[CANCER RESEARCH 56, 5579–5582, December 15, 1996]

Advances in Brief

Molecular Analysis of the *FHIT* Gene at 3p14.2 in Lung Cancer Cell Lines[1]

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Abstract

Chromosome 3p is frequently deleted in various cancers including examples in the lung. A novel gene, termed *FHIT*, was recently isolated from the fragile site at 3p14.2, with aberrant transcripts being reported in lung cancer tumor specimens. To avoid overlooking tumor-specific altered transcripts due to contaminating normal cells in primary tumors, *FHIT* alterations were examined in 41 lung cancer cell lines in the present study. Lack of detectable expression or exclusive expression of aberrantly spliced transcripts, often accompanied by intragenic homozygous deletions, were observed in 7 of 24 non-small cell lung cancers (29%) but in 0 of 17 small cell lung cancers (0%). Extensive reverse transcription-PCR-single-strand conformation polymorphism analysis revealed polymorphisms and alternative splicing but failed to identify point mutations. These results suggest distinct mechanisms for *FHIT* alterations in lung tumorigenesis and that further studies of this interesting gene are warranted.

Introduction

Accumulating evidence indicates that genetic changes in tumor suppressor genes play a major role in the pathogenesis of lung cancers (1–5). Cytogenetic and molecular analyses have revealed frequent chromosomal deletions on 3p, 5q, 8p, 9p, 11p, 13q, 17p, 18q, and 22q, suggesting the presence of tumor suppressor genes in the affected chromosomal regions (6–13). Indeed, we and others have reported genetic alterations of possible target tumor suppressor genes in lung cancers, including *p16* on 9p, *Rb* on 13q, *p53* on 17p, and *DPC4* on 18q (1–5). The search has now been intensified for isolation of the involved tumor suppressor genes in other chromosomal regions.

The 3p deletion has been shown to be the most frequent alteration in lung cancers, occurring even in mild dysplasia, an early precursor lesion (6–8, 14). Three distinct chromosomal regions, 3p25, 3p21.3, and 3p12-p14, are suspected to be locations of tumor suppressor genes (8). However, the *VHL* gene at 3p25, which is inactivated in a considerable fraction of renal cell cancers, was found to be only rarely mutated in lung lesions (15). Although homozygous deletions are frequent at 3p21.3, a strong indication of the presence of a tumor suppressor gene (16–20), no solid candidate for the tumor suppressor gene(s) on 3p21.3 has as yet been identified.

With regard to the 3p12-p14 region, Ohta *et al.* (21) recently identified a novel gene, termed *FHIT*, that spans the fragile site locus at 3p14.2, *FRA3B*. Disruption of *FHIT* was found in kindred with a reciprocal t(3;8)(p14.2;q24) chromosomal translocation segregating with disease in a family with renal cancers, with aberrantly spliced *FHIT* transcripts also being frequently expressed in digestive tract cancers (21). In addition, aberrant *FHIT* transcripts were also recently reported by Sozzi *et al.* (22) to be present in 80% of SCLC[3] and 40% of NSCLC tumor specimens, although the question of why they were much less abundant than normal-sized transcripts in most of the affected lung cancer tissues was not answered.

Our previous studies on inactivation of various tumor suppressor genes in lung cancer indicated that mutations found in cell lines faithfully reflect those in the corresponding primary tumors (2, 4, 5, 23). In the present study, we therefore conducted molecular analysis of *FHIT* alterations in 41 lung cancer cell lines to avoid the inevitable influence of admixed inflammatory and stromal cells when examining primary tumor specimens. This can complicate interpretation by masking the presence of tumor-specific altered transcripts.

