Prognostic Relevance of Fragile Histidine Triad Protein Expression in Patients with Small Cell Lung Cancer

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ABSTRACT

Purpose: The fragile histidine triad protein (FHIT) is a putative tumor suppressor in patients with lung cancer. In this study, we examined the prognostic value of FHIT expression for survival in patients with small cell lung cancer (SCLC).

Experimental Design: As assessed by immunohistochemistry using formalin-fixed, paraffin-embedded tissue sections, tumors of 225 patients with SCLC were retrospectively evaluated for FHIT expression. The influence of FHIT staining intensities as well as the proportion of FHIT-positive cells within a tumor was taken into consideration for univariate and multivariate survival analysis.

Results: FHIT expression was observed in 61.8% of the SCLC tumors. Lack of FHIT was significantly associated with a shorter survival time for the patients with a median of 157 ± 18 days compared with 210 ± 18 days for those patients with FHIT-positive tumors (P = 0.0061). Furthermore, the proportion of FHIT-positive cells within the tumor was related to survival. Patients with tumors of <25% FHIT-positive cells had the worst survival of 155 ± 21 days compared with 217 ± 19 days for patients with a proportion of ≥25% of FHIT-expressing tumor cells (P = 0.0016). In contrast to the proportion of FHIT-positive cells within the tumor, no significant difference in survival was observed when different FHIT staining intensities (weak versus strong) were considered (median survival of 208 ± 17 versus 234 ± 34 days, P = 0.665). Multivariate analysis using Cox regression including 11 variables confirmed the prognostic significance of FHIT expression next to performance status, tumor stage, and lactate dehydrogenase.

Conclusion: The presence of FHIT was correlated with a better prognosis for patients with SCLC.

INTRODUCTION

Recent advances in cancer research resulted in a better understanding of the pathophysiology of small cell lung cancers (SCLC). Molecular analysis has shown that between 90% and 100% of SCLC and between 50% and 80% of non–small cell lung cancer (NSCLC) tumors have loss of heterozygosity on chromosome arm 3p (1, 2). At least three distinct regions of loss of heterozygosity (3p25, 3p21.3, and 3p14) have been reported. The fragile histidine triad (FHIT) gene, located on 3p14.2, has been proposed to be involved in the development of lung cancers. The 16.8-kDa FHIT protein consists of 146 amino acids and is a human orthologue of the fission yeast Schizosaccharomyces pombe protein, a diadenosine 5′,5′-P₂,P₄-tetraphosphate hydrolase (3). Whereas the exact molecular pathway of FHIT signaling is unknown, the FHIT substrate complex seems to act as a signaling molecule (4) involved in the regulation of p53-independent apoptosis (5) and cell cycle control (6). Restoration of FHIT expression induced apoptosis and suppressed tumorigenicity of lung cancer cells in vitro (7) and in vivo (8). Within primary NSCLC tumors, immunohistochemical analysis of FHIT expression revealed greater frequency of FHIT loss in squamous cell carcinomas in comparison with adenocarcinomas. In contrast to NSCLC, only 19 primary SCLCs and 28 SCLC cell lines were systematically examined for FHIT expression. Between 30% and 50% of primary SCLC tumors and 20% and 47% of SCLC cell lines were FHIT positive (9–11). Because of the relatively small number of patients examined, there are no data with regard to the relationship between FHIT expression and patient survival. In this study, we examined the prognostic value of FHIT expression for survival in 225 patients with SCLC. The influence of FHIT staining intensities as well as the proportion of FHIT-positive cells within a tumor was taken into consideration for univariate and multivariate survival analysis.

