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Fragile Histidine Triad Expression in Oral Squamous Cell Carcinoma and Precursor Lesions

Omar Kujan,¹ Richard Oliver,¹ Luca Roz,⁴ Gabriella Sozzi,⁴ Noel Ribeiro,² Robert Woodward,² Nalin Thakker,^{1,3} and Philip Sloan¹

Abstract **Purpose:** Fragile histidine triad (FHIT) expression in precursor oral lesions (POL) and oral squamous cell carcinomas (OSCC) was studied with regard to (a) the frequency of loss of FHIT expression, (b) whether loss of FHIT expression correlates with degree of dysplasia in POLs, (c) whether FHIT loss predicts high-risk POLs that are more likely to transform, and (d) whether FHIT loss in OSCCs correlates with survival.

Experimental Design: Ninety-four POLs and 86 OSCCs were immunostained for FHIT. Survival analysis was done for cases with validated clinical outcomes.

Results: By optimizing the immunostaining protocol, we found that FHIT is expressed in a distinctive strong nuclear and weak cytoplasmic pattern in oral tissues. Loss of FHIT expression was found in 42 of 94 (45%) POLs and in 66 of 86 (77%) OSCCs. We observed a statistically significant positive correlation between frequency of FHIT loss and increasing grade of dysplasia ($\chi^2 = 13.8$; degrees of freedom = 4; $P = 0.008$). Loss of FHIT expression in POLs that progressed to malignancy was more frequent than in those that did not [17 of 25 (68%) versus 12 of 29 (41.4%), respectively]. This difference was statistically significant ($\chi^2 = 3.8$; degrees of freedom = 1; $P = 0.046$). In OSCCs, loss of FHIT staining indicated a worse prognosis (survival rate, 36.2%) than when positive FHIT staining was observed (survival rate, 50%), but the difference was not statistically significant ($P = 0.546$, Kaplan-Meier, log-rank).

Conclusions: FHIT seems to localize to both nuclear and cytoplasmic domains. FHIT inactivation occurs early in oral carcinogenesis and may be useful molecular marker for progressive dysplastic oral lesions.

Oral squamous cell carcinoma (OSCC) is a major cause of morbidity and mortality globally accounting for ~275,000 new cases and >120,000 deaths per annum (1). Moreover, both incidence and mortality rates are still increasing (2) and the overall 5-year survival rate has remained static at 50% over the last 30 years (3). OSCCs, like many other cancers, show frequent (45-55%) somatic hemizygous deletions (detected as loss of heterozygosity) on the short arm of chromosome 3 (4). Deletions are also detected in precursor oral lesions (POL), which sometimes precede OSCCs (5). The target of the deletions in one specific region, chromosome band 3p14.2, seems to be the *fragile histidine triad (FHIT)* gene (6, 7). It is now well established that *FHIT* is an important tumor suppressor gene that is down-regulated in most human cancers

(6, 8). Intragenic deletions and aberrant transcripts have been reported in head and neck SCC and more specifically in OSCC, although there seems to be a disagreement about the frequency of the latter. FHIT promoter methylation associated with loss of FHIT expression has been reported in ~70% of head and neck tumors and has been shown to correlate with poor outcome (9-11).

In this study, we sought to extend the previous studies by studying FHIT expression in a large series of POLs and OSCCs. We sought to address the following questions. (a) What is the frequency of loss of FHIT expression in these lesions from patients from the United Kingdom? (b) Does the loss of FHIT expression correlate with degree of dysplasia in POLs as assessed by the new WHO classification (11)? (c) Can the loss of FHIT predict high-risk POLs that are more likely to progress to malignancy? (d) Does the loss of FHIT expression in carcinomas correlate with survival?

Materials and Methods

Patients. We obtained ethics approval for the study from a National Health Service Local Research Ethics Committee (reference no. 02/104). Our total study sample series consisted of 190 formalin-fixed, paraffin-embedded oral mucosal biopsies from the period 1993 to 2003, which we retrieved from the archives of the Unit of Oral Pathology, Manchester Dental Hospital (Manchester, United Kingdom; ref. Table 1). Two pathologists reviewed all cases to confirm diagnosis;

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Table 1. Number of oral epithelial samples that were enrolled in the study

Type of investigated tissue	No.
Normal oral mucosa	10
Mild dysplasia	10
Moderate dysplasia	10
Severe dysplasia	10
CIS	10
Oral epithelial dysplasia cases that showed malignant transformation	25
Oral epithelial dysplasia cases that showed no OSCC progression	29
OSCC	86

we graded the degree of dysplasia in POLs according to the WHO 2005 classification (12) and staged the tumors according to the International Union Against Cancer tumor-node-metastasis classification (13) by consensus reporting between two observers (O.K. and P.S.).

