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The *FHIT* Gene at 3p14.2 Is Abnormal in Lung Cancer

Gabriella Sozzi,*† Maria Luisa Veronese,* Massimo Negrini,* Raffaele Baffa,* Maria Grazia Cotticelli,* Hiroshi Inoue,* Silvana Tornielli,† Silvana Pilotti,† Laura De Gregorio,† Ugo Pastorino,† Marco A. Pierotti,† Masataka Ohta,* Kay Huebner,* and Carlo M. Croce*

*Kimmel Cancer Center
Jefferson Medical College
Philadelphia, Pennsylvania 19107
†Istituto Nazionale Tumori
20133 Milan
Italy

Summary

To determine the role of the *FHIT* gene, which encompasses the fragile site at 3p14.2, we analyzed 59 tumors of the small cell and non-small cell type by reverse transcription of *FHIT* mRNA, followed by PCR amplification and sequencing of products. Allelic losses affecting the gene were evaluated by microsatellite polymorphism analysis and genomic alterations by hybridization using cDNA and genomic probes. Small cell lung tumors (80%) and non-small cell lung cancers (40%) showed abnormalities in RNA transcripts of *FHIT*, and 76% of the tumors exhibited loss of *FHIT* alleles. Abnormal lung tumor transcripts lack two or more exons of the *FHIT* gene. Small cell lung cancer tumors and cell lines were analyzed by Southern blotting and showed rearranged BamHI fragments. These data suggest a critical role of the *FHIT* gene in lung carcinogenesis.

Introduction

Lung cancer is a major cause of mortality worldwide, and the overall survival rate has not improved significantly in the last 20 years. The understanding of the molecular pathogenesis of this disease may help to prevent it and to provide new and more sensitive means to diagnose and treat lung cancer patients.

Alterations in oncogenes and tumor suppressor genes in small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC) have been described, the most frequent targets being alterations of p53 (Takahashi et al., 1989; Chiba et al., 1990; Mitsudomi et al., 1992) and retinoblastoma genes (Harbour et al., 1988; Xu et al., 1994) and allelic deletions of the short arm of chromosome 3 (3p) (Kok et al., 1987; Naylor et al., 1987; Rabbitts et al., 1989). In addition to cytogenetically visible deletions (Whang-Peng et al., 1982; Testa et al., 1994), loss of heterozygosity at loci on 3p has been reported in nearly 100% of SCLCs (Kok et al., 1987; Naylor et al., 1987; Brauch et al., 1987; Yokota et al., 1987) and in 50% or more of NSCLCs (Brauch et al., 1987; Yokota et al., 1987; Rabbitts et al., 1989; Hibi et al., 1992; Yokoyama et al., 1992; Horio et al., 1993), strongly suggesting the presence of at least one tumor suppressor gene in this chromosomal region.

However, the observation that allelic losses often involve most of 3p has hampered the isolation of the involved gene(s). Candidate loci have been identified such as the von Hippel–Lindau gene, located at 3p25, which was subsequently found to be rarely mutated in lung cancer cell lines (Sekido et al., 1994). Other loci located in a region within 3p21 were reported to be sites of recurrent homozygous deletions in SCLC (Daly et al., 1993; Kok et al., 1993, 1994). In addition, transfer of subchromosomal fragments of the region 3p21.3–3p21.2 to tumor cell lines has suggested tumor suppressor activity (Killary et al., 1992; Daly et al., 1993). More proximal deletions in the 3p12–3p14 region have also been reported (Rabbitts et al., 1989, 1990; Daly et al., 1991; Hibi et al., 1992).

Very recently, we identified the human *FHIT* gene (for fragile histidine triad) using an exon trapping strategy from cosmids covering a specific region at 3p14.2 involved in homozygous deletions in epithelial cancer cell lines (Kastury et al., 1996; Ohta et al., 1996). *FHIT*, a member of the histidine triad (HIT) gene family, is a highly conserved gene homologous to a group of genes identified in prokaryotic and eukaryotic organisms. The FHIT protein shows 69% similarity to a Schizosaccharomyces pombe enzyme, diadenosine 5′,5′′′-P^1,P^4-tetraphosphate (Ap_4A) asymmetrical hydrolase (Huang et al., 1995), which cleaves the Ap_4A substrate asymmetrically into ATP and AMP.

The *FHIT* gene is disrupted by the t(3;8) chromosomal translocation observed in a family with renal cell carcinoma and contains the *FRA3B* fragile site and the target of homozygous deletions in various human cancer-derived cell lines. Moreover, the study by Ohta et al. (1996) demonstrated that *FHIT* gene abnormalities often occur in primary digestive tract cancers.

Considering that the highest frequency of allelic deletions in lung cancer is on 3p and that tumors linked to carcinogenic exposure could be especially susceptible to breakage at this site, we have analyzed a large series of lung cancers of the small cell and non-small cell type for alterations of the *FHIT* gene.

The results indicate abnormalities of this gene in nearly all SCLCs and in a high proportion of NSCLCs, suggesting a role for the *FHIT* gene in lung carcinogenesis.

Results

RT–PCR and cDNA Sequence Analysis of SCLC

To study abnormalities in *FHIT* transcripts from tumors and normal tissues, we reverse transcribed mRNAs and amplified the cDNAs by nested polymerase chain reaction (PCR) as described in Experimental Procedures. We studied 14 primary tumor samples and three cell lines (83L, H128, and U2020). In two cases, matched normal lung parenchyma was also analyzed. Of the 14 tumors analyzed by reverse transcription–PCR (RT–

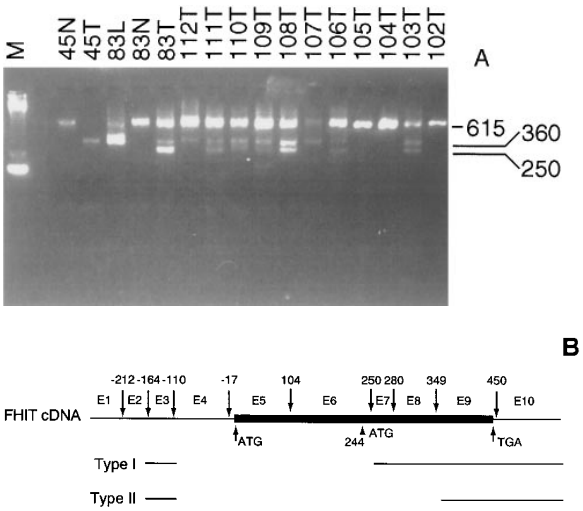

Figure 1. Expression of the *FHIT* Gene in SCLC

(A) Expression of the *FHIT* gene by nested RT–PCR analysis in SCLC tumors (T) and matched normal (N) tissues. Case 83L indicates a cell line established from the tumor 83T. Sizes of the amplified products are shown at the right.

(B) A schematic presentation of the aberrant transcripts of type I and II observed in tumor tissue of SCLCs. The top line shows the intact *FHIT* cDNA sequence. The closed bar represents the coding region within the untranslated regions. The positions of splice sites are shown by downward arrows, according to the nucleotide numbers. Type I transcripts lack exons 4 to 6, while type II transcripts lose exons 4 to 8.

