Molecular biology of lung cancer

Gabriella Sozzi

Istituto Nazionale Tumori, Divison of Experimental Oncology A, I-20133 Milan, Italy

Introduction

It has been established that lung cancer arises as a consequence of the accumulation of multiple somatic genetic changes (10–20 mutations) involving critical genes whose protein products control cell proliferation, differentiation and apoptosis. These genes include protooncogene (positive growth regulators), tumour suppressor genes (negative growth regulators) and genes involved in apoptotic control. In addition, other more generalised changes are chromosomal rearrangements such as deletions and non-reciprocal translocation, microsatellites (DNA repeat sequences) instability, deregulated expression of telomerase and angiogenesis (Fig. 1).

Before the appearance of a clinically overt lung cancer, a series of morphologically distinct preneoplastic changes such as hyperplasia, dysplasia and carcinoma *in situ* can occur in the bronchial epithelium, as a result of the chronic exposure of the bronchial epithelium to carcinogens, a phenomenon termed 'field cancerization effect'. The preneoplastic cell may contain several molecular genetic abnormalities identical to those observed in lung cancer cells (Fig. 1). Risk factors that identify normal and premalignant bronchial tissue at risk for malignant progression can thus be defined at a molecular level.

Identification and characterisation of the genetic changes that drive lung cancer development and progression can provide us with a variety of molecular markers that may ultimately redefine the criteria for cancer diagnosis and provide new tools for early detection, through the application of sensitive techniques that detect molecular changes in accessible biological specimens and for developing novel targeted cancer and pre-cancer therapies.

Genetic alterations in invasive tumours and precancerous lesions

Oncogene activation

Protooncogenes are normal cellular counterparts of dominant oncogenes and encode proteins that are positive effectors of the transformed phenotype. The activation of protooncogenes occur through a different mechanism that target only one allele such as chromosomal translocation, gene amplification, point mutations and constitutive overexpression that lead to a gain of function effect. Protooncogenes that are involved in transduction of a mitogenic signal from the cell membrane to the nucleus are abnormally activated in lung cancer. In particular, tyrosine kinase receptors are overexpressed in non-small cell lung cancer (NSCLC), and include the activation of epidermal growth factor receptor (EGFR) that occurs in about 50% of NSCLC, predominantly of the squamous cell type (SQC) [1,2]. HER2/neu overexpression observed in about 30% of adenocarcinoma (ADC) [3,4] and cKIT, with its ligand, stem cell factor, are expressed in 70% of small cell lung cancer (SCLC). In addition, the *MET* protooncogene and its ligand Scatter Factor (HGF/SF) are consistently

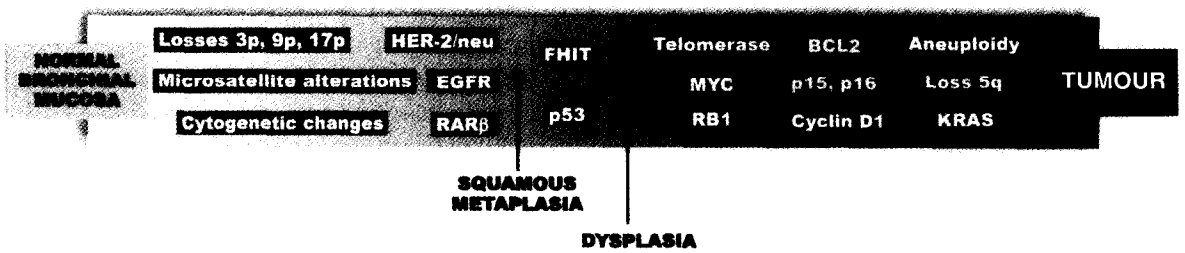

Fig. 1. Sequential molecular changes during lung cancer pathogenesis.

activated in 25% of lung tumours, where they possibly contribute to the invasive growth of tumour cells [5]. Of interest, EGFR overexpression was found in 30/62 (48%) of precancerous lesions associated with invasive tumours in a series of 34 patients [1] suggesting that EGFR expression could be an early marker of lung carcinogenesis. In accordance with this hypothesis is the observation that both EGFR and HER2/neu were already overexpressed in distant, normal-appearing bronchial mucosa specimens of lung cancer patients [6]. Overall activation of tyrosine kinase receptors and autocrine/paracrine growth mechanisms in lung tumours suggest the possibility of developing new therapeutic strategies directed at interrupting tumour cell growth such as the use of tyrosine kinase inhibitors [7].