PATIENTS AND METHODS

Patients with SCLC. Formalin-fixed, paraffin-embedded tissue sections from 225 patients with primary SCLC were used for immunohistochemical examination of FHIT. The specimens were obtained from the Institute of Pathology of the University of Düsseldorf. Biopsies from the primary lung tumor were taken at initial clinical presentation before treatment. The original diagnosis of SCLC was confirmed by two different experienced pathologists (W.M. and H.G.) before the biopsy was accepted for...
this study. The histopathologic diagnosis was based on H&E stains as well as immunohistochemical stains (cytokeratin and chromogranin A). The patients enrolled in the study were patients from the University of Duesseldorf and associated academic hospitals of the University of Duesseldorf between 1983 and 2003. Clinical data of the patients were collected from chart review with given approval from the ethics committee of the University of Duesseldorf. The baseline characteristics of the patients are presented in Table 1. The survival time in days was calculated from the date of histopathologic diagnosis and the performance status of the patient was evaluated by WHO criteria [graded from fully active (grade 0) to completely disabled (grade IV)]. The grouping of the tumor-node-metastasis (TNM) subsets (T, primary tumor; N, regional lymph nodes; M, distant metastasis) into the SCLC stages I to IV was based on the revised version of the International System for Staging Lung Cancer adopted by the American Joint Committee on Cancer and the Union Internationale Contre le Cancer (12). Chemotherapy was done as a first-line treatment with an average of four chemotherapy cycles (median, 5.0±2.8 cycles; range, 1-12) in 87.3% of evaluable SCLC patients. Patients were uniformly treated using a combination of etoposide 120 mg/m² d1, Adriamycin 45 mg/m² d1, or epirubicin 65 mg/m² d1 and cyclophosphamide 1000 mg/m² d1 (every 3 weeks), which was the preferred chemotherapeutic regimen (62.4%). A platinum-based combination (either cisplatin 90 mg/m² d1 or carboplatin 300 mg/m² d1) with etoposide (150 mg/m² d1-3; every 3 weeks) was given in 15.2%, whereas other combinations were given in 9.7% of patients.

**Immunohistochemistry.** As previously described by Ramp et al. (13), a standardized immunohistochemical staining procedure was done for FHIT detection using a polyclonal antibody raised against full-length human FHIT (Zymed, San Francisco, CA). Briefly, fresh tumor tissue specimens were immediately formalin-fixed following bronchoscopy or computer-tomographic–supported thoracal puncture. Samples were paraffin-embedded, cut in 2- to 4-μm sections on poly-L-lysine–coated slides. Following deparaffinization with xylene (15 minutes), sections were rehydrated through decreasing concentrations of ethanol (100%, 96%, and 70%) and washed for 5 minutes. For antigen retrieval, the specimens were heated in citrate buffer (pH 6) using a pressure cooker for 15 minutes. Endogenous peroxidase activity was eliminated by incubation with 3% hydrogen peroxide for 15 minutes and unspecific binding of biotin and avidin was blocked using a blocking solution (Dako, Hamburg, Germany) for 15 minutes on each slide. After intensive washing for 5 minutes, slides were incubated with 1% bovine serum albumin (Sigma, St. Louis, MO) to block unspecific binding of the primary antibody. The anti-FHIT antibody (Zymed, San Francisco, CA) was diluted 1:200 to a final concentration of 1.25 μg/mL and slides were incubated with anti-FHIT antibody for 1 hour. After washing, a streptavidin horseradish peroxidase detection kit (Dako) containing 3,3’-diaminobenzidine solution as substrate was used for immunohistochemical staining according to the manufacturer's instructions. Appropriate positive controls (kidney) were additionally evaluated in each run. All slides were simultaneously assessed by two investigators (N.R. and L.P.) using a double-headed discussion microscope. In a final discussion round all slides were reviewed and the results were confirmed by a third investigator (H.G.). The staining intensities for FHIT were evaluated semiquantitatively and classified into three groups. The first group showed equal or stronger cytoplasmic staining intensity compared with the positive control. The second group showed weaker staining intensity of FHIT compared with the positive control. The third group showed no immunohistochemical evidence of FHIT expression. Generally, the staining intensity and the proportion of FHIT-positive cells was uniform within the slide. For those cases with a difference in staining intensity, the strongest intensity was chosen for the assessment of FHIT. Furthermore, to evaluate the prognostic influence of the proportion of FHIT-positive cells in contrast to their expression level, tumors were divided into five categories: none of the tumor cells expressed FHIT, FHIT positive tumor cell number between 1% and 25%, >25% and 50%, >50% and 75%, and >75%. The percentage of positive cells was estimated in agreement with all three observers. For the estimation, all tumor areas of one to four biopsies from bronchoscopy were included.

