Fragile Histidine Triad Gene Inactivation in Lung Cancer
The European Early Lung Cancer Project

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Rationale: Fragile histidine triad (*FHIT*) is a tumor suppressor gene involved in the pathogenesis of lung cancer.

Objectives: The purpose of this study was to investigate the different molecular alterations leading to the inactivation of *FHIT* gene function and to validate their use as biomarkers of risk for progression of the disease in patients belonging to the multicentric European study for the Early detection of Lung Cancer (EUELC) who were resected for early-stage lung tumors.

Methods: FHIT immunostaining was performed on 305 tumor samples. The methylation status of *FHIT* promoter was assessed by nested methylation-specific polymerase chain reaction (MSP-PCR) in 232 tumor and 225 normal lung samples of which a subset of 187 patients had available normal/tumor DNA pairs. Loss of heterozygosity (LOH) at the *FHIT* locus was analyzed in 202 informative cases by D3S1300 and D3S1234 microsatellite markers.

Measurements and Main Results: Lost or reduced FHIT expression was found in 36.7 and 75.7% of the tumor samples, respectively. Methylation of the *FHIT* promoter was found in 36.7% of tumor and 32.7% of normal lung samples, whereas LOH was detected in 61.9% of the tumors. A strong association with complete loss of FHIT expression was present when methylation and LOH were analyzed together ($P = 0.0064$). Loss of FHIT protein expression was significantly more frequent in squamous cell carcinoma histotype ($P < 0.0001$) and in smokers ($P = 0.008$). *FHIT* methylation in normal lung was associated with an increased risk of progressive disease (OR, 2.27; $P = 0.0415$).

Conclusions: Our results indicate that different molecular mechanisms interplay to inactivate FHIT expression and support the proposition that *FHIT* methylation in normal lung tissue could represent a prognostic marker for progressive disease.

Keywords: lung cancer; *FHIT* gene; methylation; prognostic biomarker

Lung cancer is one of the most common malignancies in the world and represents the leading cause of cancer-related deaths in industrialized countries. The overall 5-year survival rate for lung cancer is less then 15% largely due to the late stage at

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

The loss of fragile histidine triad (FHIT) protein expression was frequently observed in primary non–small cell lung cancer. The FHIT gene function is inactivated by different biological mechanisms as promoter methylation and loss of heterozygosity at the FHIT locus.

What This Study Adds to the Field

The association between FHIT methylation in normal tissue and prognosis suggest that FHIT methylation in normal lung could represent a prognostic marker for early recurrence.

which most patients are diagnosed. Because survival of patients surgically resected for early-stage lung cancer is variable, the detection of additional prognostic parameters could be of importance to better predict the outcome of the disease and to offer the optimal therapeutic treatment to the patients.

Fragile histidine triad (*FHIT*) is a tumor suppressor gene that spans the FRAB3B common fragile site at chromosome 3p14.2 and is frequently altered in lung cancer (1). Restoration of FHIT expression in cell lines lacking endogenous protein restrains tumor formation *in vivo* and *in vitro* through induction of apoptosis and inhibition of cell cycle progression (2). Homozygous deletions and loss of heterozygosity (LOH) at the *FHIT* locus have been frequently reported in lung cancer cell lines and primary tumors (3–7); aberrant *FHIT* mRNA transcripts have been detected in 40 to 80% of tumor samples (8), whereas mutations are rare. Loss of FHIT protein expression is observed in almost 70% of primary tumors, mainly in those arising in smokers, and in 90% of preinvasive lesions (9). The promoter region around exon 1/intron 1 of the *FHIT* gene contains CpG islands that have been shown to be hypermethylated in lung cancer and methylation was associated with loss of *FHIT* mRNA and protein expression (10, 11). Promoter methylation is critical for regulation of FHIT expression insofar as treatment with 5-aza-2′-deoxycytidine restores the unmethylated status of the *FHIT* promoter and the reexpression of *FHIT* mRNA in epithelial cell lines (12).

