

Allelic Deletion Analysis of the *FHIT* Gene Predicts Poor Survival in Non-Small Cell Lung Cancer

Louise Burke, Mohammed A. Khan, Andrew N. Freedman, Akihiko Gemma, Marek Rusin, Douglas G. Guinee, William P. Bennett, Ned E. Caporaso, Marian V. Fleming, William D. Travis, Thomas V. Colby, Victor Trastek, Peter C. Pairolero, Henry D. Tazelaar, David E. Midthun, Lance A. Liotta, and Curtis C. Harris¹

National Cancer Institute, Bethesda, Maryland 20892 [L. B., M. A. K., A. N. F., A. G., W. P. B., N. E. C., L. A. L., C. C. H.]; Institute of Oncology, 44-100 Gliwice, Poland [M. R.]; University of Utah Medical Center, Salt Lake City, Utah 84148 [D. G. G.]; Armed Forces Institute of Pathology, Washington, DC 20306 [M. V. F., W. D. T.]; Mayo Clinic Scottsdale, Scottsdale, Arizona 85259 [T. V. C.]; Mayo Clinic, Rochester, Minnesota 55905 [V. T., P. C. P., H. D. T., D. E. M.]

Abstract

The fragile histidine triad (*FHIT*) gene at chromosome 3p14.2 is a candidate tumor suppressor gene linked to cancers of the lung, breast, colon, pancreas, and head and neck. Reports of frequent allelic deletion and abnormal transcripts in primary lung tumors plus recent evidence that it is targeted by tobacco smoke carcinogens suggest that it plays an important role in lung carcinogenesis. Non-small cell lung carcinoma still maintains a poor 5-year survival rate with the stage of disease at presentation as a major determinant of prognosis. We examined for allelic deletion at the *FHIT* locus in a series of 106 non-small cell lung carcinomas for which a full clinical, epidemiological, and 5-year survival profile was available. We found an allelic deletion frequency of 38% at one or two intragenic microsatellites. Allelic deletion of *FHIT* was related to tumor histology with 4 of 20 adenocarcinomas (20%) displaying loss of heterozygosity (LOH) compared with 12 of 22 (55%) nonadenocarcinomas ($P = 0.03$). We found that 63% of tumors with LOH of *FHIT* also had *p53* missense mutations whereas only 26% with LOH had wild type *p53* negative sequence ($P = 0.02$). We also found a significant trend toward poorer survival in patients with LOH of at least one locus of the *FHIT* gene (log rank, $P = 0.01$). This survival correlation is independent of tumor stage, size, histological subtype, degree of differentiation, and *p53* mutation status. Our data support the hypothesis that the loss of the *FHIT* contributes to the molecular pathogenesis of human lung cancer and is an indicator of poor prognosis.

Introduction

The presence of a tumor suppressor gene(s) on the short arm of chromosome 3, important in the development of lung carcinoma, is intimated by the high frequency of allelic deletions involving this chromosomal region (1-5). The recent cloning of the *fragile histidine triad* (*FHIT*) gene at 3p14.2 by Ohta *et al.* (6) and the subsequent reports demonstrating frequent allelic deletion, aberrant *FHIT* transcripts in primary and cultured lung cancers (7, 8), and evidence that *FHIT* suppresses tumorigenicity in cancer cells (9) support the contention that *FHIT* is a tumor suppressor gene. This, together with the recent observation by Sozzi *et al.* (10) that there is more *FHIT* allelic loss in carcinomas from smokers than nonsmokers, strengthens its involvement in the multistage development of lung carcinoma. The *FHIT* gene is expressed at a low level in all human tissues tested and encodes a 1.1-kb mRNA (6, 11). The *FHIT* gene contains 10 exons, 5 of which (exons 5-9) constitute the open reading frame encoding a 16.8-kDa protein (6, 11), which, as a member of the histidine triad

