

Loss of Fhit Expression Is a Predictor of Poor Outcome in Tongue Cancer¹

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Abstract

Abnormalities of *FHIT*, a candidate tumor suppressor gene located at 3p14.2, have been found frequently in multiple tumor types, including head and neck squamous cell carcinoma. To determine the role of *FHIT* in tongue cancers, Fhit expression was determined by immunohistochemistry studies in tissue samples from 41 patients with stages II–IV squamous cell carcinomas of the tongue. All of the patients underwent curative surgical treatment with a median of 83 months of follow-up care. We found that 28 (68%) of the 41 tumor specimens demonstrated a lack of or significantly decreased staining for Fhit. Fhit expression tended to be stronger in well-differentiated tumor areas than it was in poorly differentiated areas, although this trend was not statistically significant. There was no significant correlation between Fhit expression and a patient's age or sex or the histological grade or clinical stage of disease. As expected, clinical stage and nodal involvement correlated with prognosis. Interestingly, patients whose tumors demonstrated low levels of or no Fhit expression had a significantly shorter time of disease-free survival than those whose tumors had high Fhit expression ($P = 0.035$, by log-rank test). This prognostic value of Fhit was independent of other clinical parameters, including stage of disease and nodal status. We conclude that Fhit plays an important role in the development of squamous cell carcinomas of the tongue and that loss of Fhit expression may be an independent prognostic indicator for clinical outcome in patients with this tumor type.

Introduction

Head and neck cancers account for 3–5% of all malignancies in Western countries, with cancer of the oral cavity accounting for 30% of all head and neck cancers. In the United States alone, it was estimated that 30,200 new cases of oral cancer would be diagnosed in 2000, with an estimated 7,800 deaths (1). It was estimated that 6,900 new cases of tongue cancer would be diagnosed in 2000, with an estimated 1,700 deaths (1). Although for early-stage tumors excellent cure rates can usually be achieved, for advanced stage disease, the 5-year survival rate is only 40–60% and has hardly improved in the last two decades (2). To further improve the survival rate in this group of patients, their prognostic classification based on tumor biology will be crucial. Such classification might help clinicians to make the appropriate management decisions for each subset of patients.

The inactivation of tumor suppressor genes and the amplification or overexpression of oncogenic factors have been implicated in the

multistep progression of HNSCC⁴ carcinogenesis (3). LOH has been found frequently at multiple chromosome sites, including 3p, 9p, 11q, 13q, and 17p in HNSCC (4). The *FHIT* gene, located at 3p14, contains FRA3B, the most common fragile site in humans, and is frequently the target of homozygous deletions in many human cancer cell lines, including HNSCC. The Fhit protein is homologous to Ap4A hydrolase from the yeast *Schizosaccharomyces pombe* (5), and it also exhibits Ap3A activity in enzymatic assays (6). Fhit tumor suppressive function, however, appears to be independent of its hydrolase activity (7, 8).

FHIT has been investigated extensively as a candidate tumor suppressor gene in many tumor types, including those of the digestive tract, cervix, lung, breast, and HNSCC (3, 5, 9–11). A few studies have examined abnormalities in *FHIT* gene expression and Fhit protein expression in OSCC (12–14). Specifically, in one study (12), Fhit expression in OSCC was evaluated using immunohistochemistry, and a reduction or loss of Fhit in samples from 21 (66%) of 32 patients was noted; however, no prognostic value was found. It is noteworthy that the clinical features and outcomes of HNSCC are variable, depending on the anatomical site, and that the previous studies were not designed to investigate the role of Fhit in patients with HNSCC at specific sites (12–15).

To determine whether loss of Fhit expression occurs specifically in SCCs of the tongue, we examined tumor samples from 41 patients with SCCs of the tongue (stages II–IV) for Fhit expression using immunohistochemistry studies. We then analyzed Fhit expression status with clinical parameters to determine whether it has any prognostic significance in this homogeneous population. We found that loss of Fhit expression occurred in 28 (68%) of 41 tumors, and that this loss of Fhit is an independent adverse prognostic factor for disease-free survival.

Materials and Methods

Study Population. Specimens of OSCC of the tongue were obtained from archived tissue samples of surgically resected pathological stages II–IV tumors from 41 patients treated at The University of Texas M. D. Anderson Cancer Center (Houston, TX). Surgical specimens were collected between 1991 and 1994. All patients were treated by surgery and received a median of 83 months of follow-up care after surgical treatment. Survival data were available for all patients; the minimum length of follow-up care was 10 months. The study population consisted of 26 men and 15 women. The mean age of patients was 56.2 (SD, ± 11.4) years.

