Loss of FHIT Function in Lung Cancer and Preinvasive Bronchial Lesions

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Abstract

We previously cloned and characterized the tumor suppressor gene FHIT (fragile histidine triad) at chromosome 3p14.2 and found that this gene is altered by deletions in human tumors, including lung cancer. To assess the frequency and specificity of inactivation and its relevance in a clinical setting, we have produced antibodies against the Fhit protein and studied its expression in a series of non-small cell lung cancers and normal bronchial mucosa and a spectrum of preinvasive lesions by immunohistochemistry.

The data indicate that the loss of Fhit protein is the most frequent alteration in non-small cell lung cancer (73%) and precancerous lesions (93%), is significantly higher in the tumors of smokers (75%) than in those of nonsmokers (39%; \( P < 0.0005 \)), and is an independent and more frequent event than p53 overexpression in tumors and precancerous lesions (73 versus 46%). The percentage of cases lacking Fhit expression was higher in the squamous type compared to adenocarcinoma (87 versus 57%; \( P < 0.00001 \)), whereas other histotypes (large cell, mucopidermal) showed an intermediate value (69%).

Loss of Fhit expression in a very high percentage of primary lung carcinomas is a frequent event in the progression of bronchial lesions. The overall high frequency and precocity of Fhit loss in lung carcinogenesis and the development of the presently described immunohistochemical approach suggest a potential use of this gene in the early detection of lung cancer and in chemopreventive studies as an intermediate biomarker.

Introduction

Deletions of the short arm of chromosome 3 are considered critical events in the pathogenesis of lung cancer (1-3). The observation of 3p alterations in preinvasive lesions of the bronchus suggests that one or more tumor suppressor gene(s) may act as gatekeepers for lung carcinogenesis (4-8). FHIT, the tumor suppressor gene at 3p14.2 (9), has been implicated in the development of bronchial carcinomas and precancerous lesions (7, 8). The involvement of specific tumor suppressor gene(s) in any 3p region has not been demonstrated. Therefore, the identification of Fhit alterations in these tumors types may provide useful markers for early diagnosis of lung cancer and for the assessment of individuals at higher risk for this type of tumor. Inactivation of the suppressor gene p53 and overexpression of EGFR as well as more generalized allelic losses (LOH) at several chromosomal regions on the short arm of chromosome 3 have been reported in preinvasive lesions (4, 5, 7, 8, 26, 27). However, in spite of the high frequency of LOH on 3p in precancerous lesions (7, 8), the involvement of specific tumor suppressor gene(s) in any 3p region has not been demonstrated. This would be extremely important to increase the sensitivity of the molecular assays of preinvasive lesions in routine clinicopathological diagnosis as well as to better clarify the pathogenesis of lung tumors.

Materials and Methods

Study Patients. From January 1974 to December 1990, 608 consecutive patients at the Division of Thoracic Surgery of the Istituto Nazionale Tumori (Milan, Italy) underwent surgical resection for pathological stage I (pT1-2N0M0) NSCLC (28, 29). After pathological review, 474 cases were considered adequate for the present analysis, and new sections were obtained from paraffin blocks. The remaining cases were excluded due to the inadequacy of paraffin blocks or a diagnosis other than NSCLC (carcinoid, SCLC). The main clinical features of these patients are listed in Table 1. The perioperative mortality rate was 3.9%. The vast majority of the patients were treated with SCLC and NSCLC (18-20). In addition, Fhit protein expression was markedly reduced or absent in the great majority of primary SCLCs and in tumor-derived cell lines showing FHIT gene abnormalities (21). The transfection of a FHIT transgene into lung, renal, and gastric cancer cell lines lacking endogenous FHIT because of homozygous deletions resulted in a very low yield of Fhit-expressing clones (22).

Moreover, the ability of wild-type Fhit transfectants to form tumors in nude mice was suppressed (22), supporting a role for FHIT as a tumor suppressor gene.

The crystallographic structure of Fhit, a diadenosine 5',5''::P, P-polyphosphate hydrolase (23), suggests that in its active form, FHIT is bound to its preferred substrate, Ap3A, in a dimeric state (24). This binding seems to be critical for tumorigenicity because the hydrolase "dead" Fhit mutant protein still suppresses tumorigenicity, suggesting that hydrolase activity is not required for tumor suppression (22). Although the biological function of the Fhit protein is still unknown, it is conceivable that FHIT plays a role in cell proliferation and/or apoptotic pathways.

