Loss of Fhit Is Frequent in Stage I Non-Small Cell Lung Cancer and in the Lungs of Chronic Smokers

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Abstract

Abnormalities of FHIT, a candidate tumor suppressor gene at 3p14.2, have been found frequently in multiple tumor types including non-small cell lung cancer (NSCLC). To investigate whether FHIT inactivation plays a role in early lung tumorigenesis, Fhit levels were determined by immunohistochemistry in tumors from 87 patients with stage I NSCLC and in 372 bronchial biopsy specimens from 86 chronic smokers without evidence of malignancy. We found that 49% of NSCLC specimens demonstrated significantly decreased staining or lack of staining for Fhit. However, Fhit expression status was not significantly associated with disease-free survival or overall survival. Analysis of a subset of 76 specimens on which microsatellite analysis at the FHIT locus was performed did not show a strong association between loss of heterozygosity at FHIT and Fhit expression, suggesting the presence of complex mechanisms of Fhit inactivation. Of 372 bronchial biopsy specimens from chronic smokers, 86 biopsies (23%) exhibited decreased Fhit expression or lack of Fhit expression. In 37 of 86 (43%) subjects, decreased Fhit expression or lack of expression was observed in at least one biopsy site. Loss of Fhit expression was significantly higher in bronchial metastatic lesions (23 of 49 lesions, 47%) than in histologically normal bronchial epithelium (63 of 323 specimens, 20%; \( P < 0.001 \)). Smokers with a metaplasia index of $\geq 15\%$ had a higher frequency of loss of Fhit expression than those with a metaplasia index of $\leq 15\%$ (\( P = 0.015 \)). Interestingly, current smokers had a higher rate of loss of Fhit expression than former smokers (\( P = 0.002 \)). Our data indicate that Fhit expression is significantly reduced in a substantial number of early-stage NSCLC and preneoplastic lesions in chronic smokers. The association between cigarette smoking and Fhit expression suggests a role for FHIT in the initiation of smoking-related lung tumorigenesis.

Introduction

Lung cancer remains the leading cause of cancer-related mortality in the United States (1). It was estimated that 171,600 new cases of lung cancer would be diagnosed in 1998, with an estimated 158,900 deaths (1). The 5-year-survival rate of 14% for patients with lung cancer has improved little over the past decade (1). Even patients with pathological stage I NSCLC\(^1\) have only a 60% survival rate at 5 years. Two concepts, field cancerization and multistep tumorigenesis, are essential in understanding the underlying mechanisms of carcinogenesis of the aerodigestive tract. Field cancerization was first proposed by Slaughter and Skejkal in 1953 (2), who suggested that a whole tissue field exposed to common carcinogens, such as cigarette smoke, is at risk for the development of malignancy due to diffuse injury over time. Multistep tumorigenesis refers to the multistep process in which genetic events accumulate and result in malignant transformation, as well illustrated in the colorectal cancer model (3).

It is estimated that between 10 and 20 genetic events are required for lung tumorigenesis (4). Numerous genetic alterations have been identified that occur at high frequencies in NSCLC. These include p53 gene mutations (5, 6), K-ras proto-oncogene mutations (7), inactivation of the Rb gene (8), and alterations in p16, CDC25, cyclin D1, and FHIT (9–13). The FHIT gene is located at 3p14.2 (14), a region frequently lost in multiple tumor types. Loss of Fhit expression has been found to occur frequently in multiple tumor types including NSCLC (10, 15). To determine whether loss of Fhit occurs early and plays an important role in lung tumorigenesis, we analyzed Fhit expression in primary tumors from 87 patients with stage I NSCLC and in 372 bronchial biopsy specimens from 86 chronic smokers without evidence of malignancy. We found that Fhit expression is absent or markedly reduced in 49% of 87 primary tumors in patients with stage I NSCLC and in 23% of 372 bronchial biopsy specimens from chronic smokers. We also found that loss of Fhit expression was associated with bronchial metaplastic changes, patients’ MI, and current smoking status.