Immunohistochemical Staining for Fhit Protein. Paraffin-embedded, 4- μ m-thick tissue sections from all 41 primary tumors were stained for the Fhit protein using a primary rabbit polyclonal anti-gluthione *S*-transferase-Fhit antibody (kindly provided by Drs. Carlo Croce and Kay Huebner, Kimmel Cancer Center, Philadelphia, PA). Deparaffinization of all sections was performed through a series of xylene baths, and rehydration was performed

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⁴ The abbreviations used are: HNSCC, head and neck squamous cell carcinoma; OSCC, oral squamous cell carcinoma; LOH, loss of heterozygosity; Ap4A, diadenosine 5',5''-P¹,P⁴-tetraphosphate hydrolase; Ap3A, diadenosine 5',5''-P¹,P³-triphosphate hydrolase.

through graded alcohols. To retrieve the antigenicity, tissue sections were treated three times with microwaves in 10 mM citrate buffer (pH 6.0) for 5 min each time. The sections were then immersed in methanol containing 0.3% hydrogen peroxidase for 20 min to block the endogenous peroxidase activity and were incubated in 2.5% blocking serum to reduce nonspecific binding. Sections were incubated overnight at 4°C with primary anti-glutathione *S*-transferase:Fhit antiserum (1:500). The sections were then processed using standard avidin-biotin immunohistochemistry, according to the manufacturer's recommendations (Vector Laboratories, Burlingame, CA). Diaminobenzidine was used as a chromogen, and commercial hematoxylin was used for counterstaining. Adjacent normal-appearing epithelium within the tissue sections served as a positive internal control.

Representative areas of each tissue section were selected, and cells were counted in at least four fields (at $\times 200$). Immunohistochemical staining was classified, as reported previously (16), as either negative, if no staining or positive staining was present in $<10\%$ of the cells, or positive, if $>10\%$ of the cells stained positively. All slides were evaluated and scored independently by two investigators (J. I. L. and J. C. S.), who were blinded to the clinical information pertaining to the subjects.

Statistical Analysis. Survival curves for disease-free survival time and overall survival time were estimated by the Kaplan-Meier method, and the resulting curves were compared using the log-rank test. Disease-free survival time was calculated from the date of surgery to relapse or death from cancer-related causes. Fisher's exact test or the χ^2 test was used to analyze the association between two categorical variables. All tests were two sided. $P < 0.05$ was considered to be statistically significant. Multivariate analysis was performed according to the Cox proportional hazards model. Because of the sample size, two covariates were analyzed at a given time.

Results

In normal squamous epithelium, staining for the Fhit protein was present and served as an internal positive control. This staining was more prominent in the stratum spinosum and in areas of keratin differentiation, with minimal or no staining in the basal and parabasal cells (Fig. 1A). The staining was cytoplasmic. The intensity of the staining in the normal-appearing epithelium could be classified as moderate to strong. Staining was also seen in the excretory ducts of the underlying minor salivary glands.

Most cases showed a sudden change from normal epithelium to carcinoma, with the normal epithelium having positive expression and the areas of carcinoma having variable expression for Fhit. Few specimens also demonstrated areas of dysplasia with variable expression (Fig. 1B). Fhit staining among the tumor specimens was heterogeneous, displaying diffusely negative or rare positive scattered cells to diffusely positive or a mixed pattern of both weak and strong staining, suggesting that phenotypic heterogeneity is a major feature in SCCs of the tongue (Fig. 1, C-F). If 10% or more of the cells were positive for Fhit, the case was considered to have positive Fhit expression (16). Among the heterogeneously stained specimens, carcinoma cells were more prominently positive in the better-differentiated areas, especially near areas of keratinization (Fig. 1, C and E).

According to our scoring criteria, loss of Fhit expression was noted in 28 (68%) of the 41 SCC tongue specimens. Positive expression was observed in 13 (32%) of the tumors. Table 1 shows the relationships between expression of Fhit and clinicopathological factors. The fre-