PCR), 11 (79%) showed the presence of abnormal transcripts (Figure 1). Cell lines H128 and U2020 had a normal-sized product, whereas 83L presented two abnormal transcripts. Analysis of the amplified transcripts from the primary tumors consistently revealed two abnormal bands of ~360 bp (type I) and ~250 bp (type II). Seven cases displayed both type I and II abnormal transcripts, whereas four cases showed only the type I band. In two samples (Figure 1A, case 107 and Figure 2A, case 45), as well as in the tumor-derived cell line (Figure 2A, case 83L), the normal-sized transcript was undetectable, while in the other nine cases a normal-sized band of varying intensity was observed.

In one patient (Figure 2A, case 83), we examined the primary tumor, a tumor-derived cell line, and a normal lung specimen. Whereas in the normal lung only the normal transcript was detected, the primary tumor exhibited the type I and II abnormal transcripts together with a normal-sized transcript, and the tumor-derived cell line displayed the type I abnormal transcript and a novel band of ~420 bp (Figure 2), possibly generated following in vitro subculturing. Accordingly, cytogenetic analysis of this cell line revealed extensive chromosomal instability resulting in the presence of dicentric and tricentric chromosomes, telomeric associations, and double minutes. Fluorescence in situ hybridization analysis with a painting probe of chromosome 3 showed the occurrence of several structural rearrangements of this chromosome, including a translocation of the 3p arm with a breakpoint in 3p14–3p21 (data not shown). Interestingly, in the cell line the normal-sized transcript was undetectable, suggesting that the normal-sized product

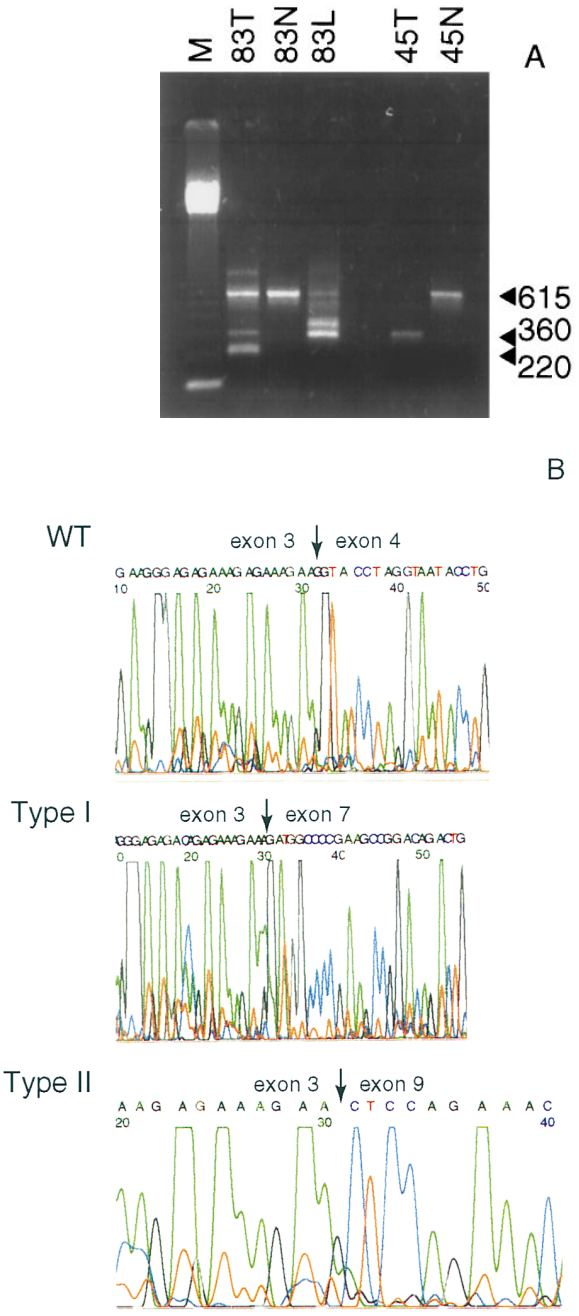

Figure 2. Expression of the *FHIT* Gene in SCLC and Sequences of *FHIT* Transcripts

(A) *FHIT* amplified products observed after nested RT–PCR of mRNA from tumor (T) and normal (N) tissues of case 45 and from tumor, normal, and cell line (L) samples of case 83. Arrowheads show the sizes of the amplified products.

(B) Sequences of the type I and II abnormal transcripts observed in SCLCs. Arrows indicate junctions between exons 3 and 4 in the wild-type transcript (WT), between exons 3 and 7 in the abnormal transcripts of type I, and between exons 3 and 9 in transcripts of type II.

observed in the primary tumor reflected the presence of normal cells infiltrating the tumor specimen.

Abnormal and normal-sized bands were separated

on agarose gel, excised, and sequenced (Figure 2B). Sequence analysis of the aberrant bands revealed that the type I transcript corresponded to absence of exons 4 to 6 (nucleotides −111 to 249) of the published *FHIT* cDNA sequence (GenBank accession number U46922), resulting in a junction between exons 3 and 7 of the *FHIT* cDNA. A loss of exons 4 to 8 (nucleotides −111 to 348), resulting in fusion of exons 3 and 9, was found in the type II transcript. In each aberrant transcript the fusion junctions coincided with splice sites.

Since both types of aberrant transcripts lacked exon 5, which contains the initial methionine codon of the *FHIT* open reading frame (Ohta et al., 1996), and type II transcripts also lacked nearly the entire coding region, including exon 8 containing the highly conserved HIT motif (Ohta et al., 1996), it is likely that these aberrant fusion transcripts could not encode functional proteins.

Sequence analysis of the normal-sized bands revealed that they contained the normal *FHIT* cDNA, possibly reflecting the contribution of normal cells infiltrating the tumor specimens. Cell line H128 displayed a normal-sized transcript; however, sequence analysis revealed a frameshift mutation, resulting from deletion of nucleotide 15 (Figure 4).

Sequencing of the normal-sized RT–PCR product amplified from normal tissues (cases 45 and 83) revealed the presence of the wild-type *FHIT* transcript.

RT–PCR and cDNA Sequence Analysis of NSCLC

RNA from 45 primary NSCLC tumors and matching normal lung parenchyma samples were similarly studied; 18 of 45 tumors (40%) and the lung adenocarcinoma cell line A549 displayed aberrant RT–PCR products. A detailed description of these results is summarized in Table 1.

The RT–PCR-amplified products from the transcripts present in these tumors consisted of one or two abnormal bands, always accompanied by a normal-sized transcript (Figure 3A). All the paired normal lung RNAs from the same lung cancer cases showed the presence of

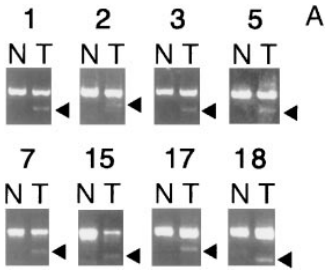

Figure 3. Expression of the *FHIT* Gene in NSCLC and Sequences of *FHIT* Transcripts

(A) Expression of the *FHIT* gene by nested RT–PCR analysis in NSCLC tumors (T) and paired normal (N) tissues. Arrowheads indicate the amplified abnormal products.