Materials and Methods

Study Population. Primary NSCLC specimens were obtained from archived tissue specimens of surgically resected pathological stage I tumors from 87 patients treated at the M. D. Anderson Cancer Center (Houston, TX). Clinical and pathological features of the study population are listed in Table 1. Surgical specimens were collected between 1975 and 1990. Survival data were available for all patients for at least 5 years. The study population consisted of 62 males and 25 females. The mean age of patients was 64.0 years. Histological subtypes included 37 adenocarcinomas, 36 squamous cell carcinomas, 8 bronchoalveolar carcinomas, 3 large cell carcinomas, 1 adenosquamous carcinoma, and 2 unclassified tumors.

A total of 372 bronchial biopsies were obtained from 86 chronic smokers (72 current and 14 former smokers) who were enrolled in a chemoprevention protocol at the M. D. Anderson Cancer Center between 1993 and 1998, before any intervention. Chronic smokers were defined as subjects who had smoked $\geq 1$ pack/day for $\geq 20$ years and who were currently smoking at the time of enrollment in the study or may have quit at any time before enrollment. Current smokers were defined as subjects who were smoking at the time of enrollment or who had stopped smoking within a year before enrollment. Former smokers were defined as subjects who had stopped smoking $> 12$ months before enrollment. Thirty-eight percent (33 of 86) were female, and 62% (53 of 86) were male. The median number of pack-years for all subjects was 30.5; the median number of pack/day was 1.2. The median age of subjects was 54 years. All patients underwent fiber-optic bronchoscopy. Random endobronchial biopsies were obtained from six predetermined sites, including the right upper lobe bronchus, the right middle lobe bronchus, the right lower lobe

Received 6/29/99; accepted 8/18/99.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1754 solely to indicate this fact.

\(^{1}\)Supported in part by American Cancer Society Grant RPG-98-054 (to L. M.), American Cancer Society-Clinical Oncology Career Development Award 96-41 (to F. R. K.), M. D. Anderson Trainee and Student Resources Research Grant (to J. E. T.), National Cancer Institute No1 Grant (to W. K. H.), U19 CA 68437 Grant (to W. K. H.), and National Cancer Institute Cancer Center Grant P30 CA16620 (to the M. D. Anderson Cancer Center). W. K. H. is an American Cancer Society Clinical Research Professor.

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\(^{3}\)The abbreviations used are: NSCLC, non-small cell lung cancer; LOH, loss of heterozygosity; DFS, disease-free survival; OS, overall survival; MI, metaplasia index.
Table 1 Fhit reactivity in stage I NSCLC tumors according to clinicopathological features of patients

<table>
<thead>
<tr>
<th>Fhit reactivity</th>
<th>Total</th>
<th>Negative (%)</th>
<th>Positive (%)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean age</td>
<td>64.0 yrs</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age range</td>
<td>39–63 yrs</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age &lt; 60 yrs</td>
<td>25 patients</td>
<td>16 (64%)</td>
<td>9 (36%)</td>
<td>0.08a</td>
</tr>
<tr>
<td>Age ≥ 60 yrs</td>
<td>62 patients</td>
<td>27 (44%)</td>
<td>35 (56%)</td>
<td></td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>62 patients</td>
<td>32 (52%)</td>
<td>30 (48%)</td>
<td>0.52b</td>
</tr>
<tr>
<td>Female</td>
<td>25 patients</td>
<td>11 (44%)</td>
<td>14 (56%)</td>
<td></td>
</tr>
<tr>
<td>Smoking history</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Smokers</td>
<td>76 patients</td>
<td>39 (51%)</td>
<td>37 (49%)</td>
<td>0.68</td>
</tr>
<tr>
<td>Nonsmokers</td>
<td>6 patients</td>
<td>2 (33%)</td>
<td>4 (67%)</td>
<td></td>
</tr>
<tr>
<td>Data not available</td>
<td>5 patients</td>
<td>2 (40%)</td>
<td>3 (60%)</td>
<td></td>
</tr>
<tr>
<td>Histology of tumors</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adenocarcinoma</td>
<td>37 patients</td>
<td>17 (46%)</td>
<td>20 (54%)</td>
<td>0.29c</td>
</tr>
<tr>
<td>Squamous cell carcinoma</td>
<td>36 patients</td>
<td>21 (58%)</td>
<td>15 (42%)</td>
<td></td>
</tr>
<tr>
<td>Bronchoalveolar</td>
<td>8 patients</td>
<td>1 (13%)</td>
<td>7 (87%)</td>
<td></td>
</tr>
<tr>
<td>Large cell carcinoma</td>
<td>3 patients</td>
<td>2 (67%)</td>
<td>1 (33%)</td>
<td></td>
</tr>
<tr>
<td>Adenosquamous</td>
<td>1 patient</td>
<td>1 (100%)</td>
<td>0 (0%)</td>
<td></td>
</tr>
<tr>
<td>Unclassified</td>
<td>2 patients</td>
<td>1 (50%)</td>
<td>1 (50%)</td>
<td></td>
</tr>
<tr>
<td>5-year-survival</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OS</td>
<td>87 patients</td>
<td>60%</td>
<td>50%</td>
<td>0.368</td>
</tr>
<tr>
<td>DFS</td>
<td>86 patients</td>
<td>70%</td>
<td>65%</td>
<td>0.69</td>
</tr>
</tbody>
</table>

