Loss of Fhit Expression in Head and Neck Squamous Cell Carcinoma and Its Potential Clinical Implication

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ABSTRACT

Purpose: **Abnormalities of *FHIT*, a candidate tumor suppressor gene, have frequently been found in multiple malignancies, including head and neck squamous cell carcinoma (HNSCC). To define its role in HNSCC treated with surgery and postoperative radiotherapy (PORT), the Fhit protein expression status was investigated in 80 patients enrolled in a prospective Phase III clinical trial addressing the dose and fractionation regimen of PORT.**

Experimental Design: **Immunohistochemical staining of HNSCC tissue sections for Fhit expression was performed. The Fhit expression status was correlated with the clinicopathological characteristics and clinical course. The median follow-up duration was 4.9 years.**

Results: **Loss of Fhit expression was found in 52 of the 80 study patients (65%). There was not a significant association between Fhit expression and clinical characteristics. Patients whose tumor exhibited negative Fhit expression had a significantly worse 5-year overall survival duration [hazard ratio = 0.49; 95% confidence interval, 0.23–1.03; $P = 0.05$ (log-rank test)] than did those whose tumor exhibited positive Fhit expression. One third of the patients with a Fhit-negative tumor had distant metastasis during the follow-up period. Paradoxically, patients classified as high risk who had a Fhit-negative tumor experienced locoregional recurrence less often (18%) than did high-risk patients who had a Fhit-positive tumor (33%).**

Conclusions: **Loss of Fhit expression is a poor prognostic indicator in patients with HNSCC. However, tumors lacking Fhit expression may be more sensitive to PORT and therefore more susceptible to locoregional control.**

INTRODUCTION

Head and neck squamous cell carcinoma (HNSCC) accounts for 2–3% of all malignancies in Western countries (1), with a much higher incidence in geographical regions such as India and southern Asia. Radiotherapy is an effective initial treatment for early- to intermediate-stage HNSCC, whereas a combination of surgery and postoperative radiotherapy (PORT) is usually required for advanced HNSCC. Organ preservation protocols developed in recent years further emphasize the role of radiotherapy for HNSCC. However, the 5-year survival rate in patients with advanced HNSCC, which ranges from 40% to 60%, has not changed significantly over the past two decades (2). Locoregional recurrence and distant metastasis remain common causes of mortality in patients with advanced HNSCC, despite the use of aggressive combinational treatment strategies.

Development of HNSCC occurs in multiple steps and involves the inactivation of important tumor suppressor genes. *FHIT*, which is located at chromosome 3p14.2, is a tumor suppressor gene (3, 4) that is frequently deleted in human cancers, including HNSCC (5–10). Loss of Fhit protein expression has also been reported in some precancerous lesions of the lung, oral cavity, and esophagus and been found to be associated with exposure to environmental carcinogens, such as smoking and alcohol consumption (11–13).

In HNSCC, deletion of the *FHIT* locus has been reported in 40–60% of tumors and cell lines (7, 13–16), whereas loss of Fhit protein expression has been found in 40–70% of tumors (16–18). We showed previously that loss of Fhit expression is associated with a poor clinical outcome in patients with oral squamous cell carcinoma treated with surgery alone, suggesting that Fhit may play an important role in oral cancer progression (19). However, it is unclear whether Fhit plays a role in HNSCC treated with radiation. To define the role of Fhit in HNSCC treated with surgery and PORT, we analyzed the Fhit expression status in tumor samples obtained from 80 patients with HNSCC who were enrolled in a multicenter Phase III clinical trial and underwent treatment at The University of Texas M. D. Anderson Cancer Center. Our data indicate that patients whose tumor showed loss of Fhit expression and were treated with surgery and PORT had a poor overall survival. However, we found that tumors lacking Fhit expression may be more sensitive to PORT, resulting in more favorable locoregional disease control.