To dissect the contribution of the different biological mechanisms leading to *FHIT* gene inactivation we looked at the association among molecular alterations (LOH and promoter methylation) and FHIT protein expression in a large series of early-stage lung tumors with prolonged follow-up collected in the multicentric European study for the Early detection of Lung Cancer. Hypermethylation of the *FHIT* promoter was also evaluated in normal lung of these patients, and the association between *FHIT* alterations in tumors and normal lung and the

clinical outcome of the patients was studied in an attempt to provide a molecular tool useful for identifying individuals at higher risk of relapse or second primary tumors. Some of the results of these studies have been previously reported in the form of an abstract (13).

METHODS

Study Population

A cohort of individuals with a diagnosis of early stage (I–II) lung cancer and who underwent surgical resection was included in the European study for the Early detection of Lung Cancer (EUELC). The patients were recruited before surgery in 12 cancer centers throughout Europe from 2002 to 2006 and were subsequently followed-up every 6 months for the duration of this study. Inclusion criteria are detailed in the online supplement. Tissue and biological specimens collected were sent from all the recruiting centers to the centralized European Bronchial Tissue Bank (EBTB) located in Liverpool, UK. A web-based database was set up (MACRO) to hold all clinical, epidemiological and follow-up data, as well as information on tissue and biological specimens collected in the study.

From the EUELC cohort of 913 patients, 359 cases were selected for this analysis on the basis of the availability of material for molecular analyses and clinical information for an average follow-up period of 18 months. To assess the molecular risk factors for disease progression, the patients were classified in two groups: those with early relapse, recurrence, metastasis, or second primary lung cancer (with a disease-free period of 6 mo after surgery) were defined as progressive disease group (PD); patients that remained tumor-free during the study were defined as disease-free group (DF). The characteristics of study population are summarized in Table 1.

Tissues Collection and DNA Extraction

All tissues were freshly collected during surgery and stored at −80°C in the EBTB bank. As a routine practice for TNM staging nontumor lung specimens are sampled at a distance from the tumor to guarantee that the tissues are free from cancerous cells, atelectasis, and obstructive pneumonia (14).

Frozen normal and tumor tissues were cut into 20 × 4 μm sections. Tumor sections were stained with H&E and microscopically reviewed for tumor cell content. Excessive normal tissue was removed to ensure 80% or more tumor cell content.

For LOH analysis, normal control DNA was extracted from peripheral blood lymphocytes. DNA extraction from tissue samples

TABLE 1. CHARACTERISTICS OF STUDY POPULATION

Patient Characteristic	First Set (n = 176)	Final Set (n = 359)
Sex		
Male	155 (88.1)	301 (83.8)
Female	21 (11.9)	58 (16.2)
Age, yr	63.8 ± 9.2	63.9 ± 9.1
Smoking history		
Never-smoker	9 (5.1)	16 (4.5)
Current and former-smoker	158 (89.8)	332 (92.5)
Missing	9 (5.1)	11 (3)
Histology		
Squamous carcinoma	84 (47.8)	165 (46)
Adenocarcinoma	90 (51.1)	183 (51)
Other	11 (3)	2 (1.1)
Stage		
Stage I	118 (67)	232 (64.6)
Stage II or higher	55 (31.3)	121 (33.7)
Missing	3 (1.7)	6 (1.7)
Event		
DF	90 (51.1)	241 (67.1)
PD	86 (48.9)	118 (32.9)

Definition of abbreviations: DF = disease-free group; PD = progressive disease group.

Values are n (%) or mean ± SD.

and lymphocytes was performed using DNeasy 96 Blood and Tissue kit (Qiagen, Studio City, CA) following supplier's protocol.

Immunohistochemistry

Immunohistochemical staining was performed on formalin fixed paraffin–embedded biopsies with the rabbit anti-FHIT antibody (18–0219, Zymed Laboratories, South San Francisco, CA) at a dilution of 1:100 on Ventana automated immunostainer (Ventana, Tucson, AZ). For antigen retrieval, 5 minutes of microwave heating in ethylenediaminetetraacetic acid buffer was repeated twice. FHIT immunoreactivity was classified according to a score obtained by multiplying two parameters: the percentage of positive cells (from 0 to 100) by the intensity mean level (from 0 to 3) for a global score of 0 to 300. Normal bronchial and alveolar epithelial cells, used as internal controls, showed a strong FHIT expression score of 200 (score 3) or greater. An extensive loss of FHIT expression immunostaining was considered for tumors with scores of 50 (score 1) or less, and reduced protein expression was considered for scores of 50 to 200 (score 2). For the purpose of statistical comparisons, a score of 0 versus 1–3 or a score of 0–1 versus 2–3, was used to obtain homogeneity of the classes and to achieve a meaningful biological assessment.