superfamily, behaves like a typical 5',5''-P¹,P³-triphosphate hydrolyase (Ap₃A; Ref. 12). The gene resides in a fragile site, FRA3B, which is commonly targeted for cancer cell-specific homozygous deletions as well as the t(3;8) translocation identified in familial renal cell carcinoma (Refs. 6, 11; Fig. 1). Tumor suppressor activity was shown recently by Siprashvili *et al.* (9), who demonstrated that the expression of the *FHIT* protein in xenotransplanted human tumor cells suppressed their tumorigenicity in athymic nude mice although the mechanism for this tumor suppressor activity remains unknown. Deletions of 3p occur in bronchial progenitor lesions (5), and Tornielli *et al.* (13) postulated that the loss of *FHIT* might be useful as a genetic marker. Furthermore, it has also been suggested that deletions of 3p in primary small cell lung carcinomas represent an unfavorable prognostic marker (14). NSCLC² still maintains a poor 5-year survival, and many cases are at an advanced stage when diagnosed. We examined 106 cases of primary NSCLC for which a full clinical, epidemiological, and 5-year survival profile was available and assessed the significance of allelic deletion for the *FHIT* gene in primary resected tumors with particular emphasis on its predictive value as a prognostic indicator.

Materials and Methods

Patients and Tissue Samples. Matched primary NSCLCs and normal tissues were obtained from 106 consecutive surgically treated patients at the Mayo Clinic, Rochester, Minnesota. These samples were collected between 1991 and 1992, and the survival data of at least 5 years are available on all patients. Histological subtypes included 29 SCCs, 55 ADCAs, and 22 large cell carcinomas. For the purposes of the study, two tumors with more than one component (*i.e.*, adenosquamous) were classified according to the predominant histological component (*i.e.*, ADCA). A full epidemiological profile including demographics, family history, occupational exposure, and medical history was available on each patient (15, 16). The study population consisted of 63 males and 43 females; 102 of the 106 cases had a history of smoking, and a detailed profile of each smoking history was available. Because the study population consisted of surgically treated patients, the majority of the tumors were in the stage 1 and stage 2 categories. Because this study is an ongoing research project, data from other genetic markers and cell cycle proteins were available for analysis including: (a) *p53* (15) and *K-ras* mutations;³ (b) expression of cyclin D1,³ p21^{waf/cip1} (17), C-erbB-2 (15), TGF- β ,³ Bax/Bcl-2 (18); and (c) genetic polymorphisms of CYP1A1, CYP2E1, and GSTM1 (19).

Allelic Deletion Analysis. Fifty- μ m sections from ethanol-fixed and paraffin-embedded tissue were dewaxed and microdissected. After microdissection of the tumor tissue, genomic DNA was isolated from tumor and normal tissue by proteinase K and phenol chloroform extraction using standard protocols. Analysis of allelic deletion used a PCR-based approach of microsatellite polymorphism analysis using microsatellite markers within and flanking

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¹ To whom requests for reprints should be addressed, at Laboratory of Human Carcinogenesis, National Cancer Institute, Building 37, Room 2C05, 37 Convent Drive MSC 4255, Bethesda, MD 20892-4255. Phone: (301) 496-2048; Fax: (301) 496-0497; E-mail: Curtis_Harris@nih.gov.

² The abbreviations used are: NSCLC, non-small cell lung cancer; LOH, loss of heterozygosity; SCC, squamous cell carcinoma; ADCA, adenocarcinoma; nt, nucleotide.

³ Manuscript in preparation.

the gene locus at 3p14.2 were used. The microsatellite sequences were obtained from the gdb-genome database site (hosted by Johns Hopkins University School of Medicine) using the Internet web site <http://gdbwww.gdb.org/gdb/>. Oligonucleotide primers were designed to generate PCR products less than 200 nts using MacVector Algorithms. The loci examined were D3S1300, D3S1234, D3S1313, D3S1312 and D3S1295 (Fig. 1, Table 1). D3S1300 is located within intron 5 of the *FHIT* gene locus close to exon 5, which is the first coding exon of the gene. This location also coincides with the region of common loss in a variety of tumor cell lines. D3S1234 is located in intron 7; D3S1295 and D3S1313 are located telomeric to the gene, the latter positioned at 3p14.3. Centromeric at 3p14.2 is D3S1312. All of the microsatellites, excluding D3S1312 for which a volume of 100 μ l was used, used PCR reaction volumes of 50 μ l for the assay. The PCR reaction tube mix contained 25 pmol of each primer, 2–2.5 mM deoxynucleotide triphosphates, standard 10 \times PCR buffer [100 mM Tris-HCl (pH 7.3), 15 mM MgCl₂, 500 mM KCl, and 0.01% w/v gelatin], and 1.25 units of AmpliTaq DNA polymerase (Perkin-Elmer Corp.). Reaction mixes of 50 μ l were overlaid with mineral oil and placed in a Perkin-Elmer Corp. thermocycler when the heating block reached 94°C. PCR conditions for each set of primers were optimized using standard denaturing and extension temperatures of 94°C and 72°C, respectively. The annealing temperatures, number of PCR cycles, and expected PCR product sizes in nts for each microsatellite are the following: (a) D3S1300—60°C, 25 cycles, 126 nts; (b) D3S1234—53°C, 30 cycles, 111 nts; (c) D3S1313—52°C, 28 cycles, 121 nts; (d) D3S1295—60°C, 30 cycles, 135 nts; and (e) D3S1312—58°C, 28 cycles, 130 nts. All PCR products were resolved on 5% metaphor agarose gels (FMC Bioproducts), and ambiguous cases were analyzed in addition by 10% nondenaturing PAGE using ethidium bromide staining and UV illumination. Allelic loss for informative cases was scored if the intensity of one or more bands was reduced by 50% or more in the tumor DNA when compared with the nontumor DNA sample (Fig. 2). Validation of this nonisotopic technique was performed by analyzing a coded set of matched tumor and nontumor DNA samples previously analyzed by Dr. G. Sozzi using standard isotopic protocols.