Signal transduction is initiated by the binding of extracellular growth factors to their receptors, leading to receptor dimerisation, which initiates a cascade of protein activation. The *KRAS* gene and the related genes *NRAS* and *HRAS* encode proteins termed p21, which are attached to the inner surface of cell membrane, and possess guanine triphosphate, GTPase, activity and function in signal transduction. Activating point mutations in the *RAS* genes inactivate their GTPase activity and promote cell growth. Most activating *RAS* mutations in human tumours occur in codons 12, 13 and 61. In lung cancer, *RAS* mutations are found most often in *KRAS* codon 12, less often in codons 13 and 61 and are rare in *NRAS* and *HRAS*. Even though published studies contain discrepancies, likely arising from methodological differences in mutation detection, a few conclusions can be drawn: *KRAS* mutations are most common in ADC (20–30%), where very sensitive assays may detect up to 50% of mutations [8]. In SQC, a few *RAS* mutation-positive cases are reported, whereas they occur very rarely in SCLC. Whereas *RAS* mutations seem to be associated with smoking habits and occupational exposure [9], the presence of *RAS* mutation has been reported to identify a poor-prognosis subgroup [10].

Concerning the precocity of *RAS* mutations, several groups reported that samples of AAH (atypical alveolar hyperplasia), which may be a precursor to lung adenocarcinoma, harboured *KRAS* codon 12 mutations; all these AAH samples were from patients with ADC [11]. However, the presence of *RAS* mutations in only a fraction of the tumour cells suggest that they are not early events, but more likely arise in subclones of the tumour and thus are acquired after clonal growth is established [12]. Therapies based on inhibiting RAS function, particularly inhibition of ras farnesyltransferase, the enzyme that catalyses the binding of the ras p21 protein to the inner surface of the plasma membrane, which is critical for normal and oncogenic activity of RAS, are novel antitumour agents.

The *MYC* nuclear protooncogene is one of the ultimate nuclear targets of the signal transduction cascade. *MYC* activation via gene amplification or transcriptional dysregulation occurs very frequently in SCLC [13], particularly in cell lines with 'variant' phenotype where it is related to adverse survival [14]. However, it has been reported that deregulated *MYC* expression is present also in 50% of NSCLC tumours and in a proportion of associated preneoplastic lesions suggesting an early role for *MYC* in lung cancer development [15].

The *BCL2* gene is not strictly considered an oncogene and it is involved in the inhibition of programmed cell death (apoptosis). *BCL2* is expressed in more than 80% of SCLC and it has been also found in 25% and 12% of SQC and ADC, respectively. In SQC, *BCL2* expression was associated with worse survival [16].

Cytogenetics 3p, LOH and genetic instability

Allelic loss (LOH) is considered one step, usually the first one, necessary to inactivate a tumour suppressor gene (TSG). It is usually considered significant in the presence of a mutation in the retained allele. Alternatively, since some TSG, like *p16* and *FHIT*, are inactivated by homozygous deletions and contamination of normal cells in the tumour samples could mask their detection in primary tumour specimens, allelic loss is accepted as a preliminary indication of TSG inactivation in primary tumours, even in the absence of mutation in the second allele. Allelic imbalances are thus widely used to assess genetic changes and have been used primarily to identify regions on specific chromosomes that contain putative tumour suppressor genes. LOH studies on NSCLC tumours have demonstrated recurrent losses at 3p, 5q, 9p and 17p, sites that coincide with the location of an established or suspected TSG [17].

Deletions of the short arm of chromosome 3 are considered critical events in the pathogenesis of lung cancer. In 1982 Whang-Peng et al. [18] first reported a deletion of 3p in all 16 SCLC cell lines. The most common pattern of deletion was an interstitial deletion of 3p (14–23). In contrast, Wurster-Hill [19] reported a chromosome 3p deletion in only 3 of 15 cases studied. Subsequently, other groups have reported data on cell lines and primary tumours confirming that a cytogenetic deletion of 3p was present in the majority of the SCLCs [20–24]. The cytogenetic analysis of the other histological types

of lung tumours, grouped together as NSCLC, has revealed complex karyotypes with frequent loss and rearrangements of chromosome 3p [25–30].

In addition to cytogenetically visible deletions, LOH has been observed in nearly 100% of SCLC [31–33].