NSCLC represents a major cause of mortality worldwide; it accounts for 75-80% of all lung tumors and includes three main histological subtypes: (a) adenocarcinoma; (b) squamous cell carcinoma; and (c) large cell carcinoma (25). The progression from normal epithelium to invasive neoplasia develops through the occurrence of morphological lesions of various grades, which are considered risk factors for tumor development. The detection of molecular changes in these samples would thus provide useful markers for early diagnosis of lung cancer and for the assessment of individuals at higher risk for this type of tumor. Inactivation of the suppressor gene p53 and overexpression of EGFR as well as more generalized allelic losses (LOH) at several chromosomal regions on the short arm of chromosome 3 (3p) have been reported in preinvasive lesions (4, 5, 7, 8, 26, 27).

However, in spite of the high frequency of LOH on 3p in precancerous lesions (7, 8), the involvement of specific tumor suppressor gene(s) in any 3p region has not been demonstrated. This would be extremely important to increase the sensitivity of the molecular assays of preinvasive lesions in routine clinicopathological diagnosis as well as to better clarify the pathogenesis of lung tumors.

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The abbreviations used are: SCLC, small cell lung carcinoma; NSCLC, non-SCLC; EGFR, epidermal growth factor receptor; LOH, loss of heterozygosity; GST, glutathione S-transferase; RT, room temperature; CIS, carcinoma in situ.
Immunohistochemistry. Staining was performed on paraffin sections with the use of a rabbit polyclonal anti-GST-Fhit antiserum (15, 22). The specificity of this antiserum was previously demonstrated by immunoblot and by the immunocytochemical detection of exogenous Fhit protein in transfected Cos and human tumor cell lines (15, 22). Additionally, absorption of the antiserum with purified GST did not reduce the detection of endogenous and exogenous Fhit (21). To determine the relationship and the temporal sequence of Fhit and p53 alterations, consecutive sections from tumor and bronchial mucosa specimens that were immunostained were serially cut and stained with anti-Fhit serum, anti-p53, and Mib1 antibody directed against a nuclear proliferation antigen to help complement the assessment of the grading of the preinvasive lesions.

Briefly, formalin- or Bouin-fixed paraffin-embedded tumor and bronchial mucosa sections were serially cut and mounted in poly-L-lysine (Sigma, St. Louis, MO)–coated slides, deparaffinized in xylene, and rehydrated in graded alcohols. Endogenous peroxidase activity was blocked by treatment for 30 min with 0.3% hydrogen peroxide in methanol. Antigen enhancement was performed by steaming the sections at 100°C in 5 mmol/liter sodium citrate buffer in distilled water (pH 6.0) for 2 min. The slides were cooled under tap water and washed three times in 0.05 M PBS-0.1% Triton and then incubated with blocking serum (normal goat at a 1:4000 dilution in PBS). D07 monoclonal antibody (at a 1:400 dilution in PBS-1% Triton) was used for the detection of p53, and a 1:200 dilution of biotinylated goat antirabbit or antisheep antibodies was used for the detection of anti-Fhit serum. Sections were washed again three times in PBS-0.1% Triton and then incubated with secondary antibody (biotinylated goat antirabbit or antismouse IgG diluted at 1:200 and 1:100, respectively, in PBS-1% BSA-0.1% sodium azide; Dako) for 30 min at RT. The slides were incubated overnight with primary rabbit polyclonal anti-GST-Fhit serum at a 1:4000 dilution in PBS, D07 monoclonal antibody (at a 1:400 dilution in PBS-1% BSA-0.1% sodium azide; Ylem-Novocacia, Newcastle upon Tyne, United Kingdom) and Mib1 (diluted 1:100 in PBS-1% BSA-0.1% sodium azide; Immunotech, Marseille, France). The slides were washed again three times in PBS-0.1% Triton and then incubated with secondary antibody (biotinylated goat antirabbit or antimouse IgG diluted at 1:200 and 1:100, respectively, in PBS-1% BSA-0.1% sodium azide; Dako) for 30 min at RT. After washings in PBS-0.1% Triton, a final incubation with streptavidin-conjugated horseradish peroxidase (Dako) at a dilution of 1:300 in PBS for 30 min at RT was performed, and after subsequent PBS washings, peroxidase activity was detected by aminoethyl carbazole for 10 min in the dark. The slides were counterstained with Caruzzi hematoxylin. Staining without the antibody was routinely performed as a negative control procedure.