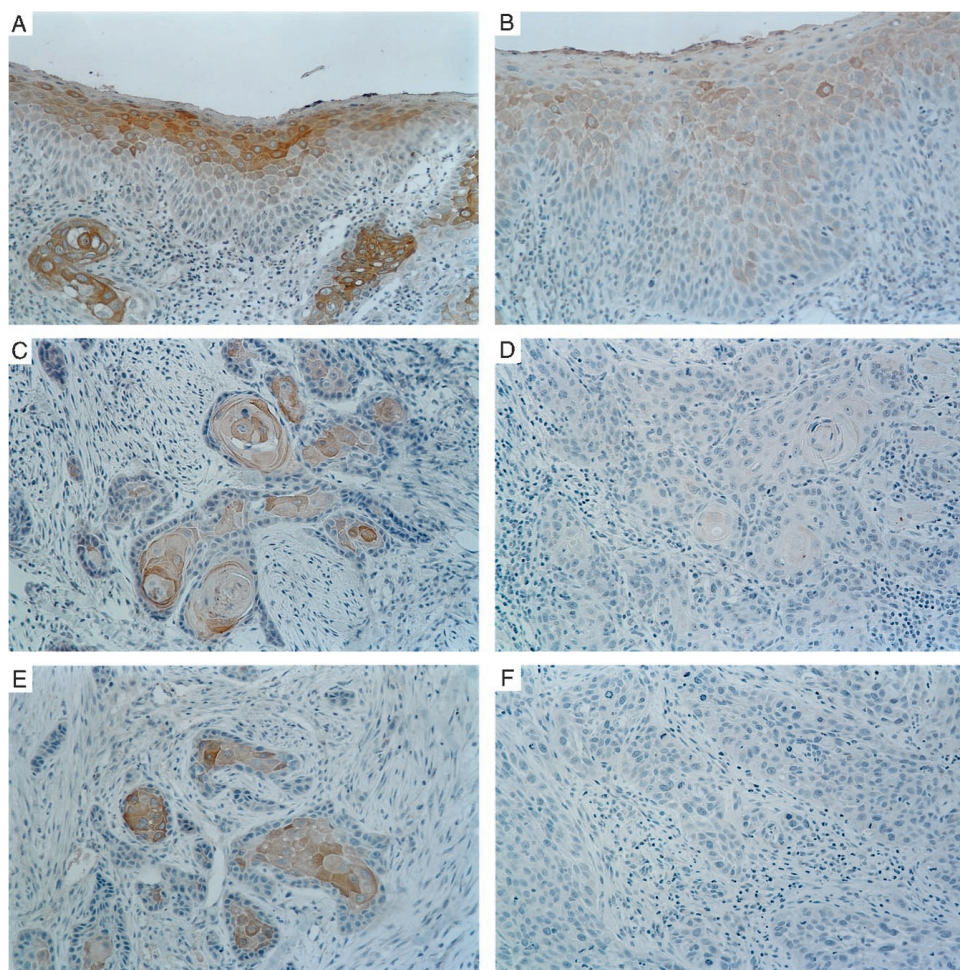


Fig. 1. Immunohistochemical staining patterns of Fhit in SCCs of the tongue. A, normal stratified squamous epithelium with prominent Fhit staining in the stratum spinosum and stratum superficiale and prominent Fhit staining in the underlying carcinoma cells ($\times 200$). B, moderate epithelial dysplasia showing staining in the superficial cells but not in the basal and parabasal cells. C and D, well-differentiated tumors were usually positive (C), although negative cases were also observed (D). E, positive Fhit staining was usually observed in the better-differentiated areas of the tumor nests, with weak expression in the periphery. F, moderate to poorly differentiated tumors were usually negative for Fhit expression.

Table 1 *FHIT* status and clinicopathological characteristics of tongue SCC tumor samples from 41 patients

	Total	Fhit negative	Fhit positive	P
Total no. of patients	41	28 (68%)	13 (32%)	
Age	Mean 56.2 years			
≤50	14	9 (64%)	5 (36%)	0.691
>50	27	19 (70%)	8 (30%)	
Sex				
Men	26	17 (65%)	9 (35%)	0.734
Women	15	11 (73%)	4 (27%)	
Histological grade ^a				
I	22	12 (54%)	10 (46%)	0.090
≥2	18	15 (83%)	3 (17%)	
Nodal status ^b				
N ₀	20	14 (70%)	6 (30%)	0.736
N ₁₋₃	20	13 (65%)	7 (35%)	
Stage				
II	15	11 (73%)	4 (27%)	0.734
III and IV	26	17 (65%)	9 (35%)	
Metastases or recurrence	11	10 (91%)	1 (9%)	
Death	18	15 (83%)	3 (17%)	
Cancer-related death	11	10 (91%)	1 (9%)	

^a One tumor specimen was of the baseloid type; therefore, its histological grade was not included in the analysis.

^b The nodal status of one patient was indeterminate (N_x) and therefore was not included in the analysis.

quency of Fhit expression did not differ significantly in different age groups or by sex. A precise evaluation of tobacco and alcohol use was not available in a subset of our patients; therefore, analysis of Fhit expression in relation to tobacco use or alcohol consumption was not performed. An association between tumor grade (G1 *versus* G2–3) and Fhit expression was noted. Of the moderately or poorly differentiated tumors, 15 (83%) of 18 were Fhit negative, whereas only 12 (55%) of 22 well-differentiated tumors were Fhit negative ($P = 0.090$). Consistent with previous reports (13), there was no significant relationship between Fhit expression and the clinical stage of the disease or whether patients had nodal disease (Table 1).