In contrast, the first Restriction Length Polymorphism Fragment (RFLP) analysis of allelic losses in NSCLC showed that LOH of 3p were present in only 4 out of 15 informative patients [32]. A subsequent study indicated that LOH at a locus located in 3p21 (DNF15S2) occurred in 100% of NSCLC [33]. The analysis of a much larger number of primary tumours definitely demonstrated that loss of alleles on 3p is a common event (>70%) also in NSCL tumours [34,35].

These findings were further supported by a molecular mapping of deletion sites on 3p in 24 SCLC tumours, where a significant reduction in the frequency of LOH was found at some, but not all, the 3p loci tested [36]. In fact, a different pattern of LOH at 3p loci was observed between SCLC and NSCLC, the latter being characterised by a preferential loss of alleles at loci located in 3p21 compared to the loss of loci in a more distal 3p region observed in SCLC. However, the hypothesis of a common regulatory element located on 3p, whose loss could lead to tumours in different tissues, was also considered.

As an initial step towards positional cloning of the TSGs on 3p, a detailed analysis of the minimum deleted region(s) on 3p was performed with a large number of RFLP probes [13] and lung cancer samples (48 informative cases) [37]. Three distinct regions (3p25, 3p21.3 and 3p14-cen) appeared to be frequent targets for deletions (100% of SCLCs and 80% of NSCLC). Of note, Whang-Peng et al. [38], have reached a similar conclusion by classic cytogenetic analysis of 31 NSCLC.

The first homozygous deletion in SCLC was identified in cell line U2020 [39]. As mentioned before, homozygous deletions in DNA of cell lines complement LOH results and facilitate determination of deletion boundaries. This submicroscopic homozygous deletion involved the D3S3 locus on 3p14-13 and was estimated to involve 4–7 Mb of DNA [40,41]. Further studies have shown that the U2020 deletion spans approximately 8 Mb and is located in 3p12-13 [42]. A physical map of the region homozygously deleted in the U2020 cell line at 3p12, including the location of putative CpG islands, has been constructed. Adjacent to one of these islands, a new gene has been identified and cloned, *DUTT1*, a member of the neural cell adhesion molecule family. Homozygous deletions occurring in lung and breast carcinomas would result in a truncated protein [43].

Several groups have subsequently described homozygous deletions involving 3p21 in SCLC cell lines. Yamakawa et al. [44], found a homozygous deletion involving a single cosmid marker in 3p21.3-22 in five SCLC cell lines and a gene related to the integrin α subunit (α_{RLC}) isolated from this deletion was identified, although no mutation was observed in SCLC [45]. However, this gene was aberrantly upregulated in SCLC tumours and cell lines suggesting still a possibility of misregulation of the gene by different mechanisms. A homozygous deletion in 3p21.3-p21.2 was found in the SCLC cell line NCIH740 [46] involving markers that were located in a chromosomal 3p fragment previously shown to exhibit tumour suppressor activity in a mouse fibrosarcoma cell line [47]. Tumour suppressor activity for the entire chromosome 3 in a lung adenocarcinoma cell line was also demonstrated [48].

Several genes in the 3p21 homozygous deletion were isolated. They included: the aminoacylase gene (*ACY1*), which showed reduced expression in about 20% of SCLC cell lines [49,50]. The acylpeptide hydrolase gene (*APEH*), corresponding to the D3F15S2 polymorphic locus most frequently deleted in SCLC, found overexpressed in one of four SCLC cell lines in a single study [49], but not in subsequent analyses [51]. The ubiquitin-activating enzyme E1 (*UBE1L*) (formerly D8) which showed a reduced expression in SCLC cell lines [51,52]; the guanine nucleotide binding protein (*GNAI2*) which was revealed to be universally expressed in SCLC cell lines [46] and two human zinc finger genes *ZnF16* and *ZnF3* [53]. However, no convincing evidence of alteration at genomic, transcriptional and translational levels in lung cancer was provided for any of these genes and they were thus excluded as candidate TSGs. The *hMLHI* DNA mismatch repair gene which is located immediately adjacent to the homozygously deleted region in 3p21.3 showed no involvement in the deletions identified in lung tumours [54]. More recently, a new *Semaphorin* gene in the common deletion region of 3p21.3 of three SCLC cell lines has been identified [54]. Even though reduced expression levels of this gene were reported in two SCLC cell lines, no further evidence has been provided in order to demonstrate a tumour suppressor activity. Recently, an 80 Kb clone from this same chromosomal region (3p21.3) was shown to suppress tumour growth *in vivo* in a mouse fibrosarcoma cell line [55]. Thus far, no solid candidate for the tumour suppressor gene(s) in 3p21 region has yet been identified.