**Statistical Methods.** The Kaplan-Meier survival analysis with log rank test was done to compare clinical parameters with FHIT expression. Two sided _t_ test was done to compare clinical variables of FHIT-positive and -negative groups. For multivariate analysis, a Cox regression model with a forward stepwise selection was used. Pearson bivariant correlation analysis was done to evaluate a correlation between FHIT expression or proportion of FHIT-expressing cells to clinical parameters. Statistical analysis was done using SPSS software 12.0. Significance is defined as _P_ < 0.05 and the respective values are given in the text. Immunohistochemical stains were done without prior knowledge of clinical parameters.

### Table 1. Baseline characteristics of patients with SCLC (N = 225)

<table>
<thead>
<tr>
<th>Patients (%)</th>
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<tr>
<td>Sex</td>
</tr>
<tr>
<td>Male</td>
</tr>
<tr>
<td>Female</td>
</tr>
<tr>
<td>Smoking status</td>
</tr>
<tr>
<td>Smoker/former smoker</td>
</tr>
<tr>
<td>Nonsmoker</td>
</tr>
<tr>
<td>Not evaluable</td>
</tr>
<tr>
<td>WHO performance status</td>
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<tr>
<td>0</td>
</tr>
<tr>
<td>I</td>
</tr>
<tr>
<td>II</td>
</tr>
<tr>
<td>III</td>
</tr>
<tr>
<td>Not evaluable</td>
</tr>
<tr>
<td>Stage</td>
</tr>
<tr>
<td>Ia, b</td>
</tr>
<tr>
<td>Ila, b</td>
</tr>
<tr>
<td>Ila, b</td>
</tr>
<tr>
<td>IV</td>
</tr>
<tr>
<td>Not evaluable</td>
</tr>
<tr>
<td>LDH (units/L)</td>
</tr>
<tr>
<td>Hemoglobin (g/dL)</td>
</tr>
<tr>
<td>Thrombocytes (μL)</td>
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<tr>
<td>Leukocytes (μL)</td>
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</table>

*Mean ± SD.
RESULTS

Immunostaining of FHIT in SCLCs. FHIT expression was observed in 139 (61.8%) of 225 examined tumors, whereas a weak and strong cytoplasmic staining intensity was found in 71 (51.1%) and 68 (48.9%) of 139 FHIT-positive SCLCs, respectively. No FHIT expression was seen in 86 (38.2%) cases (Fig. 1).

Because an inhomogeneous staining pattern was observed in 21% of the SCLCs, the proportion of positive tumor cells was assessed for each sample of 225 SCLC tumors. Proportions of FHIT-positive cells between 1% and 25%, between >25% and 50%, between >50% and 75%, and >75% were observed in 6 (2.7%), 12 (5.3%), 30 (13.3%), and 91 (40.5%) of all patients with SCLC, respectively, whereas 86 cases (38.2%) were FHIT negative (Fig. 2). For SCLC cases with a heterogeneous staining pattern no biopsy-to-biopsy variation concerning the absolute percentage of cells staining positive for FHIT was observed.

Prognostic Value of FHIT Expression Using Kaplan-Meier Survival Curves. In an attempt to evaluate the prognostic value of FHIT expression in patients with SCLC Kaplan-Meier survival curves were chosen for survival analysis. Median and mean survival rates for all 225 patients were 194 ± 16 and 321 ± 43 days; 1- and 5-year survival rates were 23.9% and 2.6%, respectively. To evaluate the impact of different FHIT staining intensities on survival, the survival curves of the three groups showing no, weak, or strong FHIT staining intensities were compared.

