Loss of heterozygosity and tumour suppressor gene alterations in human cancer: studies on FHIT and NF2 genes in lung cancer, malignant mesothelioma and gastrointestinal stromal tumours

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Several genetic alterations are involved in the development of human cancer. These alterations include changes both in tumour suppressor genes and oncogenes as well as microsatellite alterations, i.e. microsatellite instability and loss of heterozygosity (LOH).

We examined microsatellite instability and LOH in lung cancer tumours from smokers using 16 microsatellite markers locating at 9 different chromosomes. None of the tumours showed microsatellite instability. However, LOH was detected in 30% of the cases with at least one of the markers. In particular, allele losses at chromosomes 3p and 9p occurred frequently.

The FHIT gene (at 3p14.2) has been proposed to be a molecular target for carcinogens. As frequent allelic loss at 3p was seen in our series of lung tumours, we studied aberrant expression of the Fhit protein and LOH of the FHIT gene in tumours from tobacco smokers and patients with lung cancer attributed to asbestos exposure. Reduced Fhit expression was detected in 62% and LOH in 22% of the cases. An association was seen between FHIT allele loss and advanced disease or poor differentiation of the tumours. The Fhit protein expression was decreased both in the asbestos-exposed and non-exposed cases as well as in current and former smokers. These data support the significance of FHIT inactivation in the development of lung cancer.

Human malignant mesothelioma (MM) cell lines were examined for allele loss with several microsatellite markers from three known tumour suppressor gene regions (22q/NF2 gene, 9p/CDKN2A gene, 3p/ FHIT gene) and from two other frequently deleted areas (14q and 6q). We observed a high frequency of concurrent LOH at multiple loci. In particular, LOH in the NF2 gene region was detected in all (7/7) cell lines studied. At the other chromosomal sites analysed, allele loss was detected in 43-71% of the cases. The frequent detection of LOH at several sites simultaneously supports the view that the accumulation of multiple genetic changes commonly occurs in the malignant transformation of MM cells.

Gastrointestinal stromal tumours (GISTs) are the most common mesenchymal tumours of the digestive tract. The etiology of these tumours is still unknown. We examined loss of heterozygosity of chromosome 22q in 42 GISTs, and 69% of the cases showed LOH at least with one of the microsatellite markers used. Allele loss at 22q was significantly more frequent in tumours with high mitotic activity (considered as malignant) than in tumours with low mitotic activity (considered as benign). In addition, the cases with LOH in all the 22q loci studied had a 4.6–fold risk for recurrence as compared to those tumours with no LOH at 22q. Five of the GIST cases were studied for NF2 gene mutations by sequencing analysis and the Nf2 protein expression was analysed in nineteen tumours. No mutations were detected and the expression of merlin was positive in 84% (16/19) of the tumours analysed. The LOH findings suggest that tumour suppressor gene(s) involved in the development of this neoplasm may be located in chromosome 22, but the candidate gene NF2 is not likely to participate in the pathogenesis of GIST.

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CONTENTS

LIST OF ORIGINAL ARTICLES 7
ABBREVIATIONS 8
INTRODUCTION 9
REVIEW OF THE LITERATURE 11
Genetic alterations in human cancer 11
   Oncogenes 12
   Tumour suppressor genes 12
   Microsatellite alterations 13
      Microsatellite instability (MSI) 13
      Loss of heterozygosity (LOH) 15
Lung cancer 17
   General aspects 17
   LOH and candidate tumour suppressor genes at 3p 18
   FHIT gene 22
Malignant mesothelioma (MM) 27
   General aspects 27
   Allelic deletions and tumour suppressor genes in MM 28
   NF2 gene 29
   CDKN2A gene 31
Gastrointestinal stromal tumours (GISTs) 32
   General aspects 32
   LOH and candidate tumour suppressor genes in GIST 33
AIMS OF THE STUDY 35
SUBJECTS AND METHODS 36
Study subjects 36
Methods 37
   DNA extraction and polymerase chain reaction (PCR) 37
   Loss of heterozygosity (LOH) analyses 38
   Immunohistochemical studies 38
   NF2 gene analysis 39
   Statistical analyses 39
RESULTS 40
Microsatellite instability in lung cancer 40
FHIT gene alterations in lung cancer 40
  LOH at the FHIT gene region 40
  Fhit expression 41
Genetic alterations in malignant mesothelioma 44
  LOH at multiple loci 44
  Nf2 protein expression 46
  Fhit expression 46
Loss of heterozygosity and the NF2 gene in GIST 46
  LOH at 22q 46
  NF2 gene mutation analysis 47
  Nf2 protein expression 47
DISCUSSION AND CONCLUSIONS 49
ACKNOWLEDGEMENTS 57
REFERENCES 60
LIST OF ORIGINAL ARTICLES

This thesis is based on the following original articles, which are referred to by their Roman numerals in the text. Some unpublished results are also presented and discussed.


ABBREVIATIONS

APEX  apurinic endonuclease gene, alternatively APE
CDK    cyclin dependent kinase
CDKN2A cyclin-dependent kinase inhibitor 2A gene
CGH    comparative genomic hybridisation
CI     confidence interval
c–kit  a proto–oncogene encoding the KIT protein
CpG    neighboring cytosine and guanine residues on the same DNA strand in the 5’→3’ direction
DUTT1  a novel NCAM (neural cell adhesion molecule) family member gene, alternatively ROBO1
FHIT   fragile histidine triad gene
FRA3B  fragile 3B site
GIST   gastrointestinal stromal tumour
hMLH1  human mutL homolog 1 gene
hMSH2  human mutS homolog 2 gene
HNPPCC hereditary nonpolyposis colorectal cancer
LOH    loss of heterozygosity
MM     malignant mesothelioma
MMR    mismatch repair
mRNA   messenger ribonucleic acid
MSI    microsatellite instability
NF2    neurofibromatosis type 2 gene
NSCLC  non–small cell lung cancer
OGG1   8-oxoguanine DNA glycosylase gene
OR     odds ratio
p16    the product of CDKN2A gene
PCR    polymerase chain reaction
PET    paraffin embedded tumour
RARβ   retinoic acid receptor-β gene
RASSF1A ras association domain family 1A gene
SCLC   small cell lung cancer
t(3;8)  translocation (3;8) site
INTRODUCTION

Cancer is a disease where the regulation of normal cell proliferation and cell death has been disturbed. Cancer cell division is uncontrolled and the cells are eventually able to invade surrounding tissues. The development of cancer is a multistep process that involves a series of genetic changes. Tumours are characterised by abnormalities in chromosomes and multiple genes, some of which have been demonstrated to have an essential role in tumourigenesis. Mutations of oncogenes and tumour suppressor genes as well as increased genetic instability are among the major genetic events occurring in carcinogenesis (Strachan and Read, 1999).

There are multiple genetic changes involved in carcinogenesis, especially cancers related to environmental exposure, forming a very complex pattern. For many types of cancer, the lack of clinically useful tests for early diagnosis is an unresolved problem and in fact is the main reason why a disease like lung cancer has such poor prognosis. Despite major advances in cancer treatment in the past two decades, the prognosis of patients with lung cancer has improved only minimally, with overall 5-year survival rates ranging between 10 and 15% (Greenlee et al., 2000).

One characteristic of most tumours is genetical instability observed at the nucleotide or chromosomal level. The instability of short tandem repeat sequences, called microsatellite instability, was first observed in hereditary nonpolyposis colon cancer (HNPCC) patients (Aaltonen et al., 1993). Microsatellite instability is the result of uncorrected replication error attributable to mutations in mismatch repair genes. The tissue specificity of microsatellite instability has been speculated to be an indication of tissue-specific mutagens or the existence of tissue-specific genes which are targets of those spontaneous mutations which provide a growth advantage to that specific cell type (Eshleman & Markowitz, 1995). Another commonly observed type of genetic alteration in neoplastic tissue is loss of heterozygosity (LOH) where one allele of a gene is inactivated in most cases by mutation, and the functional allele is deleted. Allelic loss at a certain region of chromosome is known to indicate the presence of a tumour suppressor gene. Due to the high level of specificity, loss of heterozygosity is often invaluable as a marker for diagnosis and prognosis of cancer (Thiagalingam et al., 2002).
In this study, the genetic alterations in three different somatic human cancers have been examined. Microsatellite instability that is characteristic to HNPCC was not seen in environmental lung cancer, malignant mesothelioma (MM) or gastrointestinal stromal tumours (GISTs). In contrast, loss of heterozygosity was discovered to be a common form of genetic abnormality in the cancers studied. Particularly allelic loss at the short arm of chromosome 3 was frequently observed in lung cancers, and loss of chromosome 22q was frequent both in malignant mesothelioma and gastrointestinal stromal tumours. At these sites are the locations for two known tumour suppressor genes, namely \( \text{FHIT} \) gene at 3p14.2 and \( \text{NF2} \) gene at 22q12. Fhit protein expression was analysed in lung cancer and malignant mesothelioma and found to be decreased in both types of cancer. An association between tumours from smoking and asbestos–exposed lung cancer patients and decreased production of the \( \text{FHIT} \) gene was seen. In malignant mesothelioma cells concurrent allele losses at several sites were detected which supports the view that the accumulation of multiple genetic hits is characteristic to malignant transformation of MM cells.

A high frequency of allelic loss on chromosome 22q has been detected in many human cancers, including lung cancer, mesothelioma, colon carcinomas and breast carcinomas. The \( \text{NF2} \) gene mutations have been screened in different tumour types but, so far, only in the case of mesotheliomas there is strong evidence for inactivation of the \( \text{NF2} \) gene (Lutchman & Rouleau, 1996). As loss of heterozygosity at 22q was frequently detected we examined \( \text{NF2} \) gene alterations in GISTs. However, no evidence of \( \text{NF2} \) gene involvement in GIST development was shown. Alterations of chromosomal arms other than chromosome 14, especially 1p, 9p, and 22q, have been shown to occur in GIST, in particularly in malignant forms of the disease (Kim et al., 2000; Marci et al., 1998). This may imply that there is a cumulative effect of deletions of several tumour suppressor genes responsible for the development of this neoplasm (el-Rifai et al., 1996) and that these changes occur as late events related to tumour progression and malignant transformation.
Genetic alterations in human cancer

Both environmental and endogenous factors can interact at various steps in tumour development and progression. In most cancers, environmental factors are involved in carcinogenesis, but there is also clear evidence for genetic factors being involved in cancer susceptibility (Strachan and Read, 1999). Hereditary cancer cases are estimated to comprise approximately 1–5% of all cancers (Arver et al., 2000; Cornelisse & Devilee, 1997; de la Chapelle & Peltomaki, 1995; Gertig & Hunter, 1998; Lindblom & Nordenskjold, 2000). The majority of cancers are a consequence of external factors that act in conjunction with both genetic and acquired susceptibility. Several environmental exposures (such as tobacco smoke, occupational exposure, air pollution and diet) have clearly been associated with many cancers. Of all the environmental causes, tobacco smoking is the most important single risk factor, causing many types of cancers, in particular, lung cancer.

Though only a small minority of all cancer cases are caused by an inherited gene mutation, molecular studies of these cases have been of great help in increasing the general knowledge of the basic mechanisms underlying cancer development (Cornelisse & Devilee, 1997). As the penetrance of the disease is known to be influenced by a number of factors, such as environmental exposures, the estimation of the shared role of genetic and environmental factors in the development of cancer is very difficult (Fearon, 1997). Genetic and environmental components in different cancers have been evaluated by twin studies. Statistically significant effects of heritable factors were observed for prostate cancer (42%), colorectal cancer (35%), and breast cancer (27%) (Lichtenstein et al., 2000). In a study by Hemminki et al. (2001) the genetic component was estimated to be 10% in colon cancer, 14% in lung cancer and 18% in melanoma while the contribution of the non-shared environment was 67-71% in these cancers (Hemminki et al., 2001; Lichtenstein et al., 2000).
Oncogenes

Oncogenes are mutated forms of proto-oncogenes. The normal function of many of these genes is to control the cell cycle by stimulating cell proliferation. Activating gain-of-function mutations of proto-oncogenes may cause malignant transformation (Cornelisse & Devilee, 1997; Karp et al., 1996). Abnormal activation can occur in many different ways, e.g. by chromosomal translocation resulting in gene combination, by point mutations resulting in an altered protein product, or by amplification resulting in overexpression of the oncoprotein which again results in excessive cell proliferation (Cornelisse & Devilee, 1997; Munger, 2002). Although oncogenes are involved in many different types of cancer, they rarely seem to play a central role in hereditary cancer.