The only definite 3p linked TSG is the Von Hippel-Lindau (*VHL*) gene, at 3p25. Von Hippel-Lindau disease is a familial cancer syndrome, dominantly

inherited, that predisposes affected individuals to a variety of tumours and as such renal cell carcinoma. The *VHL* gene was shown to be mutated in the germ-line of VHL disease families [56]. The gene is also inactivated in a considerable fraction of sporadic renal cancer [57], but only rarely mutated in lung tumours [58].

The region 3p14 is frequently lost, not only in lung tumours [37], but also in tumours of different type such as renal [59,60] breast [61,62] and oral cancer [63,64] and is the site of homozygous deletions in a range of cancer-derived cell lines [65–67]. It also contains the 3p break of a hereditary renal-carcinoma associated translocation, t(3;8) (p14.2;q24), which has been shown to segregate in a family with early onset of bilateral and multifocal clear cell renal carcinoma [68]. This translocation region has been studied by several groups [69–72]; the translocation break was cloned, and a cDNA mapping just telomeric to the 3p14.2 translocation has been suggested as a human renal carcinoma gene [73]. The receptor protein-tyrosine phosphatase γ (*PTP*γ) gene has been mapped at 3p21-14 and suggested as a candidate tumour suppressor gene [71]. Although one *PTP*γ allele was lost in 50% of lung and renal carcinoma tumour samples analysed, the gene was normally expressed in lung tumour cell lines [71]. The localisation of *PTP*γ was subsequently refined to 3p14.2 centromeric to the t(3;8) breakpoint [72], but the 6 Kb coding sequence was not disrupted by the translocation.

The critical role of 3p deletions in lung cancer has been highlighted by the demonstration that allelic losses occur at the very earliest preneoplastic stages of lung cancer. In fact, molecular studies of preinvasive bronchial lesions have found a high frequency of 3p deletions in carcinoma in *situ* and dysplasia [74–76] and also in epithelial hyperplasia [77]. Moreover, cytogenetic deletions of the short arm of chromosome 3 have been detected in normal-appearing bronchial mucosa distant from the tumour [6,78]. These deletions were present in multiple lesions throughout the respiratory epithelium including bronchi, bronchioles and alveoli and the persistence of these molecular changes has been correlated with the evolution of the disease [76].

It has been shown that LOH at 3p, 9p and 17p are associated with the earliest stages of lung cancer pathogenesis being detected not only in precancerous lesions [74,79–81], but also in the normal bronchial mucosa of current and formers smokers without lung cancer [82,83].

These observations suggest that one or more tumour suppressor gene(s) may act as gate-keepers for lung cancer carcinogenesis and are also consistent with the multistep model of carcinogenesis and a 'field-cancerization' effect due to the chronic exposure of the bronchial mucosa to carcinogenic damage such as tobacco smoke, resulting in an increased risk to develop multiple, separate foci of neoplasia.

Genomic instability is considered a hallmark of cancer. Analysis of microsatellite markers in tumour tissue compared with its normal counterpart is used to determine genomic instability in the form of microsatellite alterations (MA). Microsatellite alterations have been reported to occur in lung cancer at various frequencies, ranging from 6 to 66% [84–86], and although the mechanism underlying microsatellite alterations is currently unknown, they may serve as clonal markers for early cancer detection [87,88]. In fact, a recent study identified MA in bronchial lavage specimens of 35% of patients with lung cancer, but also in 23% of individuals without clinical evidence of bronchial neoplasia [89].

Inactivation of tumour suppressor genes

TP53

TP53 was cloned in 1983 and six years later the first *TP53* mutation in a human lung cancer was discovered [90]. The gene functions as a 'guardian of the genome' in normal cellular physiology. In fact, in response to DNA damage, oncogene activation (*MDM2*), viral infection (human papilloma virus (HPV), SV40, adenovirus E1B), hypoxia and oxidative stress, *TP53* regulates the transcription of genes that control cell cycle and apoptosis, and is also directly involved in DNA repair processes [91,92].