*P for the comparison between the percentage of Fhit negativity in younger patients versus older patients.

bP for the comparison between the percentage of Fhit negativity in males versus females.

P for the comparison between the percentage of Fhit negativity in smokers versus nonsmokers.

aP for the comparison between the percentage of Fhit negativity in adenocarcinoma versus squamous cell carcinoma.

One patient whose medical record was not available for determination of DFS was excluded from the analysis.

bronchus, the left upper lobe bronchus, the left lower lobe bronchus, and the carina. Forty-nine of 372 specimens contained bronchial metaplasia; the remainder did not contain any histological abnormalities. The MI was calculated for each subject; the MI was defined as the number of biopsies exhibiting metaplasia divided by the total number of biopsies, with the quotient multiplied by 100%.

Immunohistochemical Staining for Fhit Protein. Paraffin-embedded, 4-μm-thick tissue sections from all 87 primary tumors and 372 bronchial epithelial biopsies from 86 chronic smokers were stained for the Fhit protein using a primary rabbit polyclonal anti-glutathione S-transferase-Fhit antibody (kindly provided by Drs. Carlo Croce and Kay Huebner, Kimmel Cancer Center, Philadelphia, PA). Deparaffinization of all sections was performed using a 25.5-gauge steel needle under a stereomicroscope. Approximately 1000–2000 cells were dissected from each section. After microdissection, DNA was extracted as described previously (16). The markers used for microsatellite analysis were D3S1234 and D3S1481 on 3p14 within the Fhit locus (Research Genetics, Huntsville, AL). Microsatellite analysis was performed as described previously (16). LOH was defined as a >50% decrease in signal intensity by visual inspection in one of the two alleles compared to that in the corresponding normal control. The results were interpreted by two independent observers (J. E. T. and L. M.), and agreement was reached after discussion for any with discrepancies. If either of the two markers demonstrated LOH, then the patient was considered to exhibit LOH at the Fhit locus.

Statistical Analysis. Survival curves were estimated by the Kaplan-Meier method, and the resulting curves were compared using the log-rank test. Fisher’s exact test and χ2 test were used to analyze the association between two categorical variables. P < 0.05 was considered to be statistically significant.

Immunohistochemical analysis was performed in a blinded manner with respect to the clinical information for the subjects, both for the stage I NSCLC patients and for the chronic smokers.

Results

Loss of Fhit Expression in Stage I NSCLC. Immunohistochemical staining was performed on 87 tumor sections from 87 stage I NSCLC cases. In normal bronchial tissues, cytoplasmic granular staining of Fhit was observed in bronchial epithelial cells (Fig. 1, A and B). We found that 49% (43 of 87) of pathological stage I NSCLC tissue sections showed decreased or negative staining for Fhit (score, ≥3), whereas 51% of NSCLC tissue sections showed at least moderate levels of staining (score, >3). The scoring system we applied is similar to that used by Greenspan et al. (17) and Hadaczek et al. (18) using the same antibody. Other studies have used a scoring system that classified tumors as positive, mixed, or negative for Fhit immunoreactivity (19, 20). We did observe a mixed pattern in many tumors and bronchial epithelial specimens with different staining intensities within the same section; thus, we used an immunoreactivity score that takes into account both the intensity of cells stained and the percentage of cells stained. In tumors and epithelial specimens displaying a mixed pattern of staining, the highest intensity of staining within the tumor was used as the intensity score. Examples of Fhit expression patterns in stage I NSCLC primary tumors are shown in Fig. 1. It should be noted that the Fhit positive category represents a range of intensities and extents of Fhit expression, many of which may be consistent with LOH.