We studied three main groups of samples:

1. Group 1: this consisted of 50 cases with 10 specimens each from 5 different categories: normal oral epithelium, POLs with mild, moderate, and severe dysplasia, and carcinoma *in situ* (CIS).
2. Group 2: this consisted of 54 unifocal POLs with epithelial dysplasia and known clinical outcomes. Of these, 19 cases were diagnosed with mild dysplasia, 14 with moderate dysplasia, 16 with severe dysplasia, and 5 cases as CIS. This set was divided into two main groups according to the clinical outcomes. Twenty-five unrelated cases, in which the patients subsequently had developed invasive OSCC, and 29 unrelated cases, in which the patients did not develop OSCC during the period 1993 to 2004. Follow-up ranged from 2 to 97 months (mean, 29.9 months; SD, 25.4). Of these, 22 (40.7%) patients were female and 32 (59.3%) were males.
3. Group 3: this consisted of 86 OSCCs. Of these, 61 cases were treated with surgery by the same consultant at North Manchester General Hospital (Manchester, United Kingdom) during the period 1993 to 1998 and followed up for at least 5 years (range, 1-102; mean, ± 50.8 ; SD, ± 33.4 months). The clinical details of the samples are shown in Table 2. The overall 5-year survival rate was 39.3% (range, 34.2-87.7%; mean, ± 57.5 %; SD ± 5.1). The remaining 25 cases were included in the observational study of FHIT expression but were excluded from the survival analysis.

FHIT immunohistochemistry. An optimization study of FHIT immunostaining was done on human normal oral mucosa ($n = 5$), submandibular salivary gland ($n = 5$), and OSCCs ($n = 10$) accessioned in the routine service but not included in the subsequent study. We used a primary rabbit polyclonal anti-glutathione S-transferase-FHIT antibody (Zymed Laboratories, South San Francisco, CA), together with a secondary mouse biotinylated link and a streptavidin-horseradish peroxidase conjugate (DAKO LSAB kit, DAKO, Glostrup, Denmark) with diaminobenzidine as the chromogen. Under optimal conditions with antigen retrieval by microwaving for 15 minutes in 10 mmol/L citrate buffer (pH 6), FHIT was strongly expressed in the nucleus of normal basal and parabasal oral epithelial cells and in 4 of 10 SCCs, accompanied by cytoplasmic staining of variable intensity. In the carcinomas, all stromal cells expressed strong nuclear and moderate cytoplasmic staining. In contrast to previously published studies that reported exclusively cytoplasmic localization of FHIT, we showed a nuclear and cytoplasmic localization of the FHIT protein by a variety of biochemical and immunohistochemical methods.⁵ The optimized

method was used to immunostain all of the included cases. The stromal cells were used as a positive internal control and for negative control we omitted the primary antibody.

We categorized FHIT immunoreactivity as 'negative' or 'low expression' (no staining or immunoreactivity staining present in <10% of tumor cells) or 'positive' (immunoreactivity in $\geq 10\%$ of tumor cells) as described previously (9).

Statistical analysis. Kruskal-Wallis, ANOVA, and χ^2 tests were used to test the association of FHIT protein expression with different variables of the analyzed lesions. Survival curves for overall survival time were estimated by the Kaplan-Meier test, and the resulting curves were compared using the log-rank test. Multivariate analysis was done

Table 2. The clinicopathologic characteristics of the OSCC patients with known follow-up data

Characteristics	No. cases (%)
Total number of valid cases	61 (100)
Age (y)	
≤ 50	2 (3.3)
> 50	59 (96.7)
Gender	
Male	42 (68.9)
Female	19 (31.1)
Stage	
I	13 (21.3)
II	10 (16.4)
III	22 (36.1)
IV	16 (26.2)
Histologic grading	
Well-differentiated SCC	25 (41)
Moderately differentiated SCC	17 (27.9)
Poorly differentiated SCC	18 (29.5)
Basaloid SCC	1 (1.6)
Tumor size	
T ₁	14 (23)
T ₂	24 (39.3)
T ₃	9 (14.8)
T ₄	14 (23)
Nodal status	
N ₀	38 (62.3)
N ₁	14 (23)
N ₂	9 (14)
Metastases	
M ₀	54 (88.5)
M ₁	7 (11.5)
Recurrence in neck	15/61 (24.6)
Recurrence in primary site	11/61 (18)
New primary tumor	12/61 (19.7)
Death	37 (60.7)
Oral cancer-related death	22/37 (59.5)
Death related to other diseases	15/37 (40.5)
Smoking habits*	
Smoker	34 (55.7)
Nonsmoker	13 (21.3)
Not recorded	14 (23)
Alcohol habits*	
Drinker	36 (59)
Nondrinker	11 (18)
Not recorded	14 (23)
Site	
Tongue	26 (42.6)
Floor of mouth	16 (26.2)
Maxilla	3 (4.9)
Buccal mucosa	4 (6.6)
Retromolar trigone	7 (11.5)
Gingiva/alveolus	5 (8.2)

*Data are incomplete for all patients.