Replacement of wild-type (wt) *TP53* into human lung cancer cells carrying a *TP53*-inactivated gene, was shown to suppress both in vitro and *in vivo* tumour growth [93,94]. Preclinical studies and a recent phase I clinical trial of *TP53* gene therapy in lung cancer have demonstrated that adeno-mediated transfer of wt *TP53* inhibits lung cancer growth in patients [95].

TP53 mutations, disrupting its activity by either loss of the normal function or often by gaining oncogenic properties, are common in lung cancer. The mutational spectra of SQC, large cell, ADC and SCLC are different. *TP53* mutations are found in 80% of SCLC, 70% of SQC, 60% of large cell, but only 40% of ADC. G–T transversions, thought to result from bulky molecules found in tobacco smoke, are the prevalent mutation in large cell, SQC carcinoma and SCLC (43–49%), but they are less common in ADC consistent with a weaker association of ADC with cigarette smoking. The mutational

spectra also vary by smoking history: ADC display a lower percentage of G–T transversion (13%) and a higher percentage of C–T transitions (56%) [91]. Recently, a direct aetiological link between a defined chemical carcinogen and human lung cancer has been provided by the demonstration of preferential formation of benzo(a)pyrene adducts at specific *TP53* codons (157, 248, 273) that are the major mutational hot-spots in lung tumours [96].

Missense mutations often prolong the half-life of the p53 protein leading to raised protein levels which can be detected by immunohistochemistry. Taking advantage of these observations, several studies attempted a correlation between overexpression of p53, or *TP53* mutation by molecular analysis, and survival of lung cancer patients. Conflicting results were obtained since *TP53* alterations resulted in a negative prognostic indicator, in studies that included a small number of cases and, also, in studies with a short follow-up duration, moreover these results were not confirmed in larger clinical series with multivariate analyses [2].

The analysis of precancerous lesions occurring in lung cancer patients and also in patients without invasive tumour, disclosed the occurrence of *TP53* alterations detectable at both immunohistochemical and molecular level in dysplastic tissue such as moderate and severe dysplasia [74,79,97]. Composite IHC data show p53 protein accumulation in 0% normal bronchial mucosae, 7% of squamous metaplasia, 25% of mild dysplasias, 32% of moderate dysplasia, 69% of severe dysplasias, 57% of carcinoma *in situ*, 70% of microinvasive carcinomas and 76% of fully invasive lung carcinoma [98]. Overall, these findings indicate that *TP53* alterations characterise intermediate phases of lung carcinogenesis, thus representing a suitable marker for early diagnosis and an intermediate biomarker for chemoprevention studies.

Rb, p16, p15 and cyclin D1 genes

Rb, *p16*, *p15* and *cyclin D1* genes are critical checkpoints of the G1/S cell cycle boundary. The *Rb* gene, located at band q14 of chromosome 13, is the first isolated tumour suppressor gene and encodes a nuclear phosphoprotein of 110 kDa which in its hypophosphorylated form induces G1 arrest. Rb phosphorylation allows the transition through G1/S and the entrance into cell cycle. Specific cyclin-cyclin dependent kinase complexes mediate Rb phosphorylation, such as the cyclin D1-cdk4 proteins, which are negatively regulated by the *p16 INK4/MTS1* gene. Thus *Rb* and *p16*, as well as *cyclinD1/PRAD1*, interplay as key genes in cell cycle control at the G1 checkpoint. Their alterations are critical in lung cancer pathogenesis. In fact, *Rb* loss of function is achieved in 90% of SCLC and 30% of primary NSCLC by loss of protein expression which in several cases is accompanied by structural gene abnormalities such as point mutations, deletions, or splicing abnormalities [99–102]. Conversely, inactivation of *p16INK4/MTS1* located at chromosome band 9p21, through mutation, homozygous deletion and methylation which downregulate its expression, is reported in more than 30% of NSCLC, but very rarely in SCLC [103–106]. Another CDK inhibitor, the *p15INK4B/MTS2* gene, localised close to *p16* on chromosome 9p21, is altered in a subset (20%) of NSCLC [107]. However, LOH at loci localised on 9p21-22 occurs also in SCLC and much more frequently in NSCLC than the frequency of inactivation of the *p16/p15* genes. Thus, the presence of other tumour suppressor gene(s) on 9p has been hypothesised. Overexpression of cyclin D1, resulting in Rb phosphorylation through its interaction with CDK4, is an alternative way to disrupt the G1 checkpoint control in NSCLC [108], whereas SCLC show low cyclin D1 levels. In conclusion, the G1/S growth control pathway represents a crucial step in lung carcinogenesis that is altered through alternative molecular mechanisms in SCLC and NSCLC tumours.