MATERIALS AND METHODS

Study Population. Eighty patients with HNSCC who underwent treatment at The University of Texas M. D. Anderson Cancer Center between August 1991 and March 1995 as

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part of a multicenter prospective randomized trial were enrolled in this study. There were 59 men and 21 women with a mean age of 56.4 ± 10.8 years. All of the patients had histologically proven squamous cell carcinoma in the oral cavity, oropharynx, larynx, or hypopharynx deemed likely to be cured with a combination of surgery and PORT. All of them had a Zubrod performance status of 0–2 and underwent surgery, including primary tumor resection and neck dissection. Three risk categories were created based on surgical and pathological findings, including the primary tumor site, the surgical margin status, perineural invasion, the number and location of positive lymph nodes, and the presence of extracapsular spread (ECS). Patients without any adverse pathological factors or with one factor other than ECS were considered to have a low or intermediate risk of recurrence (the low-risk group including clinically defined low- and intermediate-risk groups); these patients did not undergo PORT and received only conventionally fractionated radiotherapy to a dose of 57.6 Gy in 32 fractions over 6.5 weeks. Patients with ECS or more than two adverse pathological factors were considered to have a high risk of recurrence (high-risk group); these patients received PORT to a dose of 63 Gy in 35 fractions under conventional fractionation over 7 weeks or accelerated fractionation over 5 weeks (20). The patients underwent follow-up examinations every 2–3 months over the first year, every 3–4 months over the second year, and every 6 months thereafter. The median follow-up duration was 4.9 years (range, 22–83 months).

Immunohistochemical Staining for Fhit Protein Expression. Paraffin-embedded, 4-μm-thick tissue sections from all 80 primary tumors were stained for Fhit protein expression with a primary rabbit polyclonal anti-glutathione *S*-transferase-Fhit antibody (provided by Carlo Croce and Kay Huebner; Kimmel Cancer Center, Philadelphia, PA). The slides were baked at 55°C for 1 h, deparaffinized in a series of xylene baths, and rehydrated in graded alcohol. To retrieve their antigenicity, the tissue sections were placed in 10 mM citrate buffer (pH 6.0) and heated in a microwave three times for 5 min each. After being immersed in 0.3% hydrogen peroxide in methanol for 20 min, the tissue sections were blocked with 2.5% blocking serum to reduce the staining background. Sections were incubated overnight at 4°C with a primary anti-glutathione *S*-transferase-Fhit antibody (1:500). The sections were then processed by using a standard avidin-biotin system for immunohistochemical staining according to the manufacturer's recommendations (Vector Laboratories, Burlingame, CA). Diaminobenzidine was used as a chromogen, and hematoxylin was used for counterstaining. The adjacent noncancerous epithelium within the tissue sections served as an internal positive control.

Representative areas of each tissue section were selected, and cells were counted in at least four fields (×200). Immunohistochemical staining was classified into two groups as described previously: negative (no staining or positive staining in <10% of the cells) and positive [positive staining in ≥10% of the cells (12, 19)]. All of the slides were evaluated and scored by two investigators (J. I. L. and A. K. E-N.), who were blinded to the clinical information.

Statistical Analysis. Survival curves were estimated with the Kaplan-Meier method and compared with those obtained with the log-rank test. Survival duration was calculated from the date of surgery to relapse or death. Fisher's exact test or χ^2 test was used to analyze the association between two categorical variables. All of the tests were two-sided. $P \leq 0.05$ was considered statistically significant.

RESULTS

The normal squamous epithelium generally showed positive staining for Fhit protein expression; specifically, there was more prominent staining in the stratum spinosum and areas of keratin differentiation but almost no staining in the basal and