Bisulphite Conversion and Methylation-specific Polymerase Chain Reaction

The methylation status of the promoter region of the *FHIT* gene was evaluated by methylation-specific polymerase chain reaction (MSP-PCR) after DNA modification with sodium bisulphite (EZ DNA Methylation Gold Kit, Zymo Research, Orange, CA) (15). MSP analysis for the *FHIT* gene investigates the genomic areas that represent the best targets for functionally relevant methylation identified into intron 1 (*see* online supplement for further details on METHODS).

LOH Analysis

Allelic LOH analysis at the *FHIT* locus on 3p14.2 was performed by studying the combination of two microsatellite alterations, D3S1300 and D3S1234. Thirty nanograms of DNA from tumor tissue and matching lymphocytes were used for the analysis, as previously reported (16, 17).

Statistical Analysis

Fisher exact test was performed to test independence of variables. Distributions of demographic variables (including sex and smoking status) between PD cases and DF controls were evaluated by chi-square test. Differences between PD cases and DF controls in age and self-reported pack-years were tested using the Student's t test. When the data distribution significantly deviated from normal, the Wilcoxon rank sum test was performed. Odds ratios (OR) and 95% confidence interval (CI) were calculated as estimates of relative risk. Odds ratios associated with each risk factor were calculated using the conditional logistic regression model while adjusting for confounders. A test for trends was performed by coding the categories in successive integers and using the likelihood ratio test statistics with one degree of freedom. Statistical analyses were performed with the statistical analysis package SAS for Windows, version 8.02 (SAS Institute, Cary, NC).

Risk estimation for disease progression according to different variables was initially performed on a set of patients with a 1:1 ratio of PD:DF (n = 176, first set). To achieve optimal matching in assessing epidemiological and molecular risk factors for disease progression in the final dataset (n = 359), the conditional logistic regression for matching design was used. PD cases and DF controls were matched with a 1:2 ratio using the SAS language procedure (*see* online supplement). Patients were matched on follow-up times (at least as long as the event time for PD subjects) and also on center, sex, age (±6 y), histological subtypes, and N stage.

RESULTS

Biological Mechanisms of Inactivation of *FHIT* Gene and Their Correlation with FHIT Protein Expression

From the series of 359 cases originally selected for biomarker analysis the number of samples analyzed in each *FHIT* assay

was limited to subsets on the basis of specimen type availability in the EBTB bank. Thus, immunohistochemistry (IHC) analysis of FHIT protein expression was performed in 305 cases with formalin-fixed, paraffin-embedded (FFPE) tissue, DNA for methylation analysis was available from 232 tumor and 225 normal lung tissues, while LOH analysis was performed in a subset of 228 tumor-normal pairs.

FHIT protein expression. Immunostaining was performed on sections from 305 paraffin embedded tumor samples (Figure 1). Reduced protein expression (a score of 0–2) was found in the majority (75.7%) of the tumor samples, whereas extensive loss of FHIT expression (a score of 0–1) was also detected in a significant subset (36.7%). Frequencies for each score of FHIT expression are reported in Table 2. Loss of FHIT protein expression (a score of 0–1) was significantly higher ($P < 0.0001$) in squamous cell carcinoma (82/137, 59.9%) than in adenocarcinoma (30/168, 17.9%), whereas expression was not associated with pathological tumor-node-metastasis (pTNM) stage. A significant association was observed between expression and smoking habits: loss of FHIT expression was significantly greater ($P = 0.008$) in former and current smokers (221/287, 77%) than in never-smokers (6/14, 42.9%).