Statistical analysis was carried out using the SPSS/PC 7.5 for Windows statistical package. Although laboratory analysis was performed for five microsatellites, for statistical analysis we defined *FHIT* LOH as LOH at D3S1300 and/or D3S1234, which are located within the *FHIT* gene locus. The association between *FHIT* LOH and other genetic aberrations, and clinicopathological features were assessed using Fisher exact tests and χ^2 tests. Associations were considered statistically significant if two-tailed *P*s were <0.05. Kaplan-Maier curves were used to estimate survival probability as a function of time, and patient survival differences were analyzed by the log-rank test. A proportional hazards model was used to estimate and test the influence of *FHIT* LOH, other genetic aberrations, and clinicopathological features on prognosis.

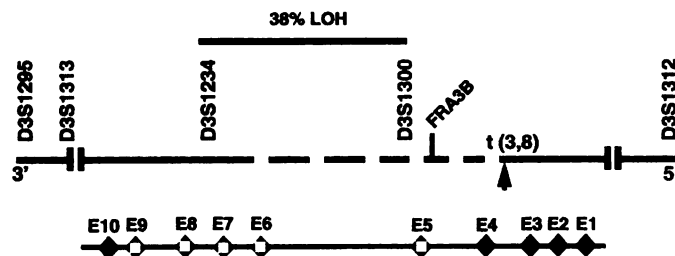


Fig. 1. Schematic representation of chromosome 3p14.2 landmarks and *FHIT* exons and introns. *FHIT* includes ten exons (diamonds, labeled E1–E10); five exons are transcribed (E5–E9), and five are not transcribed (E1–E4 plus E10). The *FHIT* coding sequences span the FRA3B fragile site (dashed line) and the t(3;8) translocation point linked to familial renal cell carcinoma. The D3S1300 and D3S1234 microsatellites occur within introns 5 and 7, respectively. Three additional microsatellites (D3S1312, D3S1313, and D3S1295) are outside the *FHIT* coding sequences.

Table 1 Microsatellite analysis of NLCLC

Microsatellite	Cases analyzed	Informative cases	Allelic deletion (%)
D3S1312	101	36	1 of 27 (4%)
D3S1300	100	57	11 of 49 (23%)
D3S1234	98	65	11 of 58 (19%)
D3S1313	101	32	9 of 25 (36%)
D3S1295	96	48	6 of 40 (15%)

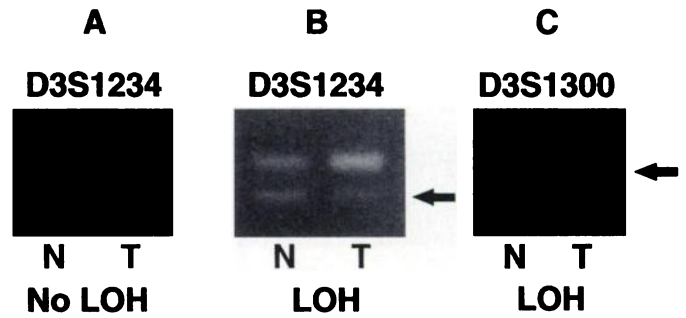


Fig. 2. Representative allelic deletion detected by nonisotopic analysis of PCR products from tumor (T) and nontumor (N) tissues. “No LOH” is shown by bands of equal intensities in both tumor and nontumor tissues (A, D3S1234); two examples of LOH are marked loss of one band in tumor tissue (B and C, arrows).