The frequency of Fhit negativity in patients with stage I NSCLC did not differ significantly according to gender, age, histological subtype, or smoking status (Table 1). However, it should be noted that the comparison between smokers and nonsmokers is limited by the small sample size of the nonsmoker group (Table 1). OS and DFS did not differ significantly between patients with Fhit-negative and Fhit-positive tumors (Table 1). In the squamous cell carcinoma and adenocarcinoma subsets, OS and DFS did not differ significantly between patients with Fhit-negative and Fhit-positive tumors.

Loss of Fhit Expression in Stage I NSCLC and LOH at the Fhit Gene. Microsatellite analysis was performed on 76 of 87 tissue sections on which immunohistochemical protein analysis was also performed. We were unable to perform microsatellite analysis on 11 of 87 tissue sections due to lack of tissue samples, lack of reliable normal tissues as controls, or the poor quality of DNA. The two highly polymorphic markers, D3S1234 and D3S1481, are located within the Fhit gene at the 3p14 locus; D3S1234 is located in intron 5 (21), and D3S1481 is located in intron 4. Fifty-nine of 76 (78%) and 44 of 76 (58%) cases were informative at the D3S1234 and D3S1481 markers,
respectively; 67 of 76 (88%) cases were informative at one or both markers. LOH at markers D3S1234 and D3S1481 were observed in 34 of 59 (58%) and 15 of 44 (34%) informative cases, respectively. Thirty-nine of 67 (58%) informative cases demonstrated LOH in at least one marker. Twenty-three of 36 (64%) tumors with negative Fhit expression demonstrated LOH at the \textit{FHIT} locus, whereas 16 of 31 (52%) tumors with positive Fhit expression demonstrated LOH at the \textit{FHIT} locus. Thus, a slightly higher proportion of tumors with loss of Fhit expression demonstrated LOH at \textit{FHIT} compared with tumors with normal Fhit expression, although this difference was not statistically significant ($P = 0.31$, \chi^2 test).

Loss of Fhit Expression in Bronchial Epithelial Specimens from Chronic Smokers. Loss of Fhit expression was observed in 86 of 372 (23%) bronchial epithelial biopsy specimens, including 63 of 323 (20%) bronchial epithelial biopsy specimens without metaplasia, and in 23 of 49 (47%) biopsy specimens containing bronchial metaplasia ($P < 0.001$; Table 2; Fig. 2). Loss of Fhit expression in the bronchial epithelium in at least one biopsy site was demonstrated in 37 of 86 cases (43%), including 8 of 31 (26%) of the cases with a metaplasia index of $\leq 15\%$ and in 29 of 55 (53%) of the cases with a metaplasia index of $>15\%$ ($P = 0.015$).

Interestingly, all biopsy specimens containing metaplasia were from current smokers. In bronchial biopsies without metaplasia, loss of Fhit expression was observed in only 4 of 59 (7%) biopsy sites from former smokers and 59 of 264 (22%) biopsy sites from current smokers ($P = 0.006$, Fisher’s exact test), compared to 23 of 49 (47%) biopsy sites with metaplasia from current smokers. When analyzed by case, loss of Fhit expression was observed in at least one biopsy site from 37 of 86 (43%) subjects, including 8 of 31 (26%) of the cases with a metaplasia index of $\leq 15\%$ and 29 of 55 (53%) of the cases with a metaplasia index of $>15\%$ ($P = 0.015$).