Bronchial and tumor sections were routinely stained with nonimmune rabbit serum (1:4000 dilution) to control for nonspecific antibody binding. Slides did not show immunoreactivity in any of these immunohistochemical staining control assays.

Immunostaining was classified in the following three groups according to both intensity and extent: (a) negative, no staining was present, or positive staining was detected in <10% of the cells; (b) weak, staining was moderate in all of the cells (i.e., less intense than that in the internal control), or a range of 10–30% of the cells stained positive; and (c) strong, immunostaining was as strong as that of the internal control and was present in >30% of the cells. Two independent readers, a pathologist (S. P.) and a scientist (G. S.), were involved in the assessment of expression.

Statistical Analysis. To examine the association of the various categorical variables, we used the χ² test with a two-sided Fisher’s exact test for the comparison of small groups. P < 0.05 was considered to be statistically significant. Statistical analysis was performed using EPI Info Version 5.

Survival curves were calculated by Kaplan-Meier estimate, using log-rank testing for the assessment of statistical significance.

Results

Loss of Fhit Expression in NSCLC. Table 1 lists the frequency of Fhit reactivity (strong and weak expression were combined as positive) in the tumor samples. Of the 474 tumors analyzed, 345 (73%) were completely negative for Fhit protein expression (Fig. 1). The percentage of negative cases was higher in the squamous-type carcinoma compared with adenocarcinoma (87 versus 57%; P < 0.00001), whereas the other histotypes (large cell carcinoma, mucopidermoid carcinoma) showed an intermediate value (69%). In the adenocarcinoma specimens with positive Fhit immunostaining, the pattern of protein expression was clearly associated with the more differentiated cells, whereas less differentiated areas within the same tumor showed weaker Fhit protein expression (Fig. 2, B and C). Fhit immunostaining in squamous tumors also showed positive reactivity restricted to the more differentiated, keratinized, and sometimes apoptotic cells, whereas the basal hyperproliferative tumor cells showed prevalent negative staining (Fig. 1, C and D). These findings demonstrate that the loss of Fhit protein expression is a very frequent change in lung tumors, particularly in the squamous type of lung carcinoma. No association was found between Fhit-negative immunostaining and the size of the tumor, whereas an association with pT1 or pT2 pathological stage (62 versus 77%; P = 0.0013) and tumor grading [49 (grade 1) versus 74% (grades 2 and 3); P = 0.009] among the patients as a whole and in the group with adenocarcinoma, respectively, was observed.

Loss of Fhit Expression and Smoking Habits. When Fhit expression was compared with smoking habits (cigarette/year and cigarette number/day), the frequency of Fhit-negative tumors in smokers was significantly higher than it was in tumors from nonsmokers (75 versus 39%; P < 0.0005; Table 2). Of note, tumors from patients who smoked for less than 20 years showed a greater frequency of Fhit-negative tumors and those with Fhit-positive tumors (40 versus 53% at 5 years: P = 0.05; Fig. 3A). When the patients with adenocarcinoma were studied separately, a slight difference in survival was observed in the patients with Fhit-negative tumors [40 versus 53% at 5 years (P = 0.05) and 33 versus 42% at 10 years (P = 0.1); Fig. 3B]. No association with overall survival was found in early-stage disease (pT1) or with time to recurrence in the whole group.

Comparison of the Frequency of Fhit Loss and of Other Alterations Observed in Lung Cancer. Absence of the Fhit protein in tumor samples (73%) was a more frequent alteration than p53 (46%), EGFR (50%), and Bcl2 (19%) overexpression (Table 3). Fhit and p53 alterations were found to be independent events, because the frequency of Fhit-negative cases in the groups of p53-positive and -negative tumors, respectively, was very similar (Table 3). The fact

Table 1 Frequency of Fhit reactivity in the tumor according to the main clinical features

<table>
<thead>
<tr>
<th>Age</th>
<th>Positive</th>
<th>Negative</th>
<th>Total</th>
<th>% Fhit negative</th>
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<td>&lt;60</td>
<td>65</td>
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<td>224</td>
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<td>60</td>
<td>64</td>
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Sex

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<td>Negative</td>
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<td>35</td>
<td>60</td>
<td>0.58</td>
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Size

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<td>40</td>
<td>116</td>
<td>156</td>
<td>74</td>
<td>0.58</td>
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<tr>
<td>Negative</td>
<td>89</td>
<td>229</td>
<td>318</td>
<td>72</td>
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pT

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<td>48</td>
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<td>Negative</td>
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<td>348</td>
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Type