(B) Sequences of the abnormal transcripts observed in NSCLC cases 2, 3, and 17. Arrows indicate the junctions of exons 4 to 5 in the wild-type products of cases 2 and 17 (2WT and 17WT) and of exons 3 to 4 in the wild-type product of case 3 (3WT). 2A shows the junction between exons 4 and 9 in the abnormal product of case 2, 3A shows exons 3 to 8 in the abnormal product of case 3, and 17A shows exons 4 to 8 in the abnormal product of case 17.

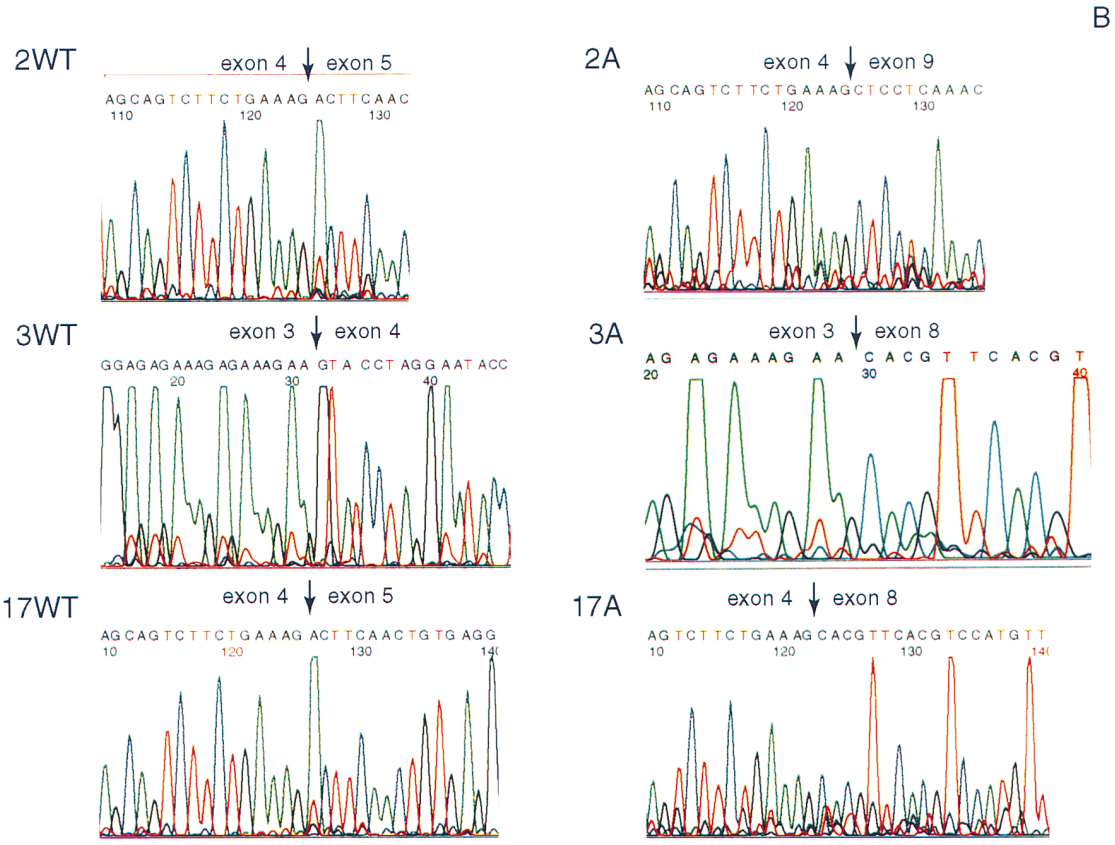

Table 1. Analysis of the *FHIT* Locus in NSCLCs

Case/Type	RT–PCR	Sequence	Loss of Heterozygosity[a]
1_{ADC}	N	Normal	Yes
	A	Deletion of exon3→7 (nt −111→249)	
2_{SQC}	N	Normal	NE
	A	Deletion of exon 4→8 (nt −17→279)	
	A	Deletion of exon 4→9 (nt −17→348)	
3_{MUCOEp}[b]	N	Normal	NE
	A	Deletion of exon 3→8 (nt −111→279)	
4_{SQC}	N	Normal	Yes
	A	Deletion of exon 4→8 (nt −17→279)	
5_{ADC}	N	Normal	Yes
	A	Deletion of exon 3→9 (nt −111→348)	
6_{ADC}	N	Deletion of exon 8 (nt 280→348) Insertion of 218 bp (nt −17)	Yes
	A	Deletion of exon 3→8 (nt −111→279)	
7_{ADC}	N	Normal	Yes
	A	Deletion of exon 3→9 (nt −111→348)	
8_{SQC}	N	Normal	Yes
	A	Deletion of exon 3→7 (nt −111→249)	
9_{SQC}	N	Normal	NE
	A	Deletion of exon 3→9 (nt −111→348)	
10_{SQC}	N		Yes
	A	NE	
11_{ADC}	N		No
	A	NE	
12_{ADC}	N		Yes
	A	NE	
13_{SQC}	N		NE
	A	NE	
14_{ADC}	N	Normal	Yes
	A	Deletion of exon 3→9 (nt −111→348)	
15_{ADC}	N	NE	NI
	A	Deletion of exon 3→9 (nt −111→348)	
16_{ADC}	N		NE
	A	NE	
	A		
17_{SQC}	N	NE	NE
	A	Deletion of exon 4→8 (nt −17→279)	
18_{ADC}	N		NE
	A	NE	

DNA and RNA from matched normal lung parenchyma and tumor tissue from 18 individuals were analyzed. Paired DNAs were tested for loss of microsatellite alleles (loss of heterozygosity) using markers spanning the *FHIT* locus; all but one of the informative tumor DNAs exhibited loss of a *FHIT* allele, as summarized in the column to the right, suggesting that the normal *FHIT* transcripts observed in some tumor RNAs (middle two columns) were derived from normal cells admixed with tumors. Cases shown are adenocarcinomas (ADC), squamous cell carcinomas (SQC), and a mucoepidermoid carcinoma (MUCOEp). Abbreviations: N, normal-sized transcript; A, abnormal transcript; NE, not evaluated; NI, not informative.

[a] Loci analyzed were *D3S1313, D3S1234, D3S4103* (ph13), *D3S1300*, and *D3S1312*.

[b] In normal lung parenchyma, deletion of exon 3→9.

the normal *FHIT* product only, except one case (case 3 in Table 1), which displayed an aberrant product differing in size from the aberrant product observed in the corresponding tumor. Sequence analysis of the aberrant fragments revealed a range of RT–PCR products with losses of various exons from 4 to 9 (Table 1; Figure 3B), including an RT–PCR product missing exons 4 to 8, resulting in a junction of exons 3 and 9 (nucleotides −111 to 348), products missing exons 4 to 6 or 7, fusing exon 3 to exon 7 or 8, respectively, and products missing exon 5 to exon 7 or 8, resulting in junctions between exons 4 and 8 or exons 4 and 9, respectively. All the types of abnormal transcripts observed lacked exon 5, the first coding exon, and half (6 of 12) of the abnormal transcripts characterized by sequence analysis also showed loss of exon 8 containing the HIT domain. The normal-sized transcripts present in the tumors appeared normal by direct sequencing of the PCR product. However, in case 6, subcloning of the normal-sized product disclosed the occurrence of three different clones: one with a normal sequence, one with an insertion of a 218 bp sequence with no significant homology to other