Tumour suppressor genes

Tumour suppressor gene products normally inhibit cell proliferation by controlling cell cycle progression or directing damaged cells into apoptosis. Thus, these genes restrict the tumourigenic and metastatic processes (Levine, 1993). According to the classical Knudson’s “two hit” model of tumourigenesis, an inactivation of both copies of a tumour suppressor gene leading to loss of function is required for the development of a neoplastic cell (Knudson, 1971). In hereditary diseases, the first hit in the germline cells represents a mutated copy of a tumour suppressor gene and the second hit is a somatic inactivation of the other allele. In cases of sporadic tumours, it has been shown that there are two somatic inactivating events that affect both copies of the tumour suppressor gene. The inactivation may happen through mutations or through epigenetic modifications (e.g. methylation) (Sugimura & Ushijima, 2000).

Knudson’s hypothesis that two hits are required for the full inactivation of a tumour-suppressor gene has been shown to be correct in many human cancers (Jones & Laird, 1999). However, as our knowledge grows about genes involved in cancer, the traditional definition of tumour suppressor genes may need to be broadened (Haber & Harlow, 1997; Le Beau et al., 1998). For instance, mismatch repair genes that maintain DNA integrity may be inactivated by a two-hit mechanism and thus act as tumour suppressors (Nicolaides et al., 1998).

The most common mechanisms of inactivation of tumour suppressor gene by two-hit mechanism known to date are presented in figure 1. Mutations in tumour suppressor
genes may be hereditary and thus present in the germline of individuals leading to genetic susceptibility to cancer, or they may be somatic when two individual inactivating events occur at the same locus (Haber & Harlow, 1997).

![Diagram of the Knudson’s “two-hit” mechanisms for the inactivation of tumour suppressor genes](image)

**Figure 1.** A schematic illustration of the Knudson’s “two-hit” mechanisms for the inactivation of tumour suppressor genes (modified from Wheeler&Bodmer, 2000).

**Microsatellite alterations**

There are two different kinds of genomic alterations that are detectable by the same laboratory techniques; namely loss of heterozygosity (LOH) and microsatellite instability (MSI). Figure 2 shows the principal characteristics and the differences in these two phenomena.

**Microsatellite instability**

Microsatellites are short tandem repeat sequences (1-6 nucleotides) of unknown function, most commonly in non-coding regions of DNA scattered throughout the human genome. Microsatellite instability is associated with slippage of DNA polymerase during DNA synthesis, resulting in extra units of repetitive sequences. The
instability of microsatellites is detected by the difference of the lengths of the repeat sequences between tumour and germline DNA from the same patient.

The first evidence that mismatch repair genes can be involved in human cancer development were genetic studies which demonstrated that tumours of patients with hereditary nonpolyposis colon cancer (HNPCC) showed frequent instability in their microsatellite sequences (Aaltonen et al., 1993; Thibodeau et al., 1993). Thereafter microsatellite instability has been observed in a number of different types of tumours, but it is clearly a specific feature for HNPCC (Peltomaki et al., 1993). The relationship between microsatellite instability and defective DNA mismatch repair (MMR) was known to occur in E. coli and S. cerevisiae and this knowledge led to the cloning of the human homologues for the mutS (hMSH2) and mutL (hMLH1) genes (Fishel et al., 1993). DNA mismatch repair is a post–replicative process which corrects the errors that have escaped the proof–reading and it is essential for maintaining genomic stability (Karran & Bignami, 1994). Today, seven human MMR genes which are thought to have a role in DNA mismatch repair and may be involved in carcinogenesis have been identified [reviewed in (Muller & Fishel, 2002)]. The majority of mutations affect the hMLH1, hMSH2, and hMSH6 genes [reviewed in (Peltomaki, 2001)]. However, mutations of MMR genes identified to date explain only about half of the HNPCC cases which suggests that still additional genes involved in mismatch repair await discovery or perhaps another completely distinct mechanism is involved (Wheeler et al., 2000).

The inactivation of the MMR genes leads to defects in the mismatch repair system. This can result in a so-called mutator phenotype, characterised by an increased frequency of mutations in tumour suppressor genes and oncogenes (Loeb, 1994). Microsatellites within the coding regions of genes important in cellular transformation, such as APC (adenomatotic polyposis), TGF-β (tumour growth factor β) receptor II, IGF-2 (insulin–like growth factor 2) receptor, TCF-4 (transcription factor 4), BAX (B–cell lymphoma 2 associated protein–1) and the mismatch repair genes themselves have been shown to be targets of microsatellite instability (Compagni & Christofori, 2000; Duval et al., 1999; Schwartz et al., 1999).

Tumours associated with carcinogen exposure, such as lung cancer, rarely show microsatellite instability (Yamasaki & Mironov, 2000). However there is also evidence that environmental carcinogens can contribute to the induction of microsatellite instability (Shridhar et al., 1994). In a mouse model with mismatch repair deficiency,
most of the mice died of lymphomas at an early age (Colussi et al., 2001). MMR-defective human or mouse cells have been shown to be tolerant to different cytotoxic agents which can accumulate in the DNA without evoking lethal effects [reviewed in (Bignami et al., 2000)].

Microsatellite instability has been widely studied in lung cancer and varying frequencies, ranging from 0% to 66% have been reported (Adachi et al., 1995; Mao et al., 1994; Merlo et al., 1994; Miozzo et al., 1996; Peltomaki et al., 1993; Ryberg et al., 1995; Shridhar et al., 1994). Interestingly, a genetically distinct form of microsatellite instability has been described in NSCLCs. According to a recent finding, instability in lung cancer occurs in tri- and tetranucleotide repeats, rather than in di- or mononucleotide repeats, which are characteristic of the tumours associated with HNPCC (Ahrendt et al., 2000; Xu et al., 2001).

Loss of heterozygosity (LOH)

As allelic loss at a certain region of chromosome is thought to indicate the presence of a tumour suppressor gene, LOH analysis is presently the most common method used to identify potential locations for these genes. Although LOH is a common phenomenon in many cancers, a frequent targeting of allelic loss at a specific chromosomal region indicates the location of a tumour suppressor gene. According to this hypothesis, there are still several as yet undiscovered tumour suppressor genes with potentially important roles in cancer development (Macleod, 2000).

Loss of heterozygosity may elicit by several different mechanisms. The genetic changes that result in LOH known to date are mitotic recombination, deletion, gene conversion, translocation, chromosome breakage, chromosomal fusion or telomeric end-to-end fusions, or loss of a whole chromosome (Thiagalingam et al., 2002). Loss of a whole chromosome is generally accomplished by nondisjunction resulting in defects in chromosome segregation (Nigro et al., 1989; Schutte et al., 1996). Recently, losses of whole chromosomes detected by allelotyping have been found to be associated with multiple copies of the remaining chromosomes in tumour cells leading to homozygosity (Thiagalingam et al., 2001). Loss of a chromosomal arm or a large fragment may be a result of a double-strand break, or reciprocal or nonreciprocal translocation, or mitotic recombination. Mitotic recombination is the result of the nonsister chromatid exchange
in mitotic cells (de Nooij-van Dalen et al., 1998; Varella-Garcia et al., 1998). Loss of one single allele or gene may be caused by a deletion resulting from two double-strand breaks or mitotic recombination involving the homologous chromosomal arms. Homozygous deletions within a gene or locus indicate that a double hit may occur targeted to specific chromosomal sites (Hahn et al., 1996; Schutte et al., 1995; Thiagalingam et al., 1996).

In a cell, multiple mechanisms have evolved to ensure the maintenance of genetic material during processes such as DNA replication and recombination or, to protect the DNA from nucleases and other adversities of intracellular byproducts (Hoeijmakers, 2001). Any defect in these cellular functions could lead to genomic instability, such as loss of heterozygosity. The main DNA damage repair pathways operating in mammals are nucleotide- and base-excision repair, homologous recombination, end joining, and mismatch repair (Hoeijmakers, 2001; Lindahl & Wood, 1999). As the outcome of DNA damage is diverse, no single repair process can cope with all kinds of damage but the network of DNA repair systems cover most of the damages provoked by environmental and endogenous genotoxicity (Cadet et al., 1997; Hoeijmakers, 2001; Lindahl, 1993).

![Figure 2](image)

*Figure 2*. Schematic illustration of microsatellite instability (MSI) and loss of heterozygosity (LOH). LOH is seen as lack of one of the allelic bands in the tumour (T) (arrow) as compared to the heterozygous normal tissue (N), and microsatellite instability appears as new allelic bands in the tumour tissue (arrows).
Lung cancer

*General aspects*

In the industrialized countries, lung cancer is the leading cause of cancer death. More than one million people die of lung cancer every year in the whole world. Despite intensive research on lung cancer, the overall survival rates have not improved significantly over the past 20 years. Almost 200 000 new cases occur in European Union countries every year. Of these, less than 10% of patients can be cured (IARC statistics, 1996). In Finland, according to The Finnish Cancer Registry, there were 1515 new cases of lung cancer among men and 474 new cases among women in 1997. The major causal factor of lung cancer is tobacco smoke, which is estimated to be responsible for 80-90% of lung cancers (IARC, 1996; WHO, 1997). Asbestos, radon, and other occupational and environmental exposures are other known etiological factors (Rom et al., 2000).

Lung tissue is composed of many different cell types, and cancers arising from the different cell types have unique characteristics. Based on the histology, lung cancers are typically divided into two major categories; i.e. small cell lung carcinomas (SCLC) and non-small cell lung carcinomas (NSCLC). Eighty percent of lung carcinomas are NSCLCs and 20% are SCLCs. In NSCLC, the most frequent cell types are adenocarcinoma (>30%), squamous cell carcinoma (~30%), and large cell carcinoma (~10%); the majority of lung cancers are however histologically heterogenous (Pass, 2000).

On the basis of current knowledge, respiratory epithelial cells require many genetic alterations both in oncogenes and tumour suppressor genes to transform them into malignant cells (Fong et al., 1999). In human lung cancer, loss of chromosomal material is commonly detected, the most frequently deleted regions being chromosome regions 3p, 9p and 17p. At these commonly altered chromosomal regions, tumour suppressor genes have been identified and alterations of these genes have been associated with human lung cancer. For instance, mutations of the TP53 gene at 17p have been detected in about 50% of NSCLCs and in 70% in SCLCs (Rom et al., 2000). The CDKN2A gene at 9p is reported to be inactivated through mutation, homozygous deletion, or methylation in about 30% of NSCLCs (Okamoto et al., 1994). The FHIT gene at 3p has
been found to be altered in about 40% of NSCLCs and 80% of SCLCs (Croce et al., 1999; Fong et al., 1997). Yet, alterations of these genes covers only a part of the lung cancer cases, suggesting that additional essential genes having an important role in the development of lung tumours are located in these frequently deleted chromosomal regions.

**LOH and candidate tumour suppressor genes at 3p**

Several regions of LOH have been identified in human lung cancer. However, despite intensive research, defining the critical molecular genetic events specific for lung cancer has turned out to be very problematic. In recent studies regarding lung cancer from 38 chromosomal arms analysed, 25 (66%) showed a high frequency of LOH (Shivapurkar et al., 1999; Virmani et al., 1998; Wistuba et al., 2000a; Wistuba et al., 1999). Overall, more than 50 different sites of frequent allelic loss (hot spots) at different loci have been reported in primary lung cancers or cell lines (Girard et al., 2000). Some of the allelic losses may be caused by genomic instability as a consequence of tumourigenesis or the presence of fragile sites at these chromosomal sites (Kohno & Yokota, 1999). Thus an inactivation of a tumour suppressor gene is not necessarily involved.