⁵ In preparation.

Fig. 1. FHIT expression in oral epithelial tissues. *A*, normal oral squamous epithelium. *B*, ducts of minor salivary gland. *C*, oral epithelial dysplasia (+). *D*, oral epithelial dysplasia (–).

according to Cox proportional hazards model. All tests were two sided. $P < 0.05$ was considered to be statistically significant.

Results

As expected, we observed uniform, strong, positive immunoreactivity for FHIT in normal surface oral epithelium in all cases. This was more prominent in the basal cell layer with the superficial layers showing little or no immunoreactivity. Indeed, a clear demarcation between the stained and non-

stained areas was found in all the cases with normal oral squamous epithelium (Fig. 1A). We also observed strong immunoreactivity in excretory ducts of the underlying minor salivary glands (Fig. 1B).

Table 3. Frequency of loss of FHIT expression in normal mucosa, POLs, and OSCCs

Lesion	Frequency of loss of FHIT expression, n/N (%)
Normal oral epithelium	0/10 (0)
POL	42/94 (45)
POL with mild dysplasia	9/29 (~31)
POL with moderate dysplasia	9/24 (~38)
POL with severe dysplasia	14/26 (~54)
POL with CIS	10/15 (~67)
OSCC	66/86 (~77)

Fig. 2. Analysis of FHIT expression in a model of the natural history of oral carcinogenesis.

Table 4. FHIT expression in progressive and nonprogressive POLs

	FHIT expression, <i>n</i> (%)		Total
	Negative	Positive	
Nonprogressive POLs	12 (22.2)	17 (31.5)	29 (53.7)
Progressive POLs	17 (31.5)	8 (14.8)	25 (46.3)
Total	29 (53.7)	25 (46.3)	54 (100)

Altogether, we studied 104 cases (50 from group 1 and 54 from group 2) with variable grades of oral epithelial dysplasia or CIS and normal epithelium. Overall, 42 of 94 (45%) of POL samples showed reduced or absent FHIT expression (Table 3). We observed a statistically significant ($\chi^2 = 13.8$; degrees of freedom = 4; $P = 0.008$) positive correlation between frequency of loss of expression with increasing grade of dysplasia (Fig. 1C and D; Fig. 2; Table 3). Thus, for example, we observed loss of FHIT expression in ~31% of POLs with mild dysplasia compared with 67% POLs with CIS. OSCCs showed the highest frequency [66 of 86 (~77%)] of loss of FHIT.

In the progressive and nonprogressive POLs (group 2; Table 4), loss of FHIT expression in POLs that progressed to malignancy was more frequent than in those that did not within the defined period [17 of 25 (68%) versus 12 of 29 (41.4%), respectively]. This difference was statistically significant ($\chi^2 = 3.8$; degrees of freedom = 1; $P = 0.046$).

In the OSCCs (group 3), we found no association between FHIT expression (Fig. 3A and B) and clinical variables, including age, gender, stage, histologic grading, tumor size, lymph node metastasis, recurrence in neck, recurrence in primary site, and new primary tumor, and the clinical outcomes (Table 5). We were unable to correlate FHIT expression with tobacco or alcohol consumption as complete data were not available.

We also correlated FHIT expression with survival in this group using Kaplan-Meier analysis with log-rank test. The mean follow-up time for the 61 subjects was 50.8 months (range, 1-102 months). At the end of the follow-up period, 37 patients had died.

Twenty-two subjects of 37 had died from oral carcinoma. Moreover, 11 subjects had relapsed by the time of the last

follow-up. The survival analysis was done on 61 patients and took into account the following variables: FHIT expression, age, gender, histologic type, tumor size, nodal involvement, metastases, and stage. The overall survival rate was 39.4%. A Kaplan-Meier curve showed that patients with either negative or low expressed FHIT staining had a worse prognosis (survival rate, 36.2%) than those of patients with positive FHIT staining (survival rate, 50%). However, this difference was not statistically significant ($P = 0.546$, log-rank; Fig. 4).