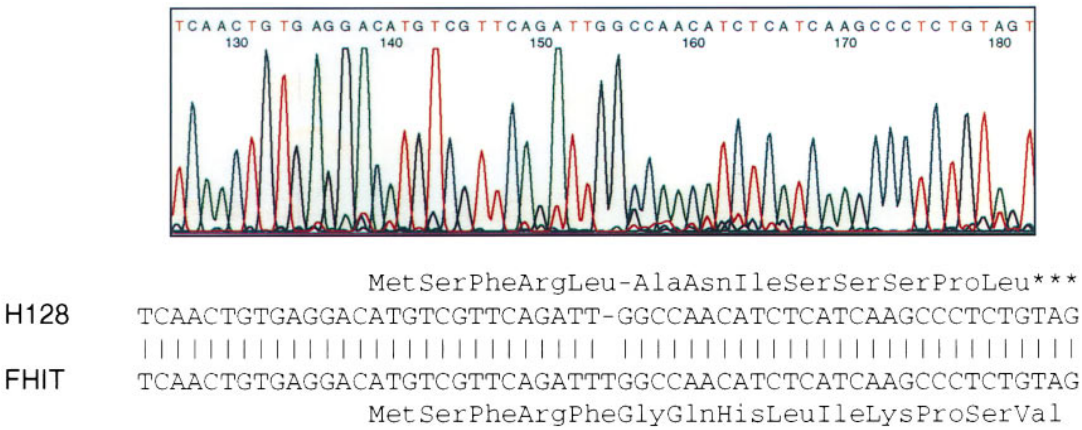

Figure 4. Sequence Analysis of the *FHIT* Transcript in H128 Cells

A frameshift mutation at nucleotide 15 of the open reading frame is shown in the upper section. Comparison between H128 and normal *FHIT* cDNA sequences showing the frameshift mutation in H128 (lower section). Three asterisks indicate a termination codon.

genes, and a third clone missing exon 8 (nucleotides 280 to 348).

In cell line A549, the abnormal product showed loss of exon 4 and resulted in a splice site junction between exons 3 and 5.

Sequence analysis of the normal-sized transcript amplified from RNA of the normal tissue of these patients revealed a normal *FHIT* cDNA sequence. A small alternatively spliced region at the beginning of exon 10 from nucleotides 450 to 460 (outside the open reading frame of the *FHIT* gene) was observed in the normal-sized transcript present in the tumor and in the corresponding normal tissue of several patients.

Southern Blot Analysis

To characterize genomic alterations that might be responsible for production of the aberrant mRNA forms, we performed Southern blot analysis of DNAs from cell lines and primary tumors. The *FHIT* cDNA does not exhibit internal BamHI restriction sites; thus, hybridization of nearly full-length labeled *FHIT* cDNA to Southern blots of BamHI-cleaved cellular DNA should reveal a BamHI fragment for each of the ten *FHIT* exons, assuming a BamHI site in each intron. In fact, such hybridization revealed eight BamHI fragments, ranging in size from greater than 23 kb to 4 kb in normal cellular DNA (data not shown), as exemplified by the U2020 lane of Figure 5. An altered cDNA hybridization pattern after BamHI cleavage would mean that a DNA alteration had occurred near a *FHIT* exon, i.e., within an exon-containing BamHI fragment. Digestion of DNAs with BamHI and hybridization with the full-length *FHIT* cDNA probe revealed abnormal restriction patterns in 4 out of 15 cases (Figure 5). Tumors 111T and 104T lacked bands of 16 kb and 11 kb, respectively. The A549 cell line revealed loss of the 11 kb fragment and a rearrangement affecting a band of 17.5 kb, whereas in cell line H128 one fragment of 16 kb was absent.

To search for specific genomic regions where rearrangements could have occurred, competitive suppression hybridization was performed, using as probes cosmids covering large intronic regions surrounding exon 5 (B4 and 76), exons 6 and 7 (05#1), and exon 8 (05#9) (Figure 6A). The results of these experiments are shown in Figure 6B. BamHI digestion detected two fragments of about 23 kb and 15 kb, respectively, recognized by probe B4. The 15 kb fragment showed a polymorphism generating two smaller fragments of about 8 and 6.5 kb in several cases (Figure 6B, lanes 1 and 4). When probed with cosmid B4, the lung adenocarcinoma A549 cell line showed loss of both the 23 and 15 kb fragments and presented a rearranged band of about 18 kb (Figure 6B, lane 2), whereas a normal restriction profile was observed after hybridization with cosmid 76 (Figure 6C, lane 2), indicating a large deletion in intron 4. This finding is in keeping with the result of the RT–PCR analysis of *FHIT* transcripts in this cell line, which showed absence of exon 4 and a splice junction between exons 3 and 5 of the *FHIT* cDNA. In H128 DNA, hybridization with cosmid 05#1, covering a region of ~40 kb encompassing exons 6 and 7, detected the absence of the largest band and two abnormal restriction fragments of ~23 kb and 18

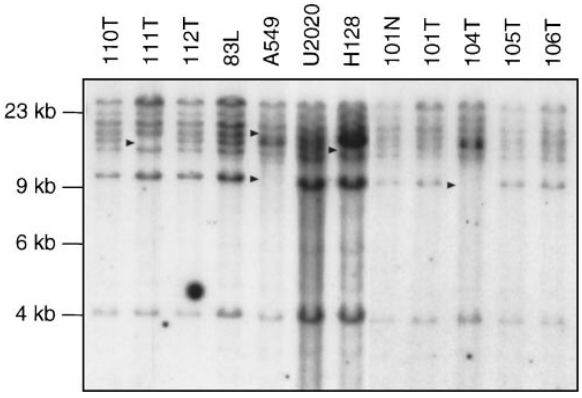

Figure 5. Genomic Rearrangements of the *FHIT* Gene in Primary Tumors and Cell Lines

Southern blot hybridization of BamHI-cleaved DNAs from cell lines and tumors (T) after hybridization with a full-length *FHIT* cDNA probe. Arrowheads indicate rearranged restriction fragments in cell lines A549 and H128 and in 111T and 104T.

kb, indicating an alteration to both alleles in the vicinity of exons 6 and 7 (Figure 6D).

Loss of Heterozygosity Analysis

To look for allelic losses in tumor samples, a PCR-based approach was used with primers that amplify polymorphic microsatellite markers internal to and flanking the *FHIT* gene. DNA from tumor and corresponding normal tissues from 28 NSCLC and 7 SCLC cases were analyzed for allelic losses at loci *D3S4103* (ph13) and *D3S1300* internal to the *FHIT* gene (Ohta et al., 1996) and at loci located in flanking regions, centromeric (*D3S1312* at 3p14.2) (Druck et al., 1995) and telomeric (*D3S1234* at 3p14.2 and *D3S1313* at 3p14.3) to the *FHIT* gene (Kastury et al., 1996) (see Table 2).

Loss of the *D3S4103* and *D3S1300* alleles, located in the intron between exons 5 and 6 of the *FHIT* gene (Figure 5A), was found in 19 of 25 (76%) and 11 of 12 (92%) informative cases for the two loci, respectively. At the *D3S1312* and *D3S1313* loci, 12 of 19 (63%) and 9 of 13 (69%) tumors, respectively, also showed loss of heterozygosity, indicating a large deletion in this genomic region. Overall, 11 of 12 (92%) of the informative tumors (9 of 10 NSCLC and 2 of 2 SCLC) that exhibited abnormal *FHIT* transcripts showed allelic losses at one or more of the loci tested.