A complete lack of FHIT expression was associated with worst prognosis compared with patients with FHIT expression independently from staining intensity (0.012 < P < 0.033). No significant differences were observed between the survival curves of the patients with weak and strong FHIT staining (median survival, 208 ± 17 versus 234 ± 34 days; P = 0.665), respectively. Therefore, positive stained cases were grouped together for further analysis. Patients with lack of FHIT-expressing tumors had a significant (P = 0.0061) shorter median survival of 157 ± 18 days compared with 210 ± 18 days for those patients with FHIT-positive tumors.

No differences between FHIT-negative and FHIT-positive patient group was observed with respect to gender (male versus female, P = 0.563), age (mean, 63.3 versus 62.2 years; P = 0.447) smoking status (nonsmokers versus smokers, P = 0.605), performance status (WHO 0 versus I versus II versus III, P = 0.809), tumor stage (I versus II versus III versus IV, P = 0.441), mean cycles of chemotherapy (3.51 ± 3.06 versus 4.35 ± 3.1 cycles, P = 0.11), mean hemoglobin level (13.47 ± 1.8 versus 13.72 ±
1.68 g/dL, \( P = 0.383 \)), mean platelet count \( (319,770 \pm 115,450 \text{ vs } 323,540 \pm 115,630 \text{ cells/µL, } P = 0.812 \)), mean leukocyte count \( (9,490 \pm 3,360 \text{ vs } 9,690 \pm 3,510 \text{ cells/µL, } P = 0.696 \)) and mean lactate dehydrogenase (LDH) level \( (394 \pm 329 \text{ vs } 365 \pm 346 \text{ units/L, } P = 0.595 \)).

Next, a possible influence of the proportion of FHIT-positive tumor cells was examined. Patients with 1% to 25% FHIT-expressing tumor cells had a similar poor prognosis as those patients with a complete lack of FHIT with a median survival of 87 ± 80 and 157 ± 18 days \( (P = 0.165) \), respectively. In contrast, median survival of the patients was improved when more than 25% of the tumor cells were FHIT positive. Similar median survival of 203 ± 40, 210 ± 52, and 217 ± 25 days was found for those tumors showing a FHIT-positive cell proportion of >25 to 50%, >50 to 75%, and >75% \( (0.644 < P < 0.934) \), respectively (Fig. 3A). Consequently, two groups were compared to evaluate the quantitative impact of FHIT expression in SCLC: tumors with a FHIT-positive cell proportion of ≤25% and >25%. A significant difference in median survival of 155 ± 21 versus 217 ± 19 days was observed for the two groups \( (P = 0.0016) \), respectively (Fig. 3B). Both groups did not show statistical differences with respect to parameters such as patients’ gender, age, smoking status, performance status, tumor stage, cycles of chemotherapy, hemoglobin level, platelet or leukocyte count, or level of LDH.