LOH at multiple sites of the short arm of chromosome 3 (3p) is one of the most common findings in lung cancer, occurring in about 80% of NSCLCs (Hibi et al., 1992) and in nearly 100% of SCLCs (Hibi et al., 1992; Kovatich et al., 1998). Three distinct regions of loss of 3p in lung cancer have been identified; 3p25, 3p21.3, and 3p14-cen indicative of the existence of more than one different tumour suppressor gene located on this chromosomal arm (Devereux et al., 1996). A progressive increase of allelic loss at 3p has been observed to occur along with cellular progress from the normal to neoplastic form which suggests that changes in this chromosomal region are relatively early events in lung carcinogenesis (Hung et al., 1995). It has also been shown that LOH at 3p occurs considerably more frequently in smokers than in those who have never smoked (Croce et al., 1999).

Several potential tumour suppressor genes have been identified at 3p. So far, only the *FHIT* (fragile histidine triad) gene at 3p14.2, has clearly been shown to be involved in the development of different types of sporadic tumours, including lung cancer (Fong et al., 1997; Sozzi et al., 1996). However, the association between the malignant behaviour
of a cell and the aberrations of the Fhit protein, which is a dinucleoside polyphosphate hydrolase, has not been fully explained as will be discussed in more detail in the next section of this review.

The von Hippel-Lindau gene (VHL) which is located at 3p25 is connected to the regulation of the transcriptional response to hypoxia (Iwai et al., 1999). This gene, which is associated with a dominantly inherited cancer syndrome von Hippel-Lindau disease (Latif et al., 1993), predisposing affected individuals to a variety of tumours, is rarely mutated in lung cancer, although this region does also show frequent LOH in lung cancer (Sekido et al., 1994). This may indicate for the presence of another tumour suppressor gene(s) involved in lung tumourigenesis in this chromosomal region.

On the same chromosomal region (3p25) resides the OGG1 (8-oxoguanine DNA glycosylase) gene, which is a base excision repair protein (Klungland et al., 1999). The OGG1 gene has been proposed to have a suppressing role in carcinogenesis (Monden et al., 1999). However, in lung cancers, only one mutation in a SCLC case has been described so far (Chevillard et al., 1998) and no mutations of the gene have been detected in NSCLC lung cancer cell lines or primary tumours (Shinmura et al., 2001).

At 3p24 maps the RARβ (retinoic acid receptor-β) gene. Retinoic acid is involved in lung development and differentiation (Grummer et al., 1994) and there is evidence that RARβ plays an important role in growth and regulation of epithelial cells and in tumourigenesis (Roman et al., 1992; Seewaldt et al., 1995; Swisshelm et al., 1994). Expression of RARβ has been demonstrated to be suppressed in many human malignancies, including lung tumour tissues and cell lines (Gebert et al., 1991; Houle et al., 1991). Loss of RARβ expression is, however, not correlated with LOH at 3p24 (Qiu et al., 2000), but methylation of the RARβ promoter region has been reported which may be the inactivating mechanism for this gene (Virmani et al., 2000).

The hMLH1 (human mutL homolog 1) gene that is involved in DNA mismatch repair is located at 3p21-p23 (Lindblom et al., 1993). Both LOH at the hMLH1 locus (43- 55%) (Wieland et al., 1996) and decreased protein expression (58%) (Xinarianos et al., 2000) have been reported to occur in a subset of NSCLCs. Mutations of the gene have not been detected in lung tumours (Benachenhou et al., 1998). However, methylation of the promoter region of the gene occurs frequently, leading to lack of expression of the
protein which may be at least one of the inactivating mechanisms of the \textit{hMLH1} gene also in NSCLC (Kane \textit{et al.}, 1997; Xinarianos \textit{et al.}, 2000).

Recently, the \textit{RASSF1A} gene (Ras association domain family 1A) at 3p21.3 was identified (Dammann \textit{et al.}, 2000). The gene has shown to be inactivated by hypermethylation of the promoter region and lack of the mRNA of the gene has been reported in a subset of lung cancer and breast cancer cell lines as well as in primary tumours of the lung, breast and kidney (Agathanggelou \textit{et al.}, 2001; Burbee \textit{et al.}, 2001; Dammann \textit{et al.}, 2000; Dammann \textit{et al.}, 2001). In addition, re-expression of the \textit{RASSF1A} transcript in lung cancer cells has been shown to inhibit tumour formation in nude mice (Dammann \textit{et al.}, 2000).

The \textit{DUTT1} or \textit{ROBO1} gene (roundabout homolog 1) at 3p12, is a member of the family of genes encoding neural cell adhesion molecules (Sundaresan \textit{et al.}, 1998). Thus, \textit{DUTT1} may be another candidate gene with a role in the development of lung tumours, although no mutations in this gene have been reported so far.

One further gene residing in the 3p region frequently showing allele deletion in lung cancer cell lines is the \textit{BAP1} (\textit{BRCA} associated protein-1) gene at 3p21.3. The gene product is ubiquitin hydrolase. This enzyme enhances BRCA1-mediated inhibition of breast cancer cell growth. It has been suggested to be a tumour suppressor gene which functions in the BRCA1 growth control pathway; intragenic homozygous deletions of this gene have been detected in lung carcinoma cell lines (Jensen \textit{et al.}, 1998).

In figure 3 the candidate tumour suppressor genes are showed with approximate location in the schematic map of the short arm of chromosome 3.
Apart from lung cancer, deletions and other rearrangements of 3p have frequently been detected in many other cancers including malignant mesothelioma tumours and cell lines (Gibas et al., 1986). In addition to these two pulmonary cancers, LOH at 3p occurs in several other cancer types such as endometrial and cervical cancer (Jones & Nakamura,
1992), head and neck cancer (Maestro et al., 1993), oral squamous cell carcinoma (Partridge et al., 1996) and renal cancer (Giaccone, 1996). As novel genes in this chromosomal region are identified, it is most probable that several tumour suppressor genes important for carcinogenesis in general will be discovered on this chromosome. In all, the short arm of chromosome 3 appears to harbour a number of genes potentially inactivated during tumour development. In the following section one of the most interesting candidates, the FHIT gene, is dealt within more detail, as it was selected to be the target of our molecular analysis of lung cancer patients.

**FHIT gene**

*Gene structure*

The human FHIT gene (fragile histidine triad), identified in 1996, is composed of 10 exons, of which five (exon 5 through 9) encode the protein (Sozzi et al., 1996). The gene is a member of the histidine triad (HIT) gene family and the protein has diadenosine 5’, 5”-P1, P3-triphosphate (Ap3A) hydrolase activity that cleaves Ap3A into ATP (adenosine triphosphate) and AMP (adenosine bis–monophosphate) (Barnes et al., 1996). Diadenosine tri- and tetra-phosphates are synthesised in response to cellular stress such as starvation and heat shock conditions and they have a role in cell cycle arrest in prokaryotes (Glover, 1998). Within the FHIT gene locates the t(3;8) chromosomal translocation breakpoint that is involved in familial renal carcinoma (Smith et al., 1998) and the most common fragile site of the human genome, FRA3B, which is susceptible to rearrangements induced by a variety of environmental carcinogens (Bugert et al., 1997; Mimori et al., 1999; Wang et al., 1999).

Figure 4 shows the organisation of the FHIT gene with approximate locations of the FRA3B region, the microsatellite markers used in our studies and the t(3;8) translocation breakpoint.
Figure 4. The organisation of the FHIT gene (modified from Pass et al., 2000). The approximate locations of the FRA3B site (grey bar), microsatellite markers studied (D3S1234, D3S1300, D3S1481; article II) and the chromosomal translocation breakpoint t(3;8) are designated. Open boxes (1-4, 10) describe the non-coding exons and filled boxes (5-9) the protein coding exons of the gene.

**FHIT gene alterations in lung cancer**

Several different kinds of abnormalities of the FHIT gene, namely homozygous deletions, abnormal transcripts, deletions and insertions, loss of protein expression, and aberrant methylation have been found to occur in lung cancers (Fong et al., 1997). In
addition, *FHIT* alterations have been detected in a large number of different human tumour types, such as hepatocellular carcinoma (Yuan *et al*., 2000), gastric adenocarcinoma (Capuzzi *et al*., 2000), cervical carcinoma (Yoshino *et al*., 2000), prostate cancer (Guo *et al*., 2000), and pancreatic cancer (Sorio *et al*., 1999). Interestingly, *FHIT* gene alterations appear to occur frequently, particularly in cancers which are associated with exposure to known carcinogens (Nelson *et al*., 1998; Sozzi *et al*., 1997a). Therefore, it has been suggested to be a molecular target of environmental carcinogens (Huebner *et al*., 1997). Although *FHIT* alterations have frequently been reported in many types of tumours, the biological and clinical significance of these changes is not clearly understood and the precise mechanism of Fhit action is still unclear.

**LOH at FHIT region**

Loss of heterozygosity in the *FHIT* locus has been reported both in lung tumours and lung cancer cell lines (Sozzi *et al*., 1996). The frequencies of allele loss vary between the histopathological types, being less frequent in adenocarcinomas (20%) than in nonadenocarcinomas (55%) (Burke *et al*., 1998). LOH has shown to be more frequent in lung cancers of smokers than nonsmokers, suggesting that *FHIT* may be a target of the carcinogens present in tobacco smoke (Sozzi *et al*., 1997a). In addition, LOH at *FHIT* occurred in 54% of cases of oral squamous cell carcinomas (Pateromichelakis *et al*., 2000) and in 63% of cases of oesophageal cancer (Menin *et al*., 2000) which are other cancer types associated with tobacco smoking. Allelic loss at *FHIT* region have been shown to occur frequently in the histologically normal bronchial epithelium of chronic smokers which suggests that alterations at this gene may be early events in lung cancer (Mao *et al*., 1997; Thiberville *et al*., 1995; Tseng *et al*., 1999; Wistuba *et al*., 2000b).

There are conflicting evidence for the association of LOH at the *FHIT* region and clinical status and/or survival of lung cancer patients. No association between LOH at 3p14 or within the *FHIT* gene in primary NSCLC and clinical status or survival was seen in two studies (Fong *et al*., 1997; Marchetti *et al*., 1998), whereas a significant trend towards poorer survival of patients with LOH was reported by Burke and co–workers (Burke *et al*., 1998).
Altered protein expression

Expression of Fhit protein is detectable in most tissues, and the highest levels of expression are found in epithelial cells and tissues (Ohta et al., 1996). Since the antibody against Fhit protein was produced (Sozzi et al., 1997b), several immunohistochemical studies assessing changes of Fhit protein expression in lung cancer have been performed. In the study of Sozzi et al. (1997b), the absence of Fhit protein expression was seen in 39% of NSCLCs and an additional 18% showed a mixed protein expression (showing both positive and negative tumour cells), while 73% of SCLCs were negative for Fhit protein expression (Sozzi et al., 1997b). Reduction or absence of Fhit expression is also observed in precancerous lesions detected in the bronchial mucosa samples, suggesting that loss of Fhit expression is an early step in lung carcinogenesis (Sozzi et al., 1997b). Similar to LOH, reduced Fhit protein expression is often observed in those cancers which are associated with environmental carcinogens, such as oesophagus (Menin et al., 2000), colorectal (Hao et al., 2000), bladder (Baffa et al., 2000), gastric (Capuzzi et al., 2000), and renal carcinomas (Hadaczek et al., 1998). In agreement with this, a marked reduction of Fhit protein expression was observed more frequently among patients with a smoking history than among nonsmokers (Tseng et al., 1999). These observations further support suggestions that the FHit gene might be a common target for carcinogen exposure and it may be involved in the development of several neoplasms caused by environmental carcinogens.