Discussion

Three distinct chromosomal regions of 3p, which include 3p25, 3p21.3–3p21.2, and 3p12–3p14.1, are believed to harbor a gene(s) involved in lung cancer on the basis of the high frequency of allelic loss in primary tumors and defined homozygous deletions in lung cancer–derived cell lines; extensive efforts have been made to define small, common regions of loss to isolate tumor suppressor genes in these chromosomal regions. It has also been reported that microcell-mediated transfer of human chromosome 3 suppresses tumorigenicity of the A549 lung adenocarcinoma cell line (Satoh et al., 1993).

Deletions of 3p constitute particularly useful genetic markers, since several studies have reported that they occur in the early stages of lung carcinogenesis, such as bronchial dysplasia and metaplasia (Sundaresan et al., 1992; Sozzi et al., 1991; Hung et al., 1995). Moreover, it has been suggested that allelic loss on chromosome 3p in primary SCLC represents an unfavorable prognostic factor (Horio et al., 1993).

This study describes the occurrence of abnormalities in transcripts of the *FHIT* gene, located at 3p14.2, in at least 80% of SCLCs and 40% of NSCLCs, with 76% of the informative cases also exhibiting loss of one *FHIT* allele. Since the RT–PCR nested amplification would detect only internal alterations in the *FHIT* gene transcripts, this is a conservative estimate of the frequency of *FHIT* gene alteration in SCLC and NSCLC.

The aberrant lung tumor transcripts were missing two or more exons of the *FHIT* gene. While in NSCLCs a varying pattern of abnormal transcripts was detected, in SCLCs the amplified transcripts were either missing exons 4 to 6 or exons 4 to 8. Both types result in loss of exon 5, containing the initial methionine codon (Ohta et al., 1996), with the second type also showing loss of exon 8 containing the highly conserved HIT domain (Ohta et al., 1996).

Two cases of primary SCLC and a tumor cell line lacked the normal-sized transcript, which was also underrepresented in the remaining cases. In addition, one primary tumor exhibited a normal and two abnormal transcripts, while a normal-sized product was not amplified from the cell line established from this tumor. These observations suggest that in the SCLC RNA the wild-type transcript could have derived from admixed normal cells.

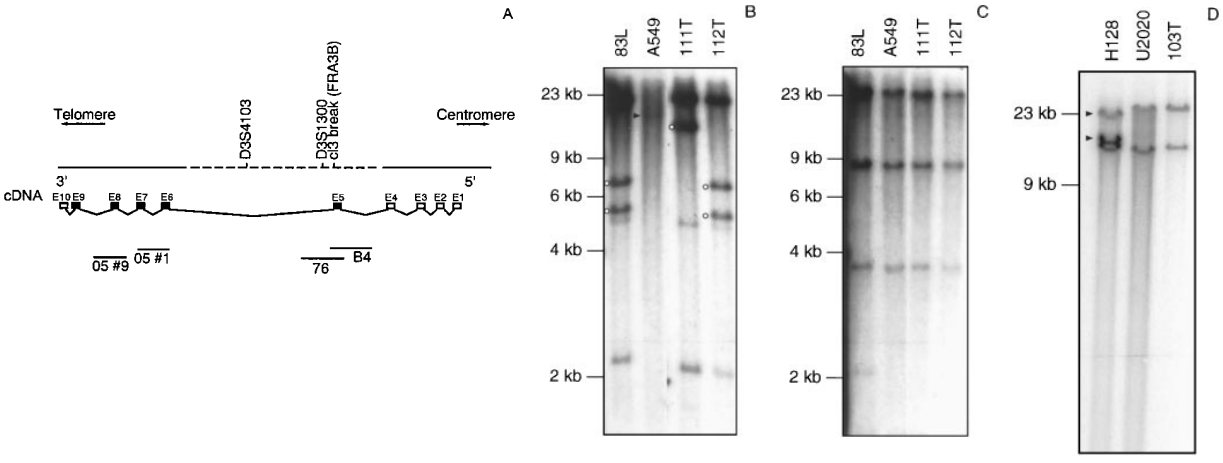

Figure 6. Genomic Alterations Detected by Hybridization of Cosmid Probes

(A) A schematic presentation of the *FHIT* gene organization showing the position of the internal microsatellite markers, the *FRA3B* represented by the hybrid cl3 break, and individual cosmids encompassing *FHIT*-coding exons (Ohta et al., 1996).

(B) Southern blot hybridization of BamHI-cleaved cell line and tumor DNAs with cosmid B4. Arrowhead points to the rearranged restriction fragment in cell line A549. Open circles indicate the polymorphic BamHI fragments observed after hybridization with cosmid B4.

(C) Southern blot hybridization of the same DNAs with cosmid 76.

(D) Southern blot hybridization with cosmid 05#1. The two arrowheads indicate the abnormal restriction fragments observed in cell line H128.

Table 2. Frequency of *FHIT* Allele Loss in Lung Tumors

D3S1313 (p14.3)	*D3S1234* (p14.2)	*D3S4103* (*FHIT*)	*D3S1300* (*FHIT*)	*D3S1312* (p14.2)
9 of 13 (69%)	18 of 25 (72%)	19 of 25 (76%)	11 of 12 (92%)	12 of 19 (63%)

Microsatellite loci flanking (*D3S1313* and *D3S1312*), within (*D3S4103* and *D3S1300*), or near the *FHIT* gene (*D3S1234*, between *D3S1313* and *D3S4103*, but exact position relative to the telomeric side of the *FHIT* gene is unknown) were tested for loss in SCLC and NSCLC DNAs for which matched normal DNA was available. The number of tumor DNAs with loss, divided by the number informative, is shown under each microsatellite marker. Results for all tumors are combined; note that the highest frequency of loss was observed at the *D3S1300* locus, which is within intron 5 of the *FHIT* gene.

In RNAs from NSCLCs, the abnormal RT–PCR-amplified products were less abundant than the normal-sized RT–PCR-amplified products. However, the apparently normal-sized transcripts could consist of a mixture of cell clones carrying small alterations not visible as discrete bands in agarose gel. Accordingly, subcloning of the normal-sized product in one NSCLC (case 6) revealed, besides the normal *FHIT* product, the presence of two different clones, one with a deletion in exon 8 and the other with an insertion of 218 bp downstream of exon 4. The abundance of normal-sized product in NSCLC could also be related to the heterogeneous, often multifocal nature of these neoplasms, which arise as a consequence of the chronic exposure of the entire bronchial "field" to carcinogens, resulting in the presence of different cell clones carrying different genetic changes (Kim et al., 1993; Barsky et al., 1994; Ebina et al., 1994). In addition, the tumor samples contained variable amounts of normal stromal tissue (stromal infiltration is known to occur in non-small cell tumors; Rabbitts et al., 1989). The complete allele loss seen in the SCLC cell line and several SCLC primary specimens and the lack of complete allele loss in NSCLC supports this interpretation. It is also possible that the abnormal transcripts are less stable than the wild-type product.