Table 2 Fhit reactivity in bronchial epithelial specimens in chronic smokers

<table>
<thead>
<tr>
<th></th>
<th>Fhit negative</th>
<th>Fhit positive</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Percentage of total biopsy sites</td>
<td>86/372 (23%)</td>
<td>286/372 (77%)</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>Histologically normal bronchial epithelium</td>
<td>63/323 (20%)</td>
<td>260/323 (80%)</td>
<td></td>
</tr>
<tr>
<td>Bronchial metaplasia</td>
<td>23/49 (47%)</td>
<td>26/49 (53%)</td>
<td></td>
</tr>
<tr>
<td>Percentage of total biopsy sites</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Former smokers</td>
<td>4/59 (7%)</td>
<td>55/59 (93%)</td>
<td>0.006*</td>
</tr>
<tr>
<td>Current smokers</td>
<td>59/264 (22%)</td>
<td>205/264 (78%)</td>
<td></td>
</tr>
<tr>
<td>All subjects*</td>
<td>37/86 (43%)</td>
<td>49/86 (57%)</td>
<td></td>
</tr>
<tr>
<td>Subjects with MI$^f$ $\leq 15%$</td>
<td>8/31 (26%)</td>
<td>23/31 (74%)</td>
<td>0.015*</td>
</tr>
<tr>
<td>Subjects with MI$^f$ $&gt;15%$</td>
<td>29/55 (53%)</td>
<td>26/55 (47%)</td>
<td></td>
</tr>
</tbody>
</table>

* Fhit negative is defined as an immunoreactivity score of $\leq 3$.

* Fhit positive is defined as an immunoreactivity score of $>3$.

* The $P$ for the comparison of the percentage of biopsy sites with Fhit negativity in histologically normal bronchial epithelium and bronchial metaplasia.

* The $P$ for the comparison between the percentage of biopsy sites with Fhit negativity in histologically normal bronchial epithelium of current versus former smokers.

* Percentage of Fhit negativity or positivity in at least one biopsy site in each subject.

* MI = the number of biopsies exhibiting metaplasia divided by the total number of biopsies, with the quotient multiplied by 100.

* The $P$ for the comparison of the percentage of Fhit negativity in at least one biopsy site in subjects with MI $\leq 15\%$ and MI $> 15\%$. 
specimen in 35 of 72 (49%) current smokers compared to 2 of 14 (14%) biopsy specimens from former smokers (P = 0.02, Fisher’s exact test).

Discussion

The role of the FHIT gene as a tumor suppressor gene has been controversial. Tumor suppressor genes may be inactivated by several mechanisms, including intragenic deletions, point mutations, loss of a whole chromosome, or genetic recombination (22). One feature of FHIT is the virtual absence of point mutations (23, 24). Furthermore, a number of investigators have reported the coexistence of wild-type transcripts along with aberrant FHIT transcripts in tumors (10, 14, 23). Additionally, aberrant FHIT transcripts have been found in nonmalignant tissues (25–27). However, multiple lines of evidence suggest that FHIT is a tumor suppressor gene. First, FHIT is located in a region of the genome that is known to be rearranged and/or deleted in multiple tumor types (10, 14, 28–30). Homozygous deletions of the genetic region have been identified in multiple types of tumors; in some cases, the deletions included FHIT exons (14, 31). Furthermore, aberrant FHIT transcripts and/or decreased protein expression have been identified in multiple tumor types, including renal cell, cervical, and lung cancers (15, 17–20). It has been demonstrated that transfection of wild-type FHIT genes into cancer cell lines lacking FHIT suppressed tumorigenicity in nude mice (32). More recently, Ji et al. (33) found that overexpression of the FHIT gene in lung and head and neck cancer cell lines induced apoptosis, caused cell cycle arrest in S phase, and suppressed tumorigenicity.

We observed frequent loss of Fhit protein expression in early-stage NSCLC, with 49% of specimens demonstrating a decrease or lack of Fhit protein staining. This frequency of decreased or absent protein expression is similar to that observed in NSCLC by other investigators (15, 19, 20). However, we did not find a significant association between the lack of Fhit expression and survival in stage I NSCLC. This is consistent with a recent report by Sozzi et al. (19). However, Tomizawa et al. (20) found that lack of Fhit expression in Japanese patients with NSCLC predicted a poorer outcome. The lack of correlation between Fhit protein inactivation and survival in our study and in that of Sozzi et al. (19) may be due to a role of FHIT at an early stage in lung carcinogenesis, whereas different scoring methodologies and patient populations may account in part for the discordance between our results and those of Tomizawa et al. (20). Additionally, the latter study included a larger proportion of adenocarcinomas, a factor that may have contributed to the different outcome of a subset of patients in this study (20).