Materials and Methods

Cell Lines. Fourteen SCLC and 9 NSCLC cell lines given the prefix ACC-LC were established in our laboratories at the Aichi Cancer Center in Japan, mostly from metastatic lesions in Japanese patients. Derivations and culture conditions of these cell lines have been reported previously (23–25). These are named ACC-LC-48, -49, -51, -61, -66, -67, -80, -87, -96, -97, -170, -171, -172, and -321 in the SCLC cases and ACC-LC-71, -73, -93, -94, -174, -176, -314, -319, and -323 in the NSCLC cases. Two sets of NSCLC cell lines which were independently established at different points of time from different sites of patients were included in this panel (from patient T. S., ACC-LC-94 and -174; from patient M. S., ACC-LC-314 and -319). In addition to our own cell lines, we also analyzed 18 (3 SCLCs and 15 NSCLCs) examples which were generously provided by Drs. L. Old and M. Akiyama (Memorial Sloan-Kettering Cancer Center and Radiation Effect Research Foundation, respectively).

PCR Amplification, SSCP, and Sequence Analyses of cDNAs. The primers used for RT-PCR analysis of *FHIT* expression were S1 (sense), 5′-TCCGTAGTGCTATCTACATC and AS3 (antisense), 5′-CATGCTGATTCAGTTCCTCTTGG. We used single-stage PCR amplification instead of a nested PCR strategy to avoid possible overrepresentation of shorter transcripts, and the PCR amplification consisted of 40 cycles (94°C for 0.5 min, 55°C for 0.5 min, and 72°C for 1 min) after the initial denaturation step (94°C for 5 min) in the presence of 5% DMSO. The integrity of the cDNAs used in the present study was checked with PCR using the following actin specific primers: ActS1 (sense), 5′-GACTACCTCATGAAGATC and ActAS1 (antisense), 5′-GATCCACATCTGCTGGAA. The PCR products were electrophoresed in 1.5% agarose gels, and those demonstrating altered size were cloned into pBluescript SKII(−) for sequence analysis. Plasmid DNAs were prepared from several independent clones and sequenced as described previously (19).

RT-PCR-SSCP analysis was conducted essentially as described previously (19). In brief, PCR amplification using random primed cDNAs was performed using oligonucleotide primers in the presence of [^{32}P]dCTP, followed by electrophoretic separation on 6% nondenaturing polyacrylamide gels both in the presence and absence of 5% glycerol at room temperature. The primer pairs used to amplify the *FHIT* cDNAs were: S1 (sense) and AS1 (antisense), 5′-ATCGGCCACTTCATCAGGA; S2 (sense), 5′-CAGTGGAGCGCTTCCATGA; AS2 (antisense), 5′-TGCCATTTCCTCCTCTGAT; and S3 (sense), 5′-CAGAAACATGACAAGGAGGA and AS3 (antisense).

Received 9/16/96; accepted 10/29/96.

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[1] This work was supported in part by a Grant-in-Aid for the Second Term Comprehensive 10-Year Strategy for Cancer Control and a Grant-in-Aid for Cancer Research from the Ministry of Health and Welfare, Japan, a Grant-in-Aid for Scientific Research on Priority Areas from the Ministry of Education, Science, Sports and Culture, Japan, and by the Vehicle Racing Commemorative Foundation.

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[3] The abbreviations used are: SCLC, small cell lung cancer; NSCLC, non-small cell lung cancer; RT, reverse transcription; SSCP, single-strand conformation polymorphism.