The significance of decreased Fhit protein expression and its influence on the progression of the disease is not clear. Microsatellite analysis at the FHit locus did not show a strong association between LOH at FHit and Fhit expression, suggesting that the protein inactivation may be due to mechanisms other than allelic deletion, such as methylation (Tseng et al., 1999; Zochbauer-Muller et al., 2001). Unlike LOH at FHit locus, reduction of Fhit protein expression has been associated with poorer survival of NSCLC patients with stage I, irrespective of the histological types of tumours. This result suggests that Fhit-negative tumours may be more aggressive than Fhit-positive tumours (Tomizawa et al., 1998).
**FHIT mutations, aberrant transcripts and hypermethylation**

The existence of point mutations is considered to be one of the defining criteria for candidate tumour suppressor genes. Overall, point mutations of the *FHIT* gene have been detected very rarely in all of the types of tumours analysed, supporting another mechanism for the inactivation of the gene (Nelson et al., 1998). So far, the most frequently observed point mutations are those of invasive cervical carcinomas (19%; 7/48) (Yoshino et al., 2000).

Abnormalities in *FHIT* mRNA transcripts have been described in several tumour types. Although the mechanism for the aberrant transcripts are currently unclear, it has been suggested to be due to abnormal splicing and processing of the transcripts (Fong et al., 1997). Abnormalities of transcripts in lung cancer have been reported in approximately 60% of cases (Fong et al., 1997; Tokuchi et al., 1999). Most frequently, aberrant transcripts have been detected as result from splicing of two or more exons of the *FHIT* gene (Fong et al., 1997; Sato et al., 1999; Sozzi et al., 1996). Interestingly, in addition to abnormal transcripts, also an additional normal-sized transcript is often present in lung tumours (Fong et al., 1997; Sozzi et al., 1996) and conversely, abnormal *FHIT* transcripts are a common finding in normal lung tissue (Tokuchi et al., 1999).

Aberrant methylation of *FHIT* gene is detected frequently in esophageal squamous cell carcinomas (Tanaka et al., 1998), in NSCLC and in breast cancers (Zochbauer-Muller et al., 2001). A significant correlation between *FHIT* methylation and loss of expression by Northern blot was seen for lung cancer and by immunostaining for lung and breast cancers. As 45% of the tumours lacking protein expression were not methylated, it is presumed that methylation is only one of the mechanisms inactivating the second allele (Zochbauer-Muller et al., 2001).

**FHIT as a tumour suppressor gene**

Cancer cell-specific homozygous deletions within a gene and lack of expression of the protein product are considered as hallmarks of tumour suppressor genes. In this respect, the *FHIT* gene is a strong candidate to be a tumour suppressor gene playing an important role in major human cancers (Siprashvili et al., 1997). Although the *FHIT* gene is altered in many human cancers as described previously, its status as a tumour
suppressor gene has remained controversial, particularly since functional studies have provided contradictory results (Michael & Rajewsky, 2001).

Overexpression of the \textit{FHIT} gene in lung cancer and head and neck cancer cell lines has been observed to induce apoptosis, cause cell cycle arrest and suppress tumourigenicity (Ji \textit{et al}., 1999). In \textit{FHIT}-deficient cancer cell lines it has been demonstrated that transfection of the wild-type \textit{FHIT} gene suppressed tumourigenicity (Siprashvili \textit{et al}., 1997). In a recent study, tumour development was inhibited by oral gene transfer, using adenoviral or adeno-associated viral vectors expressing the human \textit{FHIT} gene, in heterozygous Fhit (+/-) knockout mice, that are prone to tumour development after carcinogen exposure (Dumon \textit{et al}., 2001a). In addition, virus-mediated \textit{FHIT} gene transfer has been shown to induce apoptosis and reduce tumour growth in many different human cancer cell lines, such as lung, oesophageal, head and neck and pancreatic cancer cells (Dumon \textit{et al}., 2001b; Ishii \textit{et al}., 2001; Ji \textit{et al}., 1999; Roz \textit{et al}., 2002; Sard \textit{et al}., 1999). These findings point to the possibility that Fhit expression through gene transfer techniques may be useful therapeutically.

On the other hand, several studies have provided arguments against the \textit{FHIT} gene as a tumour suppressor. The observations that deletions in the \textit{FHIT} region most often occur in the intron regions (Huebner \textit{et al}., 1998; Ohta \textit{et al}., 1996) as well as the frequent presence of aberrant transcriptions in both tumour and normal DNAs suggest that \textit{FHIT} abnormalities may not be tumour specific (Boldog \textit{et al}., 1997; Mao, 1998). In lung cancer, aberrant \textit{FHIT} transcripts have been detected in 40-80\% of cases, but aberrant transcripts have also been discovered to be expressed commonly in normal cells (Sekido \textit{et al}., 1998). In addition, as mentioned, inactivating point mutations of \textit{FHIT} are rarely observed in lung cancer (Mao, 1998; Sekido \textit{et al}., 1998). Also, questions about the significance of the \textit{FHIT} gene in carcinogenesis have been raised since Fhit null mice (Fhit -/-) were reported to be long-lived and fertile (Fong \textit{et al}., 2000).

**Malignant mesothelioma**

*General aspects*

Malignant mesothelioma (MM) is a rare neoplasm derived from the mesothelial cells. It most commonly occurs in the pleura of the lungs, much less frequently in the peritoneal and pericardial cavities and tunica vaginalis of testes (Attanoos & Gibbs, 1997). It is a
very aggressive malignancy against which the existing therapies show very poor
efficacy (Antman, 1993). Asbestos exposure is the primary etiologic factor known for
mesothelioma, and an association to occupational asbestos exposure has been
documented in about 80% of all mesothelioma cases (McDonald & McDonald, 1980).
MM is characterised by a long latency time, up to 99% of asbestos-associated cases are
reported to have a latency period of more than 15 years from first exposure to death
from the disease, with the median latent period being found to be 32 years (Lanphear &
Buncher, 1992). The long latency period and the abundant use of asbestos earlier
probably accounts for the rapid increase in the number of mesothelioma cases during
the past few decades in most industrialized countries (Attanoos & Gibbs, 1997). The
incidence has been on the increase since the 1950s’ and it has been estimated that the
incidence of mesothelioma will peak around 2010, each year there will be about 100
asbestos-related cases in Finland (Karjalainen et al., 1997). Also, the presence of naturally
occurring erionite fibres in the soil can play an important role in the development of
environmental malignant mesothelioma as is the case in South Turkey. In villages
where erionite contaminated stones were used to build the houses, a high incidence of
MM has been reported. This was shown to be due to environmental exposure to erionite
fibres (Baris et al., 1987; Dumortier et al., 2001; Metintas et al., 1999).

The long latency time suggests that multiple, accumulating somatic genetic alterations
are required for malignant transformation of mesothelial cells (De Rienzo & Testa, 2000).
There is no single genetic alteration described which is characteristic to all MMs.
However, deletions of certain chromosomal regions are detected in most MM tumours,
suggesting the location of tumour suppressor genes as having an important role in
neoplastic transformation of MM cells (Taguchi et al., 1993). Two positional candidate
genes have been identified, i.e. CDKN2A (also called p16INK4a) at 9p21 and NF2 at
22q12, which are frequently altered in MMs (reviewed in (De Rienzo & Testa, 2000)).

*Allelic deletions and tumour suppressor genes in MM*

In human MM, a wide range of recurrent chromosomal changes has been shown. Most
of the aberrations have been detected by karyotypic or comparative genomic
hybridisation (CGH) analyses which point to changes at the chromosomal level and
imbalances in DNA level in large regions (gains and losses of DNA sequences)
Among the most frequent chromosomal changes reported for MM by these methods, are deletions of chromosome arms 1p, 3p, 6q, 9p, and 22q (Flejter et al., 1989; Gibas et al., 1986; Hagemeijer et al., 1990; Meloni et al., 1992; Popescu et al., 1988; Taguchi et al., 1993; Tiainen et al., 1988; Tiainen et al., 1989). Many of the frequently deleted regions revealed by cytogenetic or CGH analysis have been defined by LOH studies. Allelic losses in the 1p21-p22 region were observed in more than 70% of MM cases, which is consistent with previous cytogenetic studies (Lee et al., 1996). Allelic loss at 3p has been detected in more than 60% of MM cases (Lu et al., 1994). LOH analyses of 6q revealed allelic loss in 61% of MM cases studied (Bell et al., 1997) and 77% exhibited LOH at 15q (Balsara et al., 1999). Also high frequencies of LOH have been detected at 13q and 14q (Bjorkqvist et al., 1999; De Rienzo et al., 2000).

Chromosome 22 was demonstrated to be deleted in human cancer as early as the 1960’s when cultured primary meningioma cells were demonstrated to express frequent loss of chromosome 22 (Zang & Singer, 1967). Later, the same chromosomal change has been reported to occur in many human malignancies, thus implying presence of tumour suppressor gene(s) that may have a role in tumourigenesis of many different cancers. In human MM, monosomy or partial deletion of chromosome 22 has been shown to be one of the most frequent chromosomal alterations both in primary tumours and cell lines (Balsara et al., 1999; Flejter et al., 1989; Gibas et al., 1986; Kivipensas et al., 1996; Tiainen et al., 1988). The frequent detection of losses of chromosome 22 in MM suggests that tumour suppressor genes critical to mesothelial cell tumourigenesis reside in it.

**NF2 gene**

The NF2 tumour suppressor gene which encodes a protein called merlin, is located in the chromosome region 22q11.2 (Rouleau et al., 1993; Trofatter et al., 1993). Mutations in the NF2 gene are responsible for the inherited neurofibromatosis type 2 (NF2) disease, an autosomal-dominant cancer predisposition syndrome in which affected individuals have an increased risk to develop tumours of the nervous system, such as schwannomas, meningiomas and ependymomas (Parry et al., 1996; Rutledge et al., 1996). The coding region of the gene is composed of 16 or 17 exons with two alternatively spliced exons (1a and 16) that are variably detected in most tissues (Gutmann, 1997). Merlin has been shown to share homology at the amino acid sequence level with the ezrin-radixin-
moesin (ERM) proteins which link the cortical actin cytoskeleton to cell membrane glycoproteins (Uhlmann & Gutmann, 2001). In addition to its growth-suppression function, merlin is known to be involved in the regulation of the motility, adhesion, and spreading of the cell, but the definite function of this protein remains to be clarified (Gutmann et al., 2001; Gutmann et al., 1999).

Since individuals with NF2 germ–line mutations develop a variety of different tumours and also mutations in tumours that are not associated with NF2 disease have been detected, the NF2 gene is proposed to act as a tumour suppressor with general importance in tumourigenesis (Bianchi et al., 1994; Lutchman & Rouleau, 1996).

An association with NF2 gene alterations has been shown in malignant mesothelioma (Bianchi et al., 1995; Cheng et al., 1999; Deguen et al., 1998; Sekido et al., 1995). In the studies examining mutations of the NF2 gene in MM cell lines and primary tumours, mutations were detected in 26–53% of the cases (Bianchi et al., 1995; Deguen et al., 1998; Sekido et al., 1995). In some of the cases that showed NF2 gene transcript alterations, no genomic mutations were detected, suggesting that aberrant splicing may be an alternative mechanism for the inactivation of the gene (Bianchi et al., 1995; Sekido et al., 1995). Also, inactivation of the gene has been detected to occur by promoter methylation (Kino et al., 2001) indicating that methylation or mutation of the promoter region may be another mechanism for inactivation of the NF2 gene. The expression of Nf2 protein has been reported to be decreased in more than 50% of the MM cell lines studied (Cheng et al., 1999). In the same study, LOH at 22q was analysed and microsatellite marker intragenic to the NF2 gene showed allelic loss in 72% of the cases, with all cases exhibiting mutation and/or aberrant expression (Cheng et al., 1999). All these data suggest that inactivation of NF2 may be involved in development of human MMMs. However, the occurrence of cases with allelic loss but no mutations of the NF2 gene suggest that other tumour suppressor gene(s) at 22q may be involved in a subset of MMMs.
**CDKN2A gene**

Frequent deletions of 9p have been detected in MM, particularly the region 9p21-p22 at which homozygous and hemizygous deletions have been revealed (Cheng et al., 1993; Taguchi et al., 1993). Within this region, tumour suppressor gene, CDKN2A and the alternatively spliced p14ARF, is located (Kamb et al., 1994; Stott et al., 1998).