FHIT promoter methylation in tumor and normal tissues. PCR amplification was successful, as assessed by the presence of a product in the "unmethylated" reaction, in 229/232 tumor tissues and in 208/225 normal tissues. MSP demonstrated the presence of methylated *FHIT* alleles in 36.7% (84/229) tumor tissues and in 32.7% (68/208) of the normal adjacent tissues examined, indicating a marginal but not significant increase of *FHIT* methylation frequency in tumor tissue ($P = 0.0749$). In the subset of 187 patients with paired normal and tumor tissue successfully analyzed we observed methylation in both tissues in 15% (28/187) of the pairs, whereas 46% (86/187) of the pairs presented only unmethylated alleles (Figure 2). Although 21% (39/187) of the pairs demonstrated *FHIT* methylation only in tumor DNA, perhaps the most intriguing group was the remaining 18% (34/187) that included cases with methylation restricted to normal lung samples. Among the cases with methylation in normal tissue 45.2% (28/62) showed methylation also in the corresponding tumor sample, whereas the remaining 54.8% (34/62) was unmethylated in the matching tumor DNA (Table 3). No significant association was observed between *FHIT* methylation in tumor and normal samples or between *FHIT* methylation status in normal and tumor samples and clinical-pathological variables like smoking habits, tumor stage, and histology.

Genomic instability of FHIT locus. LOH at the *FHIT* locus at 3p14.2 was analyzed in 228 normal-tumor paired DNAs combining two microsatellite markers: D3S1300 (intron 5) and D3S1234 (intron 7). The analysis revealed LOH at D3S1234 in 59% (92/156) of the informative (heterozygous) cases, while for D3S1300 56.2% (59/105) of the informative cases showed with LOH. The combined analysis of the two microsatellites, D3S1300 and D3S1234, resulted in 202 informative cases of which 61.9% (125/202) showed LOH at the *FHIT* locus for at least one microsatellite. As expected, there was a high frequency of LOH coincidence between these two markers. In particular, 71.2% (42/59) of "double informative samples" demonstrated LOH at both loci (Fisher's exact test, $P = 0.001$), which is in accordance with previous observations that deletions affecting the *FHIT* locus are usually large and span the entire gene.

FHIT methylation, FHIT LOH, and loss of expression by immunostaining. To investigate the relationship between FHIT expression with the potential underlying genetic (LOH at *FHIT* locus) and epigenetic (promoter methylation) determinants we comparatively analyzed data for these parameters. *FHIT* methylation and LOH resulted as independent events ($P = 1.0000$), confirming the notion that these two types of alterations represent alternative molecular mechanisms of *FHIT* gene inactivation.

A borderline association ($P = 0.0577$) was observed between *FHIT* methylation and complete loss of FHIT protein expression (a score of 0). Among the methylation-negative samples, however, 31 of 135 (23%) samples gave negative immunostaining, suggesting that mechanisms of gene silencing other than methylation should coexist.

LOH was significantly ($P = 0.0182$) associated with loss of FHIT expression (a score of 0), further supporting the relevant role of this type of molecular event in inactivating *FHIT* function.

The occurrence of both methylation and/or LOH were highly associated with loss of FHIT protein expression ($P = 0.0064$) indicating that genetic and epigenetic events concur to functionally impair FHIT expression (Table 4).

FHIT Status and Risk for Disease Progression

The association between FHIT alterations and the risk of progressive disease was assessed considering the clinical outcome of patients with a disease-free period of at least 6 months after surgery. Patients were classified in two groups: those with early relapse, recurrence, metastasis, or second primary lung cancer (with a disease-free period of 6 mo after surgery) were defined as progressive disease group (PD); patients that remained tumor-free during the study were defined disease-free group (DF). We performed a preliminary analysis in a first set of 176 patients and subsequently extended the analysis to a larger dataset of 359 patients. Only 28 patients of the first group were not included in the final dataset.