We subsequently analyzed the relationship between Fhit expression and length of survival. Fig. 2A shows a comparison of the Kaplan-Meier overall survival curves between patients whose tumors were Fhit positive and those whose tumors were Fhit negative. Patients whose tumors were negative for Fhit expression tended to have a shorter survival time than that seen in patients positive for Fhit ($P = 0.069$, by log-rank test). About 46% of the patients whose

tumors had negative Fhit expression were alive compared with 77% of the patients whose tumors had positive Fhit expression. When disease-free survival time was analyzed, patients with negative Fhit expression demonstrated a significantly worse prognosis than did patients with positive Fhit expression ($P = 0.035$, by log-rank test). Of the 13 patients whose samples were Fhit-positive, only 2 (15%) experienced disease recurrence, metastases, or cancer-related death at follow-up *versus* 13 (46%) of 28 patients whose samples were Fhit negative ($P = 0.035$; Fig. 2B). We also examined other clinical parameters in relation to survival time. As expected, clinical stage (II *versus* III–IV) and nodal status (N₀ *versus* N₁₋₃) correlated with overall survival time (data not shown). Multivariate analysis was then performed to compare Fhit expression status to these cardinal prognostic markers for survival. Given the moderate sample size in the study, multivariate analysis could only be performed between two variables at a given time. When Fhit status and stage of disease were analyzed in relation to disease-free survival time, Fhit continued to be a statistically significant marker ($P = 0.042$). When the same comparison was made between Fhit status and nodal status, Fhit again remained statistically significant ($P = 0.040$).

Discussion

FHIT, located on chromosome 3p, is a frequent target of allelic loss, genetic rearrangement, and cytogenetic abnormalities in multiple tumor types including those of the digestive tract, breast, cervix, lung, and head and neck (5, 9–11). Many investigators have demonstrated alterations of *FHIT*, such as homozygous deletions of exons at the genomic level, insertions of intronic sequence and aberrant transcripts at the mRNA level, and lack of detectable protein within these tumor types (5, 9–11).

We demonstrated previously that LOH occurs frequently at the chromosome region containing *FHIT* and that the expression of Fhit is frequently altered in HNSCCs (17). Additionally, other studies have investigated altered *FHIT* in HNSCCs at the DNA, RNA, and protein levels (18, 19). Studies using immunohistochemical analysis have demonstrated that there is a significant loss or reduction of Fhit expression in patients with HNSCCs and OSCCs (12, 15). Van Heerden’s (12) study of 32 cases of intraoral SCC demonstrated loss or reduction of Fhit expression in 21 (66%) cases. Heterogeneous staining with strongly positive Fhit expression was seen in areas of

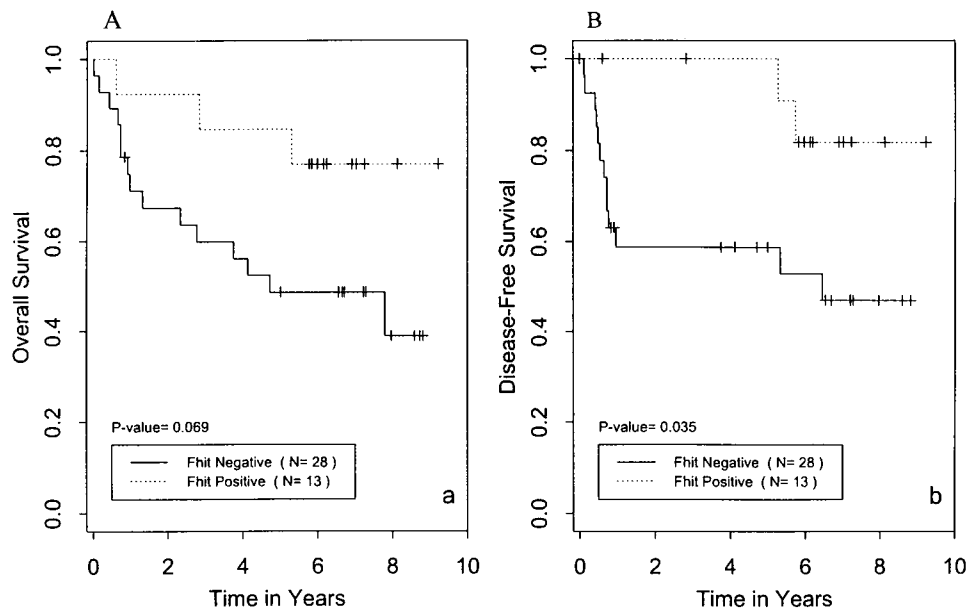


Fig. 2. A, overall survival curves of patients with tongue SCCs according to Fhit expression. B, disease-free survival curves of patients with tongue SCCs according to Fhit expression.