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<td>31</td>
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<tr>
<td>Total</td>
<td>129</td>
<td>345</td>
<td>474</td>
<td>73</td>
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* SCC, squamous cell carcinoma.
LOSS OF FHIT IN LUNG CANCER

Fig. 1. FHIT-negative immunostaining in squamous cell carcinomas. A, squamous cell carcinoma showing FHIT-negative immunostaining in tumor cells and FHIT-positive immunoreactivity in normal bronchial mucosa. B, FHIT-negative immunostaining in a squamous cell carcinoma containing FHIT-positive immunostaining in trapped bronchialized alveoli. C and D, squamous cell carcinomas showing FHIT immunoreactivity restricted to the more differentiated, keratinized cells.

that the FHIT and TP53 genes are both mutated in a large number of the same tumors suggests that the two proteins do not participate in the same biochemical pathway. p53 analysis by immunohistochemistry can underestimate the frequency of p53 alterations due to the occurrence of deletion or stop codon mutations in the p53 gene resulting in negative immunostaining; however, missense mutations (especially G to T transversions) accompanied by stabilization of the p53 protein represent the most frequent change in lung tumors (32).

Fig. 2. FHIT immunostaining modulation in adenocarcinoma. A, bronchoalveolar carcinoma showing areas with strong and weak immunoreactivity. Note the increasing cytological grading from A to C, i.e., the larger nuclear size and increased chromasia as well as mitotic rate in C, which parallels the decreased immunoreactivity of FHIT staining.
Table 2 Smoking habits and Fhit reactivity

<table>
<thead>
<tr>
<th>Fhit reactivity</th>
<th>Positive</th>
<th>Negative</th>
<th>Total</th>
<th>% Fhit negative</th>
<th>P</th>
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<tr>
<td>All cases</td>
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<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Nonsmokers</td>
<td>14</td>
<td>9</td>
<td>23</td>
<td>39</td>
<td>0.0005</td>
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<tr>
<td>Smokers</td>
<td>115</td>
<td>336</td>
<td>451</td>
<td>75</td>
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<tr>
<td>Adenocarcinoma only</td>
<td>10</td>
<td>4</td>
<td>14</td>
<td>29</td>
<td>0.049</td>
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<tr>
<td>Nonsmokers</td>
<td>74</td>
<td>108</td>
<td>182</td>
<td>59</td>
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</tbody>
</table>

Table 3 Correlation between Fhit and other markers in the tumor

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<th>p53</th>
<th>EgFR</th>
<th>Bcl2</th>
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<tbody>
<tr>
<td>Positive</td>
<td>46</td>
<td>53</td>
<td>15</td>
</tr>
<tr>
<td>Negative</td>
<td>166</td>
<td>177</td>
<td>62</td>
</tr>
<tr>
<td>Total</td>
<td>212</td>
<td>258</td>
<td>77</td>
</tr>
<tr>
<td>% Fhit negative</td>
<td>78</td>
<td>69</td>
<td>81</td>
</tr>
</tbody>
</table>
| P              | 0.018

Loss of Fhit Expression in Precancerous Lesions. The Fhit protein was clearly detectable by immunohistochemistry in the normal bronchial mucosa at the resection margin of the 309 patients analyzed. Fhit immunoreactivity was homogeneously observed in the cytoplasm of basal cells as well as in the differentiated columnar cells. The high level of Fhit expression in the normal bronchus suggests a possible role in the control of cell proliferation of bronchial cells that is disrupted during tumorigenesis. Positive Fhit immunostaining was observed in 326 hyperplastic bronchial mucosa and in 78 squamous metaplasia lesions either within the tumor or at the resection margin of the surgical specimens (Fig. 4, A and B); however, in several cases of incomplete metaplasia, Fhit immunostaining was weaker than that in the normal bronchus (Fig. 4C). The modulation of Fhit expression in the bronchial lesions of increasing grade, occurring in 34 patients, is reported in Table 4. A progressive loss of Fhit protein expression occurred in 3 of 5 cases of moderate dysplasia lesions and in 14 of 14 severe dysplasia lesions, whereas the single case of mild dysplasia analyzed showed positive immunoreactivity. (Figs. 5 and 6). Negative
immunostaining was observed in all 25 (100%) CIS lesions occurring either adjacent to the tumor or at the resection margin of the surgical samples. Overall, 17 of 20 (85%) dysplastic lesions and 42 of 45 (93%) precancerous lesions showed negative Fhit immunostaining.