Progressive disease risk in the first set of patients. In the first set of 176 patients, PD and DF groups were similarly represented,
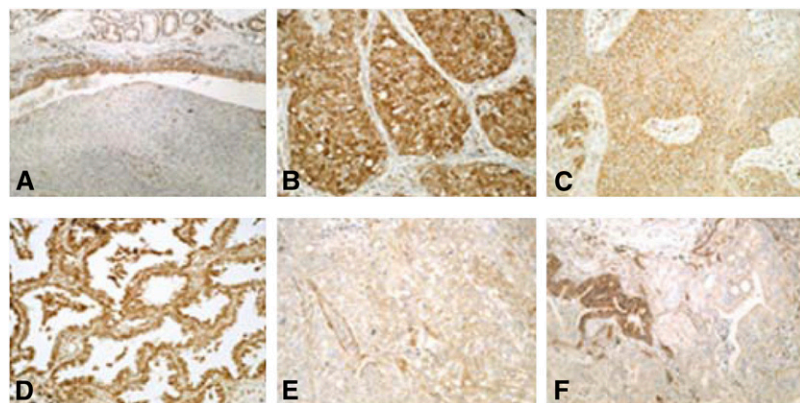

Figure 1. Fragile histidine triad (FHIT) immunostaining in lung tumor tissue. Representative immunohistochemical analysis at magnification 20×. (*A* and *F*) Loss of FHIT expression in an adenocarcinoma contrasting with the high expression of FHIT in bronchial epithelial cells. (*B, C,* and *E*) Strong, medium, and low FHIT expression in a squamous cell carcinoma, respectively. (*D*) Strong FHIT expression in the bronchioloalveolar component of an adenocarcinoma.

TABLE 2. FHIT PROTEIN EXPRESSION

Expression Score	Tumors (n = 305)	Histotype		P Value	Smoking Habits		P Value
		SCC (n = 137)	ADC and Other (n = 168)		Never (n = 14)	Current or Former (n = 287)	
0	80 (26.2)	82 (59.9)	30 (17.9)		6 (42.9)	221 (77)	
1	32 (10.5)			P < 0.0001			P = 0.008
2	119 (39.0)	55 (40.1)	138 (82.1)				
3	74 (24.3)				8 (57.1)	66 (23)	

Definition of abbreviations: ADC = adenocarcinoma; SCC = squamous cell carcinoma.

with 86 (48.9%) PD (cases) and 90 (51.1%) DF (controls) patients. All characteristics of this set of individuals are reported in Table 1. Statistical analysis for assessing the risk of progressive disease was performed according to epidemiological and clinical characteristics and molecular biological markers. No association was detected between *FHIT* methylation, LOH, or loss of protein expression in tumors and prognosis.

We found a strong association between FHIT methylation status in normal tissue, evaluated in 116 cases, and the progression of disease (OR, 4.30; *P* = 0.01). Moreover, the association remained significant after adjustment for smoking duration, life condition, and primary tumor size (OR, 7.39; *P* = 0.02).

Progressive disease risk in the final set of patients. The analyses were then expanded to a larger dataset of 359 patients having similar epidemiological and clinical characteristics. A conditional logistic regression using matching design was performed for the analysis of risk factors for progressive disease. Each PD case was matched with two DF controls, as described in METHODS. The matching was feasible for only 296 patients, where PD and DF patients represented 31.8 and 68.2% of the entire dataset, respectively. The distribution of clinical-demographic features was homogenous in PD and DF groups with the exception of the tumor stage. In the DF group 74.5% of tumors were stage I versus 47.4% in the PD group.

FHIT methylation in normal lung, evaluated in this cohort of 168 patients (Table 3), was significantly associated with an increased risk of PD (OR, 2.27; *P* = 0.0415), and the association remained significant after adjustment for tumor size (OR, 2.28; *P* = 0.0435). Analysis according to histological subtypes showed that *FHIT* methylation in normal lung was especially associated with an increased risk of having disease progression in patients with adenocarcinoma (OR, 3.54; *P* = 0.0309).
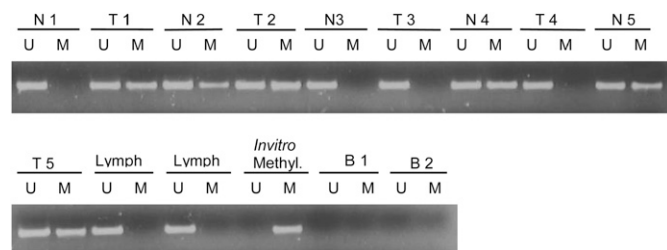

Figure 2. Methylation-specific polymerase chain reaction (MSP-PCR) for fragile histidine triad gene (*FHIT*) of normal/ tumor DNA pairs. Normal (N) and tumor (T) DNA samples were analyzed by MSP. Bisulphite-treated DNA extracted from normal lymphocytes (Linf) was used as positive control of unmethylated-DNA specific primers (U) and commercial *in vitro* methylated DNA after conversion as positive control of methylation-specific primers (M). Negative control samples without DNA were included for each PCR step (B1–B2).