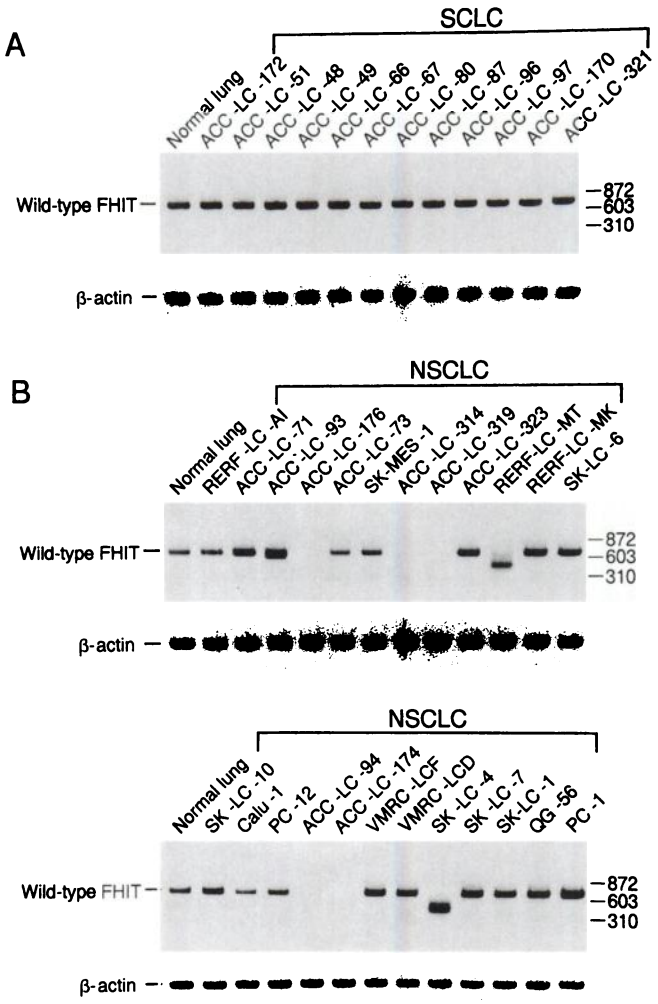

Fig. 1. Results of RT-PCR analysis of *FHIT* transcripts. *A*, expression in SCLC cell lines. All show readily detectable expression of normal size *FHIT* transcripts. *B*, expression in NSCLC cell lines. Lack of *FHIT* expression is apparent in ACC-LC-94, -174, -176, ACC-LC-314, and ACC-LC-319. Aberrantly sized transcripts are expressed in RERF-LC-MT and SK-LC-4 in the absence of normal size transcripts. PCR amplification using actin-specific primers was used as a control for the quality of the cDNAs.

The PCR amplification consisted of 40 cycles (94°C for 0.5 min, 58°C for 0.5 min, and 72°C for 1 min) after the initial denaturation step (94°C for 5 min) in the presence of 5% DMSO.

Exon-specific PCR Amplification and Southern Blot Analyses. Exon-specific PCR amplification was carried out using genomic DNAs and the following primer pairs, followed by electrophoresis on 3% agarose gels: exon 1: E1S (sense), 5′-TCTGCTCTGTCCGGTCACAG and E1AS (antisense), 5′-GGATGTTGACAGCTGGGAAT; exon 5: E5S (sense), 5′-TCAACTGTGAGGACATGTCG and E5AS (antisense), 5′-TACCACAGGTTTCCTATTCA; and exon 10: E10S (sense), 5′-TGAATTCCAGCAAAAGAGCTA and E10AS (antisense), 5′-TCACTGGTTGAAGAATACAGG.

PCR amplification consisted of 40 cycles (94°C for 0.5 min, 55°C for 0.5 min, and 72°C for 1 min) after the initial denaturation step (94°C for 5 min). Southern blot analysis using genomic DNAs digested with *Hin*dIII was performed using a PCR-generated probe covering exons 3–10 as described previously (19). Southern blot analysis and exon-specific PCR amplification were carried out in all cell lines except for ACC-LC-51, NCI-H69, VMRC-LCD, and PC-1.