A number of studies have found that alterations in the 3p locus occur frequently in bronchial preneoplastic lesions (34, 35) from chronic smokers. Sozzi et al. (19) observed loss of Fhit expression in 17 of 20 (85%) dysplastic lesions and in 25 of 25 (100%) carcinoma in situ lesions at the margins of tumor specimens from patients with lung cancer, further suggesting that inactivation of the FHIT gene may occur at an early stage in lung carcinogenesis. In this study, we have analyzed Fhit expression status in a large number of bronchial biopsies from chronic smokers. We have found that epithelium in 23% of the biopsies from 43% of smokers showed a lack of Fhit expression or a significantly reduced Fhit expression. Loss of Fhit expression was significantly more frequent in metaplastic lesions than in histologically normal epithelium (47% versus 20%, respectively) in chronic smokers. To our knowledge, our study is the first to demonstrate loss of Fhit expression in histologically normal bronchial epithelium and frequent loss of Fhit expression in bronchial metaplastic lesions from chronic smokers. Furthermore, loss of Fhit expression was more frequent in smokers with a high MI (>15%) than in smokers with a MI ≤ 15% (53% versus 26%). We have previously demonstrated that LOH at 3p14 occurs frequently in the histologically normal bronchial epithelium of chronic smokers (35). Together, these observations strongly support the notion that loss of Fhit expression occurs early in the progression from normal to premalignant lesions in the lungs of smokers. Furthermore, loss of Fhit expression appears to be strongly associated with current smoking status, suggesting that the FHIT gene may be the target of carcinogens in cigarette smoke. These data further support a potential role of the FHIT gene in smoking-related lung carcinogenesis. Recent epidemiological studies indicate that former smokers account for over 50% of lung cancer cases; former smokers have over twice the lung cancer risk of lifetime nonsmokers (36–38). These data underscore the importance of investigating the genetic abnormalities associated with smoking.
In this study, we did not find a concordance between LOH at the FHIT gene and loss of Fhit expression in NSCLC. Whereas LOH is generally associated with decreased protein expression, protein inactivation may be due to complex mechanisms other than deletion within an allele. Tanaka et al. (39) investigated such mechanisms of FHIT inactivation in esophageal cell lines and tumors and found hypermethylation of the 5′ CpG island to be an important mechanism for FHIT inactivation in some cell lines. Alternatively, abnormal protein expression may occur in the absence of genetic alterations through altered splicing fidelity (27). Additionally, posttranslational events such as ubiquitin-proteosome-dependent degradation (40) have been found to be important mechanisms of protein inactivation for other genes; however, such mechanisms have not previously been investigated for Fhit. It is also possible that microsatellite analysis may not be a sensitive method to detect homozygous deletions within the FHIT gene that have been reported in NSCLC cell lines as a mechanism of FHIT inactivation (41, 42). In fact, homozygous deletions in the FRA3B region located within the FHIT gene have been reported in certain NSCLC cell lines (41, 42).

Additionally, the scoring system that we and others have used for protein expression incorporates both the intensity of staining and the percentage of cells stained and thus includes heterogenous patterns of immunoreactivity within subsets of tumors that are classified as positive and those that are classified as negative. A tumor with an immunoreactivity score of 6 might have strong staining in a minority of cells (and perhaps loss of both alleles in most cells) or moderate expression in a majority of cells (and perhaps loss of one allele in most cells). Thus, LOH data do not provide information about mechanisms of protein inactivation that do not involve deletion.

In summary, we have demonstrated that Fhit protein inactivation is a frequent event in bronchial premalignant lesions and in stage I NSCLC, suggesting that loss of Fhit expression occurs early in the progression from normal to malignant lesions. Importantly, we found strong associations between loss of Fhit expression and cigarette smoking, metaplastic changes, and high MI, suggesting a potential role for Fhit in smoking-related lung tumorigenesis.

Acknowledgments

We thank Susan Cweren, Lakshmi Kakarala, and Shyla Kalapurakal for technical assistance with immunohistochemistry and assistance with the preparation of pathology slides.

References

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