The CDKN2A gene product p16 induces cell cycle arrest by inhibiting phosphorylation of the retinoblastoma protein, pRb (Serrano et al., 1993). The p16 protein can bind to the cyclin-dependent kinase CDK4, which normally function in regulating the cell cycle and cell division (Serrano et al., 1993). Inactivation of CDKN2A leads to loss of cell cycle regulation, that is a characteristic feature of cancer cells (Cheng et al., 1994).

Homozygous deletions and down-regulated transcript expression of CDKN2A have been shown to be frequent in cell lines derived from many different tumour types, including MM (Cheng et al., 1994; Kamb et al., 1994; Nobori et al., 1994; Prins et al., 1998). Abnormal p16 protein expression has been observed both in MM cell lines and primary tumours (Kratzke et al., 1995). In addition, re-expression of p16 in MM cells resulted in cell cycle arrest and cell death, as well as inhibition of tumour formation and tumour growth, and diminished tumour size (Frizelle et al., 1998). However, decreased expression of p16 protein has been detected also in MM cell lines that did not show any homozygous deletions of this gene (Cheng et al., 1994). This suggests that in mesothelioma tumours the CDKN2A gene may be inactivated through hypermethylation, as has been demonstrated to be the case in a subset of many other types of cancer cases (Gonzalez-Zulueta et al., 1993; Herman et al., 1995; Merlo et al., 1995).

The pattern of frequent chromosomal losses in MM suggests that multiple tumour suppressor genes are likely to be involved in the pathogenesis of this neoplasm. The identification of all of the critical somatic genetic alterations in MM and understanding how each of them contributes to the pathogenesis of this malignancy may ultimately lead to the design of more effective therapeutic strategies.
Gastrointestinal stromal tumours

General aspects

Gastrointestinal stromal tumours (GISTs) are the most common mesenchymal neoplasms of the gastrointestinal tract. They have a wide morphologic and immunohistochemical spectrum, varying from benign to multiple malignant tumours and metastasis (Allander et al., 2001; Demetri, 2001). GISTs are heterogenous with respect to differentiation and they are generally resistant to chemotherapy and radiation treatment (Breiner et al., 2000). The diagnosis of GIST is complex, and only recently have the tumours been able to distinguished by using various histological and immunohistochemical criteria from morphologically similar neoplasms of the abdomen, such as leiomyomas and schwannomas (Miettinen & Lasota, 2001; Wieczorek et al., 2001).

Tumour size, mitotic rate and, to a lesser degree, location, are the most important predictive parameters for the behaviour of GIST (Al-Bozom, 2001). According to a population-based sample, the incidence of GISTs has been estimated as 10-20/million, out of which 20-30% are malignant tumours. GISTs are rare before the age of 40 years, and the median age of diagnosis ranges between 55-65 years (Miettinen & Lasota, 2001; Miettinen et al., 1999).

GISTs have been suggested to originate from the interstitial cells of Cajal (ICC) which form a cellular network with pacemaker activity in the gut (Kindblom et al., 1998; Robinson et al., 2000; Sakurai et al., 1999; Sircar et al., 1999). The KIT transmembrane receptor tyrosine kinase, the product of the c-kit proto-oncogene is essential for the normal development of this network (Strickland et al., 2001). GISTs are characterised by expression of the KIT, and specific activating mutations in this gene have been identified in these tumours (Hirota et al., 1998). Mutations can result in constitutive KIT activity. This protein is proposed to provide growth and survival signals to GIST cells and to be crucial to the pathogenesis of the disease (Rubin et al., 2001). Mutations of c–kit occur predominantly in malignant GISTs but also more rarely in benign GISTs, but not in true smooth muscle tumours suggesting that the c-kit mutations could be used as a marker for both diagnosis and malignancy of these tumours (Lasota et al., 1999).
LOH and candidate tumour suppressor genes in GIST

Previous cytogenetic data on benign and malignant GISTs have shown losses of chromosomes 14, 15, 18, 22 and 1 as the most frequently detected aberrations (Bardi et al., 1992; Bergmann et al., 1998; Breiner et al., 2000; Marci et al., 1998; Saunders et al., 1996). Comparative genomic hybridisation has confirmed the cytogenetically observed losses of 1p, 14q, 15 and 22 as frequent chromosomal changes (el-Rifai et al., 1996; Sarlomo-Rikala et al., 1998). However, the critical genetic changes, apart from mutations of the c–kit oncogene, leading to the malignant transformation of GISTs are not well understood.

Loss of heterozygosity at 14q is a common event in many human cancers, such as neuroblastoma (Hoshi et al., 2000; Thompson et al., 2001), carcinoma of ovary (Bandera et al., 1997), and renal cell carcinomas (Herbers et al., 1997; Schwerdtle et al., 1997). In cytogenetic, CGH and LOH studies loss of genetic material at chromosome arm 14q are the most frequently occurring aberrations in both benign and malignant primary GISTs (Bergmann et al., 1998; Breiner et al., 2000; Debiec-Rychter et al., 2001; El-Rifai et al., 2000a; el-Rifai et al., 1996; Fukasawa et al., 2000; Marci et al., 1998). The deletion of 14q is thus suggested to represent early and possibly primary events in the development of GISTs (Breiner et al., 2000; el-Rifai et al., 1996). So far, no clear evidence for tumour suppressor genes that play a crucial role in tumourigenesis have been assigned at 14q, though some candidates have been proposed. A candidate tumour suppressor gene APEX at 14q11.2-q12 encodes a DNA repair enzyme called APEX nuclease or apurinic endonuclease (APE). Inactivation of the enzymes involved in DNA repair, including, would leave the damaged DNA uncorrected, thereby causing mutations (El-Rifai et al., 2000b). Another novel human gene, KIAA0008 is located at 14q22-q23. Expression of this gene has been shown in colon and breast tumour tissues. Structurally, the gene shares homology with a Drosophila melanogaster tumour suppressor gene (Bassal et al., 2001).

Deletions of 22q have been detected by CGH and LOH analysis in 21-77% of GIST cases studied (Breiner et al., 2000; El-Rifai et al., 2000b; Fukasawa et al., 2000; Kim et al., 2000). In one study, mutations of the NF2 gene were identified in two GIST cases that showed also LOH at 22q (Fukasawa et al., 2000). However this finding has not been confirmed.

Deletions of chromosome 9p have been detected by CGH and LOH analysis in 14-36% of malignant GISTs (El-Rifai et al., 2000a; Kim et al., 2000). Interestingly, no losses of 9p
were seen in benign tumours and deletions were more frequent in metastatic than primary malignant GISTs, 63% of metastasis compared to 36% of primary tumours (El-Rifai et al., 2000a). This suggests that inactivation of a tumour suppressor gene in this chromosomal region could be a late event in tumourigenesis.

In addition, frequent LOH at 1p36 has been detected and the deletion of 1p has been associated with a relatively poor prognosis and with a high mitotic index, suggesting that gene(s) at this site may be involved in the regulation of cell proliferation in malignant GIST (O’Leary et al., 1999). Many potential tumour suppressor genes are located in the region, one of which is the HKR3 gene (GLI-Kruppel family member), a strong candidate as a tumour suppressor gene in neuroblastoma. These malignant cells share certain phenotypic characteristics with the interstitial cells of Cajal which have recently been associated with GIST (O'Leary et al., 1999).
AIMS OF THE STUDY

The purpose of the present study was to examine the incidence and frequency of microsatellite alterations in human non–hereditary cancers. In addition, following a detailed LOH analysis, protein expressions of two candidate tumour suppressor genes, namely the \textit{FHIT} gene at 3p14.2 and the \textit{NF2} gene at 22q12 were studied by immunohistochemistry.

The specific aims of the study were as follows:

- to study the incidence of microsatellite instability in lung cancer (article I)

- to investigate whether loss of heterozygosity in the \textit{FHIT} gene or reduced Fhit protein expression are common in non-small cell lung cancer associated with smoking and asbestos exposure (article II)

- to evaluate the occurrence of loss of heterozygosity in human malignant mesothelioma with chromosome 22 as a special target (article III)

- to study loss of heterozygosity at 22q and the involvement of merlin, the \textit{NF2} gene product, in the development of gastrointestinal stromal tumours (article IV)
SUBJECTS AND METHODS

Study subjects

The lung cancer samples included in articles I and II were collected from patients who underwent surgery in Helsinki University Central Hospital during the years 1988-1997. The study population of malignant mesothelioma cases in article III comprise of Finnish patients referred to Helsinki University Central Hospital between the years 1993 and 1999. For all cases, the diagnosis of malignant mesothelioma was confirmed by the Finnish National Mesothelioma Panel. Detailed smoking and occupational histories of the lung cancer and malignant mesothelioma patients were defined in a personal interview conducted at the hospital. The GIST samples included in article IV were collected from the archives of the Department of Pathology, Haartman Institute, University of Helsinki. Follow-up information of the GIST patients was obtained from the Finnish Cancer Registry. In all articles, DNA samples from tumour or cell culture and corresponding normal tissues (blood or peripheral tissue) were collected and analysed, except for a few GIST cases, where normal tissue was not available.

Article I

Paired samples of primary lung carcinoma and non-tumour tissue from 93 patients (70 males and 23 females) with the mean age at diagnosis 64 years (range 38-81 years) were studied for LOH and microsatellite instability. Eighty–seven (94%) of the cases represented non-small cell lung cancers (NSCLC) and six (6%) small cell lung cancers (SCLC). Two–thirds of the patients had stage I or II disease. According to interviews, all but two of the patients were current or former smokers. The mean pack-years of cigarette smoking (the number of packs per day multiplied by the years of smoking) was 40 (range 5-116). The occupational histories of the patients were also recorded.

Article II

Alterations of the FHIT gene were studied from 110 lung cancer patients (mostly the same cases as in article I). The histopathological diagnosis of the cases was as follows: 50 squamous cell carcinomas, 43 adenocarcinomas, 3 adeno-squamous cell carcinomas, 6 large cell, and 8 small cell cancers. Disease stage was available for 87 cases of which 49% represented stage IA or IB, 23% stage IIA or IIB, 24% stage IIIA or IIIB and 3%
stage IV disease. Most of the patients were current or former smokers with the mean pack-year of cigarette smoking 43, with an average 22 cigarettes smoked per day (data was missing for two cases). Four of the patients were life-long non-smokers. Interview data on complete work history, including exposure to asbestos, were available from 92 cases. Thirty-two (35%) patients had been exposed to asbestos at work, and 60 (65%) cases were without such exposure.

Article III

Malignant mesothelioma tumours and cell lines were studied for LOH with 22 microsatellite markers from five different chromosomes. The study included seven cell cultures and 32 fresh frozen tissue specimens, five of which overlapped with the cell lines. In addition, DNA from 13 paraffin-embedded tumour (PET) tissue samples carefully trimmed to exclude normal tissue was analysed. Seven of the PET specimens overlapped with fresh tumour samples. Occupational asbestos exposure of the patients was evaluated using electron microscopic analysis or classification by an industrial hygienist (Tuomi et al., 1991).

From the same set of MM cell line and tumour samples as studied for the occurrence of LOH in article II, seven cell cultures and 21 fresh frozen tumour specimens were examined for the Fhit protein expression.

Article IV

Tumour samples from 42 GIST patients, for whom corresponding normal DNA was available in 29 cases, were analysed for LOH at chromosome 22q. The study included 26 males and 16 females, with a median age of 64 years at the time of diagnosis (range 41–87 years). Thirty–one (74%) of the tumours were located in the stomach, ten (24%) in small intestine, and one (2%) in rectum. The tumours were categorised by a pathologist into two groups according to their mitotic activity measured as the mitotic index (MI) the most commonly used prognostic marker for this tumour. Tumours with mitotic activity less than 2 mitosis/10 high–power fields (HPF) in light microscope were considered as benign and tumours with 2 or more mitoses were considered as malignant.