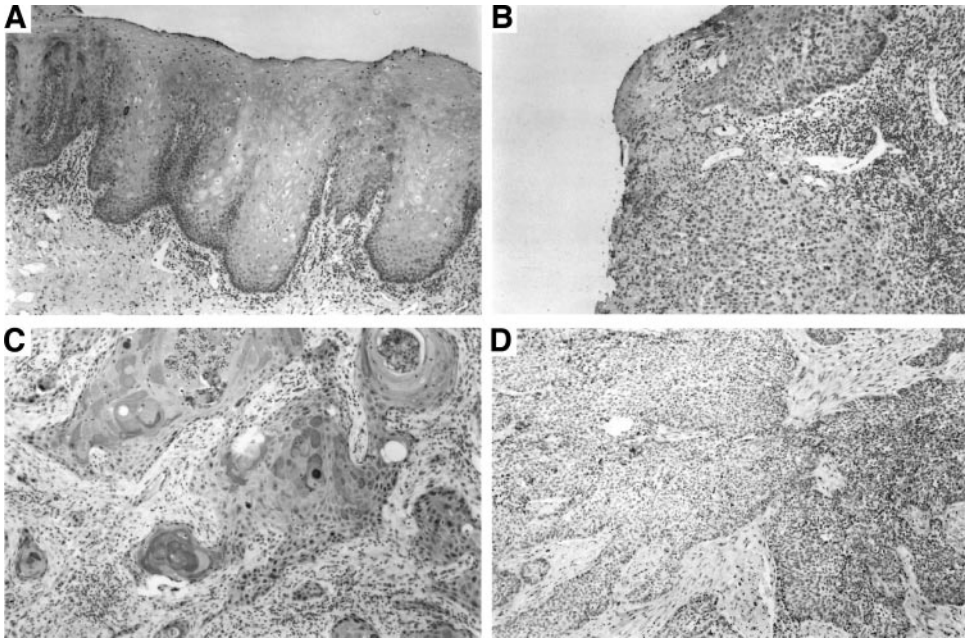
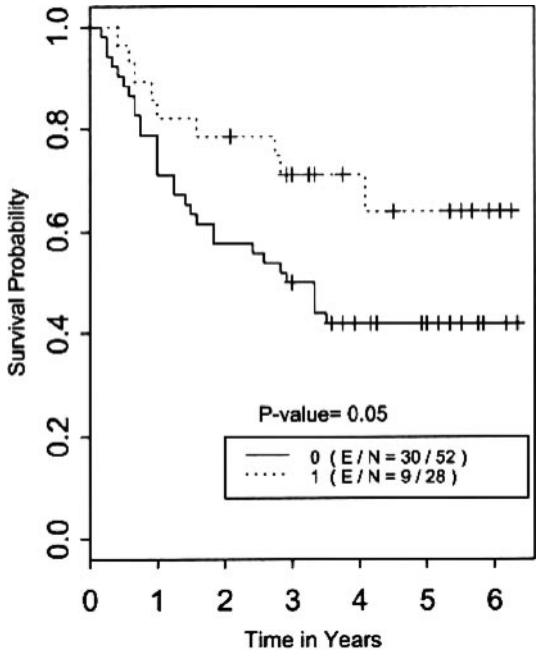


Fig. 1 Immunohistochemical staining patterns of Fhit in HNSCC (×100). *A*, noncancerous epithelium with prominent cytoplasmic Fhit staining in the stratum spinosum and stratum superficiale. *B*, sudden transition from positively stained noncancerous epithelium to negatively stained tumor. *C*, Fhit-positive staining in well-differentiated areas of the tumor nests. *D*, a moderately to poorly differentiated tumor with negative Fhit expression.

parabasal cells (Fig. 1*A*). The staining was cytoplasmic and was also seen in the excretory ducts of the minor salivary glands. In most samples in which tumor cells showed no or reduced Fhit expression (Fig. 1*D*), a sudden transition from normal epithelium having positive Fhit expression to carcinoma having no or reduced staining was observed (Fig. 1*B*). In samples with heterogenous Fhit expression, cancer cells were found to be more prominently positive in the better differentiated tumor areas, especially near areas of keratinization (Fig. 1*C*).

According to our scoring criteria, 52 of the 80 HNSCC samples (65%) were negative for Fhit; the remaining 28 samples (35%) were positive for Fhit. There was not a significant association between Fhit expression and clinical characteristics, including age, gender, tumor size, nodal status, stage, risk group, and tumor grade. Because a precise evaluation of smoking and alcohol consumption was not available for a substantial number of the patients, the potential association between Fhit expression and these factors was not examined.

We also analyzed the potential relationship between Fhit expression status and clinical outcome. Throughout the entire patient population, we found that patients whose tumor exhibited negative Fhit expression had a worse 5-year overall survival [hazard ratio = 0.49; 95% confidence interval (CI), 0.23–1.03; $P = 0.05$ (log-rank test)] than did patients whose tumor exhibited positive Fhit expression. Of the 52 patients whose tumor was negative for Fhit, 30 (58%) died during the follow-up period; in comparison, only 9 of the 28 patients (32%) whose tumor was positive for Fhit died during the follow-up period (Fig. 2). Similarly, patients whose tumor exhibited negative Fhit expression more often had distant metastases (17 of 52 patients, 33%) than did those whose tumor had positive Fhit expression (4 of 28 patients, 14%), although the difference was only marginally statistically significant [hazard ratio = 0.38; 95% CI, 0.13–1.13; $P = 0.07$ (log-rank test)]. However, the difference in disease-free survival between the Fhit-negative and -positive groups was not profound. Paradoxically, patients classified as high risk who had a Fhit-negative tumor experienced locoregional recurrence less often (7 of 38 patients, 18%) than patients in the high-risk group who had a Fhit-positive tumor (6 of 18 patients, 33%). In multivariate analysis, we found that age was the only independent factor for locoregional recurrence in the high-risk group. After adjustment for age (hazard ratio = 0.07; 95% CI, 0.009–0.52; $P = 0.01$), patients whose tumors showed Fhit expression had a higher risk of developing locoregional recurrence earlier (hazard ratio = 1.46; 95% CI, 0.49–4.38; $P = 0.50$), although the difference was not statistically significant.