Multivariate analysis was used to assess the independent influence of other prognostic variables on the survival curve. Stage, lymph node metastasis, tumor size, relapse at the primary site, and FHIT expression were included. No statistically significant associations were found between the variables and any significant effect on prognosis, except that relapse at the primary site showed association with poor prognosis (relative risk, 2.561; $P = 0.011$).

Discussion

Reduced or absent expression of FHIT has been reported in primary cancers, in cancer cell lines, and in precancerous lesions (reviewed in refs. 6, 8, 14, 15). In addition to hemizygous or homozygous deletions affecting the *FHIT* gene in cancers, epigenetic changes, such as DNA methylation and histone modification, may lead to decreased expression (reviewed in refs. 16, 17). Immunohistochemistry is a useful method for showing loss of FHIT expression in tumors where FHIT may be inactivated by a variety of molecular mechanisms. Review of published photomicrographs and descriptions of FHIT expression in normal human tissues and cancers shows that exclusively cytoplasmic expression has been regarded as typical (9, 11, 18–22). Many studies have used various antigen retrieval methods and this has typically produced intense diffuse labeling of the cytoplasm by chromogen (11, 19, 23–26). Our immunohistochemical optimization study for FHIT staining used antigen retrieval methods, including no pretreatment, proteinase K digestion, and trypsin digestion. Without pretreatment, only weak nonspecific staining was found. However, proteinase K or trypsin pretreatment resulted in diffuse cytoplasmic immunoreactivity that was similar to that published in many previous articles (11, 19, 23–26). In squamous epithelium, all layers of the epithelium were

Fig. 3. FHIT expression in OSCC. A, OSCC (+). B, OSCC (–).

Table 5. Association between FHIT expression and clinicopathologic variables in OSCC

Factors	Percentage of FHIT expression in tumor cells		P
	Negative (<10)	Positive (≥10)	
	n (%)	n (%)	
Age (y)			
≤50	2 (3.3)		0.433
>50	45 (73.7)	14 (23)	
Gender			
Male	32 (52.5)	10 (16.4)	0.813
Female	15 (24.5)	4 (6.6)	
Stage			
I	12 (19.7)	1 (1.6)	0.077
II	9 (14.8)	1 (1.6)	
III	13 (21.3)	9 (14.8)	
IV	13 (21.3)	3 (4.9)	
Histologic grading			
Well-differentiated SCC	19 (31.1)	6 (9.9)	0.455
Moderately differentiated SCC	15 (24.6)	2 (3.2)	
Poorly differentiated SCC	12 (19.7)	6 (9.9)	
Basaloid SCC	1 (1.6)	—	
Tumor size			
T ₁	13 (21.3)	1 (1.6)	0.131
T ₂	18 (29.5)	6 (9.9)	
T ₃	4 (6.5)	5 (8.2)	
T ₄	12 (19.7)	2 (3.2)	
Nodal status			
N ₀	30 (49.2)	8 (13.1)	0.848
N ₁	10 (16.4)	4 (6.5)	
N ₂	7 (11.5)	2 (3.2)	
Metastases			
M ₀	42 (68.9)	12 (19.7)	0.707
M ₁	5 (8.2)	2 (3.2)	
Recurrence in neck	10 (16.4)	5 (8.2)	0.271
Recurrence in primary site	10 (16.4)	1 (1.6)	0.227
New primary tumor	9 (14.8)	3 (4.9)	0.851
Present status			
Alive	17 (27.9)	7 (11.5)	0.352
Dead	30 (49.2)	7 (11.5)	
Oral cancer-related death	17 (27.9)	5 (8.2)	0.975
Death related to other diseases	13 (21.3)	2 (3.2)	0.308
Smoking habits*			
Smoker	25 (41)	9 (14.8)	
Nonsmoker	9 (14.8)	4 (6.5)	
Alcohol habits*			
Drinker	26 (42.6)	10 (16.4)	
Nondrinker	8 (13.1)	3 (4.9)	

*Data are incomplete for all patients.

intensely labeled, including keratin. However, when we used microwaving in citrate buffer for 15 minutes, we obtained convincing nuclear with weaker cytoplasmic staining and clean negative controls. In addition, a distinctive pattern of labeling that was restricted to the basal and suprabasal cell layers in oral epithelium was seen, consistent with inactivation of *FHIT* with progressive epithelial maturation. The findings are consistent with reports of *FHIT* localization in rat tissues where biochemical methodology showed that *FHIT* was localized to the cytoplasmic membrane and nucleus in normal tissues (27) and in human monocyte cell lineages (28). To our knowledge, our optimization study is the first to show nuclear *FHIT* immunoreactivity in human epithelial tissues. Further biochemical and immunohistochemical studies in human tissues are in progress to validate this observation.