**Clinical Parameters and FHIT Expression in Multivariate Analysis.** Next to FHIT status, other clinical parameters were also related to survival such as performance status, LDH, leukocyte count, and TNM classification using Kaplan-Meier analysis (data not shown). To evaluate whether FHIT is an independent prognostic parameter, a multivariate analysis was done using the forward selection model of the Cox regression. According to Pearson bivariate correlation analysis FHIT expression was not correlated to any other clinical variable and no statistical differences were found between the patients with FHIT-positive and FHIT-negative tumors using two-sided \( t \) test. The following 11 variables were included for multivariate analysis: gender (male versus female), age (≤60 versus >60 years), smoking status (smokers versus nonsmokers), performance status (classified into WHO 0 versus 1 versus 2 versus 3), tumor stage (classified into stage I versus II versus III versus IV), hemoglobin level (<12 versus ≥12 mg/dL), platelet count (<150,000/µL, 150,000-400,000/µL, and >400,000/µL) and leukocyte count (<3,500/µL, 3,500-11,000/µL, >11,000/µL), LDH (classified into serum levels <240 versus ≥240 units/L) as well as qualitative (FHIT positive versus negative) and quantitative FHIT expression (FHIT-expressing tumor cell proportion of <25% versus ≥25%). As a result, only FHIT-positive tumor cell proportion \( (P = 0.028) \), LDH \( (P = 0.012) \), tumor stage \( (P = 0.001) \), and performance status \( (P < 0.0001) \) were identified as independent prognostic parameters by the Cox regression model (Table 2). Of interest, the qualitative presence of FHIT was significant \( (P < 0.05) \) but not chosen as an explanatory, independent variable in the Cox regression model, whereas hemoglobin level, thrombocyte count, gender, age or smoking status were irrelevant with regard to survival time \( (P > 0.05) \).

**DISCUSSION**

In this study we show that 61.8% of 225 SCLC tumors examined by immunohistochemistry were positive for FHIT expression. To our knowledge, only three reports refer to the presence of FHIT in SCLC. Sozzi et al. (11) examined 13 primary SCLC tumors and 10 cell lines with 4 (30%) primary cases and 2 (20%) cell lines showing FHIT expression using Western blot or immunohistochemical methods. Two other reports by Pylkkänen et al. (9) and Otterson et al. (10) found higher percentages of FHIT-expressing tumors: 13 (46.4%) of 28 SCLC cell lines and 2 (50%) of 4 primary SCLCs were positive. Our study, which is in line with the results of Pylkkänen et al. and Otterson et al., indicates that the percentage of FHIT-expressing tumors in SCLC is similar to the group of adenocarcinomas of NSCLC ranging between 43% and 71% (14–17). Interestingly, in NSCLCs FHIT expression is related to tumor histology with significant lower FHIT expression in squamous cell carcinomas that were 3.6% to 42% FHIT positive (14–17).

Next, the impact of FHIT on survival in SCLC was examined. Loss of FHIT expression was significantly associated with poorer prognosis for patients with SCLC showing a median survival of 157 ± 18 days compared with 210 ± 18 days for patients with FHIT-positive tumors. Of interest, comparing survival of the patient group with weak and strong FHIT tumor expression, no statistical difference with regard to median survival was seen. This result indicates that the expression level of FHIT was irrelevant for prognosis. As a result, weakly expressing FHIT tumor cells must be graded as positive to use FHIT as a prognostic marker. Furthermore, the proportion of FHIT-positive tumor cells within the tumor had an impact on survival. Patients with ≤25% of FHIT-positive tumor cells had unfavorable prognosis, showing a median survival of 155 ± 21 days compared with 217 ± 19 days for patients with a FHIT-positive cell proportion of >25%. The prognostic significance of FHIT expression and survival was confirmed using Cox regression multivariate analysis. The presence of FHIT expression was an independent prognostic factor \( (P = 0.028) \) next to performance status, tumor stage, and LDH. Although the mean survival difference of 2 months between the FHIT-positive and FHIT-negative patient group seems marginal on first glance, one has to consider that the overall mean survival time of all patients with SCLC is 6.5 months. In this context, a predictive marker like FHIT might be
valuable to come to a decision on the still controversially discussed question of whether a patient with very limited stage SCLC should be treated with adjuvant chemotherapy or radiation after a histologically confirmed R0 resection of the tumor. Similar to our results, Toledo et al. (15) recently examined 98 primary NSCLCs with regard to FHIT expression and found a poorer survival of patients with lack of FHIT for patients with NSCLCs. Tomizawa et al. (17) reported a significant correlation between FHIT expression and improved survival in 105 NSCLC stage I tumors. These and our data underline the role of FHIT as an important gene in lung cancer biology with impact on survival, irrespective of histologic lung tumor subtype.