Results and Discussion

In the present study, the initial examination of the 41 lung cancer cell lines for the presence of alterations in *FHIT* revealed no expression in 5 NSCLC cell lines (ACC-LC-94, -174, -176, -314, and -319) established from 3 independent cases as well as altered size transcripts in 2 NSCLC cell lines (RERF-LC-MT and SK-LC-4; Fig. 1). The lack of *FHIT* transcripts in two independent pairs of cell lines (ACC-LC-94 and -174 as well as ACC-LC-314 and -319) was of particular interest because these had been derived at different time points from distinct metastatic sites in two separate patients (cases T. S. and M. S., respectively), suggesting that the change took place prior to metastasis and was maintained during disease progression. Altered size transcripts in RERF-LC-MT and SK-LC-4 were further examined by sequence analysis (Fig. 2). RERF-LC-MT exclusively expressed aberrant transcripts lacking exons 5–7. SK-LC-4 also expressed only aberrantly spliced transcripts, which included examples lacking exons 5- 8 as well as others with loss of exons 4–8 and an insertion of an extraneous 111-bp sequence between the fused exons 3 and 9. *FHIT* transcripts expressed in both cell lines were missing the central portion of wild-type *FHIT* mRNA which carries the initiation methionine codon in exon 5. Along with the absence of wild-type *FHIT* expression, these results suggest the absence of functional FHIT proteins in these two cell lines expressing aberrant transcripts. Interestingly, none of the SCLC cell lines examined in the present study carried *FHIT* alterations, showing a clear specificity in the affected histological type and marked contrast to results reported by Sozzi *et al.* (22). It is possible that the observed discrepancy might be related to different geographic and ethnic origins of SCLC patients examined in the two studies.

RT-PCR-SSCP analysis to search for subtle mutations in the *FHIT* gene demonstrated two types of distinct mobility shifts with S2 and AS2 PCR primers (Fig. 3). Sequence analysis revealed silent nucleotide substitutions at codon 294 (CAT to CAC). We also detected distinct mobility shifts in two NSCLC cell lines (ACC-LC-93 and RERF-LC-AI) using S3 and AS3 PCR primers. In comparison to the nucleotide sequence published by Ohta *et al.* (21), these two cell lines carried a single bp deletion within a short stretch of 7 Cs encompassing nucleotides 871–877, 3′ to the open reading frame of *FHIT*. Since both nucleotide changes were also present in multiple normal lung specimens, these are likely to represent polymorphisms. In addition, we also identified two forms of distinct splicings in the 3′ untranslated region of *FHIT*. Our sequence analysis revealed that the shorter form (Fig. 3, *Form 2*) lacked the 5′ end of exon 10 which corresponds to

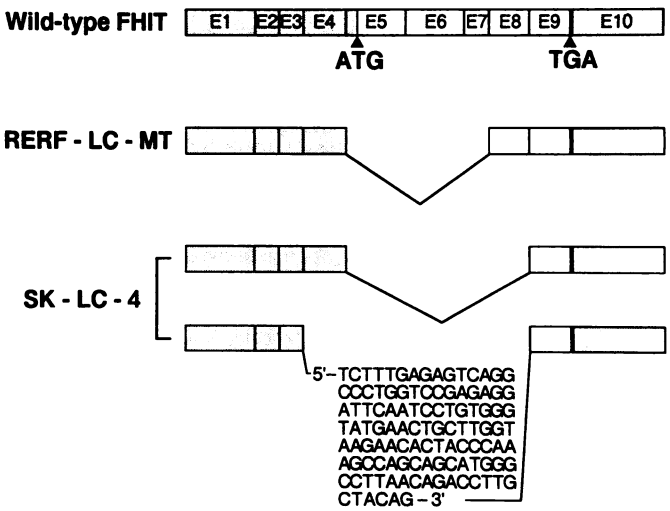

Fig. 2. Schematic illustration of aberrant *FHIT* transcripts identified in the RERF-LC-MT and SK-LC-4 cell lines. That in RERF-LC-MT is missing exons 5 through 7. In SK-LC-4 two types were found: one missing exons 5 through 8 and the other missing exons 4 through 8 with insertion of a 111-bp DNA sequence. *Open* and *shaded boxes*, coding and untranslated regions, respectively.