Results and Discussion

Allelic deletion was detected in 22 of 73 (30%) informative cases at one or more loci examined when complete data from all five microsatellites were included (Fig. 3). The allelic deletion status for each locus is detailed in Table 1. When only two intragenic microsatellites, D3S1300 and D3S1234, are considered, 16 of 42 (38%) informative tumors had LOH at one or both sites. Our rates of allelic loss at all examined markers are lower than the 76% and 45–88% reported in two previous studies (Refs. 7, 8; Table 1). These differences cannot be attributed to our nonisotopic method because we performed a validation study of nine pairs of NSCLC tumor and nontumor samples that were analyzed by routine isotopic methods and generously provided by Dr. G. Sozzi. We analyzed two microsatellites (D3S1300 and D3S1234); our results were completely concordant at D3S1300, 78% concordant at D3S1234 alone, and 89% concordant overall when both markers were combined. We conclude that the data represent an accurate rate of allelic deletion in this series of NSCLCs from American smokers.

Allelic deletion at D3S1300 and D3S1234 correlated with non-ADCA histology. Previous studies have shown that the loss of 3p14.2 is more common in SCCs than in ADCAs (1, 20). Our data are consistent with this association because 12 of the 16 cases with LOH at one or both sites were of a histological subtype other than ADCA (*P* = 0.03): 6 cases were SCCs, and 6 were large cell carcinomas. Although we did not find a positive association of *FHIT* LOH with smoking history in our series of 102 smokers and 4 nonsmokers, this histological segregation does suggest a dose effect associated with tobacco carcinogens inasmuch as the more centrally located tumors (e.g., SCCs) are exposed to greater concentrations than the more peripherally located ADCAs. The lack of correlation between *FHIT* LOH and smoking history are divergent to that of Sozzi *et al.* (10); however, there is no clear explanation for the difference. Allelic deletion of *FHIT* did not correlate with age, sex, tumor size, stage, grade, or family history of cancer including lung cancer. We did not detect a statistical relationship between allelic deletion at *FHIT* and additional biological markers previously analyzed in this series: *p53* and *K-ras* mutations, cyclin D1, p21^{waf/cip1}, C-erb-2, TGF- β , Bax, Bcl-2, CYP1A1, CYP2E1, and GSTM1 (15, 18, 19).

Although the statistical power of this study of only 106 cases was limited, allelic deletion at D3S1300 and D3S1234 was associated with *p53* missense mutations (*P* = 0.02). However, the association between *p53* missense mutations and *FHIT* LOH was independent of histological subtype. This relationship was not statistically significant when nonmissense *p53* mutations were included and there was no correlation with the mutation spectrum such as the G:C→T:A transversions that are associated with the amount of cigarette smoking (21;