In cell line H128, sequencing of the RT–PCR *FHIT* product revealed a frameshift mutation resulting from a deletion of nucleotide 15 (exon 5). This finding represents evidence of a point mutation in the coding sequence of *FHIT*; thus, in the SCLC-derived H128 cells, perhaps one *FHIT* allele is silent as a consequence of the genomic rearrangement that occurred in the region covered by cosmid 05#1, encompassing exons 6 and 7, and the other allele is mutated in the coding sequence.

In the normal tissue of the patients showing abnormal tumor transcripts, we have observed a normal *FHIT* product by PCR and sequence analysis.

Owing to the structural organization of *FHIT*, which is composed of ten small exons distributed over at least 500 kb, genomic rearrangements could best be observed by using an infrequently cutting enzyme such as BamHI. Hybridization with specific cosmids covering intronic and exonic regions revealed altered restriction patterns in some cell lines and tumor DNA. Of particular interest is the finding of a deletion and a rearranged band occurring in the fragile site region covered by cosmid B4 (Ohta et al., 1996) in cell line A549. Accordingly, this sample presented an abnormal *FHIT* transcript by RT–PCR, with loss of exon 4 and fusion of exons 3 and 5. The present data, coupled with the PCR experiments that showed amplification of all the exons in the DNA of the tumors and cell lines analyzed, suggest that the rearrangements likely occur in the large intronic regions of the *FHIT* gene. Although the exact molecular mechanisms by which these genomic rearrangements generate abnormal transcripts are still to be elucidated, these observations suggest that aberrant *FHIT* genes are the result of DNA rearrangements, particularly in the region near exon 5.

The high frequency (76%) of loss of one *FHIT* allele observed in lung tumors of both small cell and non-small cell type is noteworthy. In addition, the microsatellite marker *D3S1300*, which maps close to the first coding exon (exon 5), detected the highest frequency of allelic deletions (92%). Although we did not determine a minimal region of loss in our cases, these findings support the idea that inactivation of the *FHIT* gene could have occurred by a mechanism of loss of one allele and altered expression of the remaining one.

This model is consistent with the observation that the *FHIT* gene spans a common fragile region, *FRA3B*, where abnormalities such as deletions could be more frequent than point mutations. Tumors associated with carcinogen exposure, such as cancers of the aerodigestive tract, could be particularly susceptible to alterations of the *FHIT* gene. Owing to its etiology, lung cancer is likely to be strongly and directly associated with the effects of agents that interfere with DNA replication, such as agents in tobacco smoke. Breakage in a fragile site–containing gene as a consequence of physical, chemical, and biological agents can thus be expected. Expressivity of the *FRA3B* fragile site in peripheral blood lymphocytes of patients with cancer has been investigated; the expression of *FRA3B* appeared to be influenced by habitual tobacco smoking, and significantly higher expression was reported in lung cancer patients (Murata et al., 1992). Of particular interest was one NSCLC patient (case 3 of Table 1) with a mucoepidermoid carcinoma of the lung who subsequently developed a renal cell carcinoma. In the normal lung parenchyma of this patient, an abnormal transcript missing exons 4 to 8 was detected. This finding raises the possibility that a constitutional alteration within the *FHIT* gene could be associated with a predisposition to develop both lung and renal cancer or other types of multiple primary tumors. However, this alteration could have been somatically acquired, because carcinogen exposure can induce transformation in several fields of the bronchial epithelium through the induction of different genetic changes, which are also detectable in early preneoplastic lesions.

High levels of intracellular diadenosine 5′,5′′′-P^1,P^4 tetraphosphate (Ap_4A) have been detected at the G1–S boundary (Weinmann-Dorsch et al., 1984), and a role for

Ap_4A in the stimulation of DNA polymerase activity has been proposed (Baxi et al., 1994). It seems plausible that loss of function of the *FHIT* gene could result in the constitutive accumulation of Ap_4A and the stimulation of DNA synthesis and proliferation. Thus, loss of *FHIT* function could initiate the malignant process by stimulating the proliferation of the cells that are the precursors of digestive tract cancer and lung cancer. It has also been proposed that Ap_4A may mediate adaptive responses of mammalian cells to environmental stress, such as carcinogen exposure, by intracellular accumulation (Baker and Jacobson, 1986). In this context, altered metabolism of Ap_4A, caused by loss of normal *FHIT* activity, could interfere with this important physiological function.

An understanding of the molecular mechanisms involved in *FHIT* gene damage and of the function of the FHIT protein could provide important clues for the prevention and treatment of some of the most common cancers in humans.

Experimental Procedures

Tumors

The 59 tumors, including 25 cases of adenocarcinomas, 19 squamous cell carcinomas, 1 mucoepidermoid carcinoma, and 14 small cell lung carcinomas, were obtained from surgically resected lung cancer patients at Istituto Nazionale Tumori (Milan, Italy). A lung adenocarcinoma cell line (A549) and three small cell lung carcinoma cell lines (H128, U2020, and 83L) were also analyzed. Cell line 83L was established from small cell tumor, 83T. Among NSCLCs, 29 were in stage I, 9 were in stage II, and 6 were in stage III. The tumors were classified histologically according to the Histological Typing of Lung Tumors by the World Health Organization (1987) and staged according to the TNM classification of malignant tumors defined by the International Union Against Cancer (1987). Most cases (54 out of 59) were from male patients, and the mean age of cases at presentation was 63 years. Matched normal lung parenchyma tissue samples were taken at a most distant site from the tumor or in a different segment or lobe as a source for the normal RNA and DNA.

RNA Extraction and RT

Tumor and normal specimens were frozen immediately after surgical resection and stored at −80°C. Total mRNA was extracted from frozen tumor and normal lung tissues using guanidinium–LiCl separation (Sambrook et al., 1989) or the RNA-STAT kit (Tel TEST). cDNA was synthesized from 1 μg of total RNA. RT was performed in a 20 μl volume of 1× first strand buffer (GIBCO), 10 mM DTT (GIBCO), 500 μM dNTPs, 50 ng/μl oligo(dT), 0.3 μg/μl random primers, 16.5 U of RNAsin (Promega), and 300 U of Superscript II (GIBCO). The samples were first denatured for 5 min at 95°C and then incubated at 37°C for 60 min. The reaction was stopped by inactivating the enzyme at 94°C for 5 min. The reaction was diluted to 30 μl, and 1 μl was used for subsequent PCR amplification.

RT–PCR and cDNA Sequencing

We used 1 μl of cDNA for a first PCR amplification performed in a volume of 25 μl containing 0.8 μM of primers 5U2 and 3D2 (Ohta et al., 1996), 50 μM of each dNTP (Takara), 1x PCR buffer, and 1.25 U of Taq polymerase (Takara). The PCR reaction consisted of an initial denaturation at 95°C for 3 min and 25 cycles of 15 s at 94°C, 30 s at 62°C, 45 s at 72°C, and a final extension of 5 min at 72° using a Perkin–Elmer PCR thermocycler. The amplified product was diluted 20-fold in TE buffer, and 1 μl of the diluted reaction product was subjected to a second round of PCR amplification using nested primers 5U1 and 3D1 (Ohta et al., 1996) for 30 cycles under the above conditions. The PCR products were resolved on 1.5% ethidium bromide–stained Metaphor gel (FMC). Bands were cut from gels, and DNA was purified using a QIA quick gel extraction kit (Qiagen). Depending on the size of the PCR products, 5–50 ng of cDNA was sequenced, using primers 5U1 and 3D1, by the dideoxynucleotide termination reaction chemistry for sequence analysis on the Applied Biosystems models 373A and 377 DNA sequencers.