Methods

DNA extraction and polymerase chain reaction (PCR)

DNA was extracted from all tissue samples and cell lines with standard methods using proteinase-K digestion and phenol/chloroform purification using ethanol precipitation.
In the resection of the tumour, special care was taken to avoid contamination by normal tissue (Articles I-IV).

For all the markers, PCR amplification was performed with the same reaction conditions. 100 ng of genomic DNA template was amplified with radioactive label in a volume of 10 µl. After five minutes at 95°C, thirty-five cycles were performed 1 min at 95°C, 1 min at 52-58°C, and 1 min at 72°C. The amplified samples were separated by electrophoresis in a 6% polyacrylamide gel (7.7 M urea). After electrophoresis, the gel was dried and exposed to x-ray film (Articles I-IV).

Alternatively, PCR amplification was performed by using fluorochrome (Cy5) labelled primers. The PCR products were electrophoresed and analysed by AllelLinks software (Article IV).

**LOH analyses**

Tumour or cell culture samples with absence or at least 50% decrease (scored by eye) in intensity of the X–ray film of one allele as compared to the normal DNA from the same individual were considered as exhibiting LOH (Articles I-IV). Alternatively, LOH was scored when a reduction of one allele was seen as a fluorescence peak having less than half the size of the same allele in normal tissue (Article IV).

**Immunohistochemical studies**

*Article II*

Expression of the Fhit protein was examined with a rabbit polyclonal antibody raised against the glutathione S–transferase–Fhit protein (received from Dr Kay Huebner, Kimmel Cancer center, Philadelphia, PA). Sections of 4-5 µm were cut from the paraffin embedded samples. Thereafter the samples were deparaffinised, microwaved in Na-citrate buffer, and immersed in 1.6 % hydrogen peroxide in methanol and after that in blocking solution to avoid endogenous peroxidase activity and unspecific binding sites. Immunostaining was carried out at 4°C over–night in a dilution of 1:4000 of the Fhit- antibody in the blocking solution, and treated with biotinylated secondary antibodies. The antibody binding sites were ultimately visualised using avidin-biotin
peroxidase complex solution. As a control, a non-specific rabbit IgG at a concentration corresponding to that of the Fhit-antibody was used (Article II).

**Article III**

Two mesothelioma and three normal mesothelial cell cultures were studied merlin (NF2 gene coding protein) expression. The protein from cell cultures was separated in polyacrylamide SDS–PAGE and transferred to nitrocellulose sheets, blocked and used for immunoblotting. The expression of merlin was detected with a polyclonal antiserum anti–SCH using secondary antibody and enhanced chemiluminescence.

**Article IV**

Expression of merlin in nineteen GISTs was examined. Immunostaining was performed using the avidin-biotin peroxidase complex method. The samples were stained by a LabVision Autostainer, and the staining intensity was scored by the pathologist (O.C.) as negative, weak, moderate or strong. In addition, a polyclonal antibody was used for detection of c-kit pro-oncogene product (CD117).

**NF2 gene analysis**

The sequences of exons 1-15 of NF2 gene were analysed from four tumours with high MI and one tumour with low MI. Genomic DNA was amplified by PCR with 15 overlapping pairs of primers and the sequencing reaction was performed using the AmpliCycle sequencing kit.

**Statistical analyses**

In article II, odds ratios (OR) and confidence intervals (CI) were calculated with the Manzel– Haenzel method for SAS Windows, release 6.12. In article IV, the statistical comparision of LOH between high and low MI at each microsatellite locus was performed by the chi–square test, using a 5% level of significance, and the odds ratios (OR) were calculated with EGRET for Windows, using 95% confidence interval (CI).
RESULTS

*Microsatellite instability in lung cancer*

Primary lung tumours from 87 NSCLC and six SCLC patients were investigated for the occurrence of microsatellite instability with 16 dinucleotide microsatellite markers from nine different chromosomes. None of the cases showed microsatellite instability with any of the markers studied (Article II).

Also when 40 malignant mesothelioma and 42 cases of GIST were studied for LOH with a total of 34 microsatellite markers, no evidence of microsatellite instability was shown in any of the cases (data not shown; articles III and IV).

*FHIT gene alterations in lung cancer*

*LOH at the FHIT gene region*

Altogether, 110 primary lung cancers were investigated for the presence of LOH at the *FHIT* gene locus at 3p14.2 with three intragenic microsatellite markers D3S1234, D3S1300 and D3S1481 (Article II). For comparison, allele loss was also studied with seven other microsatellite markers in three distinct regions of chromosome 3p (3p12-13, 3p21, and 3p25). The results of the allelic loss at different sites of 3p are shown in table 1 (unpublished results).

In all of the lung cancer cases studied, LOH at the *FHIT* gene was detected in 22% with at least one marker. Out of the 83 informative NSCLC cases, 20% showed LOH. Allele loss was clearly more frequent in SCLC (50%), however, the number of informative cases was small (n=6) which makes meaningful comparisons difficult. When the cell types of NSCLC were considered separately, allelic loss was detected more frequently in squamous cell carcinomas (24%) than in adenocarcinomas (9%) (Table 2). The frequencies of LOH at the other 3p loci studied were somewhat lower than that of the *FHIT* locus, but they did not differ significantly. LOH frequencies in all lung carcinomas were 12% at 3p12-13, 14% at 3p21, and 15% at 3p25 (unpublished data) (Table 1). Similar to the allele loss for *FHIT*, all additional markers from 3p studied indicated more frequent LOH in squamous cell carcinoma than in adenocarcinoma (Table 1).
Table 1. Summary of loss of heterozygosity (LOH) in the FHIT gene and at three distinct regions of chromosome 3p in primary lung cancers (article II and unpublished results).

<table>
<thead>
<tr>
<th></th>
<th>FHIT gene&lt;sup&gt;a)&lt;/sup&gt;</th>
<th>3p12-13&lt;sup&gt;b)&lt;/sup&gt;</th>
<th>3p21&lt;sup&gt;c)&lt;/sup&gt;</th>
<th>3p25-26&lt;sup&gt;d)&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LOH %</td>
<td>No. of positive/ informative cases</td>
<td>LOH %</td>
<td>No. of positive/ informative cases</td>
</tr>
<tr>
<td>All lung carcinomas</td>
<td>22</td>
<td>20/89</td>
<td>12</td>
<td>13/106</td>
</tr>
<tr>
<td>Non-small cell lung carcinomas</td>
<td>20</td>
<td>17/83 (10/42)</td>
<td>10</td>
<td>10/101</td>
</tr>
<tr>
<td>Squamous cell carcinomas</td>
<td>24</td>
<td>3/35 (4/6)</td>
<td>16</td>
<td>8/49</td>
</tr>
<tr>
<td>Adenocarcinomas</td>
<td>9</td>
<td>3/6</td>
<td>0</td>
<td>0/43</td>
</tr>
<tr>
<td>Other&lt;sup&gt;e&lt;/sup&gt;</td>
<td>67</td>
<td>3/6</td>
<td>22</td>
<td>2/9</td>
</tr>
<tr>
<td>Small cell lung carcinomas</td>
<td>50</td>
<td>43/7</td>
<td>38</td>
<td>3/8</td>
</tr>
</tbody>
</table>

<sup>a</sup>Markers D3S1234, D3S1300 and D3S1481
<sup>b</sup>Markers D3S1284 and D3S6
<sup>c</sup>Markers D3S1289, D3S1478 and D3S966
<sup>d</sup>Markers D3S647 and D3S1038
<sup>e</sup>Includes 3 adeno-squamous carcinomas and 6 large cell carcinomas
The data for allelic loss were compared with the patients’ clinico-pathological features and with exposure to tobacco smoke and asbestos at work. Loss of heterozygosity was detected in 14% of stage IA or IB disease and 29% of stage II-IV disease, indicating a higher risk of allele loss for those cases of more advanced lung cancer (OR 2.5, 95% CI 0.9-7.4). The patients were divided into current smokers and non-smokers, the latter group including both former smokers (19 cases) and life-long non-smokers (3 cases). The combined group was formed because the number of never-smokers was too small to allow meaningful statistical comparisons. Similar difficulties have been encountered in other studies with an association observed between increase in loss of Fhit expression and extensive cigarette consumption, but without statistical significance, probably due to the small number of non-smokers (Geradts et al., 2000). LOH at FHIT gene occurred in 18% of current smokers, whereas 30% of the former or non-smokers showed allelic loss in the FHIT region. Among the patients who had a history of occupational exposure to asbestos, LOH was more frequent (25%) as compared to the non-exposed individuals (16%), but the difference was not significant (OR 1.8, 95% CI 0.5-5.9) (Table 2) (Article II).

Fhit expression

Fhit protein expression was studied by immunohistochemistry (IHC) using a polyclonal antibody. The area of positive Fhit staining was estimated in percentages of the total area of the tumour, and the intensity of staining was estimated on a scale according to the criteria described in article II. The immunohistochemical expression of Fhit protein was analysed in 53 lung cancer cases (49 NSCLCs and 4 SCLCs). In 53% of the cases, the expression was absent or clearly reduced as compared to normal epithelium. In NSCLCs, decreased Fhit expression was observed in 70% of squamous cell carcinomas and 44% of adenocarcinomas (OR 3.1, 95% CI 0.9-10.3) (Table 2) (Article II). Protein expression was analysed in relation to patients’ tobacco smoke exposure and occupational asbestos exposure and clinical features. Altered protein expression was frequent among smokers although comparison between current smokers and former or non-smokers revealed that altered expression was common in both groups (66% and 57% respectively). Aberrant Fhit expression was more frequent among cases with work-related asbestos exposure (67%) as compared to the non-exposed individuals (59%), but
the difference was not significant. Absent or markedly reduced Fhit expression did not associate with the stage of the disease, but an association was seen with loss of cell differentiation in the tumour. Of the negative cases 3% had a tumour classified as morphological grade 1, 38% had a grade 2 and 59% suffered from a grade 3 tumour (Table 2) (Article II).

**FHIT and other gene alterations**

Lung cancer cases were investigated to examine the association between mutations of TP53 or K-ras genes and FHIT alterations (either allelic loss or decreased protein expression). No significant association between FHIT aberrations and TP53 or K-ras mutation was observed (Article II).