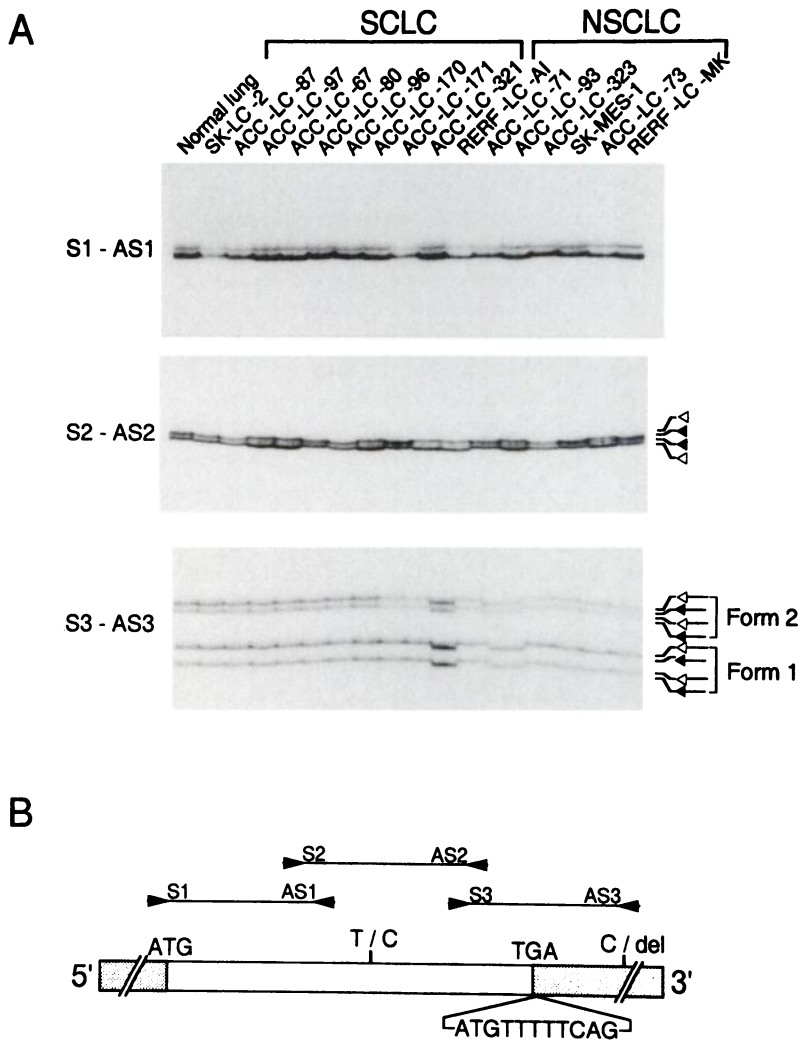

Fig. 3. Results of RT-PCR-SSCP analysis for the presence of subtle *FHIT* mutations. *A*, note the distinct mobility shifts in the middle and 3′ regions of *FHIT*. *Open arrowheads*, positions of amplified products with CAC at codon 294, whereas *filled arrowheads*, those with CAT. *Open arrows*, amplified products with a stretch of seven cytosine residues at nucleotides 871–877 as reported by Ohta *et al.* (21); *filled arrows*, those with a single bp deletion within this stretch. *Forms 1* and *2* represent previously unreported alternative splicings of *FHIT*. *B*, schematic presentation of the identified polymorphisms and alternative splicing as well as location of the PCR primers. *Open* and *shaded boxes*, coding and untranslated regions, respectively.

nucleotides 813–823 of the published sequence (21). The alternative splicing due to the use of a cryptic acceptor site in exon 10 was found to be located outside the open reading frame of *FHIT* and thus should not affect its coding capacity. It should be noted that normal lung specimens and the lung cancer cell lines thus far examined did not show significant differences with regard to the ratio of the two alternatively spliced mRNA forms.