Loss of Heterozygosity Analysis

DNAs from frozen tumor and normal tissues were extracted using standard methods (Sambrook et al., 1989). Analysis of allelic losses was performed using a PCR-based approach. Primers that amplify polymorphic microsatellite markers were used for the following loci: *D3S4103* (ph13) and *D3S1300* internal to the *FHIT* gene (Ohta et al., 1996), *D3S1234* (3p14.2), *D3S1313* (3p14.3), and *D3S1312* (3p14.2) flanking the gene. The sequences of all oligonucleotide primers are available through the Genome Data Base. We carried out 20 cycles of amplification at 55°C–60°C annealing temperature, as appropriate for each primer.

For informative cases, allelic loss was scored if the autoradiographic signal of one allele was approximately 50% reduced in the tumor DNA compared with the corresponding normal allele. The loci displaying microsatellite instability were not scored for allelic loss.

Southern Blot Hybridization

DNA (5 μg) from primary tumors and cell lines was digested with BamHI, fractionated by 0.8% agarose gel electrophoresis, transferred to Zeta-probe GT Genomic Tested membranes (Bio-Rad), and cross-linked under ultraviolet light. The following probes were used: a full-length cDNA and cosmids B4, 76, 05#1, and 05#9 (Figure 5A) (Ohta et al., 1996). Hybridization probes were prepared by random primer extension (Stratagene). Unincorporated nucleotides were removed by spin filtration in G-50 columns. The preannealing reaction consisted of 100 ng/ml of probe, 0.5× SSC, and 0.5 mg/ml sheared human genomic DNA. After heating the mixture at 100°C for 10 min, we incubated it at 65°C for 2 hr. The probe was added to the prehybridized filters at 5×10^6 to 6×10^6 cpm per milliliter of hybridization solution consisting of 5× SSPE, 5× Denhardt's solution, 0.1% SDS, 0.1 mg/ml single-stranded DNA, and 0.5 mg/ml sheared human genomic DNA.

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References

Baker, J.C., and Jacobson, M.K. (1986). Alteration of adenyl dinucleotide metabolism by environmental stress. Proc. Natl. Acad. Sci. USA *83*, 2350–2352.

Barsky, S.H., Grossman, D.A., Ho, J., and Holmes, E.C. (1994). The multifocality of bronchioloalveolar lung carcinoma: evidence and implications of a multiclonal origin. Mod. Pathol. *7*, 633–640.

Baxi, M.D., McLennan, A.G., and Vishwanatha, J.K. (1994). Characterization of the HeLa cell DNA polymerase α-associated Ap_4A binding protein by photoaffinity labeling. Biochemistry *33*, 14601–14607.

Brauch, H., Johnson, B., Hovis, J., Yano, T., Gadzar, A., Pettengil, O., Graziano, S., Sorenson, G., Poiesz, B., Minna, J., Linehan, M., and Zbar, B. (1987). Molecular analysis of the short arm of chromosome 3 in small-cell and non-small cell carcinoma of the lung. N. Engl. J. Med. *317*, 1109–1113.

Chiba, I., Takahashi, T., Nau, M.M., D'Amico, D., Curiel, D.T., Mitsudomi, T., Buchhagen, D.L., Carbone, D., Piantadosi, S., Koga, H.,

Reissmann, P., Slamon, D., Holmes, E., and Minna, J. (1990). Mutation in the p53 gene are frequent in primary, resected non-small cell lung cancer. Oncogene *5*, 1603–1610.

Daly, M.C., Douglas, J.B., Bleehen, N.M., Hastleton, P., Twentyman, P.R., Sundaresan, V., Carrit, B., Bergh, J., and Rabbitts, P.H. (1991). An unusually proximal deletion on the short arm of chromosome 3 in a patient with small cell lung cancer. Genomics *9*, 113–119.

Daly, M.C., Xiang, R.H., Buchhagen, D., Hensel, C.H., Garcia, D.K., Killary, A.M., Minna, J.D., and Naylor, S.L. (1993). A homozygous deletion on chromosome 3 in a small cell lung cancer cell line correlates with a region of tumor suppressor activity. Oncogene *8*, 1721–1729.

Druck, T., Kastury, K., Hadaczek, P., Podolski, J., Toloczko, A., Sikorski, A., Ohta, M., La Forgia, S., Lasota, J., McCue, P., Lubinski, J., and Huebner, K. (1995). Loss of heterozygosity at the familial RCCt(3;8) locus in most clear cell renal carcinoma. Cancer Res. *55*, 5348–5353.

Ebina, M., Steinberg, S.M., Mulshine, J.L., and Linnoila, R.I. (1994). Relationship of p53 overexpression and up-regulation of proliferating cell nuclear antigen with the clinical course of non-small cell lung cancer. Cancer Res. *54*, 2496–2503.

Harbour, J.W., Lai, S.L., Whang-Peng, J., Gazdar, A.F., Minna, J.D., and Kaye, F.J. (1988). Abnormalities in structure and expression of the human retinoblastoma gene. Science *241*, 353–357.

Hibi, K., Takahashi, T., Yamakawa, K., Ueda, R., Sekido, Y., Ariyoshi, Y., Suyama, M., Takagi, H., Nakamura, Y., and Takahashi, T. (1992). Three distinct regions involved in 3p deletions in human lung cancer. Oncogene *7*, 445–449.

Horio, Y., Takahashi, T., Kuroishi, T., Hibi, K., Suyama, M., Niimi, T., Shimokata, K., Yamakawa, K., Nakamura, Y., Ueda, R., and Takahashi, T. (1993). Prognostic significance of p53 mutations and 3p deletions in primary resected non-small cell lung cancer. Cancer Res. *53*, 1–4.

Huang, Y., Garrison, P.N., and Barnes, L.D. (1995). Cloning of the *Schizosaccharomyces pombe* gene encoding diadenosine 5′,5‴-P^1,P^4-tetraphosphate (Ap_4A) asymmetrical hydrolase: sequence similarity with histidine triad (HIT) family. Biochem. J. *312*, 925–932.

Hung, J., Kishimoto, Y., Sugio, K., Virmani, A., McIntire, D.D., Minna, J.D., and Gadzar, A.F. (1995). Allele specific chromosome 3p deletions occur at an early stage in the pathogenesis of lung carcinoma. J. Am. Med. Assoc. *273*, 558–563.

International Union against Cancer (1987). TNM classification of malignant tumors, Fourth Edition (Geneva: World Health Organization).

Kastury, K., Baffa, R., Druck, T., Ohta, M., Cotticelli, M.G., Inoue, H., Negrini, M., Rugge, M., Huang, D., Croce, C.M., Palazzo, J., and Huebner, K. (1996). Potential gastrointestinal tumor suppressor locus at the 3p14.2 FRA3B site identified by homozygous deletions in tumor cell lines. Cancer Res. *56*, 978–983.

Killary, A.M., Wolf, M.E., Giambernardi, T.A., Naylor, S.L. (1992). Definition of a tumor suppressor locus within human chromosome 3p21-p22. Proc. Natl. Acad. Sci. USA *89*, 10877–10881.