**Table 2. Summary of FHIT alterations in lung cancer.**

<table>
<thead>
<tr>
<th></th>
<th>LOH at FHIT gene 1</th>
<th>Decreased Fhit expression 2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>%</td>
<td>Informative cases</td>
</tr>
<tr>
<td><strong>Histology</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-small cell lung cancer</td>
<td>20</td>
<td>83</td>
</tr>
<tr>
<td>Squamous cell carcinoma</td>
<td>24</td>
<td>42</td>
</tr>
<tr>
<td>Adenocarcinoma</td>
<td>9</td>
<td>35</td>
</tr>
<tr>
<td>Small cell lung cancer</td>
<td>50</td>
<td>3</td>
</tr>
<tr>
<td><strong>Disease stage</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IA+IB</td>
<td>14</td>
<td>44</td>
</tr>
<tr>
<td>II+IV</td>
<td>29</td>
<td>42</td>
</tr>
<tr>
<td><strong>Grade</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1-2</td>
<td>14</td>
<td>35</td>
</tr>
<tr>
<td>3</td>
<td>30</td>
<td>43</td>
</tr>
<tr>
<td><strong>Smoking history</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Former and non-smokers</td>
<td>30</td>
<td>33</td>
</tr>
<tr>
<td>Current smokers</td>
<td>18</td>
<td>56</td>
</tr>
<tr>
<td><strong>Asbestos exposure</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>16</td>
<td>51</td>
</tr>
<tr>
<td>Yes</td>
<td>25</td>
<td>24</td>
</tr>
<tr>
<td><strong>P53 mutation</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>21</td>
<td>39</td>
</tr>
<tr>
<td>Yes</td>
<td>24</td>
<td>49</td>
</tr>
<tr>
<td><strong>K-ras mutation</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>19</td>
<td>60</td>
</tr>
<tr>
<td>Yes</td>
<td>20</td>
<td>15</td>
</tr>
</tbody>
</table>

1 LOH detected with at least one of the FHIT intragenic markers (D3S1234, D3S1300, D3S1481).
2 See Materials and Methods of article IV for details.
Genetic alterations in malignant mesothelioma

LOH at multiple loci

Altogether seven malignant mesothelioma cell cultures and 32 overlapping fresh frozen tissue specimens were studied for LOH in five chromosome regions (3p, 6q, 9p, 14q, and 22q), focusing on chromosome region 22q, especially the \textit{NF2} gene region of 22q12. Allele loss at chromosome 22q was detected in all (7/7) of the MM cell cultures. LOH was less consistent but still frequent in chromosomes 9p (71%), 3p (57%), 14q (43%), and 6q (43%) in the same samples. In addition to the cell lines, DNA from 22 resected tumours and 13 PET samples were analysed for LOH with three markers from 22q, but LOH was detected in only two fresh tumours and in one PET tumour sample. However, the fact that we detected LOH in MM cell cultures in very early passages may suggest that a relatively large proportion of malignant cells in the original tumours do have allele loss in 22q. The reason for the less frequently detectable LOH in DNAs from fresh tumours or paraffin-embedded samples is likely to be contamination of tumour tissue with normal cells such as stromal cells and peripheral blood lymphocytes. It is also hypothesised that cell lines may represent outgrowth of subclones from the primary tumour which may have changes somewhat different from the original tumour (Virmani \textit{et al.}, 1998). Thus, the possibility that the alterations detected in MM cell lines accured during cell culture can not be ruled out.

Figure 5 shows the schematic map of chromosome 22q indicating the microsatellite markers used in the LOH studies (Articles III and IV).
Figure 5. Map of the long arm of chromosome 22 indicating markers used in the LOH studies. The distance (megabases) from the telomere of the short arm of the chromosome of the markers is indicated in brackets. The arrow shows the location of the \textit{NF2} gene.
**RESULTS**

*Nf2 protein expression*

In a Western blot analysis of Nf2 protein, merlin, in two MM cell lines and three primary cultures of normal mesothelial cells, all normal and one MM cell line expressed the protein, but in one cell line, no protein expression was detected (Article III).

*Fhit expression*

Altogether 13 paraffin embedded MM samples were analysed by immunohistochemistry for Fhit protein expression. All tumours showed decreased immunostaining as compared to the normal bronchial epithelium or mesothelium. Fhit expression was absent or markedly reduced in 54% of the tumours, with the weakest staining occurring in poorly differentiated areas. No correlation between decreased Fhit protein expression and allelic loss at the *FHIT* gene detected as LOH was seen. (Pylkkänen et al. unpublished data).

**Loss of heterozygosity and NF2 gene in GIST**

*LOH at 22q*

Overall, 69% of GISTs showed LOH at 22q with at least one marker. In figure 5 the markers used are indicated in a schematic map of chromosome 22q. In the comparison between allelic loss and mitotic activity (MI) of tumour, LOH at 22q was more frequent in tumours with high MI (88%) compared to tumours with low MI (56%). The results demonstrate a significant (p<0.01) correlation between LOH at 22q and mitotic activity. Patient age or tumour size did not correlate with allele loss. Allele loss at 22q correlated with tumour location and disease outcome, but due to the relatively small number of tumours, the correlation did not achieve statistical significance. Tumours located in the small intestine showed more often allele loss than the tumours in the stomach (40 vs 23%; OR 3.1, 95% CI 0.2-16.7). The risk of recurrence was 4.6 fold (OR 4.6, 95% CI 0.5-49.8) for tumours with loss of entire 22q as compared to tumours with no LOH.
**NF2 gene mutation analysis**

Five GISTs with LOH at NF2 gene region were analysed for mutations of the NF2 gene by direct sequencing of exons 1-15 of the gene. The sequence analysis did not demonstrate any changes of NF2 in the GIST samples studied.

**Nf2 protein expression**

Expression of merlin was studied in 19 tumours by immunohistochemistry. Three tumours (16%) were negative, while 16 tumours (84%) revealed protein expression. No correlation was detected between immunoreactivity and mitotic activity or clinical behaviour.

In table 3 the main findings of this thesis concerning allelic deletions at different chromosomal loci and alterations of FHIT and NF2 tumour suppressor genes in different tumour types are summarised.
Table 3. The summary of the main findings of the present work by tumour type.

<table>
<thead>
<tr>
<th>Alterations(^1) (no. of microsatellite markers studied)</th>
<th>Non–small cell lung cancer(^2) (tumour samples)</th>
<th>Malignant mesothelioma (cell lines)</th>
<th>Gastrointestinal stromal tumours (tumour samples)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chromosomal region</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3p (4-6)</td>
<td>LOH (21/84; 25%)</td>
<td>LOH (4/7; 57%)</td>
<td>N.D.</td>
<td>Articles I, III</td>
</tr>
<tr>
<td>6q (5)</td>
<td>N.D.</td>
<td>LOH (3/7; 43%)</td>
<td>N.D.</td>
<td>Article III</td>
</tr>
<tr>
<td>9p (3)</td>
<td>LOH (9/74; 12%)</td>
<td>LOH (5/7; 71%)</td>
<td>N.D.</td>
<td>Articles I, III</td>
</tr>
<tr>
<td>14q (3)</td>
<td>N.D.</td>
<td>LOH (3/7; 43%)</td>
<td>N.D.</td>
<td>Article III</td>
</tr>
<tr>
<td>22q (7-12)</td>
<td>N.D.</td>
<td>LOH (7/7; 100%)</td>
<td>LOH (29/42; 69%)</td>
<td>Articles III, IV</td>
</tr>
<tr>
<td>(\text{FHIT gene})</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Allelic loss with intragenic markers</td>
<td>LOH (17/83; 20%)</td>
<td>LOH (3/7; 43%)</td>
<td>N.D.</td>
<td>Articles II, III</td>
</tr>
<tr>
<td>Protein expression</td>
<td>Reduced (31/49; 63%)</td>
<td>Reduced (7/13 tumours; 54%)</td>
<td>N.D.</td>
<td>Article II, unpublished data</td>
</tr>
<tr>
<td>(\text{NF2 gene})</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Allelic loss with intragenic marker</td>
<td>N.D.</td>
<td>LOH (5/7; 71%)</td>
<td>LOH (11/42; 26%)</td>
<td>Articles III, IV</td>
</tr>
<tr>
<td>Protein expression</td>
<td>N.D.</td>
<td>Reduced (1/2; 50%)</td>
<td>Positive (16/19; 84%)</td>
<td>Articles III, IV</td>
</tr>
<tr>
<td>Sequence analyses</td>
<td>N.D.</td>
<td>N.D.</td>
<td>No mutations (5/5; 100%)</td>
<td>Article IV</td>
</tr>
</tbody>
</table>

\(^1\) The intragenic markers used were D3S1234, D3S1300, D3S1481, D3S1313 (\(\text{FHIT gene}\)) and NF2CA3 (\(\text{NF2 gene}\)). The protein expression was analysed by immunohistochemistry.

\(^2\) The number of positive cases out of informative cases studied with the percentage is indicated in brackets. LOH; loss of heterozygosity. N.D.: no data.
DISCUSSION AND CONCLUSIONS

Most malignant tumours express multiple genetic alterations, such as mutations of oncogenes and tumour suppressor genes as well as genetic instability at both the chromosomal and DNA level. Studies on the functions of proto-oncogenes and tumour suppressor genes indicate that most of these genes mediate signal transduction pathways as well as controlling the cell cycle thus playing a critical role in cell proliferation and differentiation (Munger, 2002).

**Microsatellite instability**

Alterations of repetitive DNA sequences in tumour tissues, i.e. microsatellite instability, are one particular characteristic of hereditary non-polyposis colorectal cancer and most often due to mutations in the DNA mismatch repair genes (Wheeler *et al.*, 2000). In contrast to most human neoplasms, tumours with frequent microsatellite instability such as HNPCCs have a normal diploid karyotype (Boland, 2000). The finding that microsatellite instability is typical for HNPCC, but occurs less frequently in non-hereditary cancers, led to a still unresolved debate of whether genetic instability occurs either at the DNA or at the chromosomal level and that different types of genomic instability could be a consequence of the distinct effects of specific carcinogens (Breivik & Gaudernack, 1999; Cahill *et al.*, 1999). According to this hypothesis, different types of genomic instability lead to different mechanisms of inactivation of tumour suppressor genes or activation of oncogenes, though the consequence is the same in both events, i.e. targeting of crucial growth–controlling pathways (Cahill *et al.*, 1999).

We found no evidence of microsatellite instability in smoking related lung cancer or asbestos exposure related malignant mesothelioma or gastrointestinal stromal tumours, a neoplasm without known association to environmental carcinogens. These data suggest that genetic defects in the DNA mismatch repair pathway play a minor role in these cancers. The finding concerning lung cancer is in accordance with studies by others where total absence or only very infrequent microsatellite instability has been detected (Fong *et al.*, 1995; Peltomaki *et al.*, 1993; Rasio *et al.*, 1995). However, all the cancer types studied in this thesis were characterised by frequent LOH, which may indicate that the
tested loci may represent the location of a tumour suppressor gene that could be involved in tumour formation.

However, resistance to the cytotoxic effects of various DNA-damaging agents, including alkylating agents and chemotherapeutic agents, has been demonstrated in cell lines defective in mismatch repair (Branch et al., 1993; Glaab et al., 1998; Karran & Bignami, 1994). These observations may have practical implications for human carcinogenesis as cells defective in MMR are more resistant to the cytotoxic effects of normally toxic compounds (Glaab et al., 2000). By increasing the mutation rate through selection, there is a higher probability of mutations to occur in essential growth control genes that may promote tumourigenesis. It seems logical to assume that daily exposure via lifestyle, occupational exposure, smoking etc. will produce a similar cellular response (e.g. increased resistance and hypermutability) in MMR defective cells (Glaab et al., 2000).

**FHIT alterations in lung cancer**

In lung cancer, the high occurrence of LOH at 3p is good evidence for the presence of at least one tumour suppressor gene in this chromosomal region. Alterations of the *FHIT* gene (at 3p14.2) have been shown to be associated with exposure to environmental carcinogens and is thus suggested to have an important role in lung carcinogenesis (Inoue et al., 1997; Sozzi et al., 1997a). For example, allelic loss is shown to be more frequent in lung cancers of smokers than of the patients who have never smoked and (Sozzi et al., 1997a). In addition, an association between smoking duration and asbestos exposure and *FHIT* exon loss was seen in a study by Nelson and co-authors (1998). They hypothesised that asbestos may directly interact with the target cells in the respiratory epithelium, producing alterations at the *FHIT* locus (Nelson et al., 1998). In agreement, we found frequent decreased Fhit protein expression in the tumours of current smokers and in cases with occupational asbestos exposure. Similar results of reduced presence of Fhit protein in non–small cell lung cancer tumours have been reported by others (Sozzi et al., 1997b; Tomizawa et al., 1998; Tseng et al., 1999).