well-differentiated tumor, and weak intensity was noted in areas of poor differentiation; however, no statistically significant correlation was found between Fhit expression and tumor grade. Other studies using immunohistochemistry have also demonstrated an association between tumor differentiation and Fhit expression in breast carcinoma and in bladder carcinoma (20, 21). Findings in our study are consistent with these previous reports. The underlying etiology of this tumor heterogeneity is not well understood. Weakly stained areas may reflect genomic instability with loss of one or both alleles. Traditionally, it was thought that positive protein expression corresponds with the presence of both alleles, weak protein expression corresponds with loss of one allele, and loss of expression corresponds with loss of both alleles. It is also possible that alternative splicing of *FHIT* pre-mRNA observed in many previous studies contributes to the lack of detectable Fhit.

Regarding Fhit expression in premalignant lesions, there were too few examples of dysplasia within this study to ascertain accurately whether loss of Fhit occurs early in the multistep process of HNSCC tumorigenesis. Previous investigations of both bronchial and oral squamous epithelium using immunohistochemistry techniques showed that loss of Fhit may occur in areas of high-grade dysplasia but that Fhit is still expressed in areas of mild-to-moderate dysplasia. (12, 22) Additionally, LOH analysis has also demonstrated abnormal transcripts in two of seven premalignant lesions (13). This suggests that *FHIT* inactivation might occur at an early phase in carcinogenesis.

Regarding the 41 specimens of invasive SCC of the tongue, we demonstrated that loss of Fhit expression occurred in a large subset: 28 (68%) of 41. The survival time analysis revealed a significant correlation between loss of Fhit expression and length of disease-free survival ($P = 0.035$). The prognostic value of Fhit was retained in multivariate analysis, even when compared with well-established clinical prognostic factors such as nodal involvement or stage of disease. This suggests that *FHIT* is a potential new prognostic marker as well as a therapeutic target in SCCs of the tongue. To our knowledge, this is the first report that correlates loss of Fhit with patient outcome in HNSCCs. A possible explanation for why other investigations have been unable to correlate Fhit expression with patient outcome is perhaps the heterogeneous nature of the patient population they tested. Inclusion of all patients with OSCCs encompasses multiple sites including the alveolar ridge, retromolar trigone, floor of mouth, and the oral tongue, all of which have variable clinical behavior (12). Outcome is therefore highly site specific with respect to HNSCCs and may also reflect different tumor biology. The fact that all of the patients had tongue cancer and were treated at a single institution with lengthy follow-up care after surgery increased the statistical power of our study.

The tumor suppressor function of Fhit remains to be fully elucidated. Recently, transfection of *FHIT* in *FHIT*-negative human lung cancer and HNSCC cells was shown to induce apoptosis and inhibit cell growth (23). The mechanism through which Fhit mediates induction of apoptosis and alteration of cell proliferation is not well understood, but recent structural and functional studies have begun to provide a few clues. Although Fhit has Ap3A hydrolase activity, this activity does not appear to play a vital role in tumor suppression (7, 8). Crystallography studies have shown that binding nucleotide substrates by Fhit alters the molecular surface of the protein and that perhaps it is the Fhit-substrate complex that is the active form involved in signaling pathways and cell cycle progression (8, 24–26). Furthermore, it has been reported that the ground state of Fhit is a Fhit-PPi complex, that the level of diadenosine 5', 5''-Pⁿ-polyphosphate displaces PPi, and that this new complex signals cell death (27). Known substrates for Fhit are Ap3A and Ap4A, and alterations in

Ap3A and Ap4A levels have been associated with induction of apoptosis in cultured human cells (27, 28). Recent reports suggest that Ap4A is perhaps a more stable substrate for the Fhit-complex required for the tumor suppressor function of Fhit, whereas Ap3A is perhaps a reflection of the enzymatic activity (29) of Fhit.

In summary, we have demonstrated that loss of Fhit is frequent in SCC of the tongue. Loss of Fhit correlates with an adverse outcome in patients with this disease, suggesting that Fhit plays an important role in tongue tumorigenesis and may be an independent negative prognostic indicator for clinical outcome.

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