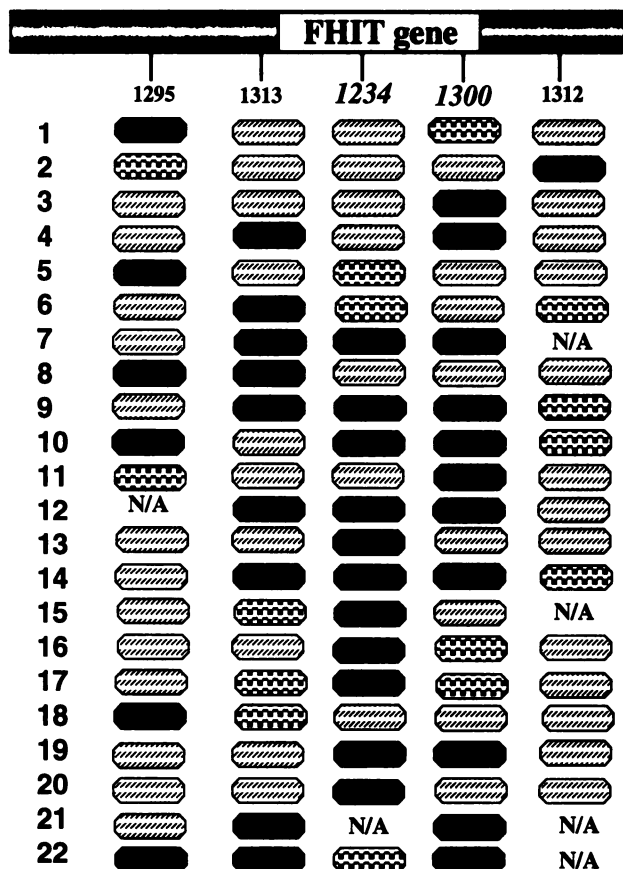


Fig. 3. Allelic loss at chromosome 3p14.2 in 22 cases of NSCLCs. When analyzed by five microsatellite markers (*i.e.*, D3S1295, D3S1313, D3S1234, D3S1300 and D3S1312), 22 tumors showed evidence of LOH at one or more sites. *Black octagons*, LOH; *checkered octagons*, no LOH; *dashed octagons*, not informative; *N/A*, not analyzed (usually because of PCR failure).

reviewed in Ref. 22). Also the association between *FHIT* LOH and *p53* immunohistochemical assessment did not reach statistical significance despite the correlation between *p53* protein accumulation and missense mutations (15). The statistical associations among *FHIT* allelic deletion, *p53* missense mutations, and non-ADCA histology might be explained by damage from tobacco smoke carcinogens. For example, *p53* mutations are more common in centrally located lung tumors (*i.e.*, small cell lung carcinomas and SCCs) than in the peripheral ADCAs (22). A plausible explanation of this observation is an increased dosage of chemical carcinogens in central airways compared with peripheral lung tissues. Chemical carcinogenesis models link carcinogen-DNA adducts to hotspots of *p53* missense mutations (23, 24), and analysis of human cancer cell deletion end points shows that the FRA3B fragile site is a common target of homologous recombination producing *FHIT* internal deletions (25). Because fragile sites are susceptible to carcinogen-induced alterations (26), it is possible that the carcinogen-driven disruption of the FRA3B fragile site produces the *FHIT* deletions observed in lung cancer. Another hypothesis is that the genomic instability that is associated with *p53* mutations (27) may precede and promote the *FHIT* deletions.

Our studies have possible clinical implications inasmuch as we observed a statistically significant trend toward poorer survival in patients with loss of one allele of the *FHIT* gene that was independent of tumor stage, size, grade, histological subtype, and *p53* mutation status ($P = 0.01$, Fig. 4). Although the biochemical mechanism by which *FHIT* exerts its tumor suppressor activity remains to be elucidated, our data linking it to poor survival, *p53* missense mutation, and

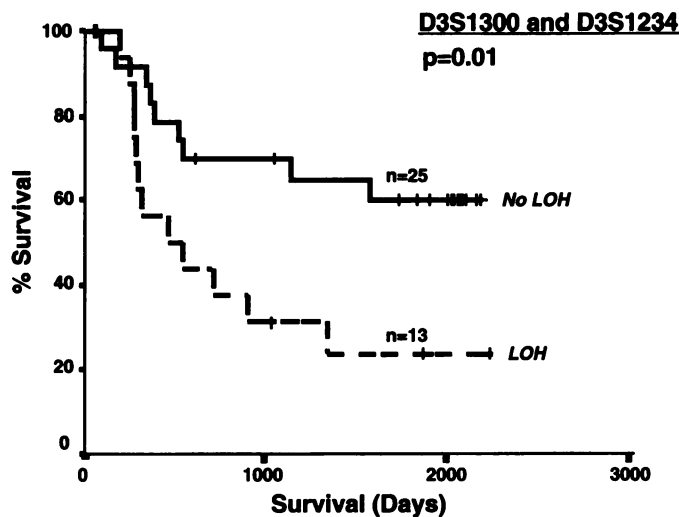


Fig. 4. LOH measured at the two *FHIT* intragenic microsatellites, D3S1234 and D3S1300, correlated with significantly reduced survival ($P = 0.01$). The locations of the microsatellite markers are indicated schematically according to available information, but precise localization is not possible at present. Specifically, the intronic placements of the two intragenic markers are not known at present.

non-ADCA histology provide additional evidence of its role in the molecular pathogenesis of human lung cancer.

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