It is interesting that the frequency of LOH at the *FHIT* gene did not correlate with the detected protein expression. One reason for this may be the contaminating non-neoplastic cells in the DNA sample that may result to false detection of LOH by PCR.
DISCUSSION AND CONCLUSIONS

technique. Another explanation for this finding may be the fact that although the
cell divisions allowing the expression of only the particular genes
necessary for that cell type (Esteller, 2000; McBurney, 1999). In cancer cells, gene
function can be disrupted by aberrant methylation patterns which alter the state of gene
expression (Esteller, 2000). Methylation explains the inactivation of tumour suppressor
genes at various frequencies. In a study by Huang et al. (1999) several hundred human
CpG islands of normal tissues and cancer cell lines were analysed for methylation
pattern, and methylation specific to the cancer cell lines was found in 10% of the cell
lines (Huang et al., 1999). On the other hand, the CDKN2A gene is reported to be
frequently methylated in many different cancers, even up to 90% of colon cancer cell
lines (Gazzeri et al., 1998; Herman et al., 1995). In addition to methylation, there are
a number of other mechanisms that can regulate gene expression. The control can occur at
transcriptional, translational or at protein level. Most genes may be regulated at multiple
levels, although control of the initiation of RNA transcription (transcriptional control) is
perhaps the most important point of control (Alberts et al., 1994; Strachan & Read, 1999).

Fhit protein expression in cancer cells can be reduced or absent, even without detectable
tumour specific DNA or RNA alterations (Boldog et al., 1997; Mao, 1998), thus the
protein expression may be down-regulated by epigenetic changes or at the post-
transcriptional level (Tanaka et al., 1998; Zochbauer-Muller et al., 2001). It is also notable,
that the FHIT gene spans the most active of the common fragile sites of the human
genome, FRA3B. In cultured cells this has been shown to respond to exposure to many
different mutagens and carcinogens (Sozzi et al., 1998). Thus it is possible that the
alterations of FHIT gene are a consequence of the sensitivity of the FRA3B, leading to
general genomic instability and aberrations of the genes present in this site (Sozzi et al.,
1996). The association between common fragile sites and genes involved in
tumourigenesis has led to the hypothesis that fragile sites may be involved in general in
the chromosomal rearrangements commonly observed in many neoplasms (Sozzi et al.,
1998). However, the biological role of common fragile sites is still unknown. As the alterations at \textit{FHIT} gene are associated with only a subset of environmental pulmonary cancers, the presence of additional still unknown genes of importance in the malignant transformation of a pulmonary epithelial cell cannot be ruled out.

\textit{LOH in malignant mesothelioma}

Although the connection between malignant mesothelioma and asbestos exposure is well documented, still only approximately 10% of individuals heavily exposed to asbestos will develop MM and on the other hand about 20% of MM patients have no clear evidence of asbestos exposure (Murthy & Testa, 1999). This may partly be explained by the fact that there is individual variation in the susceptibility to the carcinogenic effects of asbestos (Hirvonen \textit{et al.}, 1996).

The molecular mechanisms regarding carcinogenesis of mesothelial cells are largely unknown, although recent evidence suggests multiple processes involving both activation of oncogenes and inactivation of tumour suppressor genes (Testa & Giordano, 2001). Asbestos has been demonstrated to induce a significant increase of allelic loss in human mesothelioma cells in culture (Both \textit{et al.}, 1995) and extensive chromosomal aberrations have been found in mesothelioma tissues by cytogenetic analysis (Popescu \textit{et al.}, 1988; Tiainen \textit{et al.}, 1992). These data suggest that a number of molecular events may be related to the initiation and development of mesotheliomas induced by asbestos. In addition, asbestos fibres are known to generate reactive oxygen species that can cause DNA single strand breaks (Wang \textit{et al.}, 1987). This has been considered as indirect evidence for the role of free radicals in fibre induced carcinogenesis (Ollikainen \textit{et al.}, 1999). Also, it is suggested that the simian virus 40 (SV40) infection may be in connection with human malignant mesothelioma as DNA sequences homologous to SV40 has shown to be present in some MM tumours (Carbone \textit{et al.}, 1994; Testa \textit{et al.}, 1998).

In our study, we found concurrent allelic loss at multiple loci in MM cell lines, with the highest frequencies at 22q, 9p, and 3p, the chromosomal regions where the tumour suppressor genes \textit{NF2}, \textit{CDKN2A}, and \textit{FHIT} are located, respectively. In line with these findings, earlier studies (cytogenetic, CGH, and LOH analyses) have revealed several simultaneous abnormalities in human MM, one of the most frequently detected
aberrations being the loss of the long arm of chromosome 22 (De Rienzo & Testa, 2000; Kivipensas et al., 1996; Popescu et al., 1988).

It has been postulated that in malignant mesothelioma, several genetic alterations induced by asbestos accumulate over time, eventually leading to cellular malignancy. When a key regulator gene, such as CDKN2A, is affected, the cells will divide in an uncontrolled manner and additional mutations can accumulate (Rizzo et al., 2001). Once the cell has become malignant, cell growth will be rapid and the tumour will become clinically detectable. This scenario would account for the clinical observation that malignant mesotheliomas are very aggressive and eventually fast growing malignancies which develop 20-50 years after asbestos exposure (Rizzo et al., 2001).

Merlin, the NF2 tumour suppressor gene encoding protein, plays a role in cell surface dynamics and structure by linking the cytoskeleton to the plasma membrane (Hovens & Kaye, 2001). As mutations of the NF2 gene have been reported in MM cells (Bianchi et al., 1995; Cheng et al., 1999; Sekido et al., 1995) it has been proposed that inactivation of the NF2 gene could be involved in the fibre-related transformation of mesothelial cells (Lechner et al., 1997). Our present data indicating consistent LOH at t 22q in human MM, are compatible with the idea that tumour suppressor gene(s) located at this chromosomal region may contribute, either alone or in conjunction with other tumour suppressor genes, to the development of human MM. The recent completion of sequencing of chromosome 22 (Dunham et al., 1999; Lander et al., 2001) should facilitate identification of the gene(s) involved in the pathogenesis of malignant mesothelioma and many other cancers. However, as most MM specimens show a large variety of different changes at the molecular level, it is obvious that yet unidentified tumour suppressor gene(s) in genome exist. Identification of genetic alterations in MM and understanding how each of these changes contributes to the pathogenesis of this disease may have important therapeutic implications (De Rienzo & Testa, 2000).

*Genetic alterations in GIST*

Gastrointestinal stromal tumours are most common mesenchymal tumours of the gastrointestinal tract (Miettinen & Lasota, 2001). Up today, there have been few markers specific for GISTs. This has complicated the differential diagnosis of this neoplasm from tumours of smooth muscle origin. Recently, the proto–oncogene c–kit, has been
shown to be a very relevant marker as it almost invariably is expressed in GISTs (Chan, 1999; Hirota et al., 1998; Nishida et al., 1998). Mutations of this gene are frequently detected in human GISTs and transfection of the mutant c–kit into murine lymphoid cells has been shown to induce malignant transformation. This is evidence that the mutations of c–kit may contribute in the tumourigenesis of GISTs (Hirota et al., 1998; Nakahara et al., 1998). However, as only about half of GISTs show c–kit mutations (Ernst et al., 1998; Lasota et al., 1999; Moskaluk et al., 1999) alternative mechanisms leading to malignant transformation of GISTs are possible.

Molecular studies have shown that several different genetic changes occur in GISTs. The most frequent alterations in CGH and LOH studies have been revealed to be losses of chromosomes 22q and 14q, both in benign and malignant tumours (Breiner et al., 2000; El-Rifai et al., 2000a; El-Rifai et al., 2000b). In line with the CGH results indicating losses of 22q, we detected frequent LOH at 22q in GISTs, both in tumours with high mitotic activity as well as in tumours with low mitotic activity, which is an indicator of potential malignancy of these tumours. Interestingly, LOH was significantly more frequently detected in tumours with high mitotic activity. Thus, allelic loss at these chromosome sites may be a common and primary abnormality responsible for the development of GIST.

We detected no mutations of the NF2 gene in the sequence analyses comprehending five GISTs. However, NF2 gene mutations was identified in two GIST cases in a study by Fukasawa and co-authors suggesting that NF2 may act as a tumour suppressor in a subset of these tumours. The NF2 gene mutations detected occurred independently of c-kit mutations, since no correlation between NF2 mutations or LOH at 22q and the c-kit mutations was observed (Fukasawa et al., 2000).

Among the aberrant chromosomal regions, losses of 9p are of particular interest. Homozygous deletions has been found in two GISTs cases in the restricted area of 9p containing the CDKN2A gene (Kim et al., 2000). Since homozygous deletion is one of the known mechanisms for inactivation of a tumour suppressor gene, it may be possible that inactivation of CDKN2A plays a role in tumourigenesis of GISTs, at least in some cases. In a recent study, chromosomal changes in GISTs were investigated in relation to malignant behaviour and the presence of c-kit mutations (Debiec-Rychter et al., 2001). Interestingly, associations with malignancy, c-kit mutations and loss of chromosome 9 were detected (Debiec-Rychter et al., 2001). However, currently only c-kit mutations have
been shown to clearly correlate with malignancy and poor prognosis, and thus proposed to have an important role as a prognostic factor (Taniguchi et al., 1999).

Several factors contribute to genomic instability and cells accumulate many different genetic changes during carcinogenesis. The multiple forms of genetic changes may reflect cellular heterogeneity seen within individual tumours and heterogeneity between tumours of the same type. This heterogeneity is reflected as differences in the histological, karyotypic, molecular, physiological and biochemical factors that can be observed by examining different parts of the same tumour (Cahill et al., 1999). In lung cancers, it is hypothesised that the heterogeneity of bronchial tumours may be a consequence of the chronic exposure of the numerous different carcinogens, e.g. carcinogens present in tobacco, which leads to several cell clones carrying different genetic changes [reviewed in (Sozzi & Carney, 1998)]. This may be the case with other tumours also associated with environmental carcinogen exposure.

Interestingly, many of the genetic alterations detected in invasive lung tumours have been demonstrated also in the bronchial epithelium of both current and former smokers without cancer, indicating that molecular damage is already present in carcinogen-exposed bronchial cells (Mao et al., 1997; Marchetti et al., 1998; Miozzo et al., 1996). These observations may provide new methods for early detection lung cancer. In addition, identification of genetic alterations crucial for the development of lung cancer can be used for cancer prevention and for the development of the novel therapies specifically targeted to the disrupted cellular activity (Sozzi & Carney, 1998). Recent studies have also highlighted the possibility to use genetic alterations as early markers since these are detectable in plasma or serum DNA from cancer patients, including lung cancer patients (Anker et al., 1999; Chen et al., 1996; Cuda et al., 2000; Handel-Fernandez & Vincek, 1999; Sanchez-Cespedes et al., 1998; Sozzi et al., 1999).

Understanding the interplay between exposure to specific environmental carcinogens and genetic variability of important genes, will act in the ultimate goal of comprehending cancer etiology. This new knowledge will help in the identification of individuals who are at increased risk of developing cancer and will hopefully lead to better biomarkers for the early detection of cancer and cancer prevention.
Summary

In summary, the main conclusions based on the results obtained during this PhD thesis work are presented below.

Smoking related lung cancer was not observed to show microsatellite instability, as also found by others. This suggests that genetic defects in the DNA mismatch repair genes are likely to play only a minor role in lung cancer related to external exposure. Instead, frequent loss of heterozygosity, especially at chromosome 3p is a characteristic feature of lung cancer. Analysis of the FHIT gene indicated that there is a frequent decrease of Fhit protein expression, and less frequent LOH at the FHIT locus. Decreased Fhit protein expression was common in smokers and in cases with occupational asbestos exposure but the difference from the corresponding non-exposed groups was not statistically significant. These data support the proposed significance of Fhit inactivation in development of lung cancer.

Our LOH data on human malignant mesothelioma cell lines support the view that chromosomes 22q and 9p are the preferential regions for the tumour suppressor genes that may have a critical role in MM tumourigenesis. Taken together, this data and reports in the literature strongly suggest that the development of MM involves the accumulation of alterations in a number of tumour suppressor genes.

Frequent LOH detected at 22q in GISTs especially in malignant tumours suggests that a tumour suppressor gene located at this chromosomal region is involved in tumourigenesis of GIST. However, the absence of NF2 gene mutations and the presence of merlin in the tumour tissue are evidence that another, still to be identified, gene is responsible for the development of GIST.
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