Matching design analysis of this larger dataset did not indicate a significant association between FHIT status in tumor tissue and prognosis.

DISCUSSION

The overall goal of the EUELC project was to detect molecular-pathological abnormalities in early-stage primary lung tumors that would be useful for the identification of individuals at risk of developing second primary lung cancers, metastasis, and recurrences.

EUELC was one of the largest planned early lung cancer projects in Europe, and it enabled the Partners to establish protocols for assessing molecular biomarkers in early lung cancer with the view that such biomarkers maybe used in future early detection programs in Europe.

The purpose of the present study was to investigate the different types of molecular alterations leading to the inactivation of *FHIT* gene function and to validate their use as biomarkers of risk for progression of the disease in high-risk individuals.

Several studies have reported the central role of the *FHIT* gene in lung tumorigenesis, and loss of protein expression has been described in several human neoplasms of epithelial origin including lung cancer (9, 18–20). Because of the variety and complexity of the molecular and epigenetic changes affecting the *FHIT* gene, a loss or reduced FHIT protein expression, as detected by immunohistochemistry, was suggested as the most reliable test for detecting *FHIT* gene alterations. Previous studies already showed that epigenetic events could impair *FHIT* gene function in lung tumors. *FHIT* intron 1 CpG islands were found methylated in 38% of lung squamous cell carcinomas and in 57% of breast cancer DNAs and also in nonneoplastic adjacent mammary tissues (14%) and lung tissues (8%) (21). In lung squamous cell carcinomas and breast tumors, an association between methylation of *FHIT* promoter and protein expression was reported, confirming a previous study that showed a significant association between *FHIT* methylation status and loss of *FHIT* expression by Northern Blot and immunostaining in lung and breast cancer cell lines and primary tumors (10). In a small study of 30 locally advanced (stage III) lung carcinomas, no association was observed between *FHIT* LOH and loss of *FHIT* mRNA and protein expression, whereas methylation of the *FHIT* promoter correlated significantly with loss of FHIT expression at the transcript level (22).

However, previous studies have not addressed how epigenetic and genetic mechanisms interplay to inhibit FHIT protein expression and inactivate *FHIT* gene function, nor have they examined their association with prognosis using a large sample of lung cancer patients.

To dissect the molecular mechanisms leading to *FHIT* gene inactivation we investigated the relationship among the various alterations of the *FHIT* gene: allelic losses, promoter methylation, and FHIT protein expression. The high frequency of *FHIT* genetic, epigenetic, and protein alterations detected in this large

TABLE 3. METHYLATION IN NORMAL LUNG TISSUE

	Methylation Status			*FHIT* Risk for Disease Progression[‡] (*n* = 168)			
	Normal Tissue (*n* = 208)*	Tumor Tissue[†] (*n* = 187)		DF (%)	PD (%)	OR (95% CI)	*P* Value
		Unmethylated	Methylated				
Unmethylated	140 (67.3)	86/125 (68.8)	39/125 (31.2)				
Methylated	68 (32.7)	34/62 (54.8)	28/62 (45.2)	27.5	40.7	2.27 (1.03–5.00)	0.0415

Definition of abbreviations: CI = confidence interval; DF = disease-free group; FHIT = fragile histidine triad; OR = odds ratio; PD = progressive disease group
Values are n (%) unless otherwise indicated.
* Only successfully amplified samples in methylation-specific assay.
[†] Patients with paired normal and tumor tissue successfully analyzed.
[‡] DF are patients that remained tumor free during the study and PD are patients with progression of disease.

sample of early-stage lung tumors further supports a primary role of *FHIT* in lung carcinogenesis. Moreover, loss of protein expression was distinctive of smoke-related tumors and particularly related to squamous cell carcinoma.