The FHIT gene

The *FHIT* (Fragile Histidine Triad) gene is the first putative tumour supressor gene so far isolated at 3p14.2, one of the more frequently deleted regions of the short arm of chromosome 3 in lung tumours. The gene has several interesting features since it encompasses the familial kidney tumour-associated translocation breakpoint (3;8), the fragile region FRA3B, which spans introns 4 and 5, flanking the first *FHIT* coding exon 5 which is frequently targeted by homozygous deletions in cancer cells of many types, including lung. The 1.1 kb *FHIT* cDNA consists of ten small exons and is distributed over a genomic locus of about a megabase. It encodes a polypeptide of 16.8 kDa which is composed of 147 amino acids that catalyses the in vitro hydrolysis of dinucleoside polyphosphates, with Ap_3A as the preferred substrate [109]. FHIT-substrate or substrate–enzyme complexes may be involved in signalling responses to cellular stress resulting in cell-cycle arrest [110].

So far, more than 100 primary lung tumours have been analysed for abnormalities of *FHIT* expression by reverse transcriptase-polymerase chain reaction (PCR) analyses and sequencing, LOH and Southern blot, as well as by immunocytochemistry [111,112]. Abnormalities in products amplified from

FHIT transcripts were found in 80% of SCLC tumours and in 42% of NSCLC. Sequence analysis of the aberrant products showed that the absence of exons 4 or 5 through to 8 was the most common abnormality. LOH at microsatellite markers internal to the *FHIT* gene was detected in 63% of the tumour specimens. Tumours exhibiting aberrant *FHIT* amplification products also lost one *FHIT* allele, suggesting loss of function of the *FHIT* gene. In order to correlate specific *FHIT* locus DNA and RNA lesions with their effects on FHIT protein expression, we have analysed 10 lung cancer cell lines, 15 SCLC and 38 pairs of non-small cell primary tumours and bronchial mucosa specimens with molecular and immunocytochemical methodologies. By using specific antibodies against the Fhit protein, we could determine that abnormalities in the *FHIT* gene occur at DNA, mRNA and protein level and that overall a high percentage of concordance was observed between RNA abnormalities and lack of the Fhit protein expression in lung tumours and cell lines. In addition, the absence of the Fhit protein in precancerous dysplastic lesions indicated that *FHIT* is involved in the early phases of lung carcinogenesis [112].

Other investigators have confirmed that *FHIT* is altered in a significant proportion of NSCLC cell lines. Yanagisawa et al. [113] observed either lack of expression or aberrant splicing in 7 out of 24 (29%) cell lines, often accompanied by intragenic homozygous deletions. Another thorough analysis of molecular abnormalities of the *FHIT* gene in primary lung tumours, cell lines and preneoplastic bronchial lesions confirmed that *FHIT* and FRA3B abnormalities are associated with lung cancer pathogenesis [114]. The results showed that the *FHIT*/FRA3B region undergoes allele loss in the vast majority of lung cancers (occurring first at the stage of carcinoma *in situ*), which may exhibit homozygous deletions (including intragenic deletions), and very frequently express aberrant *FHIT* transcripts with concomitant downregulation of wt *FHIT* mRNA expression.

Taken together, the genetic lesions within the *FHIT* gene may be explained by the location of a fragile region within the gene, rendering the gene highly susceptible to breakage induced by carcinogens [115].

To assess the frequency and specificity of *FHIT* inactivation and its relevance in a clinical setting, we have analysed a large series of 474 primary NSCLC by IHC [116]. Of the 474 tumours analysed, 73% were negative for Fhit protein expression. The frequency of negative cases was higher in the SQC type compared to ADC. No association was found with the tumour size and no difference in the overall survival between the patients with Fhit-negative tumours and those with Fhit-positive tumours was observed. We found an association between Fhit immunoreactivity and smoking habits confirming previous results showing increased LOH within *FHIT*/FRA3B locus in lung tumours from heavy smokers compared with that recorded in tumours from never-smokers [117]. These findings suggest that *FHIT* is a preferential target of carcinogens contained in tobacco smoke and could be envisaged as an early molecular indicator of damage related to smoking in screening programmes. Fhit was always expressed in normal bronchial mucosa; however its expression was progressively lost in precancerous lesions of increasing grade from moderate to severe dysplasia and carcinoma *in situ*. The data indicated that alteration of Fhit expression occurred at the earliest clinically detectable stages of lung carcinogenesis and that the combined use of *FHIT* and *TP53* could be a remarkably sensitive tool for lung cancer screening.

