cancer cells. However, it is still possible that the variation of telomerase activity could be attributed to an admixture of normal and cancer cells or the presence of different telomerase levels within individual tumour subpopulations. Further studies, including long-term patient follow-up, are required to define the prognostic significance, if any, of varying levels of telomerase activity in lung tumours.

No correlation was found between the presence of telomerase activity and the size, stage and nodal metastasis of the tumour. Thus, telomerase may be considered to play a role in the early stages of lung cancer development. Also, there were no differences found between the presence of telomerase activity and histopathological diagnosis of the NSCLC tumours examined.

It is of interest that telomerase activity was not detected in any of the 71 adjacent normal lung tissues. This is in contrast to a previous report which demonstrated weak telomerase activity in approximately 23% of normal peripheral lung tissues from corresponding lung tumours (12). The authors of the study proposed that the presence of regenerating stem cells or damage to the epithelial cells due to exposure in chemical carcinogens found in tobacco could have led to these observations. Other studies have demonstrated either a very small incidence (approximately 4%) of weak or no detectable telomerase activity in normal lung tissues (10,11,24). Our experiments indicated that it is highly unlikely that TRAP assay inhibitors and genomic DNA are the reason for the absence of detectable telomerase activity in this subset of NSCLC studied, as well as in the adjacent normal tissues.

In this study, smoking data were available for 55 of the 71 patients. Since there were only three non-smokers and 52 current or former smokers in this series of samples, a comparison between telomerase and smokers or non-smokers could not be made. However, telomerase activity was more frequently detected in current than in former smokers ($p=0.0076$). In addition, a trend was found between telomerase positivity and a history of heavy smoking (>100 pack-years) compared with light smoking (<40 pack-years), but this was not found to be statistically significant ($p=0.06$). This is in contrast to the findings of Yashima and co-workers who demonstrated no difference in telomerase activity between former and current smokers with lung tumours (12). Our observations may indicate that certain NSCLC cases from former smokers, in this population in Northwest England, may undergo a telomerase independent initiating pathway. Telomerase activity was predominantly detected in younger (<62 years) than in older patients (>62 years), however, no significant correlation was found ($p=0.08$).

In this investigation, we have demonstrated that telomerase activity correlated with smoking status at the time of presentation in NSCLC in a UK population. A trend for a correlation between smoking exposure and telomerase activity was also found. The presence of telomerase activity in the tumours did not correlate with any other clinicopathological features. These data suggest that this subset of telomerase negative tumours from former smokers may represent a discrete population with a distinct telomerase-independent initiating pathway. Further study of telomerase activity as a diagnostic/prognostic is required. In addition, *in situ* assays for detection of the telomerase RNA (hTR) and catalytic protein (hTRT) components (25-29) may improve our understanding of telomerase regulation and its molecular initiating pathways.

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Evaluation of telomerase activity in bronchial lavage as a potential diagnostic marker for malignant lung disease

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Abstract

Telomerase is a ribonucleoprotein DNA polymerase that maintains the telomeric region of chromosomes lost during successive rounds of cell division. We used the telomeric repeat amplification protocol (TRAP) assay to examine telomerase activity in bronchial lavage (BL) samples from individuals undergoing diagnosis of lung cancer. Telomerase activity was detected in 17 (47%) of 36 samples examined. In particular, 16 (70%) of 23 BL specimens obtained from lung cancer patients showed detectable telomerase activity, while only 1 of 13 (8%) specimens obtained from patients without lung cancer demonstrated activity ($P = 0.00038$). Moreover, 9 (90%) of 10 BL specimens, which were cytologically positive for lung cancer, were also positive for telomerase activity, while 7 (54%) of 13 cytologically negative BL specimens for lung cancer showed detectable telomerase activity. Detection of telomerase activity combined with cytology were able to identify 17 (74%) of 23 lung cancer cases whereas cytology alone identified 10 (43%) of 23 such cases ($P = 0.035$). Our findings indicate that telomerase is a specific marker for malignant lung disease and a potential complementary tool to cytology in the diagnosis of certain lung cancer cases.

Keywords: Telomerase; TRAP assay; Non small cell lung cancer (NSCLC); Bronchial lavage (BL)

1. Introduction

Lung cancer is responsible for more deaths than any other cancer, resulting in about 900 000 deaths per year worldwide [1]. It is the most common malignancy in males in the UK, while it

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is the most frequent cancer in women in the Liverpool area. The highest incidence rates of lung cancer for both men and women are found in the Merseyside region of the northwest England. In the Liverpool area, the cumulative rate (0–74 years) in 1994–1995 was 11.6% for males and 7.2% for females, compared with a national average of 7.3% for males and 3.0% for females in 1992 [2,3].

Telomerase is a ribonucleoprotein enzyme which replaces chromosome ends lost during successive cycles of cell division [4]. Telomerase activity has been detected in almost all types of human cancer, including lung cancer [5–8]. Previous studies in lung cancer patients indicated that telomerase activity is present in most of such cases, ranging from 78–96% in non small cell lung carcinomas (NSCLC) while it was present in all of the small cell carcinomas tested [7–9]. Moreover, a recent study demonstrated that dysregulation of telomerase is an early event in the multistage pathogenesis of bronchogenic lung carcinomas [10]. There have been numerous studies in order to evaluate telomerase as a diagnostic marker in various types of human cancer [11,12].

In this study, we used the TRAP assay to detect telomerase activity in bronchial lavage (BL) specimens in order to establish conditions to assess telomerase as a potential diagnostic marker for malignant lung disease.

2. Materials and methods

2.1. Cell lines

The human non small cell lung cancer cell lines Calu3, H1155, HTB58, were used as positive controls for telomerase activity. All cell lines were purchased from the American type culture collection, and grown according to the conditions recommended by the supplier. Cell pellets were collected and frozen at −80°C until preparation of telomerase extracts.

2.2. Patients and bronchial lavage specimens

BL specimens were collected from 36 individuals with suspected lung cancer, who have been referred to the cardiothoracic centre in Liverpool (CTC). The criteria for referral to the CTC included; unresolved chest infection, abnormal chest X-ray, cough (>4 weeks), non specific weight loss, stridor, persistent (>3weeks) hoarse voice and other suspicious features, that would prompt referral to the lung cancer clinic. The choice of site was based on bronchoscopic findings within the large airways and ≈20 ml of saline was introduced, via the bronchoscope and then aspirated. Two to four hundred millilitres of each BL fluid were frozen in aliquots at −80°C for later preparation of protein extracts. Diagnoses of the patients from which the specimens were collected included: 20 NSCLC (five adenocarcinomas, one adenosquamous carcinoma, 11 squamous cell carcinomas, two non small cell lung carcinomas, one lung carcinoid), three small cell carcinomas, and 13 patients, who were found to have no clinical evidence of lung cancer.

2.3. TRAP assay

Each BL sample was first pelleted by centrifugation at 14000 rpm for 5 min, then lysed in 100 μl of ice-cold CHAPS lysis buffer as previously described [13]. Resulting supernatants were snap frozen in liquid nitrogen and stored at −80°C, until they were subjected to the TRAP assay. A protein concentration for each extract was determined using the BCA protein assay kit (Pierce Chemical, UK). Three different protein concentrations (2, 4 and 6 μg) were used in the TRAP reaction. The TRAP assay was performed as previously described with a few modifications [13,14]. Samples were subjected to 31 (radioactive TRAP) or 37 (non radioactive TRAP) cycles of PCR amplification. Specificity controls were performed by pre-treatment of protein extracts from BL with 0.5 μg RNase A for 30 min at 37°C, or heat inactivation at 95°C for 5 min before addition to the TRAP assay. Blank control samples containing CHAPS lysis buffer were also run. TRAP products were run on 10% non denaturing polyacrylamide gels for 1000–1100 Vh and the 6 bp DNA ladder was visualised by silver staining or autoradiography after 1–7 days exposure at

−80°C. We scored a BL sample as telomerase positive when an RNase A and or heat sensitive 6 bp DNA ladder was observed. All experiments were carried out in duplicate.

2.4. PCR controls for telomerase inhibitors

The presence of *Taq* polymerase inhibitors in the BL extracts was examined by mixing genomic DNA derived from cultured cells and protein extracts derived from the BL specimens followed by PCR amplification of either exon 1 of K-ras or exon 10 of p53. Samples which failed to amplify in the PCR reaction were subjected to two further centrifugations at 14000 rpm for 20 min at 4°C and analysed further for the presence of inhibitors. When all samples had demonstrated that no inhibitors were present, they were then examined for genomic DNA contamination. An equal volume to the one subjected to the TRAP assay from each BL specimen was used in the PCR reaction for either K-ras or p53. When all samples had demonstrated that no inhibitors and genomic DNA were present they were finally subjected to the TRAP assay.

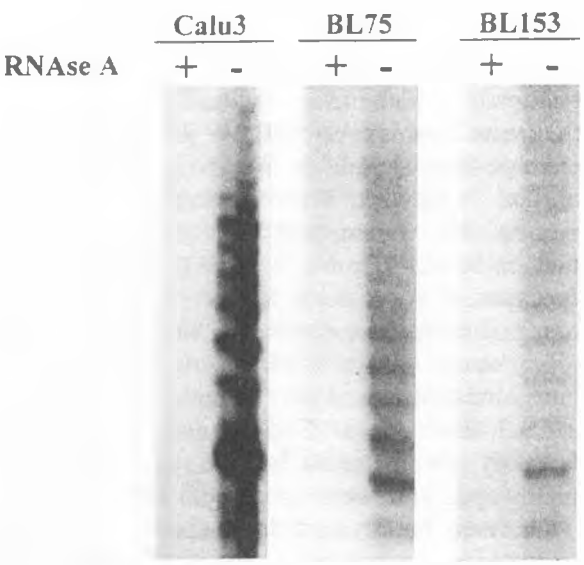

Fig. 1. Detection of telomerase activity in bronchial lavage specimens by TRAP assay. Lanes marked '+' are RNase A digestion controls for each sample. BL specimens 75 and 153 show abolishment of the TRAP assay product ladder after digestion with RNase A, indicating product is due to telomerase activity in the sample. Lane marked Calu3 is a positive control TRAP reaction using a cell extract from a lung cancer cell line.

2.5. Statistical analysis

Results were analysed using Fisher's exact test. Values of $P < 0.05$ were considered to be statistically significant.

3. Results

Protein extracts for telomerase activity were prepared from BL specimens from subjects undergoing diagnosis of lung cancer or other inflammatory lung diseases. A specimen was determined to be positive if a distinct RNase A and or heat sensitive 6 bp DNA ladder was seen in two separate assays. Examples of telomerase activity detected in BL specimen extracts are shown in Fig. 1.

Fifteen of the 36 BL specimen extracts exhibited inhibition of the PCR reactions for K-ras exon 1 or p53 exon 10. These specimens were subjected to two further centrifugations at 14000 rpm for 20 min at 4°C. Further analysis revealed that no inhibitors were present (data not shown). Overall, telomerase activity was detected in 17 (47%) of 36 BL specimens examined. In particular, 16 (70%) of 23 specimens from lung cancer patients and one (8%) of 13 BL specimens from patients with other conditions were positive for telomerase ($P = 0.00038$). Nine (90%) of ten BL specimens with positive cytology for lung cancer were positive for telomerase. One telomerase positive specimen was from a patient with cystic fibrosis. In addition, telomerase activity was detected in seven (54%) of 13 BL specimens from patients with lung cancer with a negative cytology report. Our results are summarised in Table 1. When detection of telomerase activity was combined with cytology, we could identify 17 (74%) of 23 lung cancer cases while cytology alone could only identify ten (43%) of 23 such cases ($P = 0.035$).

Table 1
Telomerase activity in BL specimens

Patient diagnoses	*n*	Telomerase positive	%
Lung cancer	23	16	70
Cytology positive[a]	10	9	90
Cytology negative[b]	13	7	54
No clinical evidence of lung cancer	13	1	8
Total	36	17	47

[a] Cytology positive, malignant cells seen.
[b] Cytology negative, no malignant cells seen.

Statistical analysis of the 36 specimens showed no significant correlation of telomerase activity in BL specimens with current smoking status, tobacco consumption, age and sex of the patient. Telomerase activity was detected more frequently in patients with squamous cell carcinomas than adenocarcinomas, ten (91%) of 11 and two (40%) of five, respectively. However, this was not found to be statistically significant ($P = 0.06$).

4. Discussion

Telomerase enzyme activity has been measured in a number of clinical specimen types that are relevant to early diagnosis of a range of cancers [15–20]. Each type of specimen provides individual challenges. In order to develop new diagnostic tools for lung cancer, it is necessary to establish conditions for the measurement of potential new biomarkers in sputum and bronchial lavage specimens [21]. We have previously demonstrated that genetic alterations in the form of microsatellite instability and/or loss of heterozygosity may be detected in BL specimens from lung cancer patients and individuals at high risk of developing lung cancer [22].

In this study, we employed the TRAP assay to detect telomerase activity in 36 BL specimens from individuals with suspected lung cancer. It is of particular interest to note that telomerase activity was detected in BL specimens from individuals who had a negative cytology report but the patient's lung cancer was diagnosed by other methods (radiology, biopsy or surgery). The latter indicates that a proportion of the cells in the BL had telomerase activity, although the specimen had no cytological evidence of malignancy. This is also in agreement with previously published reports [23,24].

In a paper by Yahata and co-workers, 16 (73%) of 22 BL specimens from lung cancer patients, assayed by an extract-based fluorescent TRAP assay, were positive for telomerase [23]. Another study reported telomerase activity by TRAP in 29 (78%) of 37 BL specimens from cancer patients, including eight which were negative by cytology [24]. In the data presented here, measurable telomerase activity was found in 16 of 23 (70%) BL specimens from patients with a final diagnosis of lung cancer. It is of note that in this study only 200–400 μl of the total volume of each BL specimen was used to prepare an extract for the TRAP assay compared to larger volumes (1–10 ml) used in previous studies [23–25].

In a previously published report, telomerase activity was detected in four bronchoalveolar lavage samples from patients with non cancerous lung diseases. Two of the telomerase-positive samples were from patients who died of aggressive pulmonary inflammation, within a month after the specimen was taken [25]. We found one telomerase-positive sample from a patient with cystic fibrosis, who died from respiratory failure 7 months after presentation. Thus, it may be argued that increased telomerase activity in bronchial specimens, may be a marker of aggressiveness in non malignant lung disease, as well as neoplasia. Since telomerase activity was detected in 16 of 23 lung cancer cases and only in one of 13 cases with a final diagnosis of benign lung disease ($P = 0.00038$), it may be argued that telomerase is a highly specific marker for malignant lung disease.

On examining the combinational results of telomerase activity and cytology 17 of 23 (74%) lung cancer cases were identified, whereas with cytology alone, ten of 23 (43%) of the lung cancer patients were identified ($P = 0.035$). However, it should also be stressed that detection of telomerase activity could identify 16 of 23 (70%) lung cancer cases. We and others have demonstrated, that telomerase activity may be detected in most

of the lung cancer cases, ranging from 78 to 96% in surgically excised tumour tissues [7–9,26]. Nevertheless, detection of telomerase activity in clinical specimens such as BL or sputum appears to be a promising complementary method to cytology for the diagnosis of certain lung cancer cases.

We have observed a trend for telomerase activity to be detected more frequently in BL specimens from patients with SqCCL (10 of 11) than with adenocarcinomas (2 of 5). This may be explained by the fact that adenocarcinomas are usually associated with peripheral airways while SqCCL are usually associated with large airways. Consequently, the latter are more efficiently sampled by bronchoscopy and BL.

In this investigation, we have demonstrated that telomerase activity is a specific molecular marker for the diagnosis of certain lung cancer cases ($P = 0.00038$) and could serve as a complementary diagnostic method to cytology ($P = 0.035$). In addition, telomerase may have a role as a marker of aggressiveness in non neoplastic inflammatory lung diseases. However, further study of telomerase activity, as a diagnostic/prognostic marker is required. Moreover, in situ assays for detection of the telomerase RNA (hTR) and catalytic protein (hTRT) components, may need to be applied to reduce the number of false negative results found in clinical specimens such as BL or sputum [27–29].

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hMLH1 and hMSH2 Expression Correlates with Allelic Imbalance on Chromosome 3p in Non-Small Cell Lung Carcinomas[1]

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ABSTRACT

DNA mismatch repair genes have been implicated in the pathogenesis and predisposition of certain malignancies through a mutator phenotype. In this study, we investigated, in 150 non-small cell lung carcinomas, the expression levels of hMLH1 and hMSH2 proteins in relation to loss of heterozygosity on chromosomes 3p and 2p, the mutational status of these genes' promoters and the hot spot exons. We have demonstrated that 88 of 150 (58.6%) tumor specimens had reduced expression levels of the hMLH1 protein, whereas 85 of 147 (57.8%) specimens had reduced expression levels of the hMSH2 protein. Reduced expression levels of both proteins were observed in 51 of 150 (34%) specimens. In adenocarcinomas, the reduction of hMSH2 expression was more frequently observed than that of hMLH1 ($P < 0.003$), whereas in squamous cell carcinoma of the lung hMLH1 expression was more frequently reduced than hMSH2 ($P < 0.006$). Reduced expression of hMLH1 correlated with allelic imbalance on loci *D3S1289* ($P < 0.0002$) and *D2S391* ($P < 0.05$). It is of note that an inverse correlation was found between hMSH2 reduced expression and loss of heterozygosity at locus *D3S1300* ($P = 0.016$). In addition, hMLH1 reduced expression was more frequently associated with heavy smokers, assessed by daily tobacco uptake ($P = 0.018$) and total smoking exposure (pack-years; $P < 0.05$). In addition, a correlation between hMLH1 reduced expression and nodal metastasis in squamous cell carcinoma of the lung was observed ($P = 0.015$). No mutations were identified in the promoters or exons examined in these two genes. These findings indicate that *hMLH1* and *hMSH2* gene inactivation is a common event in the development of non-small cell lung carcinoma and allelic loss seems to be a major genetic event involved in *hMLH1* silencing. In addition, we propose that a putative negative regulator of *hMSH2* gene may be located at the locus *3p14*.

INTRODUCTION

hMLH1 and *hMSH2* are two of the genes known to be implicated in the DNA MMR[3] system. Inactivation of the MMR machinery has been closely associated with a mutator phenotype that is a hallmark of almost all human cancers (1). Molecular defects in one or both of the genes account for a significant proportion of HNPCCs and a small proportion of sporadic colorectal cancer cases (2, 3). Inactivation of DNA MMR genes occurs in two steps, following the same pattern as in tumor suppressor genes (4). Previous studies have demonstrated that LOH at the DNA MMR loci is a frequent genetic event in human cancers, including lung cancer (5–9). It has also been shown that methylation of the promoter region of the *hMLH1* gene leads to lack of expression of the encoding protein (10–12). However, *hMSH2* promoter methylation has not been demonstrated in tumors lacking expression of the relative protein (13–15). Recent reports have indicated that reduced expression levels of the DNA MMR genes may be implicated in the pathogenesis of certain human cancers and may predict disease-free survival after primary chemotherapy (16–22). A possible role of hMLH1 and hMSH2 overexpression in the induction of apoptosis has also been suggested (23).

Multiple molecular defects have been identified to play a role in the molecular pathogenesis of lung tumors, including alterations in oncogenes and tumor suppressor genes (24–25). Mutations in the *p53* and K-*ras* genes as well as allelic losses and deletions at chromosomes 3p and 9p seem to be among the most commonly found genetic defects in carcinomas of the lung (25–29). Although the role of the *hMLH1* and *hMSH2* genes in the molecular pathogenesis of HNPCC and sporadic colorectal carcinoma has been well studied, little is known about the involvement of these genes in lung cancer. In this study, we investigated the expression levels of hMLH1 and hMSH2 proteins in relation to LOH at chromosomes 2p and 3p in NSCLC. We also examined the mutational status of the promoter regions and the most frequently mutated exons reported of the *hMLH1* and *hMSH2* genes.

MATERIALS AND METHODS

Patients. Lung tumor tissue samples were obtained from 150 patients, 59 males and 91 females, who were operated in the Cardiothoracic Center of Broadgreen (Liverpool, Merseyside, United Kingdom). The age of the patients ranged from 41–95 years (median, 65). The histology of the specimens included in this investigation was: 49 adenocarcinomas, 85 squamous cell carcinomas, 8 adenosquamous, 6 large cell carcinomas, and 2 unclassified NSCLCs. Smoking history (daily consumption, current status) was available for 111 individuals: fifty-four current smokers, 16 recently stopped smokers (1–4 years prior to presentation), 35 former smokers (≥5 years prior to presentation), and 6 nonsmokers. However, complete data for calculating the total smoking exposure was available for only 65 smokers. Total smoking exposure is expressed in pack-years:

pack-years = [(age at operation) − (age started) − (years stopped)] × (packs/day).

In this study, the patients' pack-years ranged from 17–165 (median, 69).

Immunohistochemical Detection of hMLH1 and hMSH2 Protein Expression. Protein expression was demonstrated immunohistochemically by a modified avidin-biotin complex method. Formalin-fixed paraffin process tissues were sectioned at 4-μm thickness, mounted on APES-coated slides, and dried at 37°C overnight. Sections were deparaffinized in xylene and rehydrated in a series of graded alcohols to tap water. Heat-mediated antigen retrieval was required to expose the epitopes and was performed by microwaving the sections on full power in 0.01 M citrate buffer (pH 6.0) for 15 min in a 800-W microwave oven. The sections were left to stand for 15 min to cool and then rinsed for 5 min in running tap water. Endogenous peroxidase activity was blocked by 1.5% hydrogen peroxide in methanol for 10 min. Sections were incubated in the primary antibody buffer (5% goat serum in PBS) for 20 min. Monoclonal antibodies against hMLH1 and hMSH2 (Serotec Ltd., Oxford, United Kingdom) were diluted 1:10 and 1:20, respectively, in the primary antibody buffer and incubated for 1 h at room temperature. The primary antibodies were visualized with Dako LSAB 2 Peroxidase kit (DAKO, Cam-

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[3] The abbreviations used are: MMR, mismatch repair; NSCLC, non-small cell lung carcinoma; LOH, loss of heterozygosity; SqCCL, squamous cell carcinoma of the lung; HNPCC, hereditary non polyposis colorectal carcinoma; SSCP, single-strand conformational polymorphism; HA, heteroduplex analysis.

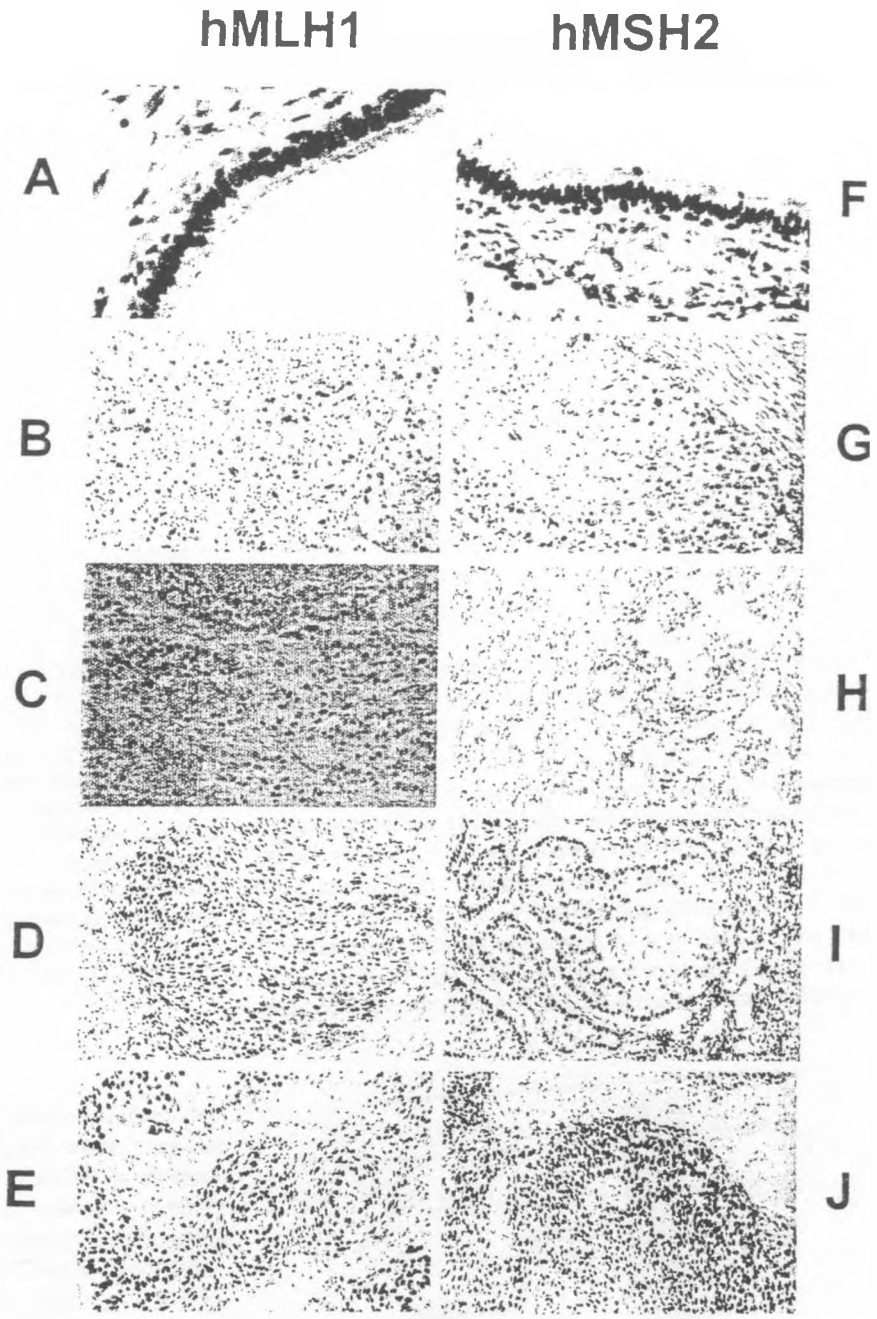

Fig. 1. Representative examples to assess hMLH1 and hMSH2 expression in lung carcinomas. *A*, absence (0) of hMLH1 expression. *B*, weak (+) hMLH1 expression. *C*, moderate (++) hMLH1 expression. *D*, strong (+++) hMLH1 expression. *E*, absence (0) of hMSH2 expression. *F*, weak (+) hMSH2 expression. *G*, moderate (++) hMSH2 expression. *H*, strong (+++) hMSH2 expression.

ridgeshire, United Kingdom). The secondary and tertiary reagents were ncubated for 30 min each and rinsed in-between each stage with 0.05 M ris-buffered saline (pH 7.6). The signal was developed with diaminobenzi- ine (Merck, Dorset, United Kingdom) and hydrogen peroxide. The sections ere counterstained with Gill's Hematoxylin. Normal mouse IgG replaced the rimary antibody as a negative control. The frequency of the nuclear staining as scored on a scale from (−) to (+++) [as absent (−), weak (+), moderate (++), and strong (+++)] without the knowledge of clinical, pathological, nd 3p LOH status data. The staining was scored by two of the authors ndependently.

DNA Extraction. Paired tumor-normal frozen tissue specimens were avail- ne from 85 individuals. Five 10-μm sections of each sample were microdis- ected to ensure presence of >75% tumor cells. Sections were lysed in 400 mM s-HCl pH, 150 mM NaCl, 60 mM EDTA, 1% SDS, and 100μg/ml proteinase and incubated at 42°C for 16 h in an orbital shaker. Deproteinization luded extraction with phenol/chloroform and chloroform. DNA was pre- ipitated by the addition of an equal volume of isopropanol. DNA was spooled o sterile microbiology loops, washed with 70% ethanol, and resuspended in 0 μl of 10 mM Tris (pH 8)-1 mM EDTA. Working stocks were prepared by old dilution in double distilled H_2O.

LOH Analysis. Four markers located proximal and distal to *hMLH1* gene (D3S1289, D3S1266, D3S1300, and D3S1304) and one marker proximal to *hMSH2* gene (D2S391) were available for 85 individuals from a previous study of ours (30). An additional marker (D2S2259) also located on 2p16 was examined in this study and added in the existing database. All fluorescent microsatellite markers were selected from the Linkage Mapping Set V2.0 (PE Applied Biosystems, Warrington, United Kingdom), and analysis was performed on a 377 ABI-PRISM automatic sequencer. The reaction conditions, details, and analysis parameters have been previously described (30).

Mutational Analysis. Mutational analysis was performed on 120 samples. Screening of the *hMLH1* promoter region and exons 9, 13, and 16 and the *hMSH2* promoter region and exons 5, 7, and 8 was performed by PCR, followed by SSCP and HA. The primers used for *hMLH1* (exons 9, 13, and 16) and *hMSH2* (exons 5, 7, and 8) and PCR amplification parameters have been described previously (31). The primers used for the amplification of the promoter regions of *hMLH1* and *hMSH2* are:

hMLH1 promoter: 5′ AGGCTCCACCACCAAATAAC 3′ (sense),
5′ CGCTGTCCGCTCTTCCTATT 3′ (antisense);
hMSH2 promoter: 5′ CCTTGCATACACCCCACCCA 3′ (sense),
5′ GCGACCCCACACCCACTAA 3′ (antisense).

Table 1 *Levels of expression of hMLH1 and hMSH2 genes in NSCLC detected by immunohistochemistry*

	hMLH1 expression				hMSH2 expression			
	Reduced			Normal	Reduced			Normal
Histology	−[a]	+	++	+++	−	+	++	+++
AdenoCa[b]	2	6	18		1	20	19	
		26		23		40		9
SqCCL	5	14	37		7	6	24	
		56		29		37		45
Other NSCLC	1	2	3		0	2	6	
		6		10		8		8
Total NSCLC		88		62		85		62

[a] −, absence of expression; +, weak expression; ++, moderate expression; +++, strong expression.
[b] AdenoCa, adenocarcinoma.

PCR reactions were performed in a 25-μl reaction volume and contained 100 ng of genomic DNA, 200 μM of each dNTP, 8 pM of each primer, 0.6 unit of BIOPRO polymerase (Bioline, London, United Kingdom), and 2.5 μl of 10× buffer [50 mM KCl, 10 mM Tris-HCl (pH 8.8), 0.1% Triton X-100, and 1.5 mM MgCl2]. Samples were subjected to 37 cycles of amplification.

For SSCP analysis, 2–4 μl of the PCR product were mixed with 10 μl of denaturing solution consisting of 80% formamide, 100 mM NaOH, 1 mM EDTA, 0.1% Bromphenol Blue, and 0.1% Xylene Cyanol FF. Samples were then heated at 95°C for 3 min, chilled on ice, and loaded onto 8–10% native polyacrylamide gels, containing 5–10% glycerol. Gels were run at 15°C for 2500–3500 V h and silver stained after electrophoresis. HA was performed as: 2–5 μl of the PCR product were denatured at 95°C for 5 min and allowed to cool down slowly. Samples were then analyzed on 8% native polyacrylamide gels and run for 1600–1800 V h. Gels were silver stained after electrophoresis.

Statistical Analysis. Fisher's exact test was used to analyze the molecular and clinicopathological data. Analysis was performed using the SPSS software.

RESULTS

hMLH1 and hMSH2 Expression in Lung Tumors. Fifteen normal lung tissues, adjacent to tumors examined in this study, were also investigated for the expression of hMLH1 and hMSH2. In all cases, normal bronchial epithelium demonstrated strong staining (+++) for both proteins (Fig. 1, *A* and *F*). Hence, tumors demonstrating strong (+++) immunoreactivity were classified as "normal expression", whereas tumors demonstrating absent, weak, and moderate immunoreactivity were classified as "reduced expression."

hMLH1 expression was examined in 150 NSCLC tissues. Sixty-two (41%) were found to have intense staining (+++; Fig. 1*E*), and 88 (59%) showed reduced (absent, weak, or moderate) staining (−, +, or ++; Fig. 1*B–D*). Of the specimens with reduced expression, 8 showed absence (−) of hMLH1 expression whereas 22 showed weak (+) and 58 showed moderate (++) expression (Table 1). On examining the NSCLC subtypes, 26 of 49 (53%) adenocarcinomas and 56 of 85 (66%) SqCCL showed reduced hMLH1 expression. (Table 1).

It is of note that hMLH1 reduced expression was more frequently found in heavy smokers (>1 pack per day) than in moderate smokers (≤1 pack per day). Forty-six of 71 heavy smokers and 13 of 32 moderate smokers had reduced hMLH1 expression (Fisher's exact test, $P = 0.018$; Table 2). In addition, hMLH1 reduced expression was more frequently found among patients with total smoking exposure higher than the median (69 pack-years: $P < 0.05$; Table 2). No association was found between the nonsmoker/former/current smoker status and hMLH1 expression levels. A correlation between hMLH1 reduced expression and nodal metastasis was found in SqCCL ($P = 0.015$). In particular, hMLH1 reduced expression was found in 29 of 51 (57%) SqCCL specimens with negative nodes and in 24 of 29 (83%) SqCCL specimens with positive nodes. No significant associations were found between hMLH1 expression and other clinicopathological parameters (age, gender, differentiation, and T stage).

hMSH2 expression was examined in 147 NSCLC tissues, because we ran short of tissue for three samples that had already been examined for hMLH1. Sixty-two (42%) were found to have strong expression (+++; Fig. 1*J*), and 85 (58%) showed reduced (absent, weak, or moderate) expression (−, +, or ++; Fig. 1, *G–I*). Nine of 49 adenocarcinomas (18%) demonstrated hMSH2 strong expression, whereas reduced expression was observed in 40 (82%). Forty-five of 82 (55%) SqCCL showed strong expression whereas 37 (45%) showed reduced expression (Table1). No significant associations were identified between hMSH2 expression and T stage, nodal metastasis, differentiation, smoking status, age, or gender of the patient.

The comparative analysis of expression levels of hMSH2 and hMLH1 in different histological types (Table 1) demonstrated that, in adenocarcinomas, hMSH2 was more frequently reduced than hMLH1, 40 of 49 and 26 of 49, respectively ($P < 0.003$). In contrast, in SqCCL, hMLH1 expression was more frequently reduced than hMSH2, 56 of 85 and 37 of 82, respectively ($P < 0.006$; Fig. 2). Simultaneous reduced expression of both hMLH1 and hMSH2 was found in 51 of 150 (34%) samples examined (Fig. 3). Samples with reduced expression of both MMR proteins, comparatively with sam-

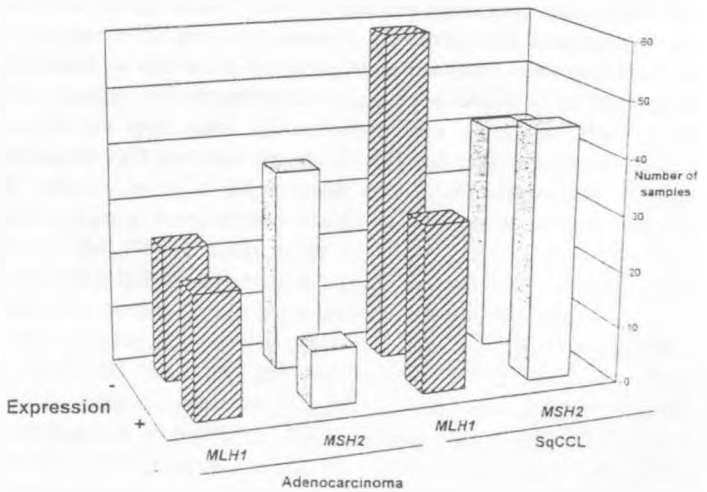

Fig. 2. Histological type-specific reduced expression of hMLH1 and hMSH2 in lung adenocarcinomas and SqCCL. hMSH2 expression was reduced more than hMLH1 in lung adenocarcinomas, whereas hMLH1 expression was reduced more than hMSH2 in SqCCL.

Table 2 *Expression levels of hMLH1 and hMSH2 proteins in lung tumors in relation to the patients' smoking exposure*

		Smoking status				Daily tobacco consumption		Total tobacco exposure[a]	
Gene	Expression level	Nonsmokers	Current	Former (≥5 yr)	Former (1–4 yr)	≤1 pack/day	>1 pack/day	<69 pack-yr	≥69 pack-yr
hMLH1	Normal	3	24	16	4	19	25	22	15
	Reduced	3	30	19	12	13	46	10	18
hMSH2	Normal	2	24	13	6	11	31	13	19
	Reduced	4	29	22	9	21	38	15	17

[a] The patients have been grouped according to their pack-year data based on the mean pack-year value found in this study (69 pack-years).

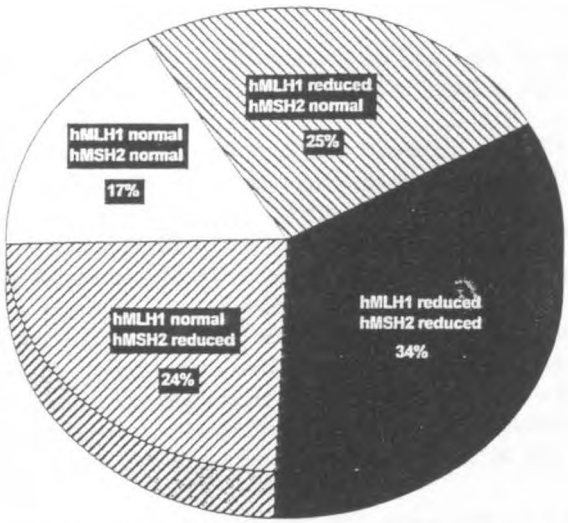

Fig. 3. Combined hMLH1/hMSH2 expression in NSCLC included in this study.

ples with reduced expression of only one of the examined proteins, did not show additional associations with any of the clinicopathological parameters examined.

Mutational Analysis of hMLH1 and hMSH2 Promoter Regions and Hot Spot Exons. Mutational analysis using SSCP and HA was performed on the promoter regions of *hMLH1* and *hMSH2* genes in 120 samples and in exons shown to have mutational hot spots in HNPCC and sporadic colorectal carcinomas (exons 9, 13, and 16 of *hMLH1* and exons 5, 7, and 8 of *hMSH2*). No mutations or polymorphisms were detected in any of the examined regions. We have also performed automated sequencing analysis for both genes in 20 randomly selected samples to check possible SSCP false negatives, however, no mutations were found.

hMLH1 and hMSH2 Expression Correlates with Allelic Imbalance at Chromosomes 2p and 3p. Eighty-five of the 150 samples examined in the current study were previously investigated for allelic imbalance using fluorescent microsatellite markers and analysis on a 377 ABI PRISM automatic sequencer (30). In this study, we have also examined an additional marker on 2p16 (*D2S2259*). LOH for the latter was identified in 26 of 60 (43%) informative cases. The comparative analysis of expression of hMLH1 and hMSH2 with allelic imbalance data of four markers on chromosome 3p (Table 3) showed that hMLH1 reduced expression correlated with allelic imbalance at the *D3S1289* locus on 3p21 (Fisher's exact test, $P = 0.00019$). No such correlation was found with the loci *D3S1304* (3p26; $P = 0.14$), *D3S1266* (3p24; $P = 0.1$), and *D3S1300* (3p14; $P = 0.57$). However, an inverse correlation was found between LOH at locus *D3S1300* (Fisher's exact, $P = 0.016$) and expression of hMSH2 protein (Table 3). In addition, a trend was observed between a higher expression level of the hMSH2 protein and LOH at loci *D3S1266* ($P = 0.063$) and *D3S1289* ($P = 0.059$), but not with locus *D3S1304* ($P = 0.55$).

No association was found between hMSH2 expression and LOH at the *D2S391* ($P = 0.28$) and *D2S2259* ($P = 0.24$) loci. However, a correlation was found between hMLH1 reduced expression and LOH at the *D2S391* ($P = 0.048$) but not at the *D2S2259* locus ($P = 0.14$).

DISCUSSION

The *hMLH1* and *hMSH2* DNA MMR genes are known to be implicated in human cancer, with colon cancer being the most well studied model. However, the information of the status of these two genes in lung cancer is limited. In this study, we have investigated the expression of the *hMLH1* and *hMSH2* DNA MMR genes in NSCLC lesions. The immunohistochemical analysis demonstrated that 59% of the examined tumors had reduced expression of hMLH1 and 58% had reduced expression of hMSH2, whereas 34% demonstrated reduction of expression in both of these genes (Fig. 3). It is of note that 82% of all examined lung tumors showed reduced expression of at least one of the two investigated genes. This is the first report on the protein expression levels of the above mentioned genes in NSCLC, and the results suggest a critical role for these DNA MMR genes in lung carcinogenesis.

It is of note that the reduction of expression of these two genes is associated with the histological subtypes; in adenocarcinomas hMSH2 expression was more frequently reduced than that of hMLH1, while the converse was observed in SqCCL (Fig. 2). Because both genes are considered to be inactivated in a two-hit model (4), we investigated the relationship between MMR gene expression levels and allelic imbalance (LOH) on 3p and 2p chromosome arms, the locations of these genes. The results indicated that reduced hMLH1 expression correlated with LOH at the *D3S1289* (3p21) locus ($P = 0.00019$). This suggests that loss of one allele of the *hMLH1* gene may be one of the major genetic events involved in its inactivation. This may explain the finding that hMLH1 expression is more frequently reduced in SqCCL than in adenocarcinomas, because the former have demonstrated a greater incidence of LOH on chromosome 3p (29–30, 32). Hypermethylation of the *hMLH1* promoter has been demonstrated in human tumors (10–14), and this most likely also contributes to changes in the gene's expression. However, such analysis was not performed on this set of our samples and remains to be elucidated in future studies. The mutational analysis of the *hMLH1* promoter region and the hot spot exons did not reveal any mutations, which is in agreement with previous reports (8, 33) and suggests that mutations are unlikely to be a major cause of *hMLH1* inactivation in lung carcinogenesis. A correlation was found between the reduced expression of hMLH1 and LOH at the *D2S391* (2p16) locus, which may suggest that hMLH1 expression regulatory gene(s) are located in this region but further studies are required to clarify this aspect.

It is of particular note that an inverse relationship between LOH at the *D3S1300* locus and hMSH2 expression was identified where hMSH2 reduced expression was more prevalent in samples retaining heterozygosity at this locus. This may imply the presence of a negative hMSH2 regulatory gene on 3p, suggestive of negative feedback mechanism; however, additional studies are required to elucidate the nature of this relationship. This inverse correlation provides a possible explanation for the lower incidence of reduced hMSH2 expression in

Table 3 *Expression levels of hMLH1 and hMSH2 proteins in lung tumors in relation to allelic imbalance on chromosomes 3p and 2p. Only informative cases (heterozygous status in normal) were included*

		D3S1304		D3S1266		D3S1289		D3S1300		D2S391		D2S2259	
Gene	Expression level	H[a]	L	H	L	H	L	H	L	H	L	H	L
hMLH1	Normal	11	11	13	16	18	13	8	18	24	11	19	10
	Reduced	8	18	7	21	3	24	9	19	13	16	15	16
hMSH2	Normal	11	15	8	24	9	25	6	26	23	14	18	17
	Reduced	8	12	12	13	12	12	11	11	14	13	16	9

[a] H, heterozygous; L, LOH.

SqCCL compared with adenocarcinomas (Fig. 2). This is possibly due to the relatively higher incidence of LOH on 3p found in SqCCL (29–30, 32).

We found no association between hMSH2 expression and LOH at the *D2S391* and *D2S2259* (2p16) loci, which suggests that allelic loss at these loci is not the main event contributing to the reduction of hMSH2 expression in NSCLC. Our results indicated no mutations in the promoter and the hot spot exons of *hMSH2*, which is in agreement with previous reports (33–35). Furthermore, no hypermethylation of *hMSH2* promoter has been demonstrated in certain human tumors (13–15), thus, inactivation of the *hMSH2* gene may rely on alternative mechanisms involving changes in its upstream regulatory genes. Recent reports have revealed p53-binding sites on the *hMSH2* promoter (36) and a possible hMSH2 expression regulatory role of p53 in leukemias (37).

hMLH1 reduced expression correlated with both higher daily tobacco uptake and total tobacco exposure (pack-years), indicating that tobacco carcinogens are implicated in *hMLH1* inactivation and, moreover, that they may have an additive effect. The lack of association with the current/former smoking status argues that smoking-related MMR inactivation may be irreversible and it is in agreement with the fact that smoking cessation does not reduce risk for lung cancer development in chronic smokers to the baseline of nonsmokers. Moreover, hMLH1 expression levels did not seem to differ between smokers and nonsmokers. Although only six nonsmokers were included in this study and a "passive smoker" status is difficult to assess, the above finding indicates that carcinogens apart from those found in tobacco may also affect hMLH1 expression. Reduced expression of hMLH1 in SqCCL correlated with nodal metastasis, suggesting that the *hMLH1* gene may contribute to a more aggressive tumor phenotype in this histological subtype. Thus, hMLH1 expression may be a useful molecular marker for the clinician when developing a treatment regimen.

The comparative analysis of hMLH1 and hMSH2 expression in the tumors in this study did not reveal any associations between the expression of these two genes. Furthermore, tumor specimens with combined reduced expression of both hMLH1 and hMSH2 proteins did not correlate with any clinicopathological parameters. Thus, no complementary role of these two proteins was demonstrated. The frequencies of the reduced expression of these two genes are different in SqCCL and adenocarcinomas, and this may be due to the different incidence of LOH on chromosome 3p in these tumor subtypes. Also, smoking seems to affect hMLH1 but not hMSH2, whereas hMLH1, but not hMSH2, correlates with nodal metastasis in SqCCL. All of the above findings support distinct roles for the two genes in lung carcinogenesis. The results suggest that at least some of the environmental and endogenous factors involved in their inactivation pathway are different. Further investigation is required to elucidate the complete pathways of inactivation of these two genes in NSCLC and reveal additional factors implicated in their regulation.

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30. Liloglou, T., Maloney, P., Xinarianos, G., Fear, S., and Field J. K. Sensitivity and limitations of high throughput fluorescent microsatellite analysis for the detection of allelic imbalance. Application in lung tumours. Int. J. Oncol., *16:* 5–14, 2000.
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34. Gotoh, K., Yatabe, Y., Sugiura, T., Takagi, K., Ogawa, M., Takahashi, T., and Mitsudomi, T. Frameshift mutations in TGFβ RII, IGFIIR, BAX, hMSH3 and hMSH6 are absent in lung tumours. Carcinogenesis (Lond.), *20:* 499–502, 1999.
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37. Zhu, Y. M., DasGupta, E. P., and Russell, N. H. Microsatellite instability and p53 mutations are associated with abnormal expression of the MSH2 gene in adult acute leukemia. Blood, *94:* 733–740, 1999.

APPENDIX IV

24/10/2000

Curriculum Vitae

George E. XINARIANOS

Personal: Date of Birth: 18/9/1971 Health: Very Good
Marital Status: Single Nationality: Greek/British Citizenship
Age: 29

Address:

Institute for Cancer Studies
University of Sheffield Medical School
Beech Hill Road, Sheffield S10 2RX, UK.
Tel: +44(0)114-271 3776/271 2374
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University Education:

November 1996-October 1999: Ph.D in Molecular Oncology (Degree anticipated in 2001). Roy Castle International Centre for Lung Cancer Research, The University of Liverpool, Medical School. Research Project: "Genetic alterations in non-small cell lung carcinomas". **Research Studentship by the Roy Castle Cause for Hope Foundation.**

September 1995-September 1996: M.Sc. in Medical Genetics with Immunology Brunel University, Department of Biology & Biochemistry. MSc Dissertation: "Telomere dynamics & telomerase activity in human cancer".

October 1991-July 1995: B.Sc. (Honours) in Medical Microbiology (Second Class with Honours Lower Division). University of Surrey, School of Biological Sciences. Final Year Project: "Production of recombinant Rubella virus antigens for screening and diagnosis".

Secondary Education:

October 1990-July 1991: Foundation Year (Validated by the Surrey University, Foundation Year Certification-Mark B^{+}). MIS English Academy, Athens, Greece.

September 1989-July 1990: 1st Post-Lyceum Centre of Piraeus, Piraeus, Greece (Subjects undertaken: Advanced Physics, Advanced Chemistry, Advanced Biology) (Post Lyceum Certification-non credit course).

September 1986-June 1989: 3rd Lyceum of Piraeus, Piraeus, Greece. (Lyceum Certification-overall mark 17.3/ 20)

September 1983-June 1986: 3rd High School of Piraeus, Piraeus, Greece. (High School Certification-overall mark 18.8/ 20).

Other qualifications:

First Certificate in English under the authorisation of Cambridge University (B grade).

Standard Certificate in English under the authorisation of Hellenic Ministry of Education (B grade).

Computing: I am very competent with PC and Macintosh computers and have adequate knowledge of several software packages, including word processors, spreadsheets, design programmes, and programmes applicable to molecular biology (restriction analysis, plasmid maps construction).

Emergency First Aid (Kays Medical, UK, 27/7/1998).

Full Clean Driving License (European Union).

Working Experience:

November 1999-Present: Research Associate. Institute for Cancer Studies, Division of Cellular Pathology & Oncology, Medical School, University of Sheffield, Sheffield, UK.

October 1996-October 1999: Molecular Oncology Unit, Roy Castle International Centre for Lung Cancer Research, The University of Liverpool, Liverpool, UK. PhD Research Project: "Genetic alterations in non-small cell lung carcinomas".

October 1994-June 1995: Molecular Genetics Laboratory, School of Biological Sciences, University of Surrey, Guildford, UK. Final Year Project: "Production of recombinant Rubella virus antigens for screening and diagnosis" (Approved by the Surrey University in part fulfilment of the requirements for the degree of B.Sc. (Honours) in Medical Microbiology).

July 1993-October 1994: Laboratory of Molecular Oncology & Biotechnology, National Hellenic Research Foundation, Institute of Biological Research & Biotechnology, Athens, Greece. Professional Training Year Project: "Application of the polymerase chain reaction as a diagnostic tool in laboratory medicine" (Approved by the Surrey University as an integral part of the B.Sc. (Honours) in Medical Microbiology).

Publications:

G. Xinarianos, T. Liloglou, W. Prime, G. Sourvinos, A. Karachristos, P. Maloney, J.R. Gosney, D.A. Spandidos, J.K. Field: Molecular alterations in the cell cycle regulators p53, Rb and p21 in relation to the expression of the DNA mismatch repair proteins MLH1 and MSH2 in non-small cell lung carcinomas. *Submitted.*

T. Liloglou, P. Maloney, **G. Xinarianos**, M. Hulbert, J.K. Field: Cancer-Specific Genomic Instability (CSGI) in bronchial lavage: A molecular tool for early lung cancer detection. *Submitted.*

S. Clayton, F. M. Scott, K. Callaghan, K. Haque, T. Liloglou, **G. Xinarianos,** S. Fear, S. Shawcross, P. Ceuppens, L. Turnbull, J. K. Field, J. C. Fox: K-ras point mutation detection in lung cancer: A comparison of two approaches to somatic mutation detection using ARMS allele specific amplification. *Clinical Chemistry. In Press.*

G. Xinarianos, T. Liloglou, W. Prime, P. Maloney, P. Fielding, J. Callaghan, J.K. Field: hMLH1 and hMSH2 expression correlates with allelic imbalance on chromosome 3p in non-small cell lung carcinomas. *Cancer Research* (2000) 60: pp 4216-422.

G. Xinarianos, F. M. Scott, T. Liloglou, W. Prime, M. Walshaw, L. Turnbull, J. K. Field: Evaluation of telomerase activity in bronchial lavage as a potential early and diagnostic marker for malignant lung disease. *Lung Cancer* (2000) 28: pp 37-42.

T. Liloglou, P. Maloney, **G. Xinarianos,** S. Fear, J. K. Field: Sensitivity and limitations of high throughput fluorescent microsatellite analysis for the detection of allelic imbalance. Application in lung tumours. *International Journal of Oncology* (2000) 16: pp 5-14.

G. Xinarianos, F. M. Scott, T. Liloglou, W. Prime, J. Callaghan, J. R. Gosney, J. K. Field.: Telomerase activity in non-small cell lung carcinomas correlates with smoking status. *International Journal of Oncology* (1999) 15: pp 961-965.

J. K. Field, T. Liloglou, **G. Xinarianos,** W. Prime, M. Walshaw, L. Turnbull: Genomic instability in bronchial lavage as a potential marker for individuals with a high risk of developing lung cancer. *Cancer Research* (1999) 59: pp 2690-2695.

D. A. Spandidos, **G. Xinarianos**, M. Ergazaki, A. Giannoudis, J. Tsamparlakis: The presence of herpesviruses in pterygium. *International Journal of Oncology* (1994) 5: pp 749-752.

M. Ergazaki, **G. Xinarianos**, M. Koffa, T. Liloglou, D. A Spandidos: Detection of human cytomegalovirus by the polymerase chain reaction in immunosuppressed and immunocompromised patients. *Oncology Reports* (1994) 1: pp 805-808.

M. Ergazaki, **G. Xinarianos**, A. Giannoudis, M. Koffa, T. Liloglou, Ch. Kattamis, D. A. Spandidos: Detection of human cytomegalovirus and Epstein-Barr virus by the polymerase chain reaction in patients with beta-thalassaemia. *Oncology Reports* (1994) 1: pp 813-816.

M. Ergazaki, **G. Xinarianos**, A. Giannoudis, N. Markomichelakis, J. Tsamparlakis, D. A. Spandidos: Detection of HSV, CMV and EBV by the polymerase chain reaction technique in patients with inflammatory eye diseases. *Oncology Reports* (1994) 1: pp 1207-1210.

M. Ergazaki, G. Sourvinos, **G. Xinarianos**, A. Giannoudis, D. Mesogiti, P. Vardas, D. A. Spandidos: Detection of Coxsackieviruses B1-6 and specific B3 RNA in serum using a combined reverse transcription polymerase chain reaction assay. Applied Clinical Microbiology and Laboratory Diagnostics (1997) 2: pp 184-189. (In Greek).

M. Ergazaki, **G. Xinarianos**, A. Giannoudis, D. A. Spandidos: Herpesviruses as pathogenic agents in childhood and their detection using molecular biology techniques. Review Article. *Paediatriki* (1994) 57: pp 36-41. (In Greek).

M. Ergazaki, **G. Xinarianos**, A. Giannoudis, D. A. Spandidos, J. Tsamparlakis: Viral implications in inflammatory eye diseases and detection using molecular diagnostics. Review Article. *Ophthalmiatriki* (1995) 43: 103-109. (In Greek).

Manuscripts in preparation:

G. Xinarianos, T. Liloglou, P. Maloney, J. K. Field: Allelic frequency of *p53* gene codon 72 polymorphism in non-small cell lung carcinomas from a UK population.

G. Xinarianos, T. Liloglou, J. Callaghan, P. Maloney, J. K. Field: Telomerase activity interactions with cell cycle regulators in non-small cell lung carcinomas.

Conference Presentations:

T. Liloglou, P. Maloney, **G. Xinarianos**, M. Hulbert, D. A. Spandidos, J.K. Field: Cancer-Specific Genomic Instability (CSGI) in bronchial lavage: A molecular tool for early lung cancer detection. 92nd Annual Meeting of the American Association for Cancer Research (AACR), New Orleans, LA, USA. *Forthcoming.*

T. Liloglou, **G. Xinarianos,** P. Maloney, J. Callaghan, P. Fielding, J. R. Gosney, J. K. Field: hMLH1 and hMSH2 expression in non-small cell lung carcinomas correlates with allelic imbalance on chromosome 3p. 91st Annual Meeting of the American Association for Cancer Research (AACR), San Francisco, CA, USA, April 2000.

G. Xinarianos, T. Liloglou, Jill Callaghan, P. Maloney, J. K. Field: Molecular investigation into the role of telomerase and its components in the pathogenesis of non-small cell lung carcinomas. Annual Meeting of the British Society of Human Genetics (BSHG). York, UK, September 1999.

J. K. Field, T. Liloglou, **G. Xinarianos,** L. Turnbull, M. Walshaw: Genomic instability in bronchial lavage specimens from individuals with no evidence of lung cancer: An early detection marker? ECCO 10, The European Cancer Conference, Vienna, Austria, September 1999.

G. Xinarianos, T. Liloglou, W. Prime, P. Maloney, P. Fielding, J. Callaghan, J.K. Field. Expression of hMLH1 and hMSH2 and allelic imbalance on chromosomes 3p21 and 2p16 in non-small cell lung carcinomas. Annual Meeting of the British Association for cancer Research (BACR). Edinburgh, UK, July 1999.

G. Xinarianos, T. Liloglou, J. Nunn, P. Maloney, G. Sourvinos, A. Karachristos, D.A. Spandidos, J.K. Field. Molecular analysis of p53 in current and former smokers with carcinomas of the upper and lower respiratory tract. 90th Annual Meeting of the American Association for Cancer Research (AACR), Philadelphia, PA, USA, April 1999.

T. Liloglou, P. Maloney, **G. Xinarianos,** S. Fear, J. K. Field: Allelic imbalance in lung cancer determined by high throughput fluorescent microsatellite assays. 90th Annual Meeting of the American Association for Cancer Research (AACR), Philadelphia, PA, USA, April 1999.

G. Xinarianos, F. M. Scott, T. Liloglou, W. Prime, Jill Callaghan, J. R. Gosney , L. Turnbull and J. K. Field: Telomerase activity in non-small cell lung carcinomas. Swiss Institute for Experimental Cancer Research Conference: Cancer and the Cell Cycle, Lausanne, Switzerland, January 1999.

J. K. Field, J. Youngson, T. Liloglou, **G. Xinarianos**, W. Prime, P. Fielding, M. Walshaw, J. R. Gosney, L. Turnbull: The Liverpool lung project: A molecular-epidemiological assessment of individuals with a high risk of developing lung cancer. 3rd World Congress on Advances in Oncology and 1st International Symposium on Molecular Medicine. Crete, Greece, October 1998.

G. Xinarianos, F. M. Scott, W. Prime, M. Walshaw, L. Turnbull and J. K. Field: Detection of telomerase activity in lung tumours and bronchoalveolar lavage. Annual Meeting of the British Association for cancer Research (BACR). Dublin, Ireland, June 1998.

J. K. Field, T. Liloglou, **G. Xinarianos**, W. Prime, L. Turnbull, M. Walshaw: Genomic instability in bronchial lavage specimens from individuals with no evidence of lung cancer: An early detection marker? 89th Annual Meeting of the American Association for Cancer Research (AACR), New Orleans, LA, USA, April 1998.

F. M. Scott, **G. Xinarianos**, W. Prime, M. Walshaw, L. Turnbull and J. K. Field: Development of telomerase activity for early detection of lung cancer: studies in human bronchoalveolar lavage fluid. 89th Annual Meeting of the American Association for Cancer Research (AACR). New Orleans, LA, USA, April 1998.

G. Xinarianos, F. M. Scott, W. Prime, M. Walshaw, L. Turnbull and J. K. Field: Telomerase activity in lung cancer and human bronchoalveolar lavage fluid. British Association for Cancer Research (BACR) Winter Meeting, London, UK, November 1997.

T. Liloglou, **G. Xinarianos,** W. Prime M. Walshaw, L. Turnbull and J. K. Field: Microsatellite instability and loss of heterozygocity in bronchial lavage specimens. British Association for Cancer Research (BACR), London, UK, November 1997.

J. K. Field, J. Youngson, H. L. Ross, L. Liloglou, F. Scott, **G. Xinarianos,** W. Prime, M. Walshaw, J. R. Gosney, L. Turnbull, D. T. Bishop, R. J. Donnelly: Molecular- Pathological assessment of individuals with a high risk of developing lung cancer. Lung cancer conference, Dublin, Ireland, 1997.

J. K. Field, T. Liloglou, **G. Xinarianos,** H. Ross, W. Prime, F. Scott, J. R. Gosney, L. Turnbull, R. J Donnelly: Microsatellite instability in non-small-cell lung cancer and bronchial lavage specimens. 88th Annual Meeting of the American Association for Cancer Research (AACR), San Diego, CA, USA, 1997.

G. Xinarianos, M. Ergazaki, J. Tsamparlakis, D. A. Spandidos: Detection of herpes simplex virus (HSV) and cytomegalovirus (CMV) in pterygium by the polymerase chain reaction technique. 3rd International Conference of the Mediterranean Society of Tumour Marker Oncology (MESTMO), Athens, Greece, 1994.

M. Ergazaki, **G. Xinarianos**, M. Koffa, T. Liloglou, D. A. Spandidos: Detection of human cytomegalovirus by the polymerase chain reaction technique. 41st Conference of the Hellenic Biochemical and Biophysical Society, Athens, Greece, 1994. (In Greek).

Chapters in books:

G. Xinarianos, T. Liloglou, J. K. Field: Detection of telomerase activity in lung tumours and bronchial lavage specimens. In Lung Cancer: Methods and Protocols. Methods in Molecular Medicine. Barbara Driscoll (ed), Humana Press Inc., New Jersey, USA. *Forthcoming.*

T. Liloglou, **G. Xinarianos,** J. K. Field: Detection of allelic imbalance in lung tumours and bronchial lavage specimens. In Lung Cancer: Methods and Protocols. Methods in Molecular Medicine. Barbara Driscoll (ed), Humana Press Inc., New Jersey, USA. *Forthcoming.*

Research Reports:

Xinarianos G: Application of the polymerase chain reaction (PCR) technique as a diagnostic tool in laboratory medicine. Professional Training Report, University of Surrey, School of Biological Sciences, 1994.

Xinarianos G: Production of recombinant Rubella virus antigens for screening and diagnosis. BSc dissertation, University of Surrey, School of Biological Sciences, 1995.

Xinarianos G: Telomere dynamics & telomerase activity in human cancer. MSc dissertation, Brunel University, Department of Biology & Biochemistry, 1996.

Xinarianos G: Genetic alterations in non-small cell lung carcinomas. PhD thesis, The University of Liverpool, Medical School, Roy castle International centre for Lung Cancer Research, 2000.

Workshops Attended:

Perkin Elmer-Applied Biosystems workshop: The face of mutation detection in the new millenium. April 1999.

British Association of Cancer Research workshop: Detection of telomerase activity in tumours. October 1998.

European Association of Tissue Culture workshop: Human cell culture: Methods & Applications Course. November 1998.

Grants Awarded:

Molecular analysis of mutator genes in sporadic colorectal carcinomas with and without genetic instability. £38,100 from MWG-BIOTECH (UK). (With Prof. M. Meuth). 2000.

Investigation into the role of genomic instability in the molecular pathogenesis of Transitional Cell carcinoma (TCC) of the Bladder. £30,000 from the British Urological Foundation. (With Prof. M. Meuth and Prof. F. C. Hamdy). 2000.

Annual meeting travel bursary from the BACR. 1999.

Conference presentation travel award from the Graduate School of Biomedical Sciences, University of Liverpool. 1999.

Travel grant from the British Society of Human Genetics (BSHG). 1999.

Workshop bursary (Detection of telomerase activity in tumours) from the British Association of Cancer Research (BACR). 1998.

Professional Bodies:

Associateship of the University of Surrey, UK.

Member of the British Association of Cancer Research (BACR), UK.

Member of the European association for Cancer research (EACR), Europe.

Associate member of the American Association for Cancer Research (AACR), USA.

Academic and working interests:

Molecular Biology and Recombinant DNA Technology.

Molecular Biology of Cancer.

Applications of Molecular Biology in understanding of pathogenicity and virulence.

Application of molecular diagnostics in clinical practice.

Identification and linkage analysis of genes responsible for human diseases.