We next examined genomic DNAs for the presence of gross alterations in the *FHIT* gene which might be responsible for the identified alterations in expression. Southern blot analysis using a cDNA probe encompassing exons 3–10 revealed homozygous losses of genetic material in four cell lines with either lack of *FHIT* expression (ACC-LC-94 and ACC-LC-174 established from the same patient) or the presence of aberrantly spliced transcripts (RERF-LC-MT and SK-LC-4; data not shown). Exon-specific PCR amplification of exons 1, 5, and 10 was then conducted to examine whether the homozygous deletions are confined within the *FHIT* locus. The results showed that these lung cancer cell lines carried intragenic homozygous deletions involving exon 5 but not exons 1 and 10 (Fig. 4). No alterations were detected by Southern blot analysis or exon-specific PCR amplification in other cell lines, including those lacking *FHIT* expression (ACC-LC-176, -314, and -319). The latter three cases may carry genetic and/or epigenetic alterations in other regions such as the large introns or the promoter region.

Detection of identical abnormalities in each of the two pairs of cell lines, which were established at different time points from distinct metastatic sites of two independent cases, suggests that *FHIT* alterations may have driven a clonal selection process prior to metastasis. The presence of intragenic homozygous deletions further lends support to the possibility that the *FHIT* gene may be the target tumor

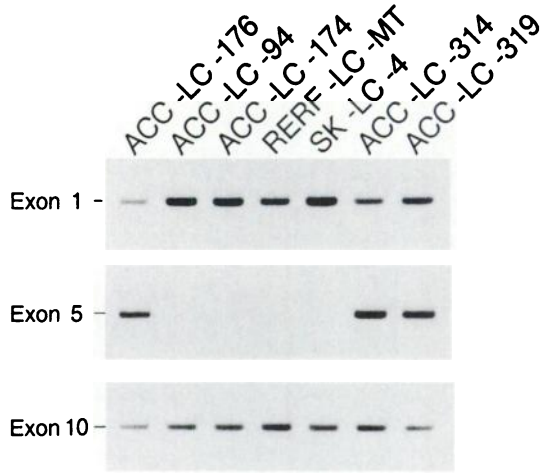

Fig. 4. Detection of intragenic homozygous deletions of *FHIT* in lung cancer cell lines. Homozygous deletions involving exon 5 are apparent in the ACC-LC-94, ACC-LC-174, RERF-LC-MT, and SK-LC-4 cell lines. Note that the ACC-LC-94 and -174 cell lines were established from a single patient: one at the time of diagnosis (cervical lymph node metastasis) and one after treatment (pleural effusion).

suppressor gene at 3p14.2 involved in lung carcinogenesis. However, it is still possible that large introns of the *FHIT* gene, which have not been completely sequenced, might contain another, yet unidentified, true target gene and that *FHIT* itself might be fortuitously involved in homozygous deletions as a mere bystander. Genes within genes have indeed been demonstrated to exist, *e.g.*, at the neurofibromatosis type 1 tumor suppressor locus (26). Alternatively, losses of genetic material at the *FHIT* locus may not be related to lung carcinogenesis at all and simply reflect instability in this genomic region. In this regard, exon 5 of *FHIT* and the surrounding genomic regions were previously shown to coincide with the constitutive and aphidicolin-sensitive 3p14.2 fragile site, *FRA3B* (21), which is known to be the most highly inducible of such sites in the human genome (27).

Here, we showed that *FHIT* is indeed altered in a significant proportion of NSCLC cell lines, with either lack of expression or aberrant splicings being found, often accompanied by intragenic homozygous deletions. The present observations provide support for a possible association between *FHIT* alterations and lung cancer development, although the observed frequency was less than that reported previously, possibly due to differences in the amplification strategies used. Additional functional studies such as introduction of wild-type *FHIT* into *FHIT*-negative lung cancer cell lines are required to answer the questions posed above and to facilitate better understanding of the molecular pathogenesis of this fatal disease. It will also be interesting to investigate whether alterations of the *FRA3B* fragile site where *FHIT* resides may have any relationship to previous exposure of lung cancer patients to risk factors such as cigarette smoking and occupational hazards.

Acknowledgments

We thank Dr. T. Hayakawa for his encouragement throughout the study.

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