Loss of Fhit Expression in Invasive Cervical Carcinomas and Intraepithelial Lesions Associated with Invasive Disease

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ABSTRACT

Allelic losses involving chromosome 3p are frequently observed in cervical cancers. Deletion mapping studies of primary cervical carcinomas have localized common regions of deletion to 3p14.2 and 3p21. The candidate tumor suppressor gene FHit has been mapped to 3p14.2, and previous studies have demonstrated reduced or aberrant FHit transcripts and reduced or absent Fhit protein expression in a large percentage of cervical cancer-derived cell lines and primary cervical carcinomas. To expand these observations to preinvasive cervical epithelial lesions and to determine whether loss of Fhit protein expression might be associated with tumor progression, immunohistochemical methods were used to examine Fhit expression in 95 invasive cervical carcinomas, 33 high-grade squamous intraepithelial lesions (HSILs) associated with concurrent invasive cancer, 38 HSILs unassociated with invasive cancer, 24 low-grade squamous intraepithelial lesions, and 22 normal cervix samples. All normal cervical epithelia and low-grade squamous intraepithelial lesions exhibited diffuse cytoplasmic immunostaining of moderate to strong intensity. Fhit protein expression was markedly reduced or absent in 67 of 95 (71%) invasive cancers, 17 of 33 (52%) HSILs associated with invasive cancer, and 8 of 38 (21%) HSILs without associated invasive cancer. The results confirm that Fhit protein expression is reduced or absent in the majority of cervical carcinomas and suggest that loss of Fhit expression often accompanies cervical tumor progression. Moreover, absent or reduced Fhit protein is observed at a significantly higher frequency in HSILs associated with progression to invasive cancer than in HSILs with unknown risk for progression (P = 0.012). These findings suggest that loss of Fhit expression in HSILs could serve as a useful marker of high-grade preinvasive lesions that have an increased likelihood of progression to invasive carcinoma.

INTRODUCTION

Cervical cancer is the third most common cancer in women, with approximately 371,200 new cases diagnosed each year worldwide (1). Overall, the ratio of mortality to incidence is 51%; hence, cervical cancer remains a significant public health concern (1). Detection of cervical carcinoma precursors and early cancers through pap smear screening programs has proven to be a very effective means with which to reduce mortality from cervical cancer (1). However, it is estimated that only 12–22% of high-grade preinvasive cervical lesions will progress to invasive carcinoma if left untreated, and morphological examination alone does not allow distinction of those lesions likely to progress from those that will regress or simply persist (2, 3). Hence, molecular markers to aid in this distinction and to enhance diagnosis of early cervical lesions would have great clinical utility.

Numerous studies have strongly implicated infection with certain “high-risk” HPVs⁵ as one of the initiating events in the development of cervical carcinoma (reviewed in Ref. 4). Although it is well accepted that high-risk HPVs are associated with cervical cancer, several lines of evidence suggest that HPV infection alone is insufficient for the malignant transformation of HPV-infected cells. For example, although infection with HPV is very common, most infected women do not develop invasive cancer (5). Those women who do develop invasive cervical cancer usually do so only after several years (6). Hence, other genetic events, such as oncogene activation or tumor suppressor gene inactivation, are likely to be required in addition to infection with HPVs for the development of cervical cancer.

⁵The abbreviations used are: HPV, human papillomavirus; HSIL, high-grade squamous intraepithelial lesion; LSIL, low-grade squamous intraepithelial lesion; LOH, loss of heterozygosity.
Analysis of allelic losses of specific chromosomal regions has been used to identify candidate tumor suppressor genes involved in the development of cancer. Several groups of investigators have analyzed primary cervical carcinomas, as well as preneoplastic lesions of the cervix, and demonstrated a particularly high frequency of allelic losses of chromosome 3p (7–11). High-resolution analysis of chromosome 3p alterations resulted in the identification of two distinct regions of allelic imbalance, 3p14.2 and 3p21, that occur in over 50% of cervical cancers (12, 13). Notably, losses of heterozygosity on chromosome 3p have also been shown to occur in preinvasive cervical lesions (12, 14).

The candidