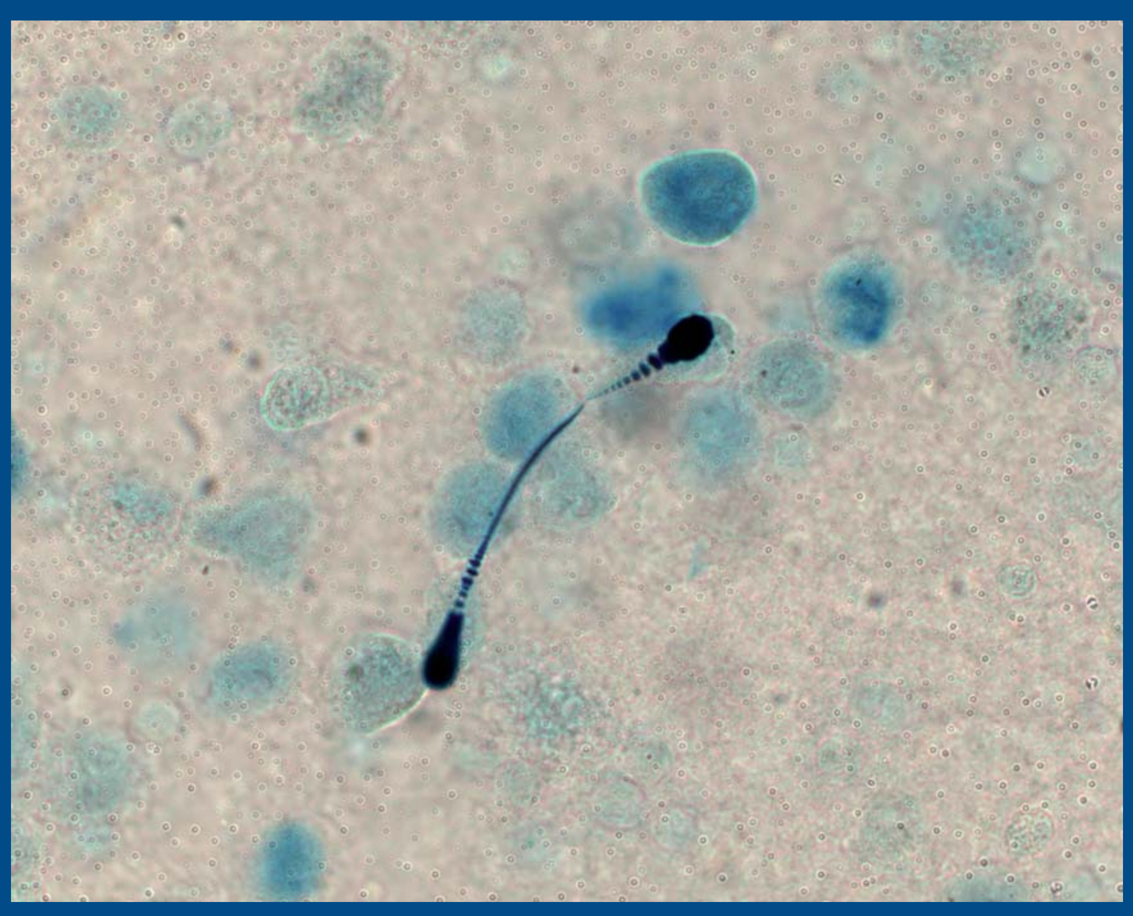
Penny Nymark

Molecular Alterations in Asbestos-Related Lung Cancer



Finnish Institute of Occupational Health

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Molecular Alterations in Asbestos-Related Lung Cancer

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Finnish Institute of Occupational Health, Helsinki, Finland

MOLECULAR ALTERATIONS IN ASBESTOS-RELATED LUNG CANCER

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ACADEMIC DISSERTATION
To be presented for public examination with the permission of the Faculty of Biological and Environmental Sciences, University of Helsinki, in the Small Lecture Hall of the Haartman Institute, Haartmaninkatu 3, Helsinki, on June 11th, 2010, at 12 o´clock noon.

Helsinki 2010

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LIST OF PUBLICATIONS

This thesis is based on the following original publications, referred to in the text by the Roman numerals I–IV as indicated below.

I Nymark P*, Wikman H*, Ruosaari S, Hollmén J, Vanhala E, Karjalainen A, Anttila S, Knuutila S. Identification of specific gene copy number changes in asbestos-related lung cancer. *Cancer Res*. 2006; 66(11):5737–43.

II Nymark P, Kettunen E, Aavikko M, Ruosaari S, Kuosma E, Vanhala E, Salmenkivi K, Pirinen R, Karjalainen A, Knuutila S, Wikman H, Anttila S. Molecular alterations at 9q33.1 and polyploidy in asbestos-related lung cancer. *Clin Cancer Res*. 2009; 15(2):468–75.

III Kettunen E, Aavikko M, Nymark P, Ruosaari S, Wikman H, Vanhala E, Salmenkivi K, Pirinen R, Karjalainen A, Kuosma E, Anttila S. DNA copy number loss and allelic imbalance at 2p16 in lung cancer associated with asbestos exposure. *Br J Cancer*. 2009; 100(8):1336–42. doi 10:1038/sj.bjc. 6605012

IV Nymark P*, Lindholm PM*, Korpela MV, Lahti L, Ruosaari S, Kaski S, Hollmén J, Anttila S, Kinnula VL, Knuutila S. Gene expression profiles in asbestos-exposed epithelial and mesothelial lung cell lines. *BMC Genomics*. 2007; 8:62.

*Equal contribution

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ABBREVIATIONS

AC	adenocarcinoma
AI	allelic imbalance
AM	alveolar macrophages
aCGH	array CGH
BAC	bacterial artificial chromosome
BEGM	bronchial epithelial cell growth medium
CCA	canonical correlation analysis
CGH	comparative genomic hybridization
cCGH	chromosomal CGH
CIN	chromosomal instability
Cip1	CDK-interacting protein 1
CNA	copy number alteration
CNC	Carney complex
CNV	copy number variation
Cy3	cyanine3
Cy5	cyanine5
DNA	deoxyribonucleic acid
ER	endoplasmic reticulum
FBS	fetal calf (or bovine) serum
FFPE	formalin-fixed paraffin-embedded
FIOH	Finnish Institute of Occupational Health
FISH	fluorescence *in situ* hybridization
GCOS	GeneChip opertaing software
GO	Gene Ontology
iGA	iterative Group Analysis
LCLC	large cell lung cancer
LCNEC	large cell neuroendocrine carcinoma

LOH	loss of heterozygosity
MAPK	mitogen-activated protein kinase
MSI	microsatellite instability
miRNA	microRNA
mRNA	messenger RNA
NCBI	National Center for Biotechnology Information
NF-κB	nuclear facor kappa-B
NSCLC	non-small cell lung cancer
p21	CDK-interacting protein 1
p53	cellular tumour antigen p53
PCR	polymerase chain reaction
RMA	Robust Multi-array Average
RNA	ribonucleic acid
RNS	reactive nitrogen species
ROS	reactive oxygen species
RR	relative risk
SAPE	streptavidin phycoerythrin
SCC	squamous cell carcinoma
SCE	sister chromatid exchange
SCLC	small cell lung cancer
SNP	single nucleotide polymorphism
SOD	superoxide dismutase
SV40	Simiar virus 40
TMA	tissue microarray
UBA1/7	ubiquitin-like modifier-activating enzyme 1/7
UPD	uniparental disomy
WHO	World Health Organization

Gene symbols are marked in *italics* and according to the guidelines of the Human Genome Organization nomenclature committee (HGNC). Detailed descriptions can be found at http://www.genenames.org/.

// ABSTRACT

Background

Asbestos is a well known cancer-causing mineral fibre, which has a synergistic effect on lung cancer risk in combination with tobacco smoking. Several *in vitro* and *in vivo* experiments have demonstrated that asbestos can evoke chromosomal damage and cause alterations as well as gene expression changes. Lung tumours, in general, have very complex karyotypes with several recurrently gained and lost chromosomal regions and this has made it difficult to identify specific molecular changes related primarily to asbestos exposure. The main aim of these studies has been to characterize asbestos-related lung cancer at a molecular level.

Methods

Samples from asbestos-exposed and non-exposed lung cancer patients were studied using array comparative genomic hybridization (aCGH) and fluorescent in situ hybridization (FISH) to detect copy number alterations (CNA) as well as microsatellite analysis to detect allelic imbalance (AI). In addition, asbestos-exposed cell lines were studied using gene expression microarrays.

Results

Eighteen chromosomal regions showing differential copy number in the lung tumours of asbestos-exposed patients compared to those of non-exposed patients were identified. The most significant differences were detected at 2p21–p16.3, 5q35.3, 9q33.3–q34.11, 9q34.13–q34.3, 11p15.5, 14q11.2 and 19p13.1–p13.3 ($p<0.005$). The alterations at 2p

and 9q were validated and characterized in detail using AI and FISH analysis in a larger study population. Furthermore, *in vitro* studies were performed to examine the early gene expression changes induced by asbestos in three different lung cell lines. The results revealed specific asbestos-associated gene expression profiles and biological processes as well as chromosomal regions enriched with genes believed to contribute to the common asbestos-related responses in the cell lines. Interestingly, the most significant region enriched with asbestos-response genes was identified at 2p22, close to the previously identified region showing asbestos-related CNA in lung tumours. Additionally, in this thesis, the dysregulated biological processes (Gene Ontology terms) detected in the cell line experiment were compared to dysregulated processes identified in patient samples in a later study (Ruosaari *et al.*, 2008a). Commonly affected processes such as those related to protein ubiquitination, ion transport and surprisingly sensory perception of smell were identified.

Conclusions

The identification of specific CNAs and dysregulated biological processes shed some light on the underlying genes acting as mediators in asbestos-related lung carcinogenesis. It is postulated that the combination of several asbestos-specific molecular alterations could be used to develop a diagnostic method for the identification of asbestos-related lung cancer.

ABSTRACT IN FINNISH – TIIVISTELMÄ

Tausta

Asbesti on tunnettu syöpää aiheuttava mineraalikuitu, jolla on tupakoinnin yhteydessä synergistinen vaikutus keuhkosyövän riskiin. Useat *in vitro*- ja *in vivo* -tutkimukset ovat osoittaneet, että asbesti voi aiheuttaa kromosomivaurioita ja muutoksia geenien ilmentymisessä. Keuhkosyövän karyotyyppi on yleensä hyvin monimutkainen ja toistuvat kromosomialueiden monistumat sekä häviämät ovat yleisiä. Tästä syystä on ollut vaikeaa tunnistaa spesifisiä molekyylitason muutoksia, jotka liittyvät pääasiassa asbestialtistumiseen. Päätavoite näissä tutkimuksissa on ollut asbestiin liittyvän keuhkosyövän tunnistaminen molekyylitasolla.

Menetelmät

Asbestialtistuneiden ja altistumattomien keuhkosyöpäpotilaiden näytteet tutkittiin käyttäen vertailevaa genomista hybridisaatiota mikrosiruilla (aCGH) ja fluoresenssi in situ hybridisaatiota (FISH), joilla havaitaan kromosomialueiden kopiolukumuutokset, sekä mikrosatelliittianalyysia, jolla havaitaan alleeliepätasapaino (AI). Lisäksi asbestialtistuneita solulinjoja tutkittiin käyttäen geeniekspressiomikrosiruja.

Tulokset

Kahdeksallatoista kromosomialueella osoitettiin kopiolukueroja asbestialtistuneiden ja altistumattomien potilaiden näytteiden välillä. Merkittävimmät erot havaittiin kromosomialueilla 2p21–p16.3, 5q35.3, 9q33.3–q34.11, 9q34.13–q34.3, 11p15.5, 14q11.2 ja 19p13.1–p13.3

(p <0,005). Muutokset 2p ja 9q alueilla karakterisoitiin tarkemmin ja varmennettiin käyttäen AI- ja FISH-analyysejä laajemmassa tutkimusaineistossa. Lisäksi mikrosiruilla tutkittiin muutokset geenien ilmentymisessä asbestialtistuksen jälkeen kolmessa eri keuhkosolulinjassa. Tutkimuksessa tunnistettiin asbestialtistukseen liittyviä geenien ilmentymisprofiileja sekä muuttuneita biologisia prosesseja. Lisäksi havaittiin solulinjoille yhteisten asbestiin liittyvien vastegeenien rikastuttamia kromosomaalisia alueita. Merkittävin asbestivastegeenejä sisältävä alue oli 2p22, joka sijaitsee lähellä aiemmin keuhkosyövissä tunnistettua asbestiin liittyviä kopiolukumuutoksia sisältävää aluetta 2p:ssa. Tässä väitöskirjassa vertailtiin myös asbestialtistuneiden solulinjojen muuttuneita biologisia prosesseja (geeniontologiatermejä) niihin muuttuneisiin prosesseihin, joita myöhemmin havaittiin asbestialtistuneiden potilaiden näytteissä (Ruosaari et al., 2008a). Yhteiset muuttuneet prosessit liittyivät proteiinien ubikitinaatioon, ionikuljetukseen ja yllättävästi hajuaistimukseen.

Johtopäätökset

Spesifisten kopiolukumuutosten ja muuttuneiden biologisten prosessien tunnistaminen asbestiin liittyvässä keuhkosyövässä valottaa taustalla olevia geenejä, jotka toimivat välittäjinä asbestin aiheuttamassa keuhkokarsinogeneesissä. Useita asbestiin liittyviä molekyylitason muutoksia voitaisiin käyttää asbestiin liittyvän ja liittymättömän keuhkosyövän erottavien diagnostisten menetelmien kehittämisessä.

INTRODUCTION

Asbestos has been used since ancient times by virtue of its flexible yet durable and fire resistant properties. However, it was not until the second half of the 19th century, that the industrial applications of asbestos began to be appreciated and its use increased markedly. The health effects of asbestos exposure had already been noted by Roman naturalists, but the first scientific indication that the fibres were associated with several severe lung diseases came at the end of the 19th century (reviewed in Liddell, 1997; Greenberg, 2004). The epidemiologic breakthroughs detailing the dangers associated with asbestos became publicized in the 1950s and 60s (reviewed in Greenberg, 1982; Newman Taylor, 2009). Nonetheless, the material continued to play an important role in the construction industry until only a few decades ago and the asbestos industry was well over 100 years old before the cancer issues were fully recognized and addressed.

Today the use of asbestos has been banned in most developed countries due to the unequivocal evidence of devastating asbestos-related diseases. However, in developing countries, asbestos is still utilized and WHO has estimated that approximately 125 million workers in the world are still being exposed to asbestos in their daily work environment (WHO, 2007). Moreover, asbestos may not only affect workers, but also their families, through the exposure of fibres brought home on shoes, clothes, skin and hair (Kilburn *et al.*, 1985). Therefore, the number of exposed may be significantly greater. Due to the long latency period of 30–40 years, asbestos-related diseases will continue to burden public health also in developed countries (LaDou, 2004). Today, 20–40% of adult men in the world are thought to have held jobs that could have entailed

asbestos exposure to some extent (Lin *et al.*, 2007). Joiners, plumbers, electricians, painters, shipyard workers, builders, engineers, and asbestos miners are at the greatest risk (Currie *et al.*, 2009).

Asbestos causes a variety of malignant pleural diseases, such as asbestosis, mesothelioma and lung cancer. Mesothelioma is commonly recognized as the primary asbestos-related cancer type. Nevertheless, it has been estimated that asbestos gives rise to an equal number or possibly even more lung cancer cases as compared to mesothelioma. Various excess lung cancer to mesothelioma ratios have been reported, but a widely cited estimate is to expect between one and two excess lung cancers for every case of mesothelioma (reviewed in Henderson *et al.*, 2004). Of all lung cancers in the world, an estimated 5–7% are attributable to occupational asbestos exposure (LaDou, 2004; Kamp, 2009).

Cancer arises from somatic cells which have been affected by successive molecular alterations occurring in a progressive, almost evolutionary, manner. These alterations evoke changes in normal cell functions, giving the cells the ability to transform into malignant derivatives. The six essential changes in cell physiology that have been proposed to dictate the malignant growth of perhaps all tumours are self-sufficiency in growth signals, insensitivity to growth-inhibitory signals, evasion of apoptosis, limitless replicative potential, sustained angiogenesis and tissue invasion and metastasis. This multistep process normally takes several years to develop (Hanahan and Weinberg, 2000). The molecular alterations can be either genetic (physical alterations of the DNA sequence, e.g. copy number alterations [CNA]) or epigenetic (e.g. promoter hypermethylation inhibiting gene transcription) leading to changes in the expression of genes critical for the maintenance of normal homeostasis. The genetic alterations may cause the duplication or even amplification of so called oncogenes encoding for molecules (e.g. proteins) able to directly or indirectly enhance the ability of the cells to become malignant. Deletion of genetic material may cause the loss of tumour suppressor genes encoding for molecules involved in the prevention of malignant transformation (Ponder, 2001). During the past few decades it has become more and more clear that it is not single oncogenes and tumour suppressors, but rather complex interactions between these genes and their products that are responsible for the changes in cell physiology

required for cell transformation. Several genes have also been found to act as both oncogenes and tumour suppressors depending on the context or stage of tumourigenesis (Bissell *et al.*, 2005). Furthermore, some humans may have inherited a genetic alteration that is “advantageous” for the development of cancer. However, the majority of cancer-causing molecular alterations are environmental and life-style related (Peto, 2001; Boffetta, 2006).

Lung cancer is a complex type of cancer and numerous genetic alterations are involved in its pathogenesis. The karyotype is chaotic, but recurrent alteration patterns have been identified during several decades of study (Balsara *et al.*, 2002). It is well known that tobacco smoking is the primary predictor of lung cancer. However, smoking in combination with asbestos exposure greatly elevates the risk of lung cancer in an almost multiplicative manner (Vainio *et al.*, 1994). Asbestos exposure alone also increases the risk of lung cancer, but asbestos workers have been reported to have the highest percentage of smokers compared to any other identifiable population; between 64–78% depending on the type of occupation (Lange *et al.*, 2006). This has proved to be one of the greatest challenges in studying asbestos-related lung cancer, i.e. the fact that two environmental exposures are often involved in the process of malignancy development. The actual cause of each individual lung cancer has been exceedingly difficult to elucidate. In addition, the long latency period from exposure to development of lung cancer makes it difficult to draw conclusions about the molecular mechanisms generating the disease. Many of the molecular alterations may be secondary or so called passenger alterations, induced by the primary possibly exposure-specific alterations, which affect the stability of the genome (Herceq *et al.*, 2007). Currently, there are no clinically useful molecular alterations that can differentiate between asbestos-related and non-related lung cancer (Kamp, 2009).

Asbestos-related lung cancer has a very dismal prognosis, as do all lung cancers, even though the risk groups are well known. The current clinical methods for screening for lung cancer in risk groups have not improved mortality during the last few decades (Silvestri *et al.*, 2009). The discovery of distinct molecular alterations related to asbestos exposure may broaden the potential of molecular diagnostics in these cancers.

Today the etiology is defined based on occupational history and pulmonary fibre count. Potential specific asbestos-related alterations could also assist in the detection of early stage cancer in risk groups. Finally, understanding the molecular basis may eventually lead to the development of specific therapeutic approaches.

REVIEW OF THE LITERATURE

1. Lung cancer

1.1 Epidemiology

During the 20th century lung cancer developed from a rare disease into a true epidemic and it is currently the most common type of cancer in terms of both incidence and mortality, being responsible for over 1.2 million deaths every year in the world. In Europe and North America, lung cancer is the primary cause of cancer related death and it is becoming increasingly more common also in Asia, Latin America and Africa (Figure 1). Overall, the rate of lung cancer among men is decreasing, while it is increasing among women (Figure 2; reviewed in Brennan *et al.*, 2000; Toh, 2009).

Smoking has been well known to be the primary risk factor for lung cancer since the 1920's (Tylecote, 1927). The risk depends largely on the amount and duration of smoking, with a clear dose-dependent response having been shown in virtually all studies. Roughly, it can be estimated that approximately 1 in 10 lifetime smokers will develop lung cancer (reviewed in Hansen, 2008).

Occupational exposures mainly target the lung through inhalation and indeed, these kinds of exposures play an important role in the risk of developing lung cancer. Metals such as arsenic, ionizing radiation such as radon gas and respirable fibers such as asbestos are some well documented exposure types that are able to induce lung cancer (Siemiatycki *et al.*, 2004). An estimated 10–15% of all lung cancers are caused by factors other than active smoking (Samet *et al.*, 2009). Asbestos exposure is the leading cause of occupational cancer in most countries and it is believed to be the second most important cause of lung cancer after tobacco smoking (Anttila *et al.*, 1993; Hagemeyer *et al.*, 2006).

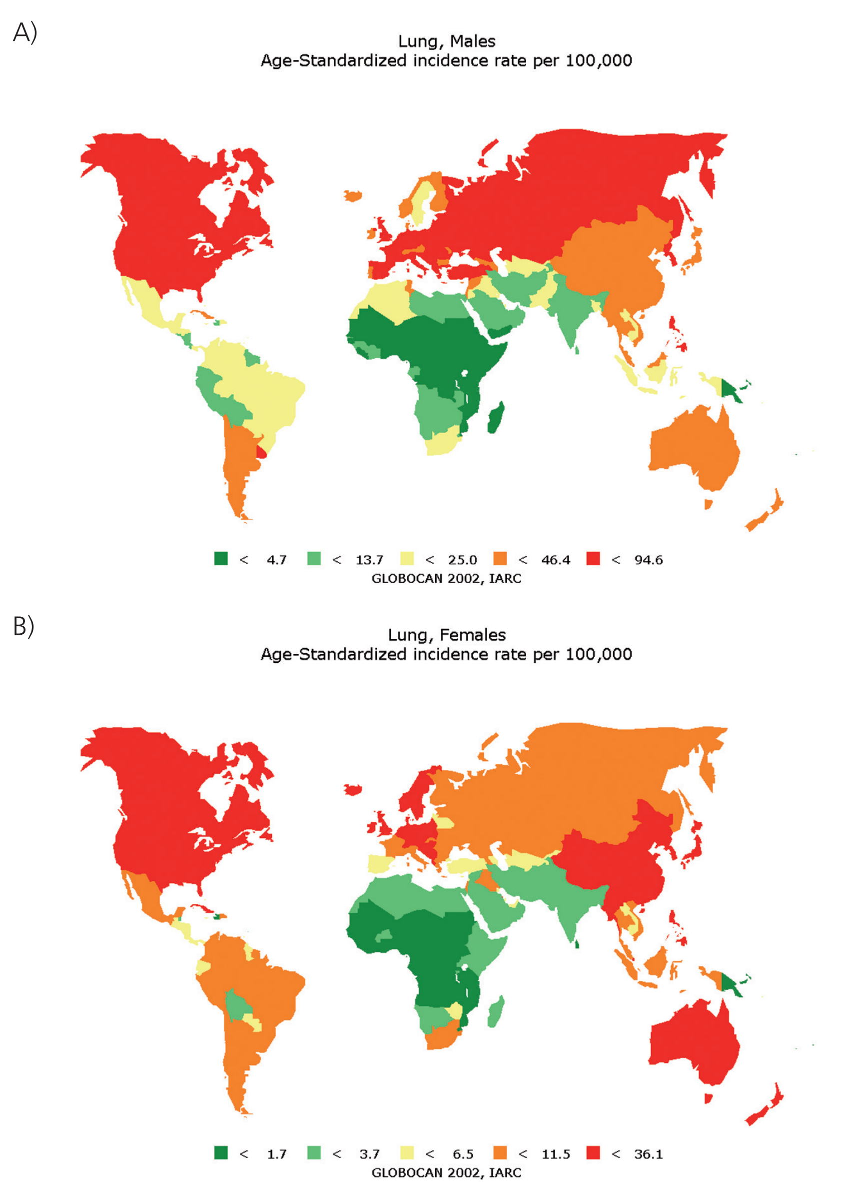


Figure 1. **Geographical distribution of lung cancer.** Distribution of lung cancer incidence in A) males and B) females. Figure produced in GLOBOCAN 2002 (Ferlay *et al.*, 2004)

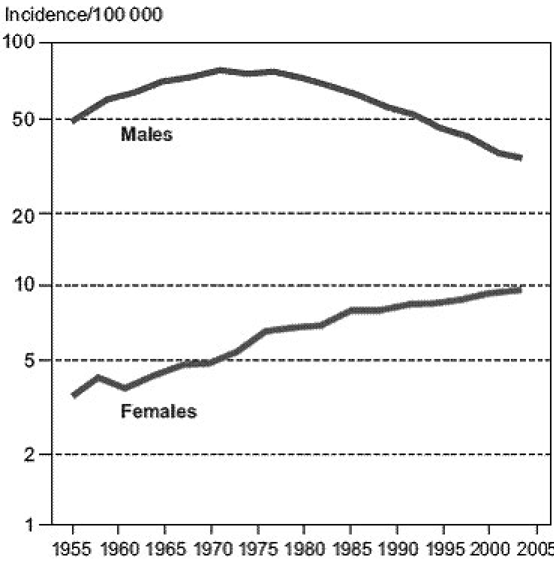


Figure 2. **Lung cancer incidence.** Lung cancer incidence in Finnish males and females between 1955 and 2005. Figure obtained from the Finnish Cancer Registry (www.cancerregistry.fi).

The prognosis for lung cancer is generally very poor, depending largely on the histology and stage at diagnosis. In approximately three-quarters of the cases, distant metastatic spread is evident at the time of diagnosis, resulting in a five-year survival of about only 15% (reviewed in Hansen, 2008). During the past decades none of the screening programs tested have shown any clear benefits (reviewed in Field *et al.*, 2008).

1.2 Histology

Lung cancer can be divided into two major histological types, non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC). This distinction is clinically important because of the differences in presentation, metastatic spread and response to therapy. NSCLC can further be divided into the major subtypes adenocarcinoma (AC), squamous cell carcinoma (SCC) and large cell lung cancer (LCLC). Minor subtypes

include adenosquamous carcinoma (AC/SCC) and large cell neuroendocrine carcinoma (LCNEC) (Hansen, 2008; Travis *et al.*, 2004).

AC accounts for around 40% of all lung cancers. AC is the predominant subtype among young, female and non-smoking patients (Lee *et al.*, 1998; Subramanian *et al.*, 2007). It has also been postulated that AC is the most common subtype among asbestos-exposed patients. However, this finding remains controversial and not all studies have reported such an association (reviewed in Henderson *et al.*, 2004). AC usually originates in the peripheral lung tissue (Hansen, 2008).

SCC accounts for about 30–35% of all lung cancers. It is strongly associated with smoking, with over 90% occurring in smokers. The primary location of SCC is central and more often in the segmental bronchi than in the lobar and mainstem bronchi (Hansen, 2008). The frequency of this type of tumour has been declining progressively, while that of AC has been increasing possibly due to the introduction of low-tar filtered cigarettes. The filters change the composition and anatomic distribution of the carcinogenic particles (LaCroix *et al.*, 2008). In addition, smokers of low-tar filtered cigarettes seem to inhale smoke more deeply than smokers of non-filtered cigarettes, which may cause the exposure of more peripheral areas of the lungs (reviewed in Hoffmann *et al.*, 1996).

LCLCs comprise between 5 and 10% of all lung cancers and are poorly differentiated tumours which often arise in the lung periphery. There are several variants of LCLC, for example LCNEC (Hansen, 2008).

AC/SCC is a combination of each histological type. It is rather rare, accounting for only approximately 0.6–2.3% of all lung cancers (Hansen, 2008).

SCLC is responsible for around 25% of all lung cancers and includes also combined SCLC, presenting cells of any NSCLC subtype to different degrees. Together with SCC, SCLC is strongly associated with tobacco smoking and often occurs in central bronchial locations (Sun *et al.*, 2007; Hansen, 2008).

1.3 Genetics and epigenetics

The continuous exposure of the lungs to carcinogens leads to the accumulation of molecular alterations and lung cancer is characterized by

its large number of genetic and epigenetic alterations, affecting almost all chromosomes (reviewed in Panani *et al.*, 2006). Many of the alterations are due to the widespread genetic instability affecting these tumours, but also recurrent alterations, apparently associated with the initiation and progression of the tumours, can be observed.

The genetic alterations are mainly unbalanced with balanced translocations being rare (Balsara *et al.*, 2002). Frequent recurrent CNA in the lung cancer genome include loss of 3p, 4, 5q, 8p, 13q and 17p and gain of 1q, 3q, 5p and 8q (Figure 3; reviewed in Panani *et al.*, 2006; Nymark, 2009; Baudis, 2009). Different histological types exhibit slightly different patterns, for example gain of 3q has been associated with SCC (Tonon *et al.*, 2005), while gain of 5p has been shown to be the most frequent change (60%) in AC (Weir *et al.*, 2007).

CNAs often lead to allelic imbalance (AI), where one of the two alleles of a gene is lost, gained or amplified. AI can also be copy number neutral, where one allele has been lost but the other duplicated, which is referred to as uniparental disomy (UPD) (reviewed in Tuna *et al.*, 2009). Loss of heterozygosity (LOH) describes the type of AI where one of the two alleles has been lost. If the other allele has already been inactivated by some other means, for instance by mutation or methylation, this may lead to a total loss of activity, e.g. of a tumour suppressor. In cancer, whole, terminal and interstitial chromosome deletions, as well as unbalanced translocations have been shown to lead to LOH (Ogiwara *et al.*, 2008). In lung cancer, AI can be detected in most of the regions with CNA, though it is especially frequent at two regions; 3p14, which contains the tumour suppressor gene *FHIT*, and 3p21.3 (Zabarovsky *et al.*, 2002).

Another type of genetic alteration is point mutation, which in lung cancer is common, especially in the genes *TP53*, *EGFR* and *KRAS*. These point mutations have, in addition to LOH at 3p, 9p21 and 17p13, been demonstrated to be early events during multistage lung carcinogenesis. Other CNA are thought to be relatively late events associated with tumour phenotype and metastatic behaviour (reviewed in Herbst *et al.*, 2008; Soh *et al.*, 2008). However, different types of exposures have been linked to different types of alterations and e.g. tobacco smoke is closely associated with point mutations and promoter hypermethylation (reviewed in Hecht, 1999), while asbestos exposure tends to be associ-

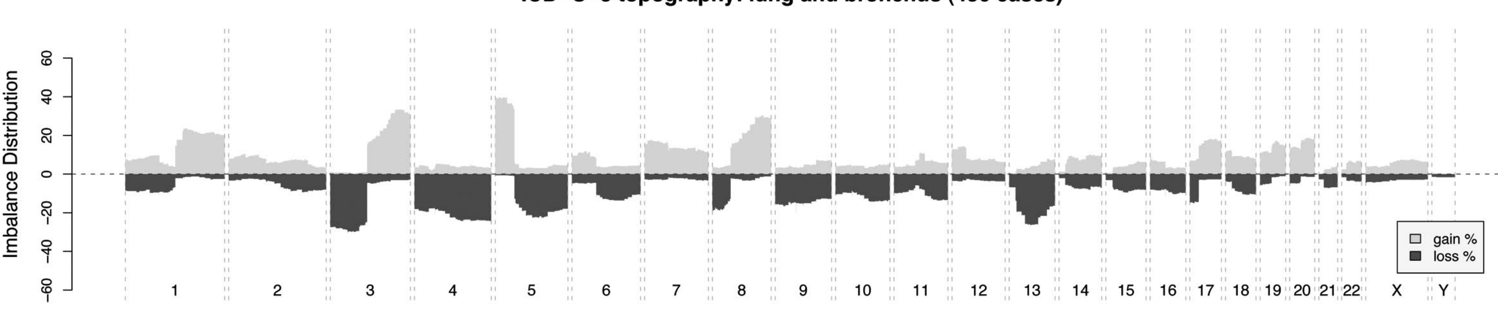


Figure 3. **Recurrent copy number alterations in lung cancer.** Figure obtained from the Progenetix database, www.progenetix.net (Baudis *et al.*, 2001; Baudis, 2009).

ated with DNA loss and chromatid breaks (Huang *et al.*, 1978; Hei *et al.*, 1992; Lohani *et al.*, 2002; Msiska *et al.*, 2009; Pelin *et al.*, 1995; Valerio *et al.*, 1980; Xu *et al.*, 2007). Therefore, the early changes in tobacco-related and asbestos-related lung cancer may be different. An example can be made of the tumours in smokers and non-smokers, in which the genetic alterations appear to differ extensively. The chromosomal regions 9p21, 12p, 16p, 16q, 17q and 19q13 have been reported to be affected by alterations more frequently in AC of never-smokers than in those of smokers (Sanchez-Cespedes *et al.*, 2001). Furthermore, *TP53* and *KRAS* mutations have been associated with lung cancer of smokers, while *EGFR* mutations are associated with those of non-smokers. These differences have been shown to be important tools in the diagnosis, prognosis, clinical follow-up and targeted therapies in lung cancer patients (reviewed in Subramanian *et al.*, 2008).

Inherited genetic alterations or polymorphisms, such as single nucleotide polymorphisms (SNP) and DNA copy number variations (CNV) may be advantageous for the development of cancer. Indeed, lung cancer susceptibility has been shown to be increased in several inherited cancer-syndromes caused by germ-line mutations in *TP53*, *RB1* and *EGFR*. Lung cancer itself is, however, rarely familial and few large families with multiple cases of lung cancer are available for genome wide association studies of SNPs and CNVs (reviewed in Herbst *et al.*, 2008). Nevertheless, a few large studies have identified an association between lung cancer susceptibility and SNPs at 6q23-25, 13q13.3 and 15q24-15q25.1 (You *et al.*, 2009; Li *et al.*, 2010; Liu *et al.*, 2008). The region at 15q contains two genes encoding subunits of the nicotine acetylcholine receptor alpha, which is regulated by nicotine exposure. Furthermore, deleting CNVs in the tobacco-carcinogen detoxifying gene *GSTM1* has been shown to increase the risk of lung cancer (Lam *et al.*, 2009). Several other genes related to the metabolism of tobacco-borne carcinogens, as well as genes associated with DNA repair and inflammation, have been studied as hereditary risk factors, but the results require verification in large study populations (reviewed in Foulkes, 2008).

The ploidy is often altered in lung cancer. Normal human lung cells show a diploid genome, but in lung cancer, the presence of a near-triploid genome is common and around 50–60% of all lung cancers can be said to be polyploid containing >57 chromosomes per nucleus (Testa *et al.*,

1992; Hoglund *et al.*, 2004). LCLC more often shows polyploidy than the other histological types (Desinan *et al.*, 1996). Polyploidy has been associated with tumour infiltration into the pleura (Desinan *et al.*, 1996).

Epigenetic alterations are defined as mitotically or meiotically heritable changes in gene expression that are not caused by alterations in the DNA sequence. DNA methylation and histone modification are the two major epigenetic alterations involved in human carcinogenesis. DNA methylation is involved in normal cellular gene expression regulation and abnormal promoter hypermethylation may lead to the silencing of a gene (Jones *et al.*, 2002). In lung cancer, promoter hypermethylation is common in tumour suppressor genes, such as *P16/CDKN2A*, *RASSF1A* and *RB1* (reviewed in Pfeifer *et al.*, 2009). Histone modifications control the accessibility of the chromatin and may inhibit or activate transcription. These types of modifications have also been implicated in the pathogenesis of lung cancer (Bowman *et al.*, 2006).

CNA, point mutations, inherited genetic variations, polyploidy and epigenetic alterations may lead to changed expression of the affected genes and subsequently to disruption of whole signalling pathways. In addition to affecting the traditional protein coding genes, these alterations may also evoke the dysregulation of other types of genes, e.g. those encoding for microRNAs (miRNAs). A new era in the field of cancer investigation was recently opened with the discovery of these non-coding RNAs, which regulate the expression of an estimated 30% of all human genes. During only a few years of study miRNAs have been shown to be extremely useful in the characterization of cancers (reviewed in Croce, 2009). Several miRNAs have been identified as being dysregulated in lung cancer (Yanaihara *et al.*, 2006). Consequently the main pathways affected in lung cancer are those involved in growth promotion (e.g *EGFR*), growth inhibition (e.g *TP53* and $p16^{INK4a}$-*RB*), apoptosis (e.g *BCL-2*) and DNA repair (reviewed in Sato *et al.*, 2007; Brambilla *et al.*, 2009).

1.3.1 Methods used in the detection of genetic alterations and gene expression

CNAs can be detected by using comparative genomic hybridization (CGH), which was originally developed by Kallioniemi *et al.* (1992). In

this method, fragmented labelled tumour DNA competes with differently labelled, fragmented control DNA in a hybridization to a normal genome. The normal genome may be in the form of a metaphase spread (chromosomal CGH or cCGH) or as several thousands of spotted fragments on a microarray (array CGH or aCGH). The ratio between the signal intensities of the different labels can then be measured automatically and over- or under-representation of genetic material in the tumour DNA is scored.

The different types of AI can be detected by utilizing e.g. microsatellites in the loci of interest and performing fragment analysis on the PCR products from those loci (Slebos *et al.*, 2004). Microsatellites are short sequence repeats in the genome, which may differ in length, i.e. number of repeats, between the two alleles. Patients that are heterozygous for a microsatellite can be analyzed for the presence of AI in that region, in the tumour DNA. The proportion of one allele relative to the other can then be measured at a given microsatellite locus. The allele ratio in the tumour DNA is compared to the allele ratio in normal DNA from the same patient. Basically, microsatellite analysis to detect AI is another way of identifying CNA in tumour samples, but also UPD can be detected with this method in contrast to CGH.

Polyploidy cannot be detected with CGH. Instead flow cytometry or karyotypic analyses for example using fluorescence in situ hybridization (FISH), are needed (D'Urso *et al.*, 2010). FISH, which was originally developed by Pinkel *et al.* (1986), uses fluorescent probes, which hybridize to whole chromosomes or to specific chromosomal regions. The probes are applied to interphase, metaphase or tissue preparations and analyzed for the presence of fluorescent signals using a microscope. CNA can also be analyzed by FISH, usually using a centromeric probe together with a locus-specific probe in dual-colour hybridizations.

Gene expression changes in lung tumours compared to normal lung tissue can be studied using high-throughput methods such as gene expression microarrays, which estimate the mRNA expression level of tens of thousands of genes (Ramsay, 1998). There are several different types of microarrays. Today, oligonucleotide arrays are a widely used type; they contain tens of thousands of short sequenced probes designed to match parts of known or predicted genes.

1.3.2 Gene Ontology (GO)

The Gene Ontology (GO) Consortium has attempted to produce a systematic description of genes and their products, classifying them into so called ontologies. There are three major ontologies, namely biological process, molecular function and cellular compartment. These three ontologies are structured vocabularies or networks of terms, where each term is a so-called "child" of one or more than one "parent" term. The child terms are more specialized, while the parent terms are more general (The Gene Ontology Consortium, 2001).

The GO terms can be used to facilitate the interpretation of data from high-throughput analysis methods such as gene expression microarrays. By using the GO annotations, it is possible to analyze genes at group level, i.e. with a common nominator such as biological process. This type of analysis is beneficial in array experiments where the expressions of several thousands of genes are monitored at the same time. Moderate changes in a group of genes operating in the same biological process could reflect significant differential expression of the whole pathway.

In lung cancer, some of the dysregulated biological processes have been reported to be cytokine-cytokine receptor interactions, focal adhesion, the MAPK signalling pathway, DNA replication and repair, protein targeting and transport as well as sodium ion transport (Chang *et al.*, 2007; Dehan *et al.*, 2007; Gusev, 2008).

2. Asbestos

2.1 Characteristics

Asbestos is a common term for industrially refined and produced fibrous silicate minerals. Asbestos can be classified into six distinct mineralogical types based on the chemical composition and physical appearance of the fibres, i.e. chrysotile, crocidolite, amosite, tremolite, anthophyllite, and actinolite. Chrysotile belongs to the serpentine group and is a curly, thin fibre. The other five belong to the amphibole group of minerals, which are longer and needle-like (LaDou, 2004).

The fibers are composed of hydrated magnesium silicates containing various amounts of iron. Amosite and crocidolite contain the largest amounts of iron (~27%) within the crystal structure, while chrysotile

contains less iron (2–6%) on the surface of its crystal structure. Iron is believed to be important in the biological effects of asbestos, since it can catalyze reactions which generate reactive oxygen and nitrogen species (ROS and RNS), which in turn induce oxidative stress in the cells (Peterson *et al.*, 1998).

2.2 Use

The use of asbestos peaked in Western Europe, North America, Japan and Australia in the 1970's when it was advertised as a miracle compound for its flexible and fire resistant properties, although the first suspicions about its harmful effects had been reported as early as 1898 (reviewed in Tweedale, 2001; Virta, 2006). Asbestos fibres can be found in a multitude of products such as heat, fire and acid resistant coatings, gaskets, pipes, cement, insulation, flooring, roofing and several other types of building material (Figure 4), as well as in lawn furniture, asphalt, car brakes and stage curtains (Figure 5; reviewed in Virta, 2005). A cigarette with a supposedly health protective filter containing crocidolite asbestos was even launched in 1952 and sold at least until 1956 (Figure 6) (Longo *et al.*, 1995).

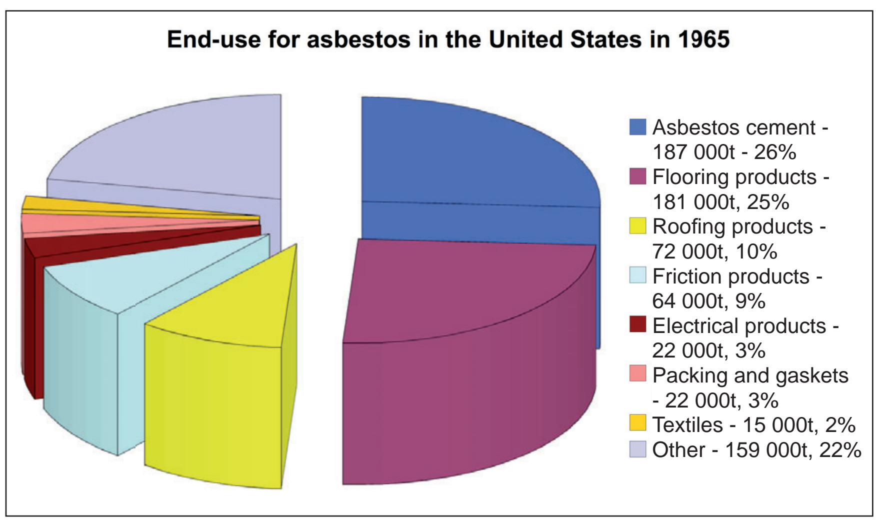


Figure 4. **End-use for asbestos in the United States in 1965.** Figure produced with data from Virta, 2005.

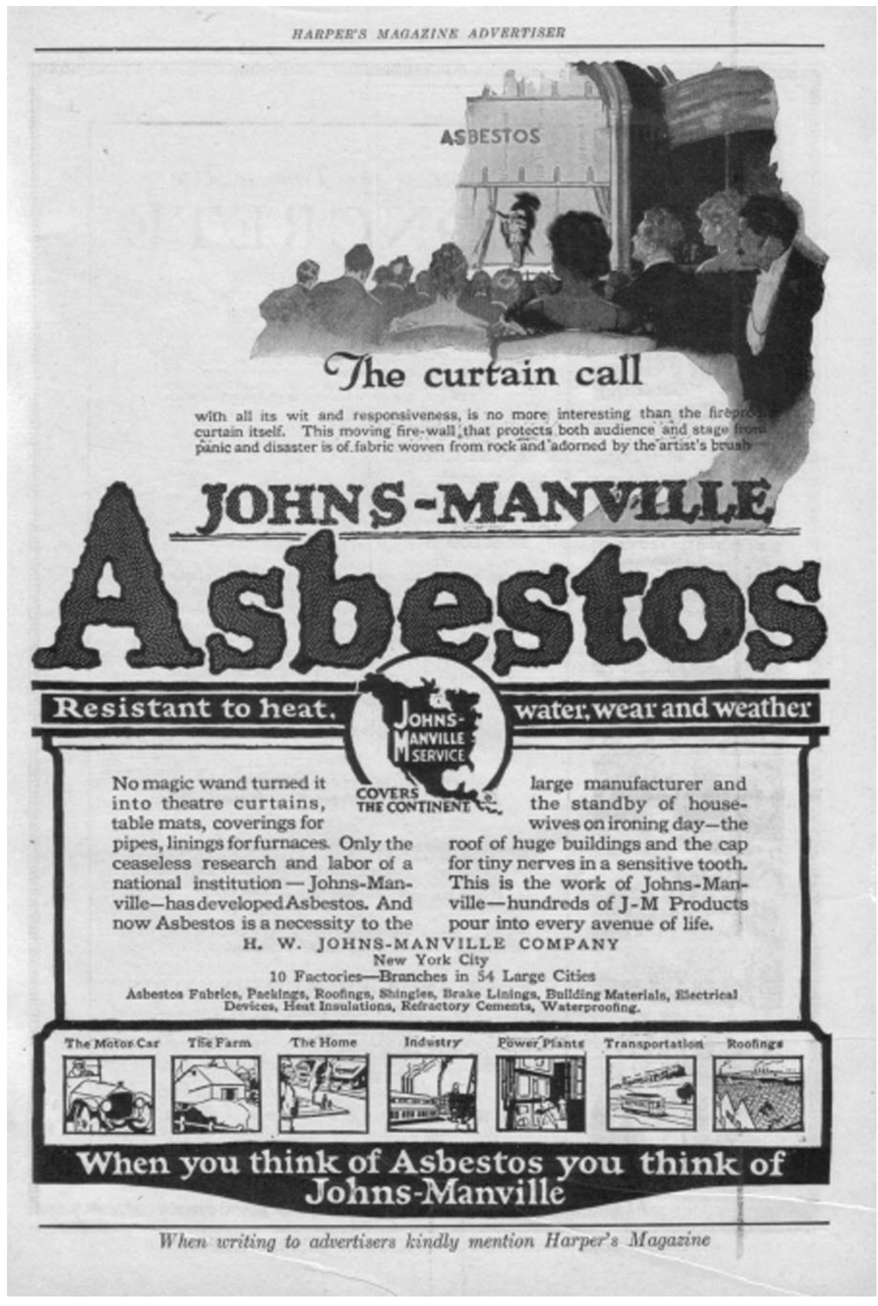


Figure 5. **A 1950's advertisement for asbestos in Harper´s magazine.** Figure reproduced with permission from the UK Asbestos Services (www.asbestosservices.com).

Today the majority of the asbestos produced is used in Eastern Europe, Latin America and Asia (Consensus Report, 1997; LaDou, 2004; Lin *et al.*, 2007). In most of the Western world and in Japan, the use of asbestos in manufacturing or building has been banned or at least subject to strict control for the last 20–30 years, and asbestos demolition work is tightly regulated by law. In Finland, the use of asbestos has been forbidden in building materials since 1988, but a complete ban on asbestos was introduced as late as 1994 (Finnish government decision 1380/1994). The leading asbestos producing countries are Russia, China, Kazakhstan, Canada and Brazil (Hetherington, 2008). Chrysotile is the most commonly used and economically important asbestos type (LaDou, 2004).

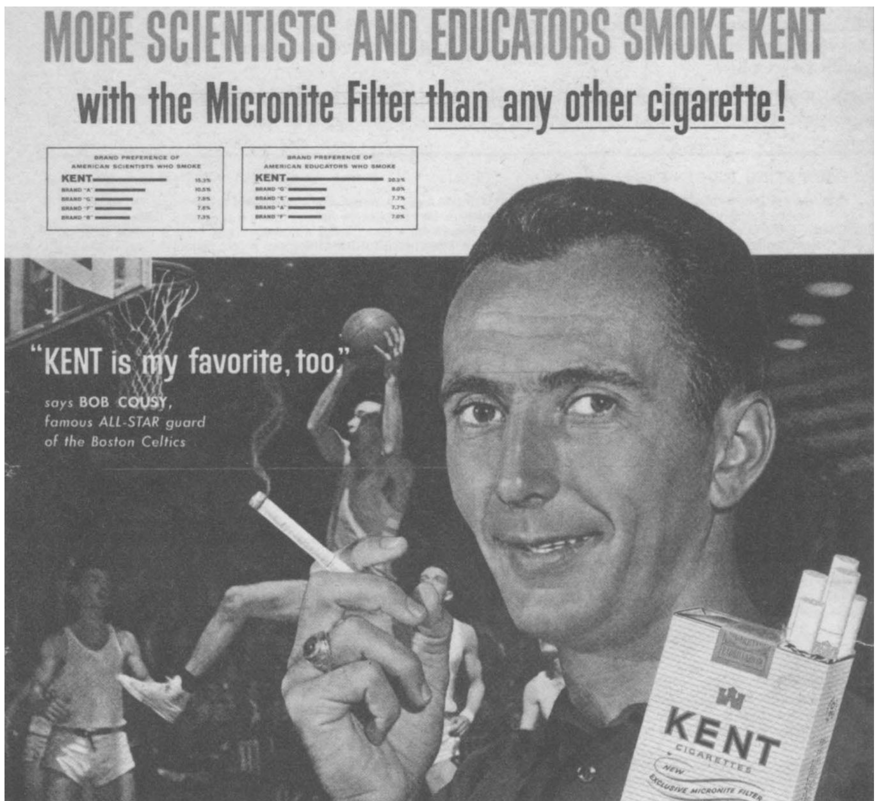


Figure 6. **A 1960 advertisement for Kent cigarettes in the *New York Mirror* magazine.** Micronite filters contained crocidolite asbestos for a few years during the 1950's. Figure reproduced under "fair use" from Levin, 1987.

2.3 Toxicity and carcinogenicity

The genotoxic and carcinogenic effects of asbestos depend largely on the fibre's chemical composition and structure as well as the cell environment (Mossman *et al.*, 1998). A number of *in vitro* and *in vivo* studies have shown that the longer the fibre, the more carcinogenic it is *per se* (Donaldson et al., 1989). However, other researchers have claimed that fibres of all lengths induce pathological responses and no type of asbestos should be considered as being non-carcinogenic, simply based on its fibre length (Dodson *et al.*, 2003). Furthermore, on an epidemiological basis, it has been difficult if not impossible to establish such a hypothesis, since asbestos workers are often exposed to a mixture of different fibre types and sizes (Anttila *et al.*, 1993).

Due to the metals, the fibre structure and their bio-persistence, the amphiboles are thought to be more pathogenic in the human body compared to chrysotile. In contrast to chrysotile asbestos, which becomes fragmented and cleared from the lungs, amphiboles are considered to be totally insoluble in human lung (Stanton *et al.*, 1972; Bernstein *et al.*, 2006). However, there is considerable controversy regarding the malignancy risks associated with chrysotile exposure. It has been estimated that several hundred times the levels of amphibole fibres are needed to induce a similar risk of malignancy with chrysotile (reviewed in Kamp, 2009). Nevertheless, there is considerable pathological as well as experimental evidence that also chrysotile is highly carcinogenic (Nicholson, 2001; Pezerat, 2009; Suzuki *et al.*, 2005). In fact, it has been established that chrysotile is as potent as crocidolite in its ability to cause lung cancer, even though it is 2–4 times less potent in evoking mesothelioma (Landrigan *et al.*, 1999).

Asbestos fibres enter into the lungs by inhalation. Once inside the lungs, the fibres are surrounded by alveolar macrophages (AM). The AM deposit a protein coating around the fibres, which are then referred to as asbestos bodies (see cover picture of this thesis). However, due to the larger size of the fibres compared to the AM, so called frustrated phagocytosis may take place, leading to elevated release of ROS and RNS (Mossman *et al.*, 1998). Amphibole fibres contain high levels of associated mono- di- and trivalent metals such as iron and it has also been proposed that asbestos is toxic by the particular way iron is bound to the fibre's surface, enabling generation of ROS and RNS (Lund *et al.*, 1992; Gazzano *et al.*, 2007).

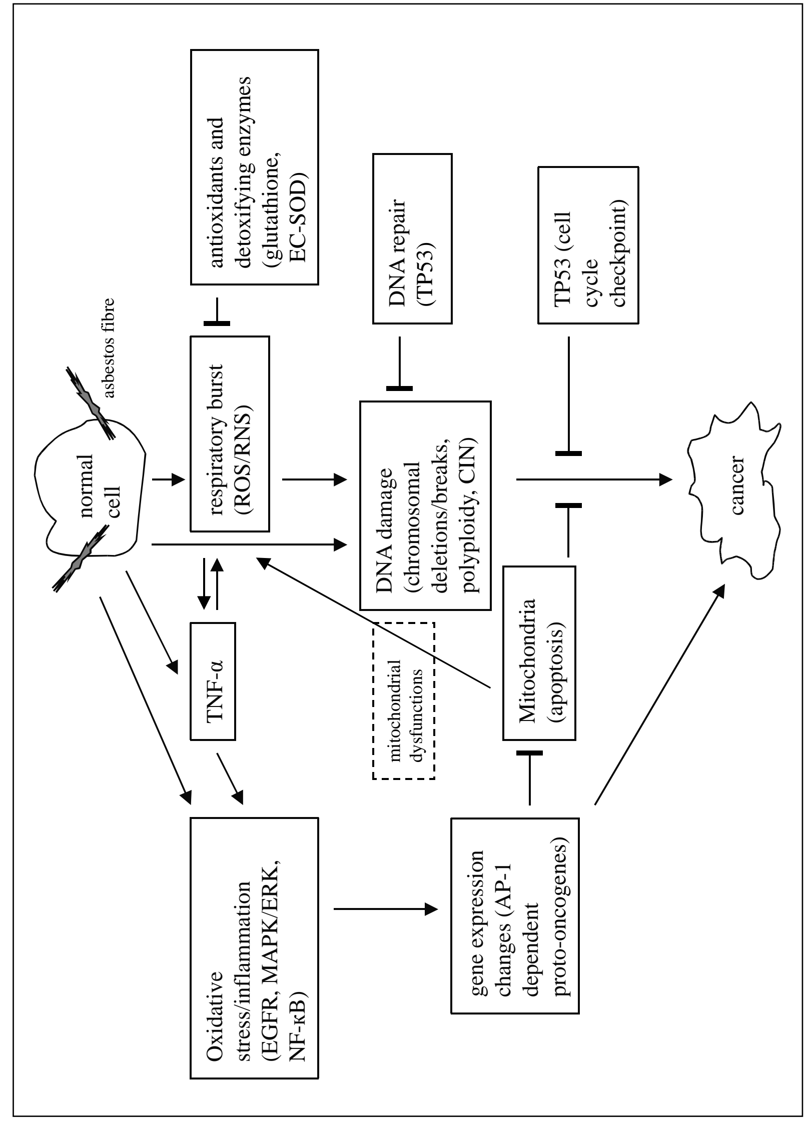


Figure 7. **Asbestos-related carcinogenic pathways in the lung.** Modified from Nymark *et al.* (2008).

In addition to the generation of ROS/RNS, the main mechanisms behind the toxic effects of asbestos are thought to be alterations in mitochondrial function, mechanical disturbance of cell cycle progression, and activation of several signal transduction pathways (reviewed in Jaurand, 1997; Mossman *et al.*, 1998; Upadhyay *et al.*, 2003; Figure 7). Many of these effects are due to the triggering of universal cellular responses, induced by several types of cytotoxic substances. However, *in vitro* studies have also shown that asbestos fibres are clastogenic (able to induce disruptions and breaks in chromosomes), even though they are not mutagenic in the Ames assay (Daniel, 1983; Hei *et al.*, 1992). These genetic alterations are thought to contribute to the carcinogenic effects of asbestos. Experimental studies as well as studies on lymphocytes from asbestos workers, have demonstrated asbestos-induced clastogenicity, involving DNA single and double strand breaks, deletions, increased sister chromatid exchanges (SCE) and formation of micronuclei (Dopp *et al.*, 1997; Dopp *et al.*, 2005; Fatma *et al.*, 1991; Fatma *et al.*, 1992; Hardy *et al.*, 1995; Lu *et al.*, 1994; Marczynski *et al.*, 1994; Hei *et al.*, 1995; Msiska *et al.*, 2009; Hei *et al.*, 1992; Huang *et al.*, 1978; Lohani *et al.*, 2002; Xu *et al.*, 2007). DNA double-strand breaks are the most severe types of DNA damage that can lead to translocations and chromosomal instability (CIN), since they are more difficult to repair than for example DNA single-strand breaks. Crocidolite asbestos has been shown to be able to induce greater amounts of DNA double-strand breaks than silica and titanium dioxide (Msiska *et al.*, 2009). In addition, asbestos has been reported to cause abnormal chromosome segregation, which can not only lead to chromosomal deletions and other DNA alterations but also to aneuploidy (Fatma *et al.*, 1991). The fibres have also been shown to sterically block cytokinesis, leading to binucleated cells and consequently polyploidy (Jensen *et al.*, 1996).

Several studies have shown that asbestos is able to induce transformation of both murine and human cells (reviewed in Barrett *et al.*, 1989). Nevertheless, the exact molecular mechanism behind asbestos-related carcinogenesis is still unresolved. It is thought to be very complex, probably involving several parallel pathways (reviewed in Nymark *et al.*, 2008).

2.4 Asbestos and tobacco smoke as co-carcinogens

Asbestos elevates the risk of contracting lung cancer in non-smokers, but the risk seems to increase even more significantly in smokers, indicating that tobacco smoke and asbestos act as co-carcinogens in a synergistic manner (Nelson *et al.*, 2002). Various joint effects ranging from less than additive to more than multiplicative have been reported, but the generally accepted model that seems to fit the best is a more than additive or less than multiplicative one (Henderson *et al.*, 2004).

Several mechanisms are likely to contribute to the synergistic effects of these two carcinogens. For example, there are studies demonstrating that cigarette smoke augments the penetration of asbestos fibres in rat tracheal explants by an oxygen radical-mediated mechanism (Churg *et al.*, 1989). Tobacco smoke may also interfere with the clearance of asbestos fibres from the lungs (Henderson *et al.*, 2004). Conversely, tobacco carcinogens are known to be adsorbed onto the surface of asbestos fibres increasing their uptake into the cells (Fournier *et al.*, 1986; Nelson *et al.*, 2002). In addition, ROS have been observed to alter the metabolism of the tobacco carcinogen, benzo[a]pyrene, by inhibiting its detoxification pathways (Flowers *et al.*, 1991). Yet another hypothesis is that asbestos fibres induce cell proliferation and thereby clonal expansion of cells with heritable tobacco carcinogen-induced alterations in critical genes (Haugen *et al.*, 1982).

3. Asbestos-related lung cancer

In numerical terms, asbestos-related lung cancer is considered to be the most important occupational cancer in the world (Karjalainen *et al.*, 1994). In Finland, there are around 2000 lung cancer cases every year and approximately 90 of these are reported to be of occupational origin and associated with asbestos.

Table 1. Relative risks of lung cancer associated with different levels of tobacco smoke and asbestos exposure. Modified from Gustavsson *et al.* (2002).

Asbestos exposure (fiber-years[1])	Smoking (cigarettes per day)			
	0	1–10	11–20	>20
	RR[2] (95% CI[3])			
0	1	10.5 (6.7–16.6)	23.3 (15.2–35.8)	45.4 (28.6–71.9)
>0–0,99	1.8 (0.6–5.5)	18.1 (8.2–40.4)	17.0 (8.8–32.7)	38.5 (17.7–83.4)
1–2,49	2.7 (0.7–9.5)	12.1 (5.1–29.3)	29.8 (15.1–58.6)	36.8 (11.9–113.7)
≥2,5	10.2 (2.5–41.2)	13.56 (4.6–40)	86.2 (28.8–258.2)	80.6 (20.2–322.0)

[1]fibers/ml x years,[2]relative risk,[3]confidence interval

3.1 Lung cancer risk associated with asbestos exposure

The first associations between asbestos exposure and lung cancer were made in 1935 (Gloyne, 1935; Lynch *et al.*, 1935). Today, there is a large body of epidemiological evidence demonstrating that asbestos exposure increases the risk of lung cancer and, together with tobacco smoke, the risk is significantly enhanced as described above in Section 2.4 (reviewed in Kamp, 2009). Not unexpectedly, the risk is highly dependent on the duration and amount of exposure as demonstrated in Table 1 (Gustavsson *et al.*, 2002). Depending on the study, the relative risk (RR) of lung cancer due to asbestos exposure has been estimated to be between 0.83 and 25, due to smoking between 1.78 and 10.85 and for both exposures combined, between 4.51 and 53.24 (reviewed in Lee, 2001; Reid *et al.*, 2006; Wraith *et al.*, 2007). Inaccuracies in both self-reported occupational exposure and in differences in the exposure level depending on the type of work have made it very difficult to determine the actual RR associated with asbestos exposure and smoking on their own or in combination. This may explain the broad range of RR in different studies (Bakke *et al.*, 2001).

Asbestos-related lung cancer is largely gender dependent, but this is due to the low numbers of female workers in the construction, shipyard and asbestos industries. The ratio of men to women among asbestos-related lung cancer patients is approximately 32:1, compared to about 4.5:1 for all lung cancers (Karjalainen *et al.*, 1994).

3.2 Clinical features

Asbestos exposure has been associated with lower-lobe and a peripheral location of the tumour as well as with AC histology in some studies (Anttila *et al.*, 1993; Karjalainen *et al.*, 1994; Paris *et al.*, 2003). In contrast, tobacco smoking has been associated with upper-lobe and central location as well as SCC and SCLC. However, the asbestos-related observations remain controversial, since not all studies have detected such associations (Lee *et al.*, 1998; reviewed in Henderson *et al.*, 2004). The contrasting results may be due to different types of exposures in different populations. For example, Finnish asbestos workers have often been exposed to larger amounts of antophyllite and crocidolite than those reported in Britain and North America, which have been primarily exposed to chrysotile (Anttila *et al.*, 1993).

Asbestosis is a type of pleural fibrosis, i.e. chronic inflammation and scarring of the lung tissue caused by the inhaled fibres (Huggins *et al.*, 2004). While it has been proposed that asbestosis must precede the development of asbestos-related lung cancer, there is considerable evidence that lung cancer can develop also without the presence of asbestosis. Indeed, asbestos has been shown to be able to act as an independent carcinogen on all the critical steps of malignant transformation of a cell, i.e. initiation, promotion and progression (reviewed in Kamp, 2009). Nevertheless, it is generally agreed that the presence of asbestosis greatly increases the risk of lung cancer in a manner that is similar to the presence of other types of pulmonary fibrosis. Whether asbestosis is simply a marker of high-dose asbestos exposure or if it is necessary for attributing an individual's lung cancer to asbestos exposure, remains a matter of debate (reviewed in Hessel *et al.*, 2005).

3.3 Molecular alterations attributable to asbestos exposure in lung cancer

The fact that most asbestos-exposed lung cancer patients are also tobacco smokers has made it very difficult to differetiate the asbestos-related molecular changes from those attributable to tobacco carcinogens by molecular epidemiology (Nelson *et al.*, 2002). However, several attempts have been made and some molecular alterations associated with asbestos have been identified. Many of the studies are experimental, but a few have also been performed on primary lung cancer samples. Some of the most noteworthy alterations, which have been reported more than once, are listed in Table 2.

3.3.1 Genetic alterations

A few CNA have been associated with asbestos exposure in lung cancer. For example, Dopp and co-workers have demonstrated on experimental level that the centromeric regions of chromosomes 1 and 9 are affected by DNA breakage following asbestos exposure in human amniotic fluid cells and lymphocytes (Dopp *et al.*, 1997; Lohani *et al.*, 2002). Another study has shown that loss of one or both copies of chromosome 5, monosomy of chromosome 19 and trisomy of chromosome 8 are common changes in five asbestos-transformed tumorigenic bronchial epithelial cell lines compared to the parental non-tumorigenic cell line. In addition, the asbestos-transformed cell lines display hypoaneuploidy with 42–44 chromosomes compared to the parental cell line with hyperaneuploidy containing 46–50 chromosomes (Suzuki *et al.*, 2001). In contrast, as mentioned before asbestos has also been shown to cause polyploidy *in vitro* (Jensen et al., 1996). Both aneuploidy and polyploidy may lead to malignant cell transformation (Storchova *et al.*, 2008).

All of the chromosomes mentioned above have also been reported to be affected by alterations to different degrees in lung cancer in general (Figure 3; Baudis *et al.* 2001; Baudis, 2009). However, based on these results, in asbestos-related lung cancer they may potentially be primary alterations appearing at an early stage of carcinogenesis. In fact, none of these chromosomes or regions appears to be affected by early changes in lung cancer in general (reviewed in Herbst *et al.*, 2008; Soh *et al.*, 2008).

Table 2. Alterations in chromosomes, genes and pathways, attributable to asbestos exposure in lung cancer. Modified from (Nymark *et al.*, 2008).

Chromosome/ biological process	Alteration	Carcinogenic association	Type of study	References
chrom. 1	break at the centromere		*in vitro*	(Dopp et al., 1997; Lohani *et al.*, 2002)
chrom. 3	LOH at 3p14 LOH at 3p21	*FHIT* exon loss possible down-regulation of tumour suppressors	primary tumour samples	(Nelson *et al.*, 1998; Marsit *et al.*, 2004)
chrom. 9	LOH/homozygous deletion at 9p21.3 break at the centromere	loss of *P16/CDKN2A*	*in vitro*; primary tumour samples	(Dopp *et al.*, 1997; Andujar *et al.*, 2010)
chrom. 19	monosomy AI and loss at 19p13	possible down-regulation of tumour suppressors	*in vitro*; primary tumour samples	(Suzuki *et al.*, 2001; Ruosaari *et al.*, 2008b)
Whole genome	Polyploidy	Aneuploidy and CIN	*in vitro*	(Jensen *et al.*., 1996; Jaurand, 1997)
Oxidative stress and inflammation	up-regulation of the NF-κB pathway	tumour promotion through transcriptional activation of proto-oncogenes (e.g. *c-myc*)	*in vitro; in vivo*	(Xie et al., 2000; Yang *et al.*., 2006 and many more as reviewed in Shukla *et al.*., 2003b)
	up-regulation of TNFα through NF-κB	enhances the interactions between cells and fibres by increasing the binding of asbestos to tracheal epithelial cells	*in vitro*	(Xie et al., 2000; Cheng *et al.*., 1999 and many more as reviewed in Shukla *et al.*., 2003b)
	phosphorylation/ activation of EGFR	accumulative ROS generation	*in vitro*	(Zanella *et al.*., 1999; Wang *et al.*., 2006 and many more as reviewed in Shukla *et al.*., 2003b)
		blocking of apoptosis		
		activation of the MAPK/ERK pathway		
		cell proliferation	*in vivo*	
	activation of MAPK/ERK pathway (e.g. *ERK* genes)	tumour promotion through activation of AP-1 dependent proto-oncogenes (e.g. *c-fos*)	*in vitro; in vivo*	(Mossman *et al.*, 2006; Wang *et al.*, 2006 and many more as reviewed in Shukla *et al*, 2003b)
	redistribution of extracellular SOD and intracellular glutathione	cell proliferation	*in vivo*	(Tan *et al.*, 2004; Fattman *et al.*, 2006)
		decreased resistance against ROS/ RNS		
DNA repair	up-regulation of p53	decreased tumour suppressor activity possibly due to mutations	*in vitro*; primary tumour samples	(Pääkkö *et al.*, 1998; Liu *et al.*, 1998 and many more as reviewed in Kamp, 2009)
Mitochondrial activity and apoptosis	activation/inhibition of *BCL2* and *BCL2*-like genes	apoptotic resistance	*in vitro*	(Narasimhan *et al.*., 1998; Kamp et al., 2002; Miura *et al.*, 2006)
	apoptotic bypass	ROS generation feedback loop through mitochondrial dysfunctions	*in vitro*	(Shukla *et al.*., 2003a; Yuan *et al.*., 2004)

As noted previously, asbestos has primarily been associated with deletions, breaks and fragments in the genome, while smoking is likely to cause point mutations and aberrant methylation of genes. The *P16/CDKN2A* gene has been found to be affected by both epigenetic (hypermethylation) and genetic (homozygous deletion and rare point mutations) alterations in lung cancer. In a recent study on primary lung cancer samples, homozygous deletions were found to correlate significantly with asbestos exposure, while methylation of the gene was confirmed to correlate with smoking (Andujar *et al.*, 2010). Interestingly, *P16/CDKN2A* is generally affected by homozygous deletions also in mesothelioma (reviewed in (Andujar *et al.*, 2010)).

Another chromosomal region, that seems to be affected more frequently in asbestos-related than in non-related lung cancer, is 3p. LOH at 3p21.3 (Marsit *et al.*, 2004) and 3p14 (*FHIT*) have been linked with asbestos exposure in primary lung cancer (Nelson *et al.*, 1998; Pylkkänen *et al.*, 2002b). However, this may be the effect of the asbestos-induced clonal expansion of cells with a tobacco smoke-related alteration, since these regions are often also affected in lung cancer without asbestos association. Reduced expression of *FHIT* has been shown to be equally frequent in both exposed and non-exposed lung cancer patients, indicating that the gene is silenced by some additional mechanism in lung cancer of non-exposed patients (Pylkkänen *et al.*, 2002b). Indeed, this gene is frequently hypermethylated in smokers with early stage SCC (Kim *et al.*, 2004).

TP53 has been one of the most extensively studied genes in asbestos-related lung cancer, as it is in cancer in general. This gene has been shown to be up-regulated by asbestos exposure and abnormal accumulation of p53 is more frequent in tumours and serum of asbestos-exposed patients compared to non-exposed subjects (Nuorva *et al.*, 1994). Some studies have associated *TP53* mutations with asbestos exposure in lung cancer (Liu *et al.*, 1998; Lin *et al.*, 2000; Panduri *et al.*, 2006; Wang *et al.*, 1995), but the association remains controversial since others have not been able to confirm these results (Husgafvel-Pursiainen *et al.*, 1999). Similarly, there are controversial results regarding the *KRAS* gene, in which mutations have primarily been associated with smoking in lung cancer. A few studies have also reported correlations between its mutation and asbestos exposure (Husgafvel-Pursiainen *et al.*, 1993; Nelson

et al., 1999; Vainio *et al.*, 1993), but not all (Hei *et al.*, 1997; Husgafvel-Pursiainen *et al.*, 1999).

Individual susceptibility to asbestos-related lung cancer has been studied to some extent. However, few studies have been able to efficiently evaluate the actual association of the genetic variation, i.e. whether it modifies the effect of the asbestos exposure or that of tobacco-smoke exposure (reviewed in Neri *et al.*, 2008). The most convincing evidence has come from a study on the *MPO* gene, in which a polymorphism referred to as the A-allele genotype conferred a protective effect against cancer development in asbestos-exposed individuals, i.e. reducing lung cancer susceptibility (Schabath *et al.*, 2002).

3.3.2 Signalling pathways

Cells depend on internal and external signals in order to maintain normal function and the balance of this microenvironment is crucial for normal homeostasis. Changes in the signalling pathways of the internal microenvironment may provide also surrounding cells with growth promoting signals. Expressional changes in central genes caused by molecular alterations in the DNA may alter these signalling pathways (Hanahan and Weinberg, 2000). Carcinogenic substances may also alter gene expression by binding to receptors or transcription factors prior to any genetic alterations. Indeed, asbestos has been shown to bind to several specific proteins involved in the cell cycle as well as in cytoskeletal and mitotic processes (MacCorkle *et al.*, 2006).

The main signalling pathways that have been found to be affected by asbestos exposure include those involved in oxidative stress, inflammation, DNA repair, mitochondrial activity and apoptosis (Figure 7 and Table 2; Upadhyay *et al.*, 2003; Jaurand, 1997). As mentioned before, many of these changes are probably the consequences of normal cellular responses to foreign toxic particles. However, they may nevertheless be involved in the carcinogenic effects of asbestos in combination with the more specific clastogenic properties of the fibres.

Asbestos induced oxidative stress and inflammation directed pathways are intertwined and complex involving the induced expression of several genes such as *TNFa* and NF-κB subunits as well as *EGFR* and *MAPK* (Figure 7, reviewed in Shukla *et al.*, 2003b). In addition, antioxi-

dant enzymes and peptides, such as superoxide dismutase (SOD) and glutathione have been proposed to be involved in the pathogenesis of asbestos-related lung cancer, causing disturbances in the oxidant-antioxidant balances and subsequently reducing the cell's capability to protect itself against ROS and RNS (Bhattacharya *et al.*, 2005; Fattman *et al.*, 2006; Pande *et al.*, 2006; Puhakka *et al.*, 2002; Tan *et al.*, 2004; Wang *et al.*, 2006).

DNA damage repair pathways are up-regulated in asbestos-treated cells (Upadhyay *et al.*, 2003). In particular, the genes *TP53* and *CDKN1A* (encoding for Cip1, also known as p21) have been found to be expressed at higher levels following asbestos exposure (Johnson *et al.*, 1997; Piao *et al.*, 2001; reviewed in Kamp, 2009). Over-expression of *TP53* should assist the DNA repair processes, however if the gene is mutated it may render the protein non-functional or malfunctional (Schetter *et al.*, 2009).

Apoptosis protects against abnormal proliferation of cells with non-repairable DNA damage. However, if the apoptotic pathways are bypassed, then the asbestos-associated dysfunctions in the mitochondrial respiratory chain maintain an increased release of ROS, creating a continuous supply of these damaging molecules (Shukla *et al.*, 2003a). Several apoptosis-related genes have been identified in asbestos-induced apoptotic resistance, especially *BCL2* and *BCL2*-related genes seem to have a central role (Kamp *et al.*, 2002; Miura *et al.*, 2006; Narasimhan *et al.*, 1998).

AIMS OF THE STUDY

Lung cancer is strongly associated with inhaled exposures such as tobacco smoke and asbestos fibres. These exposures cause extensive DNA damage on their own as well as acting together as co-carcinogens. The resulting complexity of the lung cancer genome has made it difficult to unravel specific molecular alterations associated with the different exposures. Tobacco-related molecular alterations have been identified, whereas there are no specific molecular biomarkers associated with asbestos-related lung cancer. These molecular correlations could be of importance not only for the diagnosis, prognosis and treatment of the disease, but also in the medico-legal aspects of this occupational disease.

Therefore, the specific aims of this thesis were to:

- Perform a global search for specific gene copy number alterations (CNA) differing between asbestos-related and non-related lung cancer (I)
- Validate and characterize the identified asbestos-related CNAs in a larger study population (II–III)
- Profile expressional changes in genes, pathways and specific chromosomal regions in asbestos-exposed cell lines (IV)

MATERIALS AND METHODS

1. Lung cancer patients and DNA samples (I, II and III)

All the patients used in the studies of this thesis were of Finnish Caucasian origin with histologically confirmed lung cancer. The tumours were classified according to the latest WHO classification (1999/2004). Two different collections of samples were used in the studies (I, II, III; Table 3). All samples were analyzed by electron microscopy with energy dispersive spectrometry to determine their pulmonary asbestos fibre concentration.

The first collection (subsequently referred to as collection 1) consisted of 82 fresh-frozen lung tumour and adjacent normal samples from a set of tissue samples obtained during lung cancer surgery at the Helsinki University Central Hospital in the 1980´s and -90´s. All the patients were personally interviewed about their smoking habits and work history, and provided their informed consent to take part in the study and to use their tissue. Thirty-six patients were current and 41 were ex-smokers, three were non-smokers; for two patients smoking data was missing. Forty-nine patients had less than 0.5 million fibres per gram of dry lung tissue (later referred to as fibres/g) and 44 of them did not have any known history of asbestos exposure, while 5 had a probable history of exposure. Thirty-three patients had more than 1 million fibres/g and 25 of them had a probable or definite history of asbestos exposure. The Ethical Review Board for Research in Occupational Health and Safety and the Coordinating Ethical Review Board, Helsinki and Uusimaa Hospital District (75/E2/2001) approved the study protocols.

The second collection (later referred to as collection 2) consisted of 135 patients' formalin-fixed paraffin embedded (FFPE) tumour samples, which had been obtained through surgery, bronchoscopy, lymph node biopsy or autopsy. Fifty-three patients had less than 0.5 million fibres/g and 82 patients had more than 1 million fibres/g. The samples of these patients had originally been sent to the Finnish Institute of Occupational Health (FIOH) from different regions of Finland for analysis of pulmonary asbestos fibre count as a part of the diagnostic procedure during 1985–2006. Permission to use the diagnostic samples for research purposes was given by The National Agency for Medicolegal Affairs (4476/33/300/05) and the collection of patient information for research was approved by the Ministry for Social Affairs and Health (STM/2474/2005).

Twenty-eight samples from collection 1 were included in study I (Table 3). Fourteen of the samples originated from patients with a pulmonary fibre count of 5 million fibres/g or more and a definite or probable history of asbestos exposure. The other fourteen samples originated from patients with 0.5 million fibres/g or less and no known history of asbestos exposure. Fourteen of the samples were malignant lung tumours from highly asbestos-exposed patients and 14 tumours were from non-exposed patients that were matched for age, gender, nationality and smoking history. In addition, the distribution of different tumour histologies was matched between the groups (Table 3).

In study II, 139 tumour samples from collections 1 and 2 were used. Seventy-seven samples originating from patients with a fibre count of 1 million or more fibres were included in the exposed group. Sixty-two samples originating from patients with a fibre count of 0.5 million fibres/g or less made up the non-exposed group (Table 3).

In study III, 205 tumour samples from collections 1 and 2 were used. One-hundred and nine samples from patients with a fibre count of 1 million or more fibres were included in the exposed group. Ninty-six samples from patients with a fibre count of 0.5 million fibres/g or less represented the non-exposed group (Table 3).

Table 3. Characteristics of patients and lung tumour samples used in studies I–III. More detailed information can be found in the original publications.

		Study I		Study II		Study III	
		Asbestos-exposed	Non-exposed	Asbestos-exposed	Non-exposed	Asbestos-exposed	Non-exposed
		(n=14)	(n=14)	(n=77)	(n=62)	(n=109)	(n=96)
Pulmonary fiber count[1], median (range)		11.7 (5.9–145)	0.0 (0.0–0.5)	8.2 (1.1–570)	0.0 (0.0–0.5)	6.6 (1.0–570)	0.0 (0.0–0.5)
Age at time of surgery/biopsy/death, mean ± s.d.		62.6 ± 3.2	64.2 ± 8.9	65.6 ± 7.4	62.5 ± 10.2	66.3 ± 7.8	61.2 ± 10.0
Sex (male/female)		14/0	14/0	76/1	60/2	105/4	87/9
Histology[2]	AC	5	6	32	24	39	32
	SCC	4	3	27	25	39	36
	LCLC	3	2	9	3	11	6
	SCLC	1	1	5[3]	6[3]	12[3]	13
	Other histological types	1[4]	2[5]	4[6]	4[7]	8[8]	9[9]
Stage	I	7	6	*na*	*na*	*na*	*na*
	II	1	2	*na*	*na*	*na*	*na*
	III	4	3	*na*	*na*	*na*	*na*
	IV	2	3	*na*	*na*	*na*	*na*
Smoking	ex	9	7	*na*	*na*	*na*	*na*
	current	5	7	*na*	*na*	*na*	*na*
	pack years, median (range)	34 (20–105)	47 (25–89)	*na*	*na*	*na*	*na*

Note: studies II and III contain partly the same samples and both studies include the samples used in study I; na, not available

[1] Million asbestos fibers/g of dry lung tissue

[2] AC, adenocarcinoma of the lung; SCC, squamous cell carcinoma of the lung; SCLC, small-cell lung cancer; LCLC, large-cell lung carcinoma

[3] One combined SCLC

[4] One adenosquamous (AC/SCC) carcinoma

[5] One pleomorphic lung cancer and one AC/SCC carcinoma

[6] One AC/SCC carcinoma, two large cell neuroendocrine carcinoma (LCNEC) and one combined LCNEC.

[7] Two pleomorphic lung cancer, one combined LCNEC and one AC/SCC carcinoma

[8] One AC/SCC carcinoma, three pleomorphic lung cancers, one combined LCNEC, two LCNEC and one undefined NSCLC

[9] Four AC/SCC carcinomas, two LCNEC, one combined LCNEC, one undefined NSCLC and one pleomorphic lung cancer

A minimum of 1 million fibres/g is usually considered a sign of occupational exposure to asbestos and increased risk of lung cancer (Karjalainen *et al.*, 1993; Karjalainen *et al.*, 1994). Up to 1 million fibres/g of dry lung tissue have been reported to be detected in the general population (Churg, 1997). Thus, it was decided to include cases with a fibre count of up to 0.5 million fibres/g in the non-exposed group, but cases with more than 0.5 and less than 1 million fibres/g were excluded from the study to highlight the contrast between exposed and non-exposed.

2. Cell lines (IV)

Three cell lines, A549, BEAS-2B and MeT5A, were used to study gene expression changes related to asbestos exposure. A549 is a human lung adenocarcinoma cell line, while BEAS-2B and MeT5A are human SV40-transformed immortalized bronchial epithelial and pleural mesothelial cell lines, respectively. All three cell lines are well characterized and have been widely used in pulmonary research (Fung *et al.*, 1997; Kahlos *et al.*, 2001; Pache *et al.*, 1998; Perkins *et al.*, 1999). The cells were cultured as described previously (Kinnula *et al.*, 1996; Kinnula *et al.*, 1998; Ollikainen *et al.*, 2000; Puhakka *et al.*, 2002). In brief, A549 cells (American Type Culture Collection, Rockville, MD) were cultured in nutrient mixture F-12 growth medium supplemented with 15% fetal calf serum (FBS), 100 U/ml penicillin and 100 mg/ml streptomycin at 37 °C in a 5% CO_2 atmosphere. The BEAS-2B cells (National Cancer Institute, Laboratory of Human Carcinogenesis) were cultured according to the manufacturer's instructions (bronchial epithelial cell growth medium (BEGM); Clonetics Inc., San Diego, CA). MeT5A cells (American Type Culture Collection) were cultured with RPMI 1640 medium containing 10% heat inactivated FBS, 0.003% L-glutamine, 100U/ml penicillin, and 100 mg/ml streptomycin at 37 °C in a 5 % CO_2 atmosphere.

2.1 Asbestos exposure

The cells were exposed to crocidolite asbestos in semi confluent cell cultures (2 μg/cm^2 for A549 and BEAS-2B cells and 1 μg/cm^2 for MeT5A cells; International Union Against Cancer, Johannesburg, South Africa).

The fibre doses were chosen based on previous studies in our laboratory and by others (Kinnula *et al.*, 1996; Ollikainen *et al.*, 2000; Puhakka *et al.*, 2002). Samples were collected from asbestos-exposed and non-exposed parallel control A549 and BEAS-2B cells at 5 time points, i.e. 0h (before any asbestos exposure or other treatment) and at 1h, 6h, 24h, and 48h. Additionally, a sample was collected from A549 cells at 7 days. Samples from the exposed and non-exposed control MeT5A cells were collected at 1h and 48h. The cultures and exposures were conducted on three or more separate tissue culture plates (T25 and T75), and the cells were pooled before RNA extraction to eliminate the need of biological replicate hybridizations.

3. Analysis methods

3.1 Chromosomal and array CGH (I)

DNA was isolated from 28 tumour samples from exposed and non-exposed patients and from peripheral blood from two male references using QIAamp DNA Mini Kit (QIAGEN®, Valencia, CA).

cCGH was performed on all 28 tumour samples. 1 µg of digested and labelled reference (TexasRed-5-dCTP and -dUTP) and tumour (FITC-5-dCTP and –dUTP) DNA was used for the hybridizations (NEN™; Life Science Products Inc., Boston, MA). The slides were hybridized over-night at 37°C and washed according to standard protocols (Björkqvist *et al.*, 1998b). Isis CGH program (version 3; MetaSystems GmbH, Altlussheim, Germany) was used for the analysis. Twenty metaphases were analyzed for each sample. Standard cut-off thresholds were set to <0.85 for deletions, >1.17 for gains and >1.5 for amplifications, as described by Björkqvist *et al.* (1998b).

aCGH analyses were conducted on twenty of the same samples (11 exposed and 9 non-exposed; see Table 1 in study I). cDNA microarrays (Human 1.0; Agilent Technologies, Palo Alto, CA) containing 12,814 unique clones (97% map to named human genes) were used as described by Wikman *et al.* (2005). Briefly, the hybridizations were performed with 5 µg of digested (25U Alu1/25U Rsa1) reference and tumour DNA, labelled (Cy3 dUTP-tumour, Cy5 dUTP-reference; Amersham Pharmacia Biotech, Piscataway, NJ) using a random priming method

(RadPrime DNA Labelling System; Gibco BRL, Gaithersburg, MD). After hybridization at 65°C overnight, the slides were washed, dried in a centrifuge and scanned using Agilent's DNA Microarray Scanner (G2565AA).

Feature Extraction software (Agilent Technologies) was used to measure raw signal intensities from the arrays. Measurements flagged as unreliable were removed from subsequent analysis. Additionally, faulty measurements defined by our own image analysis methods were removed (Ruosaari *et al.*, 2002). In order to identify exposure-related aberrations, the aCGH data from individual patients were analyzed at the group level by comparing gene copy number ratios of the tumours from exposed and non-exposed patients. Exposure-related areas were identified by using overlapping 0.5–1 Mbp segments that were tested for differences in copy numbers as described in the supplemental data of the original publication (I).

3.2 Microsatellite analysis for detection of allelic imbalance (II and III)

Tumour cells were harvested with a Veritas Laser Capture Microdissection Instrument (Arcturus, Mountain View, CA, USA) from 8 μm tissue sections stained with 1% toluidine blue/0.2% methylene blue solution. DNA was isolated using a PicoPure™ DNA Extraction Kit (Arcturus) following the manufacturer's instructions and stored at –20ºC.

In Study II, 15 microsatellite markers were analyzed at 9q31.3–q34.3 with an average spacing of 1.8 Mbp (base pairs 112128955–139756701, 27.7 Mbp, Figure 8a) in 52 fresh-frozen lung tumour and corresponding normal samples from the same patients (collection 1, see section 1. of Materials and Methods). The primer sequences for thirteen of the microsatellite markers (D9S1675, D9S1683, D9S930, D9S289, D9S302, D9S1776, D9S170, D9S1872, D9S195, D9S1116, D9S1831, D9S1793 and D9S1838) were obtained from the database of the National Center for Biotechnology Information (NCBI). Two additional markers were identified with Alex Dong Li´s RepeatFinder *v4.0* (http://www.genet.sickkids.on.ca/~ali/repeatfinder.html) in the region 9q33.1 (TC-repeat and AC-repeat, base pairs 121021696-121168710, NCBI Build 36.1). Primers were designed with the Primer3 software (Rozen *et al.*, 2000).

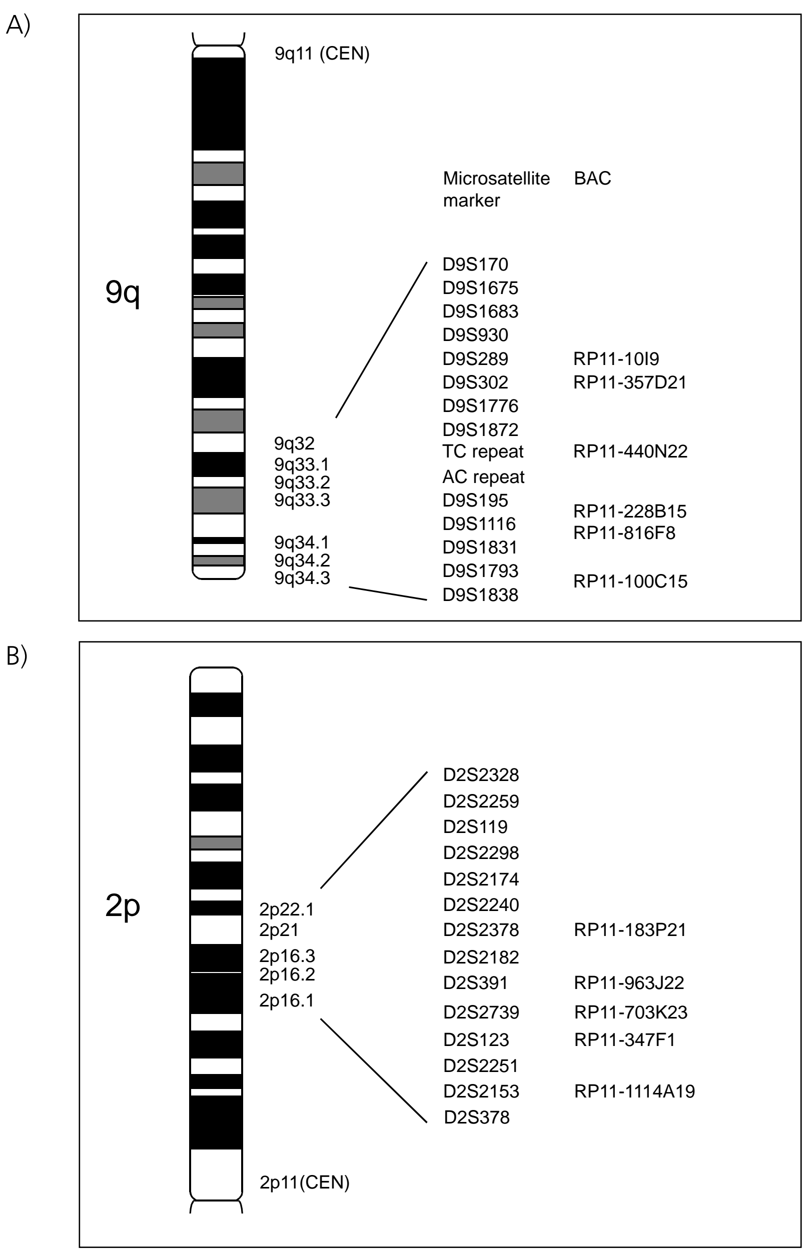


Figure 8. **Map of microsatellite markers, Bac probes and centromere probes on 9q (A) and 2p (B).** The markers and probes are aligned to correspond to each other's physical position on the chromosomes.

In Study III, 14 microsatellite markers (D2S2328, D2S2259, D2S119, D2S2298, D2S2174, D2S2240, D2S2378, D2S2182, D2S391, D2S288, D2S2739, D2S123, D2S2251, D2S2153, D2S378 from NCBI) were analyzed at 2p22–p16.1 with an average spacing of 1.2 Mbp (base pairs 40532153- 57157077, 16.6 Mbp, Figure 8b) in 27 fresh-frozen lung tumour and corresponding normal samples from the same patients (collection 1).

All primers were synthesized and labelled with FAM™, HEX™ or NED™ fluorochrome at TIB MOLBIOL Syntheselabor GmbH (Berlin, Germany). The target sequences were amplified by PCR in a volume of 10µl containing 200µM dNTPs, 700 mM of each primer, 1x PCR Buffer containing 15 mM $MgCl_2$, 0.25 units of HotStarTaq DNA Polymerase (Qiagen) and genomic DNA from normal or tumour samples. An initial 10 min, 95ºC denaturation step was followed by 40 cycles of 95ºC for 40 s, 40 s at the optimized annealing temperature, and 72ºC for 1 min. The PCR products were analyzed with a 310 and a 3100-Avant Genetic Analyzer (Applied Biosystems, Foster city, CA) according to the manufacturer's protocol.

GeneMapper Analysis Software versions 3.5 and 4 (Applied Biosystems) were used to study the lengths of the allele fragments. The alleles were defined as the two highest peaks within the expected size range. According to standard procedures, the ratio of the alleles' peak heights between a patient's tumour and normal samples was calculated for heterozygous markers. A ratio of 2.0 or higher was scored as AI.

The frequency of AI was compared at the 9q and the 2p regions between the tumours of the exposed and the non-exposed patients. Differences within histological types were tested separately if the number of cases was sufficient for testing. At 9q, tumours with more than 6 informative microsatellite markers were assigned AI at the whole region if more than 25% of the markers showed AI. In addition, AI at 9q33.1 was analyzed separately using 3 microsatellite markers (TC-repeat, AC-repeat and D9S195) covering 0.15 Mbp. Tumours with an informative result from at least 2 markers were included in the analysis and AI at the region was scored for a tumour if more than 1 marker displayed AI. In this thesis, the 2p region was analyzed in the same way as the 9q region. Thus, tumours with more than 5 informative microsatellite

markers at 2p were assigned AI at the whole region if more than 25% of the markers showed AI. Also, 2p16 was analyzed separately including 5 markers (D2S2739, D2S123, D2S2251, D2S2153 and D2S378) covering 7.7 Mbp. Tumours with an informative result from at least 2 markers were included in the analysis and AI at the region was scored for a tumour if more than 1 marker showed AI.

In the tumour DNA, the presence of novel peaks of a size that differed from those in the normal DNA by an integer number of repeat units was defined as microsatellite instability (MSI).

Parallel replicates with two to three separate PCR reactions were performed for three markers on five cases to ensure general reproducibility. If replicates were performed, an average of the ratios was used, although the results were highly congruent.

3.3 Fluorescence *in situ* hybridization (FISH) (II and III)

3.3.1 Tissue microarrays

Five tissue microarray (TMA) blocks were designed with FFPE tumour material from 24–34 patients from collection 2. Two to four tissue cores with tumour material from each patient were included on the arrays. Normal lymph node material, included in each TMA was used as normal controls.

3.3.2 Locus-specific FISH

The region 9q32-34.3 (base pairs 115085166-137867305; Figure 8a) was analyzed by fluorescence *in situ* hybridization (FISH) using six bacterial artificial chromosome (BAC) probes (RP11–10I9, RP11–357D21, RP11–440N22, RP11–228B15, RP11–816F8 and RP11–100C15; BAC PAC Resources, CHORI, Oakland, CA, USA) on 95 tumour samples from both collections (38 fresh-frozen and 57 FFPE; 48 asbestos-exposed and 47 non-exposed).

The region 2p21–p16 (base pairs 46081709–54680411; Figure 8b) was analyzed by FISH using five BAC probes (RP11–1114A19, RP11–347F1, RP11–703K23, RP11–963J22, and RP11–183P21; BAC PAC Resources)

on 151 tumour samples from both collections (67 fresh-frozen and 84 FFPE; 79 asbestos-exposed and 72 non-exposed).

Bacteria containing the BAC sequences were grown according to the manufacturer´s instructions (http://bacpac.chori.org/protocols.htm). DNA was extracted with a Qiagen Plasmid Purification Kit (Qiagen, Valencia, CA, USA) and nick-translated (Nick Translation Kit, Vysis, Inc. /Abbott Molecular, Inc., Downers Grove, IL) followed by labelling with biotin-14-dATP (Invitrogen Life Technologies, Carlsbad, CA, USA) or fluorescent Spectrum Green™ dUTP (Vysis, Inc. /Abbott Molecular, Inc., Downers Grove, IL, USA).

To ensure specific hybridization, the probes were hybridized to normal metaphases and histologically normal FFPE sample sections of lung tissue or lymph nodes. Each probe was then hybridized on pretreated TMA slides and fresh frozen tumour sections. Pretreatment of the slides was performed as follows: after paraffin removal of TMA slides and hydration of slides with fresh-frozen samples, the sections were treated in 0.01M citrate, pH 6.0 at 80 ºC for 2 hours and digested with DigestAll™3 (Zymed Laboratories Invitrogen Immunodetection, San Francisco, CA) at 37 ºC for 10 min. Denaturation, dehydrations and hybridizations were performed as previously described (Kettunen *et al.*, 2000). Biotin-labeled probes were visualized with avidin-FITC (Vector Laboratories, Burlingame, CA, USA). Post-hybridization washes were performed for directly-labelled (Spectrum Green™) probes. All hybridizations were mounted with Vectashield Mounting Medium containing DAPI (Vector Laboratories, Burlingame, CA, USA). On average approximately 100 interphase nuclei, with no overlapping nuclei, were scored (range 30-200).

Samples were analyzed without prior knowledge of the exposure status. The tissue cores on the TMA slides were analyzed without any prior knowledge of which of the cores originated from the same tumour.

At the 9q region, copy numbers for each locus were calculated by subtracting the locus-specific signal counts from the signal count of centromere 9 (FISH*Bright* 550-labeled [red], Qbiogene, Illkirch, France and Kreatech, Amsterdam, Netherlands). Samples with a locus-minus-centromere value higher than 0.9 were considered to carry a gain and with lower than -0.9, a loss. The frequency of gains and losses at each

locus was compared between the asbestos-exposed and non-exposed patients' tumours.

At the 2p region, a ratio between the signal counts for each locus-specific probe and the signal count for centromere 2 (SpectrumOrange™-labelled, Vysis Inc./Abbott Molecular Inc., IL, USA) was used to calculate the copy numbers. Samples showing a locus-centromere ratio of 1.3 or higher were considered to carry a gain and with 0.75 or lower, a loss. The frequency of gains and losses at each locus was compared between the asbestos-exposed and non-exposed patients' tumours. In addition, the prevalence of gains and losses was compared in the 2p21 and 2p16 regions separately. Differences within histological types were tested separately if the number of cases was sufficient to allow statistical testing.

The locus-specific copy numbers were calculated differently for 9q and 2p, because of the frequent trisomy of chromosome 2 detected with the centromere 2 probe. Chromosome 9 mostly showed disomy and occasionally monosomy or trisomy, detected with the centromere 9 probe. Therefore, we chose a more sensitive method for analysing locus-specific CNA at 2p than was used at 9q. The cutoff values were based on results from normal samples.

3.3.3 Centromere FISH

Centromere FISH probes for chromosomes 2 (SpectrumOrange™, Vysis Inc), 3 (biotinylated, Oncor, Gaithersburg, MD, USA), 9 (FISH*Bright*, Kreatech Diagnostics, Netherlands), 10 and 15 (Spectrum Green™- and SpectrumAqua™-labelled Vysis Inc) were used to estimate tumour ploidy in 100 tumour samples from both collections (45 fresh-frozen and 55 FFPE; 58 asbestos-exposed and 42 non-exposed). The probes were hybridized to TMA slides and slides with fresh-frozen samples and washed according to the manufacturer's instructions, respectively, at 65 ºC or 73 ºC.

Ploidy for each tumour was assessed by the average of the signal counts from three to five centromere probes. Average signal counts of above 2.5 were considered to represent polyploidy.

3.4 Gene expression microarrays (IV)

RNA from the cell line samples was extracted and purified using Qiagen RNeasy kit (Qiagen Inc., Valencia, CA, USA) and the RNA quality was measured using Agilent's BioAnalyzer (Agilent Technologies, Palo Alto, CA).

Each sample and one replicate from any time point for each cell line were hybridized to Affymetrix Human Genome U133 Plus 2.0 oligonucleotide microarrays (Affymetrix, Santa Clara, CA). Five µg of high-quality total RNA was reverse transcribed to cDNA using a Superscript Double Stranded cDNA Synthesis kit (Invitrogen, Paisley, UK). The cDNA was linearly amplified and *in vitro* transcription reactions using the BioArray high-yield RNA transcript labelling kit (T7; Enzo Life Sciences, Farmingdale, NY) were carried out to produce biotinylated CTP and UTP-labelled cRNA. The labelled and fragmented cRNA was then hybridized to the Affymetrix microarrays for 16 h at 45°C in a rotating oven (60 rpm). The arrays were washed and stained with streptavidin-phycoerythrin (SAPE) in a Fluidics station 450 (Affymetrix, 2004), and scanned with Affymetrix GeneChip Scanner 3000. The image was analyzed using the GeneChip operating software (GCOS; Affymetrix, Sacramento, CA) and Comparison Analysis was done according to the instructions provided by the manufacturer.

The arrays were scaled to the target value of 100 and absent and present calls were identified with Affymetrix Analysis Suite v. 5 (MAS 5.0). Arrays showing a background of 35-70 and housekeeping control genes signal ratios close to 1.0 were included in the data analysis.

Pre-processing of the hybridization data was performed using RMA (Robust Multi-array Average) (Irizarry *et al.*, 2003) with default settings (i.e., quantile normalization) in R. AFFX control sets and probe sets lacking GeneID information were excluded from the analyses. In addition, probe sets were exluded from the analyses when the "present / marginal / absent" expression rating system used in Affymetrix microarray analysis software (Affymetrix, 2004) (here, an open source implementation (Gautier *et al.*, 2004) of the Affymetrix algorithms was used) declared a probe set as absent in all microarrays relevant to the experiment. RMA pre-processing, designed to enhance the comparability of expression

values between separate arrays, produces a single logarithmic expression value for each probe set in the Affymetrix arrays. In the case of replicates, the mean RMA value was used in the analysis.

BioConductor package 'hgu133plus2', version 1.10.0 was used to obtain gene and chromosome band assignments (Bioconductor). Only unique assignments to properly named chromosome bands were used in the analysis.

In order to be able to interpret the large amount of gene expression data obtained from these array experiments and place it back into its biological sense, three different bioinformatics methods specifically modified to suit this experiment were performed.

3.4.1 GO analysis

A statistical analysis of Gene Ontology (GO) annotation terms was performed. A method, similar to that described by Breitling *et al.* (2004) was used to identify asbestos exposure-associated biological processes.

All cell lines were analyzed separately. Each time point was analyzed for both under- and overexpressed groups of genes. Genes were first rank-ordered according to their logarithmic fold-change values between exposed and corresponding non-exposed samples. In the case of multiple probe sets corresponding to a unique gene (GeneID), the '_at' set with the highest overall expression level in the three cell lines was chosen. If no '_at' sets were available, one of the sets was selected at random. The '_at' sets are designed to recognize one unique transcript only. Each gene was assigned to a biological process and a hypergeometric distribution was used for the statistical evaluation of enriched terms. Affected biological processes were determined by using the iGA (iterative Group Analysis) algorithm by Breitling *et al.* (2004). For a given gene class, iGA computes the minimal class-wise hypergeometric "p-value". The significance of this statistical indicator was here assessed by comparing its value against a distribution of 10 000 random permutations of the data. GO terms with a permuted p-value of less than 0.01 were considered significant.

In addition, the most detailed (with the least genes) biological processes were detected by ordering the GO terms in branches according to their parent-child relationships. It was assumed that truly significantly

affected processes should be detected on several levels of this tree-like structure. Therefore, branches with at least three affected terms ($p<0.01$) were identified and the most detailed term containing less than 100 genes was listed.

Furthermore, in this thesis, the results from this analysis were also compared with results from patient samples obtained in a later study (Ruosaari *et al.*, 2008a). In that study, a similar GO analysis was performed on gene expression data from patient samples. Briefly, differentially regulated pathways (GO terms) were identified in normal and tumour tissue of asbestos-exposed as compared to non-exposed patients. To pinpoint the most relevant findings, branches containing at least three significant GO terms were identified and the most detailed terms (containing less than 100 genes) were listed. The significant biological process terms identified in the GO and cluster (see below) analyses on the cell line data were compared with the most relevant biological process terms in the patient data.

3.4.2 Clustering

A differential expression time series for each probe set was formed by subtracting the expression values measured in the non-exposed control from the values in the corresponding asbestos-exposed sample at each time point. Probe sets were excluded from the analysis when none of the time points showed an approximate >1.4-fold ($\sqrt{2}$) difference between the non-exposed and exposed cell samples. Subsequently, genes with similar time dependent expression profiles were clustered.

After the pruning procedures, 7 538 (MeT5A), 12 436 (A549), or 16 640 (BEAS-2B) probe sets remained. Each remaining probe set was treated as the sole representative of a gene. The cluster analysis included a total of 19 710 out of the 54 675 different probe sets on the Affymetrix array, in at least one cell line.

The remaining data sets from each cell line were clustered using an algorithm specifically designed for short time series expression data (Ernst *et al.*, 2005; Korpela *et al.*, 2006). However, significant clusters were not grouped as in the original paper. Each probe set was assigned to one of 50 model profiles during the clustering procedure. Using a permutation test, the algorithm labelled some clusters as being statisti-

cally significant ($p<0.05$, Bonferroni corrected), based on their expected and realized number of probe sets.

In addition, enriched biological processes (GO) as well as enriched chromosomal regions (both referred to as "terms" in the following section) for each cluster were listed. The probability of having at least the observed number of probe sets associated with a given term, assuming a random selection of probe sets, was calculated. All probe sets on the microarray (54 675) were used as a reference set. The enrichment of the terms in each gene cluster was evaluated by computing p-values from the hypergeometric distribution.

In this thesis, the enriched GO terms identified in the significant clusters of each cell line were also compared to the results from the GO analysis in patient samples (Ruosaari *et al.*, 2008a), as described above in Section 3.4.1.

3.4.3 Enriched chromosomal regions

A canonical correlation analysis (CCA)-based method (Hotelling, 1936) was performed on the A549 and BEAS-2B cell lines combined. CCA identifies correlations, i.e. shared variations between two data sets. The MeT5A cell line was excluded from the analysis due to the scarcity of time points in comparison to the other two cell lines. Multiple probe sets corresponding to the same gene were treated as in the GO analysis described above. Based on the results from CCA the genes were ordered according to their contribution to the dependencies of the two data sets, which was measured by the squared sum of CCA projection scores. Finally, enrichment in 307 chromosome bands (Bioconductor) was tested. The p-values were evaluated by permutation testing as in the GO analysis.

RESULTS

1. Asbestos-related genetic alterations in lung cancer

1.1 Genome-wide copy number alterations (I)

Typical patterns of alterations for different histological types of lung cancer were identified with cCGH. SCLC showed the highest number of alterations, irrespective of exposure. A higher number of CNA were detected in the exposed than in the non-exposed group in all histological types, except SCC (Table 4). The tumour stage-groups I to II and III to IV also showed more CNA in the tumours from exposed patients compared to those from non-exposed patients (Table 4). The sole specific alteration that seemed to differ significantly between the asbestos-exposed and the non-exposed groups was a gain at the minimal overlapping region 2p23. This gain was present in 57% (8/14 cases) of the exposed and in 14% (2/14 cases) of the non-exposed patients' tumours (p=0.025). In 7 out of these 8 exposed cases, the gain affected also 2p22 and in 4 cases 2p21.

All the large and most prominent alterations seen with cCGH were also detected by aCGH, which in addition detected some smaller alterations, such as a relatively frequent amplification at 12q13.3–14.1 (Wikman *et al.*, 2005). The aCGH results were, however, not analyzed on an individual level, since our goal was to identify differences between the asbestos-related and non-related lung tumours. An analysis at the group level does not require any *a priori* knowledge of the type of alteration in individual cases. Thus, no patient-specific alterations were listed. A combined statistical analysis on the data revealed 18 regions, in which

the DNA copy number between the exposed and non-exposed groups differed significantly (Figure 9 and Table 3 in Study I). None of these regions seemed to harbour high-level DNA copy number changes, but instead low-level gains or losses. The median size of the asbestos-associated regions was 1.74 Mbp. The most significant differences were detected in the regions 2p21–p16.3, 5q35.3, 9q33.3–q34.11, 9q34.13–q34.3, 11p15.5, 14q11.2 and 19p13.1–p13.3 ($p<0.005$, Table 3 in Study I).

In addition, eleven fragile sites, two of which were situated at the 9q region, coincided with the 18 asbestos-associated regions ($p=0.08$).

Table 4. Median number of CNAs detected in asbestos-exposed and non-exposed patients tumours using cCGH.

	Number of samples		Median number of CNA	
	Exposed	Non-exposed	Exposed	Non-exposed
Histology				
AC	5	6	6	1
SCC	4	4	1,5	10
AC/SCC	1	1	5	4
LCLC	3	2	5	1
SCLC	1	1	23	14
Stage				
I–II	8	8	3,5	2
III–IV	6	6	6	2,5
All	14	14	5	2

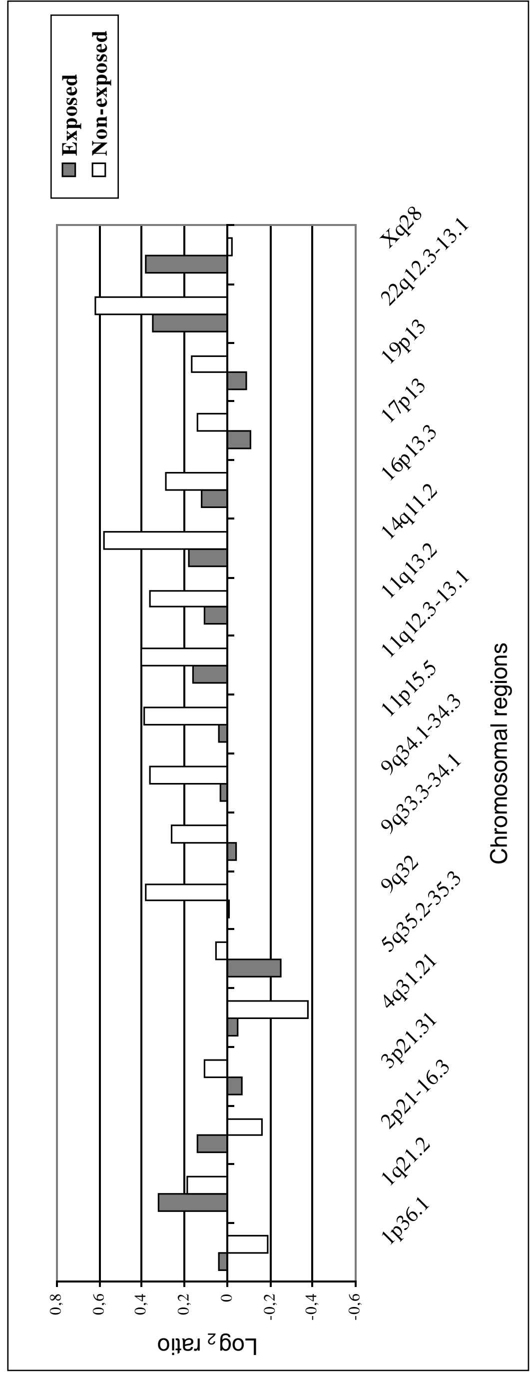


Figure 9. **Differential CNA identified with aCGH in asbestos-exposed and non-exposed patients' tumours.** The Y axis shows average $\log_2$ ratios of all probes in all samples of each group (exposed and non-exposed) and the X axis shows the chromosomal regions with significant differences between the groups.

1.2 Genetic alterations at 9q (II)

1.2.1 Allelic imbalance

Fifteen microsatellite markers were used to identify allelic imbalance (AI) at 9q31.3–34.3 in lung cancer samples from 29 non-exposed and 23 asbestos-exposed patients. The frequency of heterozygosity (i.e. informativity) for each marker correlated well with the reported degree of heterozygosity.

AI was detected in 27–74% of the samples, depending on the marker. All histological tumour types showed some degree of AI. Microsatellite instability (MSI) was detected in 47% (7/15) of the markers of one tumour with SCLC histology from a non-exposed patient. The same case had previously been identified to harbour MSI with the colon MSI marker BAT-26 and with microsatellite markers at 19p13 (Wikman *et al.*, 2007).

A higher frequency of AI was detected with all markers in the asbestos-exposed (42%–90%) than in the non-exposed (10%-65%) patients´ samples. Considering the whole region, 9q31.3–34.3, AI (AI in >25% of the markers) was detected in all (17/17) of the exposed cases and in 64% (14/22) of the non-exposed cases (p=0.005, Fisher's exact test). In addition, a small region at 9q33.1 (base pairs 121021696-121169435) was tested separately. This region included the marker showing the most significant asbestos-associated AI (TC-repeat, Table 5) compared to all the other markers, as well as two adjacent markers (AC-repeat and D9S195, Table 5). Significantly more AI (AI in >1 of at least 2 informative markers) at this locus was detected in the exposed patients' tumours (73%, 11/15) in comparison to the non-exposed patients' tumours (21%, 4/19; p=0.002, X^2-test, Figure 10). The different histological types could not be tested separately due to the small number of samples; however a similar trend could be seen among all types and especially in AC (AI in 0% [0/8] of the non-exposed and 50% [3/6] of the exposed).

Table 5. Allelic imbalance (AI) and copy number alterations (CNA) at 9q.

Chromosomal region	Microsatellite marker	Allelic imbalance				FISH probe	Copy number alteration							
		Asbestos-exposed		Non-exposed			Asbestos-exposed		Non-exposed		Asbestos-exposed		Non-exposed	
		All histological types					All histological types				NSCLC[1]			
		AI/n	%	AI/n	%		CNA/n	%	CNA/n	%	CNA/n	%	CNA/n	%
9q31.3	D9S1675	6/11	55	4/12	33									
	D9S1683	4/6	67	4/9	44									
9q32	D9S930	15/18	83	13/20	65									
	D9S289	14/17	82	13/20	65	RP11-10i9	7/20	35	9/25	36	7/17	41	9/22	41
	D9S302	13/19	68	16/25	64	RP11-357D21	5/23	22	7/25	28	4/20	20	6/21	29
9q33.1	D9S1776	5/12	42	1/10	10									
	D9S170	5/9	56	4/9	44									
	D9S1872	6/11	55	7/13	54									
	TCrepeat	12/15	80	6/17	35[2]	RP11-440N22	16/44	36	9/42	21	15/37	41	6/34	18[3]
	ACrepeat	8/10	80	5/9	56									
	D9S195	12/19	63	9/22	41									
	D9S1116	12/18	67	11/22	50									
9q34	D9S1831	10/16	63	11/24	46	RP11-228B15	5/11	45	4/11	36	5/10	50	4/10	40
	D9S1793	9/10	90	11/18	61	RP11-816F8	1/7	14	2/13	15	1/6	17	2/10	20
	D9S1838	11/16	69	12/24	50	RP11-100C15	5/23	22	6/24	25	5/20	25	6/20	30

[1] Three major histological types of non-small cell lung cancer (AC, SCC and LCLC)
[2] p = 0.01 for the frequency of AI between asbestos-exposed and non-exposed.
[3] p = 0.03 for the frequency of CNA in NSCLC between asbestos-exposed and non-exposed
bold text - microsatellite markers used to compare the frequency of AI at 9q33.1 between asbestos-exposed and non-exposed patients samples (Figure 10).

Exposure	Histology	9q33.1 TCrepeat	9q33.1 ACrepeat	9q33.1 D9S195	AI/n	AI > 1 marker
Non-exposed	AC				0/2	21%
					0/2	
					0/3	
					1/2	
					1/2	
					1/2	
					1/2	
					1/2	
	SCC				0/2	
					1/2	
					1/2	
					1/2	
					1/2	
					2/2	
					2/2	
					2/2	
					2/2	
	LCLC				0/2	
	SCLC				0/2	
Exposed	AC				0/2	73%
					1/2	
					1/2	
					2/3	
					3/3	
					3/3	
	SCC				**2/2**	
					2/2	
					2/2	
					3/3	
	LCLC				1/2	
					2/3	
					2/3	
	SCLC				**2/2**	
	AC/SCC				**2/3**	

Figure 10. **Allelic imbalance (AI) at 9q33.1 in the tumours of asbestos-exposed and non-exposed patients.** The column denoted AI/n shows the number of markers with AI/number of informative markers. Black, AI; white, no change; gray, non-informative/no result available; **bold**, AI in >1 marker.

1.2.2 Copy number alterations

Six BAC probes and a centromere 9 probe were used to analyze CNA at 9q32–34.3 with FISH. CNA at the locus were detected in 15–41% of the tumour samples, depending on the probe (losses in 5–27% and gains in 7–14%).

At 9q33.1, losses were more frequent than gains in the three major histological types of NSCLC, i.e. AC, SCC and LCLC. In the other rarer NSCLC types and in SCLC, losses and gains could be detected with equal frequencies. Thus, we decided to analyze the three major types of NSCLC as a group. SCLC and rare histological types were not tested separately due to the small number of samples.

Overall, depending on the probe, CNA at 9q were detected in 14–45% of the asbestos-exposed patients' tumours and in 15-36% of the non-exposed patients' tumours. Among the NSCLC tumours, significantly more frequent CNA at 9q33.1 (RP11–440N22) was detected in the tumours of asbestos-exposed (41%, 15/37) than in those of non-exposed patients (18%, 6/34; p=0.03, X^2-test; Table 5). The same trend could be seen among all histological types with the difference being most significant among AC, 40% (6/15) in exposed and 6% (1/16) in non-exposed (p=0.04, Fisher´s exact test). Similar results were obtained both with the fresh-frozen and the FFPE samples when analyzed separately (data not shown). A dose-dependent trend could be observed with increasing pulmonary fibre count of the patients and CNA at the locus was detected in 18% (6/34) of the tumours from non-exposed patients, in 35% (8/23) of the tumours from patients with between 1 and 9.9 million fibres/g and in 50% (7/14) of the tumours from patients with a pulmonary fibre count of 10 million fibres/g or more (p=0.03, exact Cochran-Armitage trend test, Figure 12a). The trend was also significant among the AC tumours: CNA in 6% (1/16) of the tumours from non-exposed patients, in 25% (2/8) of the tumours from patients with 1 to 9.9 million fibres/g and in 57% (4/7) of the tumours from patients with 10 million fibres/g or more (p=0.01, exact Cochran-Armitage trend test, Figure 12b). A similar, but non-significant trend (p=0.10) was seen when the SCLC and rare histological types were included in the analysis (data not shown).

1.3 Genetic alterations at 2p (III)

1.3.1 Allelic imbalance

Fourteen microsatellite markers were used to identify AI in 27 lung tumours from exposed and non-exposed patients. The heterozygosity rates of the markers correlated well with the reported frequencies.

AI was detected in 16-61% of the samples, depending on the marker. Microsatellite instability (MSI) at 2p was observed in two ACs (one asbestos-related and one non-related) and in one non-asbestos-related SCLC.

With the exception of one marker all of the other markers displayed an equal or higher frequency of AI in the asbestos-exposed patients' (18–88%) than in the non-exposed patients' (0–50%) tumours. At 2p16.3 (D2S123), AI occurred in 63% (5/8) of the asbestos-exposed and in 0% (0/5) of the non-exposed patients' tumours (p=0.08, Fisher's exact test). The markers D2S2739 and D2S2251 adjacent to D2S123 at 2p16 showed similar trends of asbestos association (Table 6).

Overall, AI was more frequent (AI in >25% of the markers) in the lung tumours of asbestos-exposed (11/13, 85%) than in those of non-exposed patients (4/12, 33%, p=0.02, Fisher's exact test). In the region 2p16, which included five markers, the difference was even more significant, 67% (8/12) of the exposed and 7% (1/14) of the non-exposed patients tumours showed AI (p=0.003, Fisher's exact test; Figure 11).

1.3.2 Copy number alterations

Five BAC probes and a centromere 2 FISH probe were used to obtain DNA copy numbers at 2p21–p16 in 151 lung tumours from exposed and non-exposed patients.

CNA were detected in 16-49% of the tumours, depending on the probe at the 2p region (losses in 1–15% and gains in 7–48%). The probes at 2p21 revealed mainly gains (33–48%, depending on the probe), while the probes at 2p16 showed losses and gains at an approximately equal frequency (losses in 7–15% and gains in 7–14%, depending on the probe). Therefore, 2p21 and 2p16 were analyzed separately.

Table 6. Allelic imbalance (AI) and copy number alterations (CNA) at 2p

Chromosomal region	Microsatellite marker	Alelic imbalance				FISH probe	Copy number loss							
		Asbestos-exposed		Non-exposed			Asbestos-exposed		Non-exposed		Asbestos-exposed		Non-exposed	
		All histological types					All histological types				Non-AC			
		AI/n	%	AI/n	%		loss/n	%	loss/n	%	loss/n	%	loss/n	%
2p22.1	D2S2328	2/11	18	1/8	13									
2p21	D2S2259	5/9	56	3/7	43									
	D2S119	5/10	50	1/8	13									
	D2S2298	4/10	40	3/9	33									
	D2S2174	3/5	60	3/7	43									
	D2S2240	5/10	50	4/10	40									
	D2S2378	7/8	88	4/10	40	RP11-183P21	2/44	5	1/38	3	1/24	4	0/23	0
	D2S2182	1/3	33	3/6	50									
	D2S391	4/7	57	3/7	43	RP11-963J22	0/37	0	1/38	3	0/22	0	0/24	0
2p16.3	**D2S2739**	8/11	73	3/9	33	**RP11-703K23**	8/70	11	1/64	2[2]	6/44	14	0/42	0[3]
	D2S123	5/8	63	0/5	0[1]	**RP11-347F1**	8/47	17	4/48	8	3/27	11	2/29	7
2p16.2	**D2S2251**	4/7	57	2/9	22									
	D2S2153	3/8	38	3/8	38	**RP11-1114A19**	5/26	19	3/28	11	4/16	25	0/14	0
2p16.1	**D2S378**	4/9	44	1/10	10									

[1] p=0.08 for the frequency of AI between asbestos-exposed and non-exposed
[2] p=0.09 for the frequency of loss between asbestos-exposed and non-exposed
[3] p=0.03 for the frequency of loss between asbestos-exposed and non-exposed
bold text - microsatellite markers and FISH probes used to compare the frequency of AI and loss, respectively, at 2p16 between asbestos-exposed and non-exposed patients samples (Figures 11 and 13)

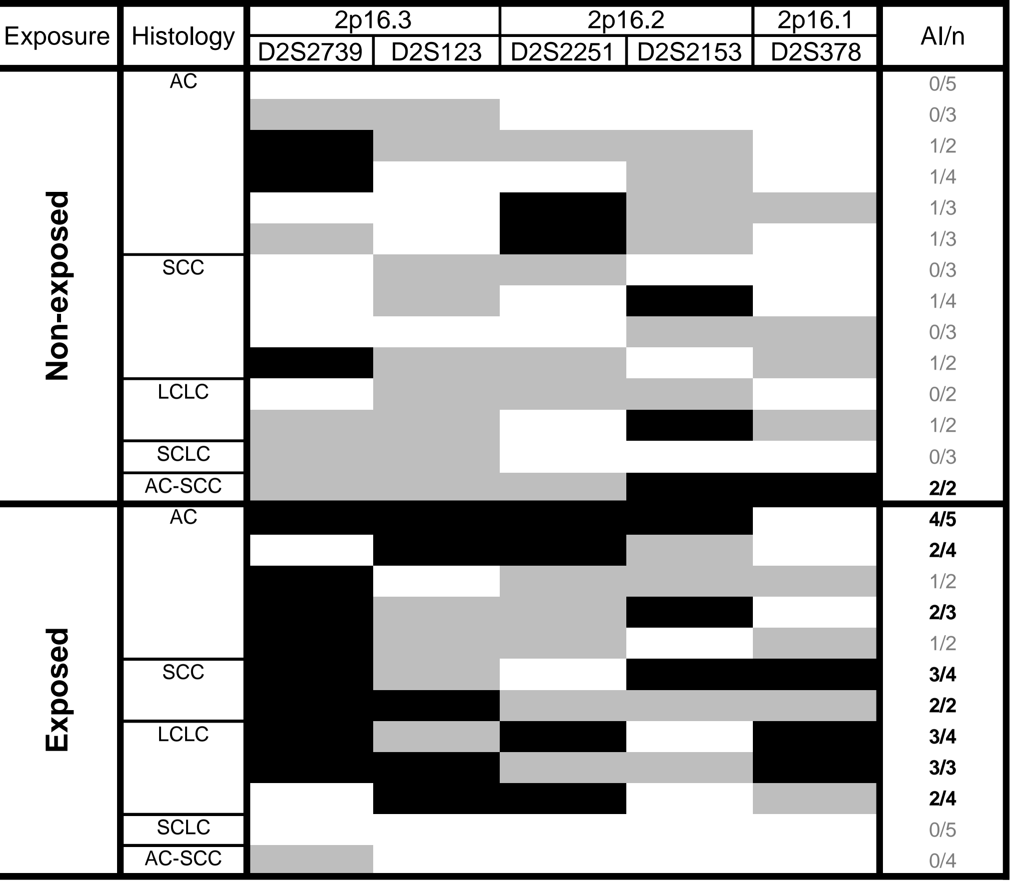


Figure 11. **Allelic imbalance (AI) at 2p16 in the tumours of asbestos-exposed and non-exposed patients.** The column denoted AI/n shows the number of markers with AI/number of informative markers. Black, AI; white, no change; gray, noninformative/no result available; **bold**, AI in >1 marker.

At 2p16.3 (RP11-703K23) more frequent copy number losses were detected in the asbestos-exposed (8/70, 11%) patients' than in the non-exposed patients' (1/64, 2%) tumours (p=0.09, Fisher's exact test, Table 6). Furthermore, the prevalence of at least one copy number loss at 2p16, including the probes RP11-703K23, RP11-347F1, and RP11–1114A19 was significantly higher in asbestos-exposed (20%, 14/70 cases) than in non-exposed patients' tumours (8%, 6/71 cases; p=0.05, χ^2 test). A borderline significant dose-dependence was seen between the losses and the level of asbestos exposure: 8% (6/71) of the non-exposed patients' tumours, 19% (9/47) of the exposed with between 1 and 9.9 million fibres/g and 22% (5/23) of the exposed with 10 million fibres/g or more showed loss at 2p16 (p=0.07, Figure 13a). Although the groups were too small to be statistically evaluated, dose-dependent trends could be seen for all histological types separately, except for AC (data not shown). Thus, the dose-dependence was tested and found to be significant for all non-AC tumours together: 4% (2/50) of the non-exposed patients' tumours, 15% (5/33) of the exposed with between 1 and 9.9 million fibres/g and 23% (3/13) of the exposed with 10 million fibres/g or more showed loss at 2p16 (p=0.03, exact Cochran-Armitage trend test, Figure 13b). In addition, the probe at 2p16.2 (RP11-703K23) displayed a significant difference in frequency of loss between the exposed and non-exposed groups among non-AC tumours (p=0.03, Fisher's exact test, Table 6).

1.4 Polyploidy (II)

Three to five centromere probes for different chromosomes were used to estimate the ploidy level in each individual tumour. Average centromere signal counts indicating polyploidy (range 2.5 to 4.9) were detected in 40% (40/100) of all tumours. A significant difference in the frequency of polyploidy was detected between asbestos-exposed (48%, 28/58) and non-exposed patients' tumours (29%, 12/42; $p<0.05$, X^2-test). The same trend could be seen among all histological types and especially among AC, 48% (11/23) in exposed and 20% (3/15) in non-exposed (p=0.08, X^2-test**)**. Similar results were obtained with both the fresh-frozen and the FFPE samples when analyzed separately (data not shown). No dose-dependent trend could be observed.

2. Asbestos-related gene expression changes in cell lines (IV)

Three different cell lines were exposed to asbestos and the changes in gene expression compared to non-exposed controls during different time points were studied using microarrays. The large set of data obtained by this type of experiment was scrutinized in three different ways to profile the expression patterns induced in the cells by the exposure.

In the first analysis (GO analysis) it was possible to detect 351 detailed Gene Ontology (GO) terms describing biological processes enriched in at least one cell line at any one time point. Each term was associated with between one and 99 genes.

The second analysis clustered genes that showed a similar expression profile during the time series in the asbestos-exposed cells compared to the non-exposed cells. The clusters could be ordered according to their significance, based on the expected and realized number of genes assigned to each cluster. The analysis revealed 12 significant clusters in A549, 16 in BEAS-2B, and 3 in MeT5A. Further interpretation and elucidation of the results focused on the three most significant clusters in each cell line. Enrichment analyses for GO terms and chromosomal loci were performed for each cluster. The clusters contained between 1085 and 2403 genes, between 10 and 56 enriched biological processes and between 8 and 23 enriched chromosomal loci.

The third analysis (CCA) was designed to identify the dependencies between the A549 and the BEAS-2B cell lines. To achieve robust results, it was decided to focus on the interpretation of gene groups rather than on individual genes. It was hypothesized that the asbestos effects would be spatially localized in the chromosomes. Therefore, it was examined whether certain chromosomal regions were enriched in the gene list obtained by CCA. These regions could potentially be specifically affected by asbestos exposure (referred to as asbestos hotspots in the following), and be common to the cell lines. The analysis revealed 21 enriched chromosomal regions containing between 1 and 71 genes contributing to the significant dependencies between the cell lines (p-value<0.03; q-value<0.38; Table 4 in Study IV). The GO terms associated with each gene were also listed in this analysis.

Although the three analyses were not directly comparable, since they focused on different aspects of the data, it was possible to find some similarities that could be considered as being most relevant. Furthermore in this thesis, the enriched GO terms in the cluster analysis and the GO analysis were compared with the most relevant GO terms (biological processes) obtained in a similarly performed GO analysis on gene expression data from patient samples (Ruosaari *et al.*, 2008a). The most relevant results from the cell line study and the unpublished results correlated with patient data are summarized below and in Table 7. The original complete results can be viewed in more detail in the original publication (IV).

2.1 Genes

Two genes, *TXNDC (TMX1)* and *BNIP3L*, were identified as highly significant in all analysis methods. *TXNDC* (*TMX1*) is located at 14q22, identified in the CCA results and was represented in a highly significant gene cluster of all three cell lines. In addition, it is one of the genes contributing to the differential expression of the GO term "positive regulation of transcription, DNA-dependent", which was downregulated in all cell lines after 48h of asbestos exposure. *BNIP3L* is located at 8p21, which was represented in the CCA results. The gene contributed to the significant downregulation of the GO term "negative regulation of survival gene product activity" after 48h of asbestos exposure in all cell lines and was present in highly significant gene clusters of all three cell lines.

2.2 Biological processes

The GO terms at the 1h and 48h time points were compared between the cell lines to identify commonly enriched biological processes. As described in the Methods Section 3.4.1, the focus was resticted to those branches of the GO tree that contained at least three enriched GO terms and listed the most detailed term in the results. No common processes were observed after 1h of exposure, whereas 10 common GO terms were identified after 48h (Table 1 of Study IV). The number of genes belonging to the most detailed process of the branch ranged from 1 to 85.

Table 7. Biological processes found to be dysregulated in both asbestos-exposed cell lines and patient samples.

GO ID	Biological process	Regulation		Other related terms significant in any cell line3 OR patient sample OR **both** (cell line AND tumour OR normal sample of patients)
		cell lines[1]	patients[2] (normal AND tumour samples)	
Ubiquitination				
GO:0006511	ubiquitin-dependent protein catabolism	represented in three gene clusters (6,8,15) of BEAS-2B	Down	**GO:0016567 protein ubiquitination** **GO:0006512 ubiquitin cycle** GO:0043161 proteasomal ubiquitin-dependent protein catabolic process GO:0000209 protein polyubiquitination GO:0031397 negative regulation of protein ubiquitination GO:0030327 prenylated protein catabolic process
G.-protein signaling				
GO:0007214	gamma-aminobutyric acid signaling pathway	represented in one gene cluster (16) of BEAS-2B	Up	**GO:0007187 G-protein signaling, coupled to cyclic nucleotide second messenger** GO:0007202 activation of phospholipase C activity GO:0007189 activation of adenylate cyclase activity by G-protein signaling pathway
GO:0007188	G-protein signaling, coupled to cAMP nucleotide second messenger	up 6h BEAS-2B	Up	
GO:0007200	G-protein signaling, coupled to IP3 second messenger (phospholipase C activating)	down 1h Met5A	Up	
GO:0007223	frizzled-2 signaling pathway	down 1h A549 up 1h BEAS-2B up 6h BEAS-2B	Up	

Table 7. contd.

tRNA metabolism				
GO:0006399	tRNA metabolism	up 1h A549	Down	**GO:0006418 tRNA aminoacylation for protein translation** GO:0006429 leucyl-tRNA aminoacylation GO:0006428 isoleucyl-tRNA aminoacylation GO:0006427 histidyl-tRNA aminoacylation GO:0006434 seryl-tRNA aminoacylation GO:0006436 tryptophanyl-tRNA aminoacylation GO:0006388 tRNA splicing GO:0042780 tRNA 3′-processing
Ion transport				
GO:0006814	sodium ion transport	up 1h BEAS-2B up 6h BEAS-2B down 1h Met5A	Up	GO:0006826 iron ion transport GO:0006825 copper ion transport GO:0006829 zinc ion transport GO:0006813 potassium ion transport GO:0006820 anion transport
GO:0006816	calcium ion transport	up 1h BEAS-2B up 6h BEAS-2B down 1h Met5A	Up	
Sensory perception				
GO:0007608	sensory perception of smell	up 48h A549 up 1h BEAS-2B up 6 BEAS-2B up 48 BEAS-2B down 1h Met5A up 48h Met5A	Up	GO:0050909 sensory perception of taste GO:0007605 sensory perception of sound GO:0050953 sensory perception of light stimulus GO:0007601 visual perception GO:0050906 detection of stimulus involved in sensory perception GO:0050896 response to stimulus
Humoral immune response				
GO:0019735	antimicrobial humoral response (sensu Vertebrata)	up 6h BEAS-2B up 48h BEAS-2B	Up	**GO:0016064 humoral defense mechanism (sensu Vertebrata)** GO:0019731 antibacterial humoral response (sensu Vertebrata)

[1] Study IV
[2] Ruosaari *et al.*, 2008a
[3] Enriched in a gene cluster or significant in the GO analysis

Furthermore, common GO terms were examined in the gene cluster and the GO analyses. Nine biological processes were identified to be significantly enriched in significant gene clusters of at least two cell lines and in at least two time points of the GO analysis (Table 2 of Study IV). The biological processes "positive regulation of transcription, DNA-dependent" and "negative regulation of survival gene product activity", observed in the cluster and GO analyses, were also associated with genes in the regions of the CCA.

In the study on patient samples, eighteen biological processes were found to be the most relevant, when searching for branches with at least three significant GO terms differing between asbestos-exposed and non-exposed patients' tumour and normal tissue (Ruosaari *et al.*, 2008a). Ten of the 18 terms were also significant in the GO or cluster analysis of at least one cell line (Table 7). One of the terms, "sensory perception of smell", was also found to be commonly up-regulated in all cell lines after 48h of exposure. The other terms belonged to branches describing ion transportation (2 terms), G-protein signalling (4 terms), ubiquitination (1 term), tRNA metabolism (1 term) and humoral immune response (1 term).

2.3 Enriched chromosomal regions

The representation of the previously identified chromosomal regions affected by asbestos-related CNA in lung tumours (I), were examined in the results of the cell line experiment. In the most significant gene cluster of A549 (cluster 5) nine of the 18 previously identified asbestos-associated chromosomal regions were enriched, i.e. 11q13, 19p13, 9q34, 16p13, 1p36, 17p13, 5q35, 3p21, and 22q13 ($p<0.01$). In addition, cluster 2 of A549 contained enrichment of genes in 5 regions corresponding to the asbestos-associated regions in lung cancer. The less significant gene clusters 3, 7, and 13 in BEAS-2B contained enrichment of genes in 3 to 5 regions and cluster 2 in MeT5A contained enrichment of genes in 5 regions corresponding to the asbestos-associated regions. The most frequently enriched regions were 19p13, 3p21, 11q13 and 9q34 in all significant clusters of all three cell lines.

Furthermore, one of the most significant regions showing differential copy number changes between the lung tumours of exposed and non-exposed patients was 2p21–16.3. In the cluster analysis it was noted that probe sets located at 2p were substantially enriched in the highly significant gene clusters 9 of A549 ($p=0.00032$) and 5 of BEAS-2B ($p=0.000114$). Notably the specific region 2p21 was enriched in the second most significant cluster (1) of A549 ($p=0.0039$). In addition, the most significant region in the enrichment analysis of the CCA results was 2p22.

DISCUSSION

1. Genomic alterations in asbestos-related lung cancer

The biopersistant asbestos fibres can be present in the lungs for decades and may, during that time, cause several molecular changes in the surrounding cells. *In vitro* experiments have shown that the most typical asbestos-induced DNA alterations are losses and breaks (Huang *et al.*, 1978; Valerio *et al.*, 1980; Hei *et al.*, 1992; Hei *et al.*, 1995; Pelin *et al.*, 1995; Lohani *et al.*, 2002; Xu *et al.*, 2007; Msiska *et al.*, 2009). During the last decade, genome wide analysis methods have proven to be the most efficient in analyzing complex tumours such as lung cancer. Here, by using whole genome aCGH and matched groups of tumour material from asbestos-exposed and non-exposed lung cancer patients, it was possible to identify a set of chromosomal regions differing in copy number between these two groups. Eighteen regions were identified to be affected by differential CNA in tumours from asbestos-exposed patients as compared to tumours from non-exposed patients and 13 of the regions seemed to be affected by losses in the exposed group (I, Figure 9). The seven statistically most significant regions identified were 2p21-p16.3, 5q35.3, 9q33.3-34.11, 9q34.13-34.3, 11p15.5, 14q11.2 and 19p13.1-p13.3.

The aCGH study was performed using reference samples from only two healthy individuals, which may have caused biased results due to the recently discovered polymorphic CNVs. There is a possibility that a duplication/deletion in the reference DNA is interpreted as a loss/gain in the tumour DNA. This is, however, unlikely in this study considering the size of the identified asbestos-related CNAs. The average size of

CNVs has been reported to be within the range of several kilobases (Redon *et al.*, 2006), while the CNA identified here were all in the range of 1 megabase or more (Table 3 in Study I). In addition, the choice of using a combined statistical analysis, where the array results from the two tumour groups were compared to each other at the group level, should eliminate the risk of detecting false positive CNAs due to CNVs, since the same references were used in both groups. The group level analysis may not, however, fully compensate for the noise present in the arrays caused by for example normal cell contamination. Therefore, there is a slight possibility that, for instance a gain in one of the tumour groups could be interpreted as a loss in the other group. Nonetheless, one of the regions which seemed to be lost in the asbestos-related group, 3p21, has previously been reported to be significantly more frequently affected by LOH in the tumours from asbestos-exposed patients compared to those from non-exposed patients (Marsit *et al.*, 2004). Furthermore, the CNAs at 19p13, which seemed to be both lost in the asbestos-related group and gained in the non-related group, were later verified with FISH in a larger study population. (Ruosaari *et al.*, 2008b.) These results indicate that at least in these regions the original array results were correctly interpreted. Previous *in vitro* experiments have shown that asbestos fibres are mainly involved in causing breaks in chromosomes 1 and 9 (Dopp *et al.*, 1998; Lohani *et al.*, 2002) as well as loss of one or both copies of chromosome 5 and monosomy of chromosome 19 (Suzuki *et al.*, 2001). Here, two asbestos-related CNAs were identified in chromosome 1, three in chromosome 9 and one each in chromosomes 5 and 19. Finally, a recent aCGH study on mesothelioma, which is tightly linked to asbestos exposure, identified frequent CNA in 17 chromosomal regions with five of them (1p36.22-36.23, 3p21.31, 9q34.11, 17p13.11 and 19p13.2) corresponding to those identified here in asbestos-related lung cancer (Ivanov *et al.*, 2009).

Another type of microarrays was used to study gene expression changes in asbestos-exposed cell lines and 21 chromosomal regions enriched with genes that contributed to the common asbestos-related responses in two exposed cell lines were identified (Table 4 in Study IV). It can be postulated that these regions may be especially prone to asbestos-induced DNA damage, i.e. asbestos hotspots, which consequently affects the expression of the genes in those regions. Interest-

ingly, the most significant region was 2p22, which is close to the 2p region affected by differential CNA in asbestos-exposed compared to non-exposed patients' tumours.

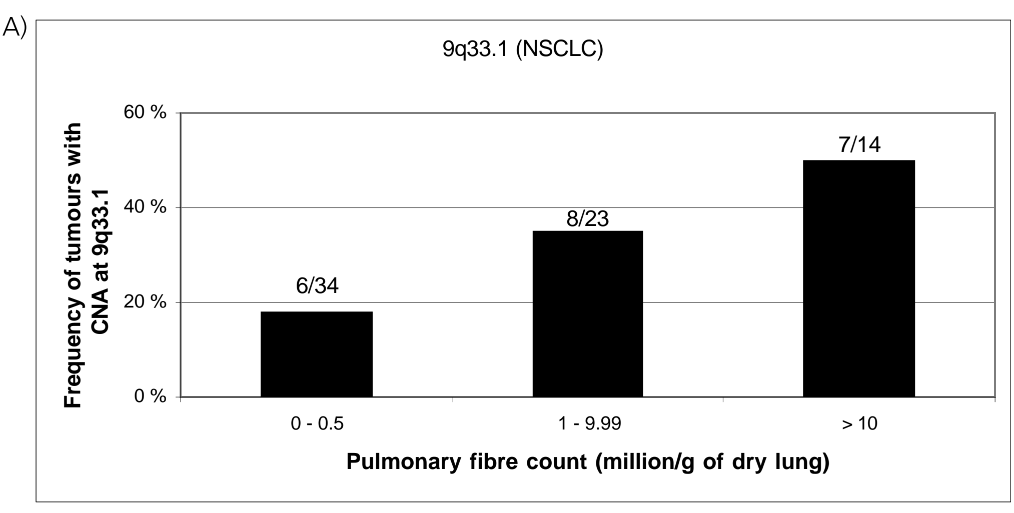
Here, the asbestos-related regions at 2p and 9q were chosen for further analysis by other methods and in larger study populations (II and III).

1.1 Allelic imbalance and copy number alterations at 9q (II)

The long arm of chromosome 9 has been characterized with losses in around 15%, gains in about 7% and AI in 40-60% of lung cancers (Baudis *et al.*, 2001; Beau-Faller *et al.*, 2003; Girard *et al.*, 2000; Merlo *et al.*, 1994). These frequencies correspond well to our results. In addition, it was possible to correlate the results with the amount of asbestos exposure of the patients. The whole 9q31.3-34.3 region harboured >25% AI in all of the exposed and in 64% of the non-exposed patients' samples. In particular, 9q33.1 was significantly more frequently affected by AI in the exposed group (73% versus 21%; Table 5 and Figure 10). Although, it is important to notice that the percentage of non-informative results at 9q33.1 was higher in the non-exposed group than in the exposed group (Figure 10), which may affect the significance of the results. However, with FISH it was also possible to detect more frequent CNA at 9q33.1 in the asbestos-exposed and these alterations showed a dose-dependent association in NSCLC (Figure 12a). Additionally, it was noted that the correlation of CNA at 9q33.1 with asbestos exposure was particularly significant among tumours with AC histology compared to other histological types, although the number of samples was small (Figure 12b).

The region 9q33.1 harbours a tumour suppressor gene, deleted in bladder cancer 1 (*DBC1*), which has been found to be down regulated by several mechanisms in many types of cancer (Gao *et al.*, 2004; Beetz *et al.*, 2005; Gronbaek *et al.*, 2008). The gene has been shown to inhibit cell proliferation by negative regulation of the G_1/S transition of the cell cycle (Nishiyama *et al.*, 2001). In a previous gene expression study on the same patient samples as the ones used here in the aCGH study (I),

A)



B)

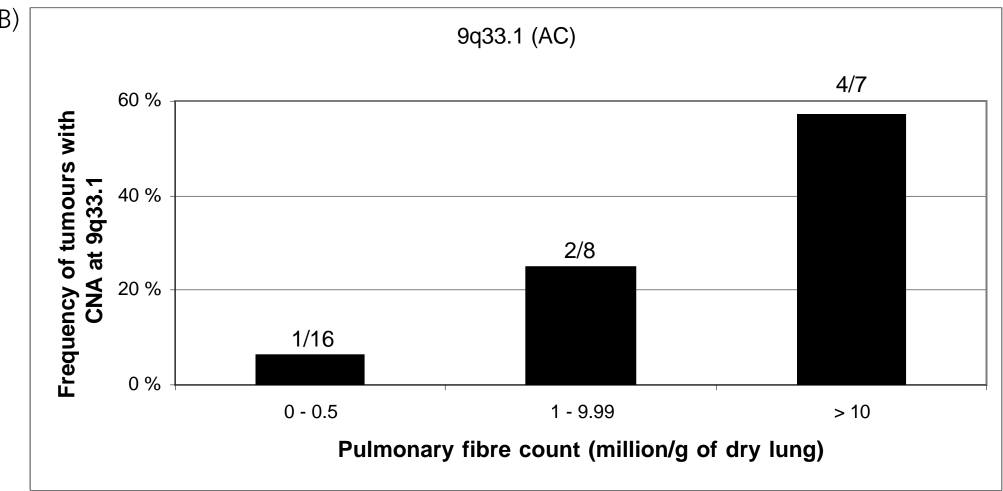


Figure 12. **Frequency of CNA at 9q33.1.** A) The major histologic tumour types of NSCLC (i.e. AC, SCC and LCLC, p=0.03) and B) AC tumours (B, p=0.01) from asbestos-exposed (>10 and 1–9.9 million fibres/g) and non-exposed (0–0.5 million fibres/g) patients. The number of samples with CNA/number of all samples is shown above each column.

low expression of *DBC1* was detected in both normal lung and tumour samples and there was no significant difference between the exposed and non-exposed groups (Wikman *et al.*, 2007). The gene has been found to be frequently methylated and down-regulated in NSCLC as well as in adjacent normal tissue of smokers (Izumi *et al.*, 2005). Thus, it is possible that *DBC1* is central to the development or progress of both asbestos-related and non-related lung cancer, but that the mechanism of down-regulation may be different in the two groups. The combination of smoking and asbestos exposure may increase the probability of a cell acquiring CNA in one allele and methylation in the other, elevating the risk of cancer according to Knudson's two-hit hypothesis (Knudson, 1971). A similar difference between asbestos-related and non-related lung cancer was recently reported for the gene *P16/CDKN2A*. LOH and homozygous deletion of this gene were found to be associated with asbestos exposure, while promoter hypermethylation was correlated with heavy smoking in lung cancer. Consequently, the gene was down-regulated in the tumours of both exposure groups (Andujar *et al.*, 2010).

CNA at 9q has been reported in 10–15% of mesotheliomas (Björkqvist *et al.*, 1998b; Lindholm *et al.*, 2007; Taniguchi *et al.*, 2007). Interestingly, two aCGH studies on mesothelioma discovered 6 of 43 cases with CNA at 9q and 3 of those cases harboured losses initiating at 9q33.1, indicating a potential breakpoint hotspot for asbestos-induced DNA damage (Lindholm *et al.*, 2007; Taniguchi *et al.*, 2007). It is also worth mentioning that the gene *TNC* (*HXB*), which resides at 9q33.1 and is involved in wound healing has been found to be down-regulated in mesothelioma cell lines (Kettunen *et al.*, 2001).

1.2 Allelic imbalance and loss at 2p (III)

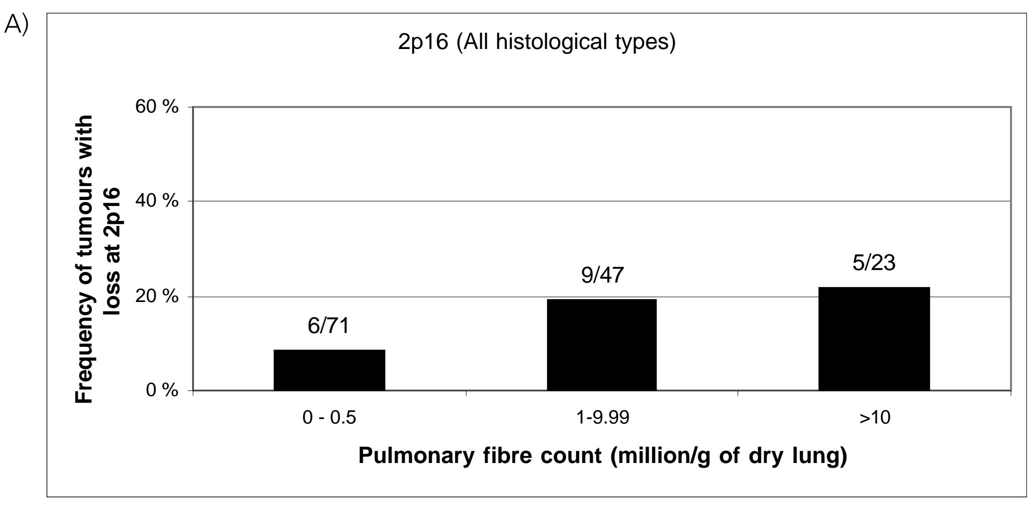
The short arm of chromosome 2 has been reported to be affected by gains in 16.5–50% depending on the study, by losses in approximately 5% (32% in SCLC) and by AI in 25–45% of lung tumours (Balsara *et al.*, 1997; Dehan *et al.*, 2007; Garnis *et al.*, 2006; Michelland *et al.*, 1999; Otsuka *et al.*, 1996; Petersen *et al.*, 1997; Shen *et al.*, 2009; Weir *et al.*, 2007; Wong *et al.*, 2002; Xinarianos *et al.*, 2000; Björkqvist *et al.*, 1998b; Yan *et al.*, 2005; Björkqvist *et al.*, 1998a). Similar frequencies were detected in our samples. Additionally, it was also possible to detect a significant

asbestos association with both copy number loss (20% vs 8% in exposed and non-exposed patients' tumours, respectively) and AI (67% vs 7%, Figure 11) at 2p16. In the AI analysis, the percentage of non-informative results at the 2p16 region was higher among the nonexposed cases (Figure 11), similarly as at the 9q33.1 region, which may affect the significance of the result. Nevertheless, a dose-dependent association was also observed between FISH-detected loss at 2p16 and asbestos exposure and the trend was especially significant among tumours with non-AC histological type (Figure 13). In the genome wide aCGH study (I) an association was detected between fragile sites and the asbestos-associated CNAs. The aphidicolin-sensitive fragile site, FRA2D is located at 2p16.2 adjacent to the microsatellite marker D2S123 (Schwartz *et al.*, 2006), which in comparison with the other markers showed the most significant difference in AI between the exposed and non-exposed groups (63% and 0%, respectively, Table 6). Thus, the fragile site may be involed in the mechanism of asbestos-induced damage in this region. It is worth mentioning that the overall frequency of AI at D2S123 (38%) correlated well with a previously reported frequency in lung cancer (30%, Wong *et al.*, 2002).

Copy number loss as well as LOH and UPD at 2p16 has been detected in other types of cancer such as ovarian carcinoma, meningioma and hereditary non-polyposis colorectal cancer (Lucci-Cordisco *et al.*, 2005; Krupp *et al.*, 2008; Plisiecka-Halasa *et al.*, 2008). The cancer syndrome Carney complex (CNC), which is associated with multiple neoplasms, has also been characterized with gains and losses at 2p21-p16 (Matyakhina *et al.*, 2003). Furthermore, the precursor of miR-216, located at 2p16.1, has been found to be down-regulated in lung cancer (Yanaihara *et al.*, 2006). It is intriguing to speculate that miR-216 is involved in the tumorigenesis of both asbestos-related and non-related lung cancer, but once again by separate mechanisms, similarly as the previously mentioned *p16/CDKN2A* gene.

In the cell line experiment (IV), significantly enriched chromosomal regions were also tested for in the most significant gene clusters, i.e. those clusters that contained more similarly expressed genes than expected. Consequently, in addition to identifying an asbestos hotspot at 2p22, genes located at 2p were found to be substantially enriched in highly significant gene clusters of the two epithelial cell lines, A549

A)



B)

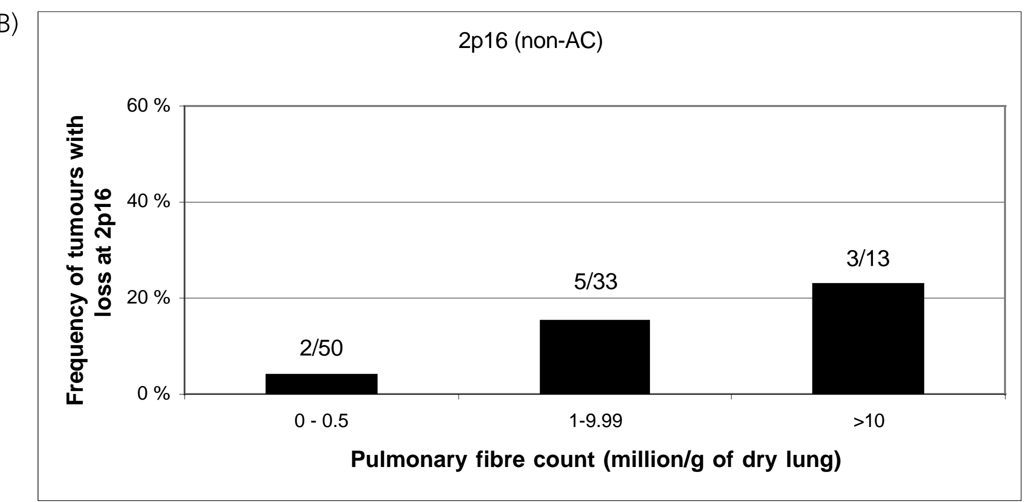


Figure 13. **Frequency of loss at 2p16.** A) All histological tumour types (p=0.07) and B) non-AC tumours (p=0.03) from asbestos-exposed (>10 and 1–9.9 million fibres/g) and non-exposed (0-0.5 million fibres/g) patients. The number of samples with loss/ number of all samples is shown above each column.

and BEAS-2B. These results could indicate that either alterations at 2p and consequently changes in the expression of the genes in the region are specifically involved in the asbestos-related cellular responses or *vice versa*. I.e. asbestos-related gene expression changes in the region may sensitize it to genomic alterations, while it is in an open conformation, allowing transcription (Obe *et al.*, 2002).

1.3 Chromosomal regions enriched with asbestos exposure response genes (IV)

Many of the regions identified in the cell line experiment as potential asbestos hotspot regions correspond to regions frequently found to be affected by CNA in mesothelioma, i.e. 4q, 6q, 8p, 8q22-23, 9p13, 14q and 15q (Kivipensas *et al.*, 1996; Lindholm *et al.*, 2007; Baudis, 2009). In particular, two regions are worth mentioning, namely 8p21 and 14q22. Both regions contain one interesting gene each. Single genes are usually of less interest in a genome wide analysis such as this one, since significant differences in the expression of single genes are expected to arise at random, due to the large amount of data. Nevertheless, these two may be mentioned due to their high significance in all analyses on all cell lines and because of their asbestos-related functions and relationships with previously identified asbestos-associated genes.

The chromosomal region 8p21 harbours the gene encoding for a BCL2/adenovirus E1B 19kD-interacting protein (*BNIP3L*). *BNIP3L* is a potential tumour suppressor, associated with hypoxia-induced apoptosis of epithelial cells (Krick *et al.*, 2005). The gene was involved in the down-regulation of the biological process "negative regulation of survival gene product activity", i.e. a proapoptotic process, in all cell lines at 48h of exposure (Table 1 in Study IV). Thus, loss of the gene may result in apoptotic bypass.

Region 14q22 harbours the thioredoxin domain containing gene (*TXNDC* also known as *TMX1*). *TXNDC* was found to contribute to the down-regulation of the biological process "DNA-dependent positive regulation of transcription" in all cell lines at 48h exposure (Table 1 in Study IV). Thioredoxin (*TXN*) is involved in the DNA-binding activity of NF-κB, which has been shown to be up-regulated by asbestos exposure (Shukla *et al.*, 2003b). *TXNDC* has been found to have *TXN*-like

functions and may in addition be involved in relieving endoplasmic reticulum (ER) stress, such as that induced by asbestos exposure (Matsuo *et al.*, 2001; Nozaki *et al.*, 2001). Thus, loss of the gene may contribute to inability of the cell to protect itself against ER stress.

1.4 Polyploidy and aneuploidy

The ploidy level of the tumours was analyzed using five centromere FISH probes and significantly more frequent polyploid tumours were detected among the asbestos-exposed (48%) than the non-exposed (29%) patients. The difference was particularly significant among tumours with AC histology compared to other histological types (48% vs 20% in exposed and non-exposed, respectively). Asbestos is known to cause chromosome missegregation and the fibres have been shown to induce polyploidy by sterically blocking cytokinesis *in vitro* (Jensen et al., 1996; Jaurand, 1997). Polyploidy has been suggested to act as a catalyst to promote further CIN and aneuploidy (Holland *et al.*, 2009), which may explain the finding that asbestos-exposed patients' tumours show a larger number of alterations as detected with cCGH (Table 4). However, it is not certain whether polyploidy leads to aneuploidy in cancers or *vice versa*. *In vitro*, asbestos seems to be able to cause both aneuploidy and polyploidy (Jensen *et al.*, 1996; Suzuki *et al.*, 2001). Thus, the chromosomal alterations detected in asbestos-related lung cancer need not only be nonspecific results of polyploidy-directed CIN, but may also be induced in specific regions by asbestos fibres before the development of polyploidy, which has been indicated by the findings at 2p and 9q in this thesis, and at 19p13 (Wikman *et al.*, 2007; Ruosaari *et al.*, 2008b).

Polyploidy may confound the analysis of CNA in specific chromosomal regions, since low level alterations may not be detected as easily by CGH as in diploid genomes. This may explain the difficulty over the years in identifying specific asbestos-associated CNA. Increased ploidy levels and thus increased overall gene copy numbers could contribute to the over-expression of many genes (reviewed in Petersen *et al.*, 2009). However, losses may not affect the expression of genes if the genome is polyploid and an extra copy compensates for the expression of genes in a region showing a one-copy loss. This may explain why asbestos-associated increased expression of the genes in the 2p region

was detected in a study examining the gene expression in the same patients as in the aCGH study (I; Wikman *et al.*, 2007) and at the same time asbestos-associated copy number losses were detected here (III). The losses were defined by calculating a ratio between the locus specific probe and the centromere of chromosome 2, which often implied trisomy or tetrasomy of the chromosome. Thus, one-copy losses would not affect the possible up-regulation of the genes in that region, caused by the polyploidy or aneuploidy.

Due to these complex alteration patterns, asbestos-related CNA may not always cause a differential expression profile compared to non-asbestos-related lung cancer, which is also demonstrated by the previously mentioned findings on the *P16/CDKN2A* and *FHIT* genes. These genes are frequently down-regulated in lung cancer, but by different mechanisms in those that are asbestos-related, than in those that are not (Pylkkänen *et al.*, 2002a; Andujar *et al.*, 2010; Nelson *et al.*, 1998). Instead the correlations are simply biomarkers of the exposure and could, regardless of their biological effects, be useful in identifying these tumours among lung cancers without asbestos association.

2. Dysregulated biological processes in asbestos-related lung cancer

Several biological processes (GO terms) that were differentially regulated in asbestos-exposed cell lines compared to non-treated cells were identified. Many of those processes are probably due to the triggering of various universal cellular responses to a toxic substance. However, by using three different cell lines it was anticipated that it would be possible to pinpoint the specific asbestos-related changes and to be able to overlook the cell-type or malignancy-associated changes. Indeed, the number of shared differentially expressed processes increased with exposure time, indicating that the response to asbestos exposure occurs through the same pathways in all three cell lines. However, with *in vitro* experiments, one can naturally never precisely reflect the actual conditions and mechanims *in vivo*. Therefore, in this thesis, the complete results from the cell line experiment were correlated with those of a similar study performed later on normal and tumour samples from lung

cancer patients with and without past asbestos exposure (Ruosaari *et al.*, 2008a). The main aim of this thesis has been to identify asbestos-related alterations in lung cancer and therefore the discussion here will focus on the processes found to correlate between the cell lines and the patient samples.

Ten biological processes were found to correlate between at least one of the cell lines and the patient samples (Table 7). Most of them were different from those found in common between the three cell lines themselves. This was to be expected since the differentially regulated processes found in the tumours of asbestos-exposed patients are anticipated to be associated with cancer, while those discovered in the cell lines are more likely to be early asbestos-related changes not yet involved in the carcinogenic processes. One should keep in mind that the development of clinical cancer associated with asbestos exposure appears decades after the initial exposure. The processes found in common between the two studies could be hypothesized to be related to the very early carcinogenic effects induced by asbestos exposure.

Many of the processes that correlated between the three cell lines at 48h have previously been reported to be asbestos-related, based on other types of *in vitro* experiments, i.e. NF-κB, MAPK and mitochondrial pathways (reviewed in Shukla *et al.*, 2003b; Kamp, 2009). These pathways were not found to be significantly dysregulated in the patient samples. However, another of the processes shared by the three cell lines at 48h, "sensory perception of smell" (Table 1 in Study IV), was also found to be highly significant in the asbestos-exposed patients' samples. It was the only up-regulated pathway detected in all three cell lines at 48h as well as at other time-points of all cell lines and it was also up-regulated in both the tumour and normal samples of the asbestos-exposed patients. This finding was slightly surprising, but the significance of its discovery was strengthened by the dysregulation of related processes in either the cell lines or the patient samples, namely "sensory perception of sound", "-taste" and "-light" (Table 7, Figure 14). Interestingly, the majority of the genes involved in the perception of smell and taste are those encoding for G-protein coupled olfactory and taste receptors. This could mean that the dysregulation of G-protein signalling, which was also highly represented in the list of processes found in both cell lines and patient samples (Table 7), may affect the regulation of these

receptors. G-proteins have been proposed to be involved in the respiratory burst (release of ROS) caused by asbestos (Elferink *et al.*, 1988). Furthermore, the genes implicated in the sensory perception of smell could be involved in other yet unidentified processes. In fact, prostate cancer has been characterized with the over-expression of an olfactory receptor, *PSGR* (Xu et al., 2000). It is also worth mentioning that three of the asbestos-related CNA regions identified in the aCGH study (I; 16p13.3, 17p13.3 and 19p13.3) contain clusters of olfactory receptors contributing to the up-regulation of the "sensory perception of smell" in the asbestos-exposed cell lines.

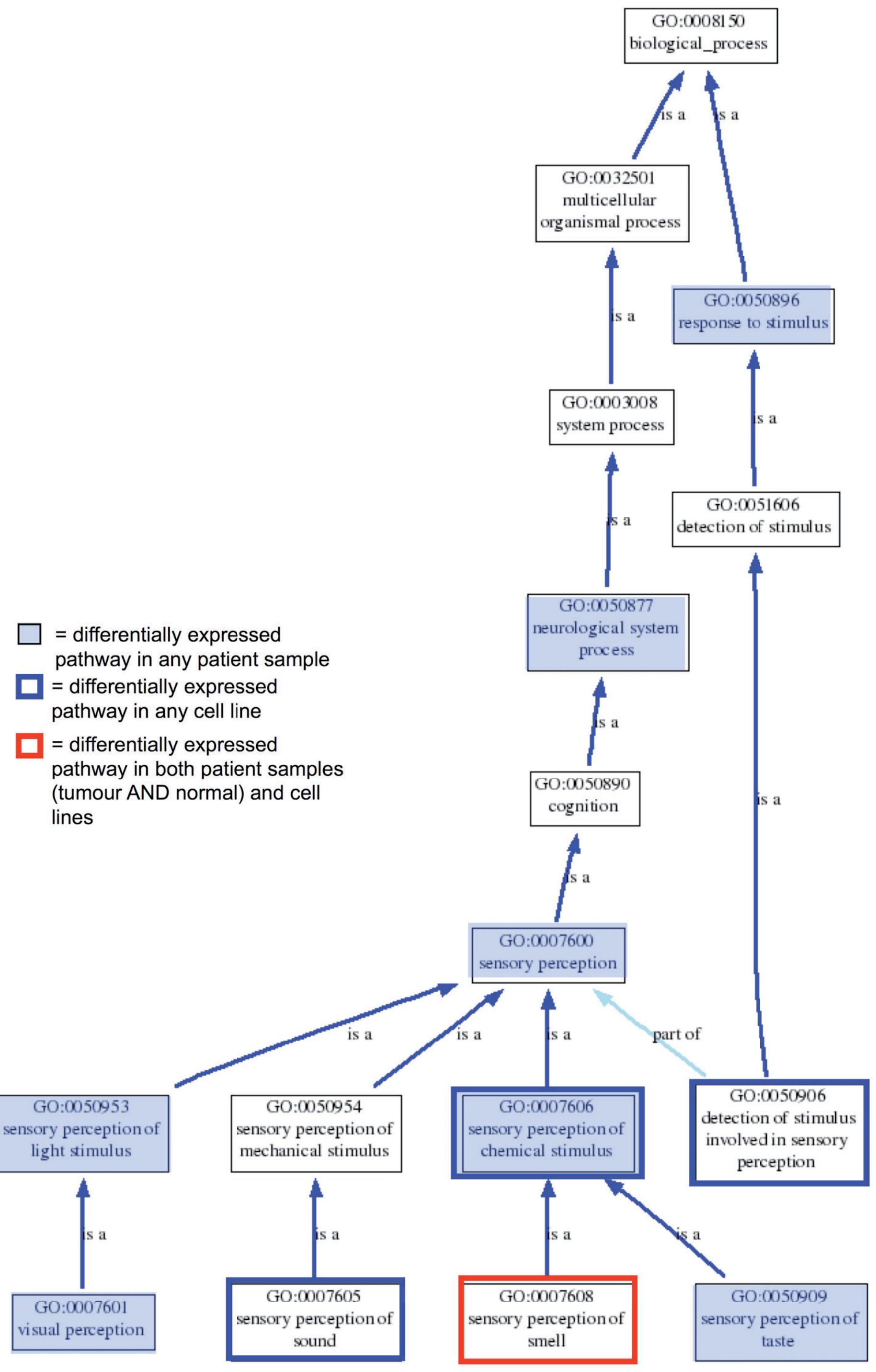
Another process dysregulated in all three cell lines at 48h, i.e. "prenylated protein catabolic process" (Table 1 in Study IV), was closely related to a process found in the patient samples. It belongs to the same parent process ("modification-dependent protein catabolism") as "ubiquitin-dependent protein catabolic process", which was down-regulated in the asbestos-exposed patients' samples (Table 7). Protein ubiquitination regulates various key cellular events, such as DNA repair, cell cycle and apoptosis and the dysregulation of this process has been linked to mesothelioma (Borczuk *et al.*, 2007). Interestingly, ubiquitination is also involved in DNA damage-activated NF-κB, which as previously mentioned is known to be up-regulated by asbestos exposure (Shukla *et al.*, 2003b; Skaug *et al.*, 2009). In addition to the process found in common between the cell lines and the patient data, six other processes related to ubiquitination were also detected in at least one of the cell lines or patient samples (Table 7). The processes were all down-regulated in both patient samples and cell lines, except for "negative regulation of protein ubiquitination", which concordantly showed up-regulation. The expression and protein levels of the ubiquitin-activating enzymes, UBA1 and UBA7, involved in the early stages of protein ubiquitination, were further investigated in the study on patient samples, but neither showed any differences between asbestos-exposed and non-exposed (Ruosaari *et al.*, 2008a). However, several hundreds of enzymes are involved in the process and the role of protein ubiquitination in asbestos-related carcinogenesis cannot be ruled out, based on these results. Indeed, the region 2p16, which was found to harbour asbestos-associated AI and copy number losses (III) contains two genes (*ASB3* and *RPS27A*) involved in protein ubiquitination as well as the previously mentioned miR-216,

which has been predicted to target the ubiquitin-conjugating enzyme *UBE2V2* (Kirschner *et al.*, 2000; Chung *et al.*, 2005; Lewis *et al.*, 2005).

Finally, the biological process of ion transport was also highly represented in both the cell lines and the patient samples (Table 7). Asbestos could be linked to the up-regulation of ion channels through ROS, which has been shown to trigger the opening of mitochondrial channels and mitochondrial depolarization. Indeed, asbestos has been reported to cause a slow and sustained increase in intracellular Ca^{2+} levels and there is some evidence to suggest that the disruption of intracellular Ca^{2+} levels could, together with ROS, be involved in the asbestos-induced DNA strand breaks (reviewed in Shukla *et al.*, 2003b). Dysregulation of ion channels has been proposed to have a role in tumourigenesis and tumour progression (reviewed in Ruosaari *et al.*, 2008a).

Other common processes between the GO analyses in the cell lines and the patient samples were involved in tRNA metabolism and humoral immune response. Both have been implicated in cancer (Tan *et al.*, 2007; Park *et al.*, 2008).

Figure 14. **Gene Ontology network.** The network illustrates the branch leading to the biological process "sensory perception of smell" (red box) found to be commonly up-regulated in asbestos-exposed cell lines and samples from asbestos-exposed lung cancer patients. Terms represented either in the cell lines, patient samples or both are marked as described in the figure. The figure has been generated using the AmiGO visualization tool (Carbon *et al.*, 2009). →


GO:0008150
biological_process
is a
is a
GO:0032501
multicellular
organismal process
is a
GO:0050896
response to stimulus
GO:0003008
system process
is a
is a
GO:0051606
detection of stimulus
GO:0050877
neurological system
process
= differentially expressed
pathway in any patient sample
= differentially expressed
pathway in any cell line
= differentially expressed
pathway in both patient samples
(tumour AND normal) and cell
lines
is a
GO:0050890
cognition
is a
is a
GO:0007600
sensory perception
is a
is a
is a
part of
GO:0050953
sensory perception of
light stimulus
GO:0050954
sensory perception of
mechanical stimulus
GO:0007606
sensory perception of
chemical stimulus
GO:0050906
detection of stimulus
involved in sensory
perception
is a
is a
is a
is a
GO:0007601
visual perception
GO:0007605
sensory perception of
sound
GO:0007608
sensory perception of
smell
GO:0050909
sensory perception of
taste

CONCLUSIONS AND FUTURE PROSPECTS

Many studies have attempted to explain the extremely high risk of lung cancer among smoking asbestos workers. Nevertheless, none of them have led to truly efficient screening methods that could reduce mortality even though the risk groups are very well known, in contrast to many other types of cancer risk groups. Furthermore, there are no molecular markers clinically available for identifying asbestos-related lung cancer even though asbestos is well known to be able to evoke alterations in the DNA, one of the hallmarks of cancer initiation and progression (Hanahan and Weinberg, 2000). Around ten years ago, it was estimated that past asbestos exposures in Western Europe alone will be responsible for at least a quarter of a million deaths from lung cancer and an equal amount of deaths from mesothelioma over the next 35 years (reviewed in LaDou, 2004). Currently, clinical identification of asbestos-related lung cancer relies on occupational history and pulmonary asbestos fibre counts and thus, diagnosis would greatly benefit from the identification of a specific molecular marker (Kamp, 2009).

In this thesis, asbestos-related lung cancer was characterized at a molecular level in an attempt to pinpoint specific asbestos-related alterations that differ from lung cancer in patients without this type of exposure. Specific chromosomal alterations were identified in 18 regions (I) and the alterations in two of the regions, 2p16 and 9q33.1, were validated and characterized in detail (II and III). It is believed that a combination of several asbestos-associated molecular changes, such as CNA, could represent a feasible method for differentiating asbestos-related lung cancers from those that are not related to asbestos exposure. Here, significant differences in the frequency of AI and CNA at 2p16 and 9q33.1 were found between the tumours of exposed and non-exposed patients. In

addition, the frequency of polyploidy was significantly higher among the tumours of asbestos-exposed patients. At 9q33.1 the differences were especially noteworthy among tumours with AC histology (Figure 12), while 2p16 exhibited the most considerable differences among the tumours with non-AC histology (Figure 13). Thus, in combination the alterations at these two regions and previously identified alterations at 19p13 (Ruosaari *et al.*, 2008b), as well as polyploidy may be useful in a test identifying asbestos-related lung cancer irrespective of histological type. Finally, we were also able to experimentally identify changes in the expression of genes in specific pathways and chromsomal regions that correlated with findings in patient samples, thus validating them for further studies (IV).

Some future prospects include (i) validating the asbestos-related chromosomal alterations in combination and in a large study population, (ii) performing transcription factor binding site analyses on the gene expression alterations in patient samples and asbestos-exposed cell lines, (iii) profiling the miRNAome in asbestos-related lung cancer, (iv) genome wide association studies of CNVs associated with asbestos-related lung cancer and (v) functional studies based on the most relevant findings.

Transcription factor binding site analyses on gene expression data can be used to identify dysregulated transcription factors that consequently cause the dysregulation of their target genes (Yap *et al.*, 2005). This kind of analysis could be performed on a group of genes showing similar expression e.g. during a time series experiment, such as the gene clusters generated here in the experiment on asbestos-exposed cell lines (IV).

MiRNAs are rapidly becoming an attractive method for profiling cancers. The miRNAome has proved to be more efficient in distinguishing between tumour histology, classifying undifferentiated tumours and predicting patient outcome, than traditional gene expression profiling of mRNAs. The majority of, if not all, cellular processes are likely to be regulated by miRNAs and changes in the expression of these genes are a hallmark of several diseases, including cancer. During the past few years several tumour suppressive and oncogenic miRNAs have been identified (reviewed in Croce, 2009). Profiling of the miRNAome in asbestos-related lung cancer would greatly add to the understanding of the molecular alterations in this type of cancer.

CNVs represent a recently discovered type of human genetic variation, i.e. the occurrence of missing or additional segments of DNA which have been found in two or more genomes of healthy individuals. CNVs are likely to play an important role in the susceptibility of cancers and may interfere with the analysis of tumour specific somatic CNAs. During the past few years much attention has been paid to human genetic variation, especially CNVs and there are several large ongoing projects to map these variations (reviewed in Dear, 2009). It would be tempting to perform genome wide association studies of CNVs associated with asbestos-related lung cancer.

Finally, the identified putative biomarkers of asbestos-related lung cancer need to be verified in larger sets of samples and their biological roles in asbestos-related lung carcinogenesis should be clarified using functional experiments. Novel high-throughput methods, such as the next-generation sequencing (NGS) techniques (reviewed in Metzker, 2010) could also be a next step in gathering a better understanding of the chromosomal alterations and their effects involved in asbestos-related lung cancer.

ACKNOWLEDGEMENTS

This work was carried out in the Health and Work Ability Centre of Expertise of the Finnish Institute of Occupational Health (FIOH) and in the Department of Pathology of the Haartman Institute and HUSLAB, University of Helsinki and Helsinki University Central Hospital. I want to thank the Heads and staff of these institutes for providing excellent research facilities. Especially, team leader **Kirsti Husgafvel-Pursiainen** is thanked for her positive attitude towards my studies and for making this work possible. I am grateful for the financial support provided by TEKES, the Finnish Work Environment Fund, Maud Kuistila Memorial Foundation, the Ida Montin Foundation, the Swedish Cultural Foundation in Finland and Nylands Nation. I wish to express my most sincere gratitude to all the people who have contributed to the completion of this thesis and I especially acknowledge:

My supervisors, **Sisko Anttila** and **Sakari Knuutila**, for support and encouragement during the past years. Sisko is thanked for believing in my ideas and for giving me great freedom and independence in my work. I would like to thank Sakari for his never-ending enthusiasm for genetic research on cancer. He has inspired me in my work, time after time.

Harriet Wikman for all her incredible support for so many years. Haiju functioned as the supervisor of my master´s thesis and since then has given me invaluable guidance during my development as a researcher. She is deeply thanked for "hiring" me to the asbestos-related lung cancer-project and for being an unofficial reviewer of this thesis.

The official reviewers, **Janna Saarela** and **Veli-Matti Kosma** for their critical review of this thesis and for their valuable comments that truly helped me improve my thesis.

All the co-authors of the original publications of this thesis, for interesting and enjoyable collaboration. Without them this thesis would not have been possible. Especially **Eeva Kettunen, Salla Ruosaari** and **Pamela Lindholm** are sincerely thanked for their major contributions to studies III, I and IV. I am also grateful to Eeva for all her excellent advice in both scientific and non-scientific topics over the years. Salla is thanked for sharing her extraordinary skills in bioinformatics. **Mervi Aavikko** is thanked for amusing discussions over the microscope and for her contribution to study III. **Leo Lahti** and **Mikko Korpela** are thanked for their invaluable roles and patience during the bioinformatic analyses and interpretation of the results in study IV. **Eeva Kuosma** is thanked for the statistical analyses in studies II and III. **Jaakko Hollmén** and **Samuel Kaski** are acknowledged for providing excellent bioinformatic expertise. I would also like to acknowledge **Antti Karjalainen**, **Kaisa Salmenkivi**, and **Risto Pirinen** for their contributions in the gathering of the patient samples utilized in studies I–III, **Esa Vanhala** for the asbestos fibre count analyses of the samples and **Vuokko Kinnula** for providing the cell line samples examined in study IV.

Ewen MacDonald, for critical linguistic revision of this thesis and for being so quick at it.

Former and present staff in the pathology lab; **Virinder Sarhadi**, **Päivi Tuominen**, **Helinä Hämäläinen**, **Jaana Kierikki**, **Sauli Savukoski**, **Tuula Stjernvall**, **Tuija Hienonen-Kempas**, **Henrik Wolff** and **Laura Teirilä** for creating a friendly and comfortable atmosphere at FIOH. Päivi is especially thanked for excellent technical assistance and for being a great roommate. I am also grateful to Virinder for all the interesting discussions and advice on scientific matters and for her friendship. Tuija is thanked for her contribution to the review article in which a version of Figure 7 in this thesis was originally published.

My fellow researchers and friends on the 2nd and 5th floors at FIOH; **Reetta Holmila**, **Miia Antikainen**, **Kati Hannukainen**, **Hanna Lindberg**, **Ghita Falck**, **Mari Kukkonen**, **Anne Puustinen**, **Satu Hämäläinen, Jaana Palomäki** and **Elina Rossi** for all the stimulating activities at work and after work. **Tuula Suomäki** is also thanked for all her help with the bureaucratical stuff at FIOH.

My colleagues and friends, former and present members of the CMG-group; **Kowan Jee**, **Linda Siggberg**, **Tarja Niini**, **Tarja Nieminen**,

Ioana Borze, **Mohamed Guled**, **Neda Mosakhani**, **Ilari Scheinin**, **Minna Koivumaa**, **Fabricio Passador**, **Anne Tyybäkinoja**, **Samuel Myllykangas, Suvi Savola, Sippy Kaur, Hanna Vauhkonen, Shinsuke Ninomiya**, **Anu Usvasalo**, **Jassu Atiye and Tuija Lundán** for all the help, exciting discussions and recreational activities. Special thanks go to **Pirjo Pennanen** for excellent linguistic review of the original manuscripts included in this thesis. I would also like to thank all the people who worked in the former diagnostics lab of cytomolecular genetics at HUSLAB for teaching me about the cytomolecular genetic methods and for making my first years in the lab so much fun. **Kirsi Autio** is especially acknowledged for proposing my recruitment as a summer trainee a long time ago, despite my extremely poor Finnish language skills.

All my great friends, especially the "biolla"-gang; **Sandra Nyholm**, **Tomas Häggvik**, **Maria Lindell**, **Mia Lindfors** and **Anna Enzerink** for the good times during our university studies. All my other magnificent friends outside the lab are thanked for creating an ideal balance in my life between work and pleasure. Especially **Nina Gestrin** and **Louise Forsman** are thanked for their life-long and unconditional friendship.

My parents **Monica Tollet-Nymark** and **Anders Nymark** for believing in everything I do. They both played a major part in my decision to become a geneticist and a researcher. Also my sister **Wilhelmina** and the rest of my fantastic family are thanked for all their endless love and support.

Above all, I thank **Mikko Kjellberg** for being so great at making me believe that I am good at what I do, even in my darkest moments of doubt, for being the best home-dad ever and for reminding me that life doesn´t have to be so serious. Finally, **Alva**, my fabulous daughter, is thanked for her ability to instantly make me forget all the stress and difficulties at work, with just one tiny little hug.

Helsinki, May 2010

Penny Nymark

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Asbestos is still a serious problem all around the world, even though the devastating health effects of breathing in the microscopically tiny mineral fibres have been known for over a century. Asbestos exposure causes a variety of severe pulmonary diseases and unfortunately due to the long latency period between exposure and development of disease, this epidemic will continue, even in countries where asbestos use has been banned for many years.

Asbestos-related lung cancer is one of the most common types of occupational cancer. It is clinically indistinguishable from lung cancer in patients with no known history of asbestos exposure and the treatment is the same for both etiologic types. Nevertheless, the molecular basis may be different and diagnosis as well as prognosis and treatment strategies may benefit from the identification of specific asbestos-related molecular alterations. In addition, these kinds of molecular correlates could be of importance in resolving some of the medico-legal issues arising from occupational diseases.

This study sheds light on the molecular alterations related to asbestos exposure in lung cancer and may point the way for the development of molecular-based clinical methods for asbestos-related lung cancer.

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ISBN 978-951-802-982-6 (paperback)
978-951-802-983-3 (PDF)
ISSN-L 1237-6183
ISSN 1237-6183

Cover photograph: A bronchoalveolar lavage sample with macrophages attempting to engulf an asbestos body, formed on a crocidolite asbestos fibre (courtesy of S. Anttila).