FHIT and its protein product have been the focus of recent debate with regard to its role in tumorigenesis in bronchial (29), esophageal (30), colorectal (31), breast (32), and cervical (33) cancer. A tumor suppressor role for *FHIT* was postulated based on the ability of *FHIT* to eliminate or reduce the tumorigenicity of tumor cells in nude and knockout mice (34, 35) and is now widely accepted (6). Several reports showed previously that loss of heterozygosity occurs frequently at the chromosome region containing *FHIT* (4, 5, 36) and that the expression of *FHIT* is frequently altered in head and neck SCC (11, 19, 21, 37, 38) and POLs (6, 22, 39).

In this study, consistent with other studies (29, 39, 40), we have shown that *FHIT* is down-regulated early during carcinogenesis and that its down-regulation is associated with increasing dysplasia grade in POLs. In addition, the loss of

Fig. 4. Kaplan-Meier curves for overall survival stratified according to the level of FHIT expression in patients with OSCC.

FHIT expression was observed more frequently in precursor lesions that developed invasive SCC. More interestingly, a large fraction of SCCs displayed reduced or absent FHIT expression. Studies on cancer evolution showed that genomic alterations that inactivate suppressor genes or activate oncogenes could relieve cells from growth constraints, allowing tumor growth (41). *FHIT* is a tumor suppressor gene but its pathway is still unclear (6, 15). The down-regulation of FHIT may reflect a growth deregulation adding proliferation advantages.

There is a clear need for improved tools to identify, at an early stage, lesions that will develop into OSCC. Study of the natural history of oral cancer and precancer should identify critical stages that can be used to develop preventive strategies or for early detection that may improve the survival rate associated with oral cancer. We attempted to study the role of FHIT in a multistep malignant transformation model of oral carcinogenesis (42). Our results showed that FHIT down-regulation is an early frequent event in oral carcinogenesis and may provide a predictor marker for disease progression and outcome alongside other conventional clinicopathologic and molecular markers that are in use. Many molecular markers have been postulated as good predictors (43, 44). However, the application of these markers has not yet translated into daily routine service. Current candidate markers lack a clear cutoff point value in determining cases that will transform into malignant disease. We suspect that using a panel of these

prognostic markers would help in improving the accuracy of predicting malignant change in POLs.

Our data showed that most invasive OSCCs displayed reduced or absent FHIT expression (76.8%), which is larger than the findings of other studies. Previous studies showed a range of 4% to 68% with absent or markedly reduced FHIT expression in OSSC (9–11, 18, 20, 21, 37). This discrepancy may depend on the specificity and sensitivity related to different FHIT antibodies, staining protocol, scoring system, and tissue collection.

A few studies have shown that absent or low levels of FHIT protein are associated with poor prognosis (reduced overall survival time) or with advanced stage in OSSC (9–11). We noted that FHIT inactivation was associated with worse prognosis in OSSC but the effect was not statistically significant. In addition, we did not find any correlation between FHIT expression levels and clinicopathologic variables, such as degree of differentiation, lymph node status, and stage. Similar findings were presented by Mineta et al. (20) and Paradiso et al. (21). In addition, the multivariate Cox regression model failed to find any significant correlation between FHIT expression and any prognostic variables, except that the relapse at the primary site showed association with poor prognosis (relative risk, 2.561; $P = 0.011$), which is a common finding in OSCC.

In the literature, there is controversy about the prognostic significance of FHIT in primary OSCC. This dispute originates from studies using either immunohistochemistry or molecular techniques that are mostly based on heterogeneous samples of OSCCs (10, 20, 21). It can be argued that the best method to evaluate FHIT expression in a primary tumor is immunohistochemistry because the proportion of cells exhibiting the protein, as well as their level of expression, can be determined and the method can be easily used in practice (10, 18, 20).

Conclusions

Our observations of the loss of FHIT protein expression in a substantial percentage of oral precursor and cancerous lesions suggest that FHIT alterations play an important role in the development of oral cancer. FHIT inactivation may be useful for predicting malignant changes in biopsies of oral precursor lesions. Cells collected from brushing the oral mucosa could also be a valuable tool for the surveillance of oral dysplasia lesions (45). Moreover, due to the high frequency of FHIT inactivation in oral carcinogenesis and the successful restoration of FHIT in cell lines and mice (35, 46–48), the *FHIT* gene could present an eligible target for gene therapy for POLs and for prevention of OSCC.

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