Several recent studies provide support for a functional activity of FHIT as a tumor-suppressing gene. Fifty-three percent of homozygous (−/−) FHIT-deficient mice developed spontaneous tumors within 2 years of follow-up compared with 8% of animals expressing wild-type FHIT (18). Further evidence supporting the hypothesis that FHIT has tumor-suppressing activity is based on gene transfer experiments. Adenoviral vector–mediated replacement of wild-type FHIT gene (Ad-FHIT) in lung cancer cell lines that lacked endogenous FHIT gene expression significantly reduced cell growth up to 80%, whereas normal human bronchial epithelial cells remained unaffected. Furthermore, lung tumor growth in nude mice that received intratumoral injections of Ad-FHIT was suppressed by 85% to 90% compared with control vector (8). Although FHIT-mediated growth suppression was associated with a G0-G1 arrest of lung cancer tumor cells and an induction of apoptosis (6) the exact molecular pathway of FHIT action is still unclear. Recently it was shown that FHIT is the physiologic target of the protein kinase Src (19). Furthermore, the apoptotic, p53-independent function of FHIT is mediated by the disruption of the inner mitochondrial transmembrane potential with the release of cytochrome c protein (5). FHIT protein is a diadenosine-tetraphosphate hydrolase and hydrolyzes diadenosine nucleotides into ADP and AMP. All conserved histidines are required for full activity and the central histidine of the triad is essential for hydrolase activity (20). Surprisingly, a hydrolase “dead” mutant gene also suppressed tumorigenicity in cancer cells and nude mice (21). Therefore, it has been suggested that FHIT binding to a thus far unknown effector or interacting protein forms a complex that acts as the tumor-suppressor molecule (4) possibly involved in apoptosis (6, 19), DNA cytoskeleton assembly (22), and repair machinery (23).

![Fig. 3](image)

**Fig. 3** Association of the proportion of FHIT-positive cells within the SCLC tumor and survival. A, different survival curves of patients were shown with regard to proportion of FHIT-expressing cells. Patients with a complete lack (dotted line) or with ≤25% FHIT-expressing cells (dashed line) had the worst survival of 157 ± 18 and 87 ± 80 days ($P = 0.165$), respectively. Survival was improved when the proportion of FHIT-expressing tumor cells were >25% (solid lines). For those tumors showing a FHIT-positive cell proportion of >25% to 50% (solid line 1), >50% to 75% (solid line 2), and >75% (solid line 3), no differences in median survival of 203 ± 40, 210 ± 52, and 217 ± 25 days were found, respectively ($0.644 < P < 0.934$). B, comparison of survival curves for patients with a tumor proportion of FHIT-expressing cells of ≤25% including negative cases (dotted line) and >25% (solid line).

<table>
<thead>
<tr>
<th>Table 2</th>
<th>Variables accepted in the forward selection model of the Cox regression as explanatory factors</th>
<th>$p^*$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Percentage of tumor cells expressing FHIT $\dagger$</td>
<td>0.028</td>
<td></td>
</tr>
<tr>
<td>Stage $\ddagger$</td>
<td>0.001</td>
<td></td>
</tr>
<tr>
<td>Performance status $\S$</td>
<td>&lt;0.0001</td>
<td></td>
</tr>
<tr>
<td>LDH $\kappa$</td>
<td>0.012</td>
<td></td>
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</tbody>
</table>

$^\dagger$ According to the score test.

$\ddagger$ Proportion of FHIT-positive cells within SCLC tumor of 0% to 25% versus >25% to 100%.

$\S$ Performance status according to WHO: 0 versus I versus II versus III.

$\kappa$ LDH serum levels ≤240 versus >240 units/L.
In conclusion, our data show that FHIT expression is present in 61% of SCLCs. The presence of FHIT was associated with a significant improved outcome for patients with SCLC, indicating that FHIT signaling plays an important role in lung cancer biology.

REFERENCES
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