Kim, S.Y., Lee, J.S., Ro, J.Y., Gay, M.L., Hong, W.K., and Hittelman, W.N. (1993). Interphase cytogenetics in paraffin sections of lung tumors by non-isotopic *in situ* hybridization: mapping genotype/phenotype heterogeneity. Am. J. Pathol. *142*, 307–317.

Kok, H., Osinga, J., Carrit, B., Davis, M.B., van der Veen, A.Y., Landsvater, R.M., de Leij, L.F.M.H., Berendsen, H.H., Postmus, P.E., Poppema, S., and Buys, C.H.C.M. (1987). Deletion of a DNA sequence at the chromosomal region 3p21 in all major types of lung cancer. Nature *330*, 578–581.

Kok, K., Hosfra, R., Pilz, A., van den Berg, A., Terpstra, P., Buys, C.H., and Carrit, B. (1993). A gene in the chromosomal region 3p21 with greatly reduced expression in lung cancer is similar to the gene for ubiquitin activating enzyme. Proc. Natl. Acad. Sci. USA *90*, 6071–6075.

Kok, K., van den Berg, A., Veldhuis, P.M., van der Veen, A.Y., Franke, M., Shoenmakers, E.F., Hulsbeek, M.M., van der Hout, A.H., de Leij, L., van de Ven, W., and Buys, C.H.C.M. (1994). A homozygous deletion in a small cell lung cancer cell line involving a 3p21 region with a marked instability in yeast artificial chromosomes. Cancer Res. *54*, 4183–4187.

Mitsudomi, T., Steinberg, S.M., Nau, M.M., Carbone, D., D'Amico, D., Bodner, S., Oie, H.K., Linnoila, R.I., Mulshine, J.L., Minna, J.D., and Gadzar, A.F. (1992). p53 mutations in non small cell lung cancer cell lines and their correlations with the presence of RAS mutations and clinical features. Oncogene *7*, 171–180.

Murata, M., Otsuka, M., Hayakawa, Y., Takahashi, E., Tsuji, H., and Hori, T. (1992). Expressivity of a common fragile site, fra(3)(p14.2), in patients with cancer and other diseases. Jpn. J. Hum. Genet. *37*, 205–213.

Naylor, S.L., Johnson, B.E., Minna J.D., and Sakaguchi, A.Y. (1987). Loss of heterozygosity of chromosome 3p markers in small-cell lung cancer. Nature *329*, 451–454.

Ohta, M., Inoue, H., Cotticelli, M.G., Kastury, K., Baffa, R., Palazzo, J., Siprashvili, Z., Mori, M., McCue, P., Druck, T., Croce, C.M., and Huebner, K. (1996). The human *FHIT* gene, spanning the chromosome 3p14.2 fragile site and renal carcinoma–associated translocation breakpoint, is abnormal in digestive tract cancers. Cell *84*, 587–597.

Rabbitts, P., Douglas, J., Daly, M., Sundaresan, V., Fox, V., Haselton, P., Wells, F., Albertson, D., Waters, J., and Bergh, J. (1989). Frequency and extent of allelic loss in the short arm of chromosome 3 in non small-cell lung cancer. Genes Chromosom. Cancer *1*, 95–105.

Rabbitts, P., Bergh, J., Douglas, J., Collins, F., and Waters, J. (1990). A submicroscopic homozygous deletion at the D3S3 locus in a cell line isolated from a small cell lung carcinoma. Genes Chromosom. Cancer *2*, 231–238.

Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989). Molecular Cloning: A Laboratory Manual (Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press).

Satoh, H., Lamb, P.W., Dong, J.T., Everitt, J., Boreiko, C., Oshimura, M., and Barrett, J.C. (1993). Suppression of tumorigenicity of A549 lung adenocarcinoma cells by human chromosome 3 and 11 introduced via microcell-mediated chromosome transfer. Mol. Carcinog. *7*, 157–164.

Sekido, Y., Bader, S., Latif, F., Gnarra, J.R., Gadzar, A.F., Linehan, M., Zbar, B., Lerman, M.I., and Minna, J.D. (1994). Molecular analysis of the von Hippel-Lindau disease tumor suppressor gene in human lung cancer cell lines. Oncogene *9*, 1599–1604.

Sozzi, G., Miozzo, M., Tagliabue, E., Calderone, C., Lombardi, L., Pilotti, S., Pastorino, U., Pierotti, M.A., and Della Porta, G. (1991). Cytogenetic abnormalities and overexpression of receptors for growth factors in normal bronchial epithelium and tumor samples of lung cancer patients. Cancer Res. *51*, 400–404.

Sundaresan, V., Ganly, P., Hasleton, P., Rudd, R., Sinha, G., Bleehen, N.M., and Rabbitts, P. (1992). p53 and chromosome 3 abnormalities, characteristic of malignant lung tumors, are detectable in preinvasive lesions of the bronchus. Oncogene *7*, 1989–1997.

Takahashi, T., Nau, M., Chiba, I., Birrer, M., Rosenberg, R., Vinocour, M., Levitt, M., Pass, H., Gazdar, A., and Minna, J.D. (1989). p53: a frequent target for genetic abnormalities in lung cancer. Science *246*, 491–494.

Testa, J.R., Siegfried, Z.L., Hunt, J.D., Feder, M.M., Litwin, S., Zhou, J.Y., Taguchi, T., and Keller, S.M. (1994). Cytogenetic analysis of 63 non-small cell lung carcinomas: recurrent chromosome alterations amid frequent and widespread genomic upheaval. Genes Chromosom. Cancer *11*, 178–194.

Weinmann-Dorsch, C., Grummt, I., Albert, W., Ferdinand, F.J., Friss, R.R., Pierron, G., Moll, W., and Grummt, F. (1984). Drastic rise of intracellular adenosine (5′) tetraphospho(5′) adenosine correlates with onset of DNA synthesis in eukaryotic cells. Eur. J. Biochem. *138*, 179–185.

Whang-Peng, J., KaoShan, C.S., Lee, E.C., Bunn, P.A., Carney, D.N., Gazdar, A.F., and Minna, J.D. (1982). Specific chromosome defect associated with human small-cell lung cancer. Science *215*, 181–182.

World Health Organization (1981). Histological Typing of Lung Tumors, Second Edition (Geneva: World Health Organization).

Xu, H.J., Quinlan, D.C., Davidson, A.G., Hu, S.X., Summers, C.L., Li, J., and Benedict, W.F. (1994). Altered retinoblastoma protein expression and prognosis in early-stage non-small-cell lung carcinoma. J. Natl. Cancer Inst. *86*, 695–699.

Yokota, A., Wada, M., Shimosato, Y., Terada, M., and Sugimura, T. (1987). Loss of heterozygosity on chromosomes 3, 13, 17 in small cell carcinoma and on chromosome 3 in adenocarcinoma of the lung. Proc. Natl. Acad. Sci. USA *84*, 9252–9256.

Yokoyama, S., Yamakawa, K., Tsuchiya, E., Murata, M., Sakiyama, S., and Nakamura, Y. (1992). Deletion mapping on the short arm of chromosome 3 in squamous cell carcinoma and adenocarcinoma of the lung. Cancer Res. *52*, 873–877.