A strong relationship among the combination of LOH and methylation with FHIT protein expression was detected in this sample. This association indicates that the mechanism of *FHIT* gene inactivation consists of both deletions and methylation events that contribute to the functional loss of expression. The lack of statistical association between LOH and methylation suggests that these two events independently affect FHIT expression.

So far no studies have analyzed the effects of *FHIT* promoter hypermethylation in normal lung as a prognostic factor, whereas cohypermethylation of *p16* and *FHIT* promoters in tumor tissue in 335 stage I NSCLC was associated with an increased risk of recurrence and poor recurrence-free survival after surgery (23).

We observed a similar frequency of methylation in normal lung and tumor tissue; however, 54.8% of patients with methylated *FHIT* in normal lung tissue lacked methylation in the corresponding tumor. This is not surprising because evidence supports, at a molecular level, an independent origin of multiple preoplastic and neoplastic lung lesions (24, 25).

The high frequency of *FHIT* methylation detected in normal lung tissue of the patients in our study suggests that this type of *FHIT* alteration is an early event in lung carcinogenesis that likely reflects an early smoke-induced epigenetic damage. Moreover, the finding that *FHIT* methylation in normal lung was associated with an increased risk of progressive disease strongly suggests that the continuous exposure to tobacco smoke creates a "permissive" environment of damaged tissue ("field cancerization" effect) and that additional genetic damage could trigger the transformation process.

Recent data also indicate that extensive DNA damage, manifested through double strand-breaks, could in part be responsible for the acquisition of aberrant gene promoter methylation during lung carcinogenesis. In particular a diminished DNA repair capacity was found to be associated with an increased

TABLE 4. ASSOCIATION AMONG *FHIT* METHYLATION, *FHIT* LOH AND COMPLETE LOSS OF FHIT PROTEIN EXPRESSION IN LUNG TUMORS

FHIT Alteration	n	Percentage in FHIT Negative Tumors* (n/n)	*P* Value[†]
Methylation	197	42.6 (23/54)	0.0577
LOH (D3S1234-D3S1300)	186	76.9 (40/52)	0.0182
Methylation and/or LOH	190	90.9 (50/55)	0.0064

Definition of abbreviations: FHIT = fragile histidine triad; LOH = loss of heterozygosity.
* Score of 0.
[†] Fisher's exact test.

methylation index in sputum from persons at risk for lung cancer (26), supporting the hypothesis that methylation in normal lung might also reflect inability to repair DNA breaks induced by tobacco injury and therefore be linked to increased cancer risk.

Our finding is in agreement with previous reports showing that the ability to identify extensive or specific patterns of genetic changes in normal and preoplastic tissues or sputum samples, may provide new methods for assessing the risk in smokers of developing invasive primary or recurrent lung cancer (27, 28).

Currently the outcome of patients that undergo resection for stage I-II lung cancer is variable and influenced by many factors. The identification of a biological marker linked to prognosis that is assayable in surgical specimens could therefore provide valuable information regarding the risk of early recurrence and help in the clinical management of those early lung cancer patients that might require more intensive follow-up or further treatment.

Recently, gene-expression profiling studies in noncancerous hepatic tissue, and subsequently in noncancerous lung tissue of patients with early-stage adenocarcinoma, have reported a cytokine gene expression signature associated with a poor prognosis. These findings showed how noncancerous tissues could be suitable specimens to identify prognostic markers for patients who are at high risk of recurrence or metastasis (29, 30).

Thus, the final analysis on the complete dataset confirmed the association between *FHIT* methylation in normal tissue and early recurrence that was already detected in a preliminary analysis on a smaller set. A statistical trend, although not significant because of the limited number of events (n = 23), was observed between methylation status in normal lung, particularly in patients with adenocarcinoma, and development of secondary primary tumors of the lung during follow-up of the patients.

These results, if validated in larger studies, support the proposition that *FHIT* methylation in normal lung tissue could represent a prognostic marker for progressive disease, and particularly for assessment of the risk of early recurrence after surgery.

In conclusion, this multicentric effort of establishing a centralized European lung tissue bank has facilitated the collection and sharing of large series of biological specimens and could prove to be a useful approach to reliably study the role of a number of different biomarkers with relevance for lung cancer prognosis or early detection.

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