Fhit = 1				
Time in Years	0	1	2	3
No. of patients at risk	28	23	22	17
No. of events		5	1	2

Fhit = 0				
Time in Years	0	1	2	3
No. of patients at risk	52	37	30	24
No. of events		15	7	5

Fig. 2 Overall survival. *0*, negative Fhit expression; *1*, positive Fhit expression.

DISCUSSION

One of the major concerns in the treatment of HNSCC is radiosensitivity, which is determined according to DNA damage from ionizing radiation and the capacity of DNA repair (21–23). Several molecular markers related to radiosensitivity have been investigated to help in selecting treatment and predicting outcome after radiotherapy (24–27). Loss of Fhit expression has been found to be a marker of poor outcome in several types of cancers, including breast (10), lung (28), colon (29), gastric (9), and tongue cancer (19). However, the relationship between loss of Fhit expression and radiosensitivity has rarely been mentioned in the literature. In this study, we demonstrated for the first time improved locoregional control in high-risk patients with Fhit-negative tumors. Although not statistically significant, these results show that loss of expression of Fhit, a product of the *FHIT* tumor suppressor gene, may paradoxically enhance radiosensitivity. This factor also attenuated the difference in disease-free survival between the Fhit-positive and -negative groups in our study.

It is not clear how Fhit influences radiosensitivity. However, there are some possible explanations. First, the fragility of the DNA structure may play a role. Because the *FHIT* gene encompasses the most active common fragile site, *FRA3B* (3), tumors with negative Fhit expression may exhibit more fragile DNA susceptible to double-strand breaks due to ionizing radiation when compared with tumors with positive Fhit expression. The second possible explanation is the growth-inhibitory effect of Fhit. In a study of a Fhit–re-expressing cell model, it was reported that the growth-inhibitory effect of Fhit could be related to induction of apoptosis and cell cycle arrest at G_0-G_1, possibly through up-regulation of the universal cell cycle inhibitor $p21^{waf1}$ (30). In another study with esophageal cell lines, accumulation of cells at S to G_2-M was observed after adenoviral *FHIT* transduction (31). Given that rapidly dividing cells and cells at G_2-M phase are generally more sensitive to radiotherapy than more slowly dividing cells and cells at other phases, cell cycle arrest may cause radioresistance (32, 33).

Therefore, tumors with positive Fhit expression may be less radiosensitive than tumors with negative Fhit expression.

Despite the better locoregional control observed in high-risk patients with a Fhit-negative tumor, loss of Fhit expression was still associated with significantly poor overall survival in our study. One third of the patients with Fhit-negative tumors had distant metastasis, which contributed to the poor result. This implies that loss of Fhit expression is also involved in the progression of HNSCC. A similar correlation between loss of Fhit expression and distant metastasis has also been observed in colorectal cancer (29). We hypothesize that, without the tumor-suppressing function of Fhit, tumor cells will be more aggressive and more likely to migrate outside the radiotherapy field before treatment. Thus, it is very important to distinguish these patients and administer systemic treatments, such as adjuvant chemotherapy or immunotherapy. On the other hand, in patients with a Fhit-positive tumor, adjuvant therapy may not be necessary due to the lower rate of distant metastasis. However, preradiotherapy radiosensitization strategies should be used to improve locoregional control. Surgery performed as primary or salvage treatment also plays an important role in this patient group.

In summary, our study demonstrated that loss of Fhit expression predicts significantly poor overall survival in patients with HNSCC and an increased rate of distant metastasis. Loss of Fhit expression in high-risk patients contributes to improved locoregional control through surgery and PORT. Therefore, Fhit expression status may be a useful biomarker to stratify patients for novel therapeutic trials, such as those of adjuvant therapy or radiosensitization. However, due to our small sample size, some of the observations were not statistically significant. Further investigation in a larger patient population is required to verify our hypothesis about the potential to use Fhit expression status as a marker for treatment selection.

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