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. The analysis 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 and precancerous lesions supports the notion that FHIT alterations play an important role in the growth control of bronchial cells. FHIT inactivation is particularly important in squamous cell carcinomas that are often associated with precursor dysplastic 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. The involvement of specific tumor suppressor gene(s) in any 3p region has not been demonstrated. This Fhit protein expression was shown to be reduced in these tumor types to be correlated with deletions within the FHIT gene (9, 13–17). Structural changes and abnormal mRNA expression have been reported in cell lines and primary tumors of SCLC and NSCLC (18–20). In addition, Fhit protein expression was markedly reduced or absent in the 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). Inactivation of the suppressor gene p53 and overexpression of EGFR as well as more general 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.

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_N0M0) 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 as if they were smokers (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).

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with surgery alone. For the present analysis, the follow-up period was updated to August 1997, with a median observation time of 64 months overall and 102 months for surviving patients. The diagnosis of NSCLC was made by histological examination according to the WHO (30) and to the Armed Forces Institute of Pathology (31).

Immunohistochemistry. Staining was performed on paraffin sections with the use of a rabbit polyclonal anti-GST-Fhit antiserum (15, 22, 23). The specificity of this antisera was previously demonstrated by immunoblot and by the immunocytochemical detection of exogenous Fhit protein in transfected Cos and human tumor cell lines (15, 22). Additionaly, 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 shown to contain precancerous lesions of various grades by morphological examination 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% hydrogen peroxide in 5033

### 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, mucoepidermoid 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 nonsmokers (75 versus 39%; P < 0.0005; Table 2). Of note, tumors from patients who smoked for less than 20 years showed an intermediate rate of Fhit-negative immunostaining (50%). The correlation between lack of Fhit expression and smoking was also observed in the subset of adenocarcinoma patients (Table 2). These results indicate that FHT ALTERATIONS preferentially occur in the tumors of heavy smokers.

There was no difference in overall survival between the patients with Fhit-negative tumors and those with Fhit-positive tumors (P = 0.5; 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] at the final incidence of Fhit-negative immunostaining (50%).

#### 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...
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>All cases</th>
<th>Nonsmokers</th>
<th>Smokers</th>
<th>Adenocarcinoma only</th>
<th>Nonsmokers</th>
<th>Smokers</th>
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<tr>
<td>Positive</td>
<td>14</td>
<td>9</td>
<td>23</td>
<td>39</td>
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<tr>
<td>Negative</td>
<td>93</td>
<td>36</td>
<td>41</td>
<td>20</td>
<td>2%</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>234</td>
<td>114</td>
<td>182</td>
<td>91</td>
<td></td>
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</table>

Table 3 Correlation between Fhit and other markers in the tumor

<table>
<thead>
<tr>
<th>Fhit reactivity</th>
<th>Positive</th>
<th>Negative</th>
<th>Total</th>
<th>% Fhit negative</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>p53 Positive</td>
<td>46</td>
<td>166</td>
<td>212</td>
<td>78</td>
<td>0.018</td>
</tr>
<tr>
<td>Negative</td>
<td>81</td>
<td>177</td>
<td>258</td>
<td>69</td>
<td>0.67</td>
</tr>
<tr>
<td>EgFR Positive</td>
<td>53</td>
<td>177</td>
<td>230</td>
<td>77</td>
<td>0.047</td>
</tr>
<tr>
<td>Negative</td>
<td>74</td>
<td>163</td>
<td>237</td>
<td>69</td>
<td>0.67</td>
</tr>
<tr>
<td>Bcl2 Positive</td>
<td>15</td>
<td>62</td>
<td>77</td>
<td>81</td>
<td>0.67</td>
</tr>
<tr>
<td>Negative</td>
<td>110</td>
<td>270</td>
<td>380</td>
<td>71</td>
<td>0.67</td>
</tr>
</tbody>
</table>

The overall frequency of the tumors showing either Fhit-negative immunostaining or p53-positive immunoreactivity was 83%; thus, the use of these two markers could be a remarkably sensitive tool for lung cancer screening. An even higher sensitivity, i.e., 91%, could be achieved by adding EGFR immunostaining of the tumor samples.

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 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.

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Fig. 5. Fhit and Mib1 immunoreactivity in bronchial lesions occurring at the bronchial resection margin of a lung cancer patient. A. a progressive decrease in Fhit immunoreactivity starting from basal cell hyperplasia to moderate/severe dysplasia and questionable early invasive squamous carcinoma (left to right). B. a progressive increase in Mib1-positive nuclei in the same lesions.
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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