A relevant step toward the classification of *FHIT* as tumour suppressor gene has been achieved by the demonstration that replacement of *FHIT* in cancer cell lines suppressed tumorigenity in vitro and in nude mice [118,119], as well as the data suggesting that *FHIT* tumour suppressor activity is related to its proapoptotic function [120].

Genetic markers for early detection in biological specimens

Despite optimal use of therapeutic resources, the improvement of cure rates has been modest during the last decade, and overall survival is only 10–15% [121]. When lung cancer is detected in an early stage, surgical resection can achieve a 60–80% five-year survival, but the majority of cases are unresectable at the time of diagnosis and no longer curable. Patients with preinvasive and microinvasive lesions detected by cytological examination of sputum show high survival rates after localised therapies [121].

In the past, screening procedures with conventional sputum cytology and chest radiography have been unable to decrease lung cancer mortality. These negative results may have resulted from either the low sensitivity of sputum cytological examination and because microscopic metastatic disease must have been present even at this early stage. Thus, more sensitive tools based on specific biological/genetic markers may provide new opportunities for early detection and screening of this disease [122].

Identification and characterisation of the genetic changes that drive lung cancer development and pro-

gression have provided us with a variety of molecular markers that may ultimately redefine the criteria for cancer diagnosis and provide new tools for early detection through the development of sensitive procedures aimed at detecting these alterations in easily accessible samples, such as blood and exfoliative material.

Genetic alterations including microsatellite changes, *p16* methylation, *KRAS* and *TP53* mutations, are detectable in body fluids such as cytological samples of the sputum and bronchial lavage [123]. Also, in circulating tumour DNA in the plasma or serum of patients with various malignancies including SCLC and NSCLC [124,125], head and neck [126,127], breast [128,129], liver [130], colon [131] and pancreatic cancer [132]. Other attractive genetic changes in lung cancer, to be potentially employed as biomarkers, are retinoic acid receptor beta (RARB) silencing by promoter methylation [133,134] and mitochondrial DNA mutations [135]. With the rapid increase in knowledge about the molecular events leading to lung cancer, it may become possible to use genetic markers to identify the early clonal phase of progression of lung cancer in high-risk populations, thus enabling cancer to be detected earlier.

In a previous study [136] we have reported that 61% of the NSCLC patients showing allele shift and LOH at critical genomic loci in tumour samples also displayed a microsatellite change in plasma, irrespective of tumour size and stage, thus suggesting that circulating tumour DNA is associated with early phases of lung tumour development. These findings may have important clinical implications. In fact, given the high prevalence of plasma DNA alterations in Stage I and II NSCLC (a potentially curable disease) and the large amount of tumour DNA released from these tumours, genetic analysis of plasma DNA could be employed for diagnostic purposes and form the basis for lung cancer screening. The sensitivity of the plasma test could be further increased by the simultaneous search for other, distinctly different genetic alterations within the same specimen. In fact, we have demonstrated by immunohistochemical studies that loss of function of *FHIT* and *TP53* genes are independent events, and 83% of early stage NSCLC show at least one of these two abnormalities [116].

KRAS mutations have been reported in plasma DNA from patients with colorectal and pancreatic cancer [131,132] indicating that a wide range of genetic changes could be found in plasma DNA. In addition, aberrant promoter methylation of the tumour suppressor gene *p16*, DNA repair (*MGMT*) and other key genes was detected in 73% of serum samples with abnormal methylated DNA in the matched tumour in lung cancer patients [125]. Very recently, it has also been reported that *p16* promoter hypermethylation and *TP53* mutations can occur in chronic smokers before any clinical evidence of neoplasia and may be indicative of an increased risk of developing lung cancer, whereas *KRAS* mutations occur exclusively in the presence of clinically detectable neoplastic transformation [137].

Together, these studies suggest that molecular analysis of exfoliated material and plasma/serum DNA may provide an effective means of screening high-risk individuals such as chronic smokers to enable earlier detection and therapeutic intervention of lung cancer.

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