A high (81%) concordance was observed between the results of Fhit immunostaining in the high-grade precancerous lesions and the invasive tumor occurring in the same patient. When the same serially cut sections containing the preinvasive lesions were immunostained for p53, an inverse correlation between Fhit and p53 immunostaining was observed in dysplastic and CIS lesions (Table 4; Fig. 6). In fact, p53 overexpression and loss of Fhit protein expression were concordant in these lesions. However, the frequency of Fhit-negative high-grade preinvasive lesions (dysplasia and CIS) was significantly higher than that of p53 overexpression (93 versus 55%; \( P = 0.00013 \)) and reflected that observed in tumors of the squamous type. Thus, the level of sensitivity of Fhit immunostaining is higher than that achieved by p53 immunoreactivity in precancerous lesions. These findings reinforce the hypothesis that FHIT may act as a gatekeeper in the lung carcinogenetic process, and the similar temporal sequence of Fhit and p53 alterations in lung carcinogenesis points to a role for both pathways in lung carcinogenesis.

Discussion

In the present study, we analyzed the largest series of lung tumors and bronchial mucosa samples reported thus far for the expression of a novel tumor suppressor gene, FHit, and its correlation with three other well-established molecular markers of lung cancer: (a) p53; (b) EGFR; and (c) BCL2. The series was homogeneous by stage and treatment and was enriched by prolonged and intensive clinical follow-up. With the number of patients available, the power of the statistical test should have detected any correlation of major significance. The results show that altered Fhit expression is the most frequent genetic change in lung tumors and is an independent biological marker. We did not observe a major impact of Fhit expression on prognosis, a finding similar to that reported for p53 abnormal expression in the same series (28). This is not surprising, because the inactivation of both genes is involved in the early phases of lung carcinogenesis and hence is more likely related to the initiation of the neoplastic process rather than to the progression to invasive tumor and distant metastasis.

Our observations of the loss of Fhit protein expression in a substantial percentage of primary NSCLCs and in precancerous lesions that were either adjacent or distant from the tumor suggest that FHit alterations play an important role in the growth control of bronchial cells. This is particularly true for squamous cell carcinoma, which is often associated with precursor dysplastic lesions of various types, where the progressive loss of Fhit protein was demonstrated to be an early event, likely leading to altered control of cell growth and/or apoptosis. The assessment of the mechanisms of Fhit action will permit the elucidation of specific pathways by which Fhit affects the tumorigenic process.

Loss of Fhit expression was significantly associated with tumors occurring in heavy smokers, a finding consistent with our previous observation of a higher rate of LOH at the FHIT locus in the lung tumors of smokers versus those of nonsmokers (33). This suggests that FHit plays a specific role as a sensor of smoke-related carcinogenic damage leading to the loss of FHit function. One has to take into account the different prevalence of female lung cancer in the United States and Northern Europe; nonetheless, the excess of male lung cancers is associated with smoking. In our data, the loss of Fhit expression among smoking females was very similar to that observed in males (65 versus 75%).

In conclusion, this study demonstrates in a large series of stage I NSCLCs, precancerous lesions, and bronchial tissues that loss of the
Cancer or cancers of the upper aerodigestive tract. Moreover, the FHIT gene product occurs at the highest frequency reported thus far for uppermost retained columnar cells of the incomplete metaplasia. D, p53-positive immunostaining in incomplete metaplasia associated with severe hyperproliferative layers showing Fhit-negative immunostaining display immunoreactive p53 nuclei. C, Fhit immunostaining in incomplete metaplasia associated with severe dysplasia adjacent to a squamous cell carcinoma. Note a weak Fhit immunostaining in the uppermost retained columnar cells of the incomplete metaplasia. D, p53-positive immunoreactivity is restricted to the severe dysplastic lesion.

FHIT gene product occurs at the highest frequency reported thus far for molecular markers in lung tumors, reaching a peak of ~90% in squamous cell carcinoma and preneoplastic lesions. The data show that an alteration of Fhit expression can occur at the earliest clinically detectable stages of the neoplastic process. Thus, immunocytochemical analysis of Fhit expression in tissue biopsy samples, sputum cytological specimens, or cells collected from brushing or bronchial lavages could be a valuable tool for early detection in screening programs as well as an indicator of response in cancer chemoprevention trials. This could be particularly true for high-risk patients such as those who have suffered a previous lung cancer or cancers of the upper aerodigestive tract. Moreover, the FHit gene could represent an eligible target for early gene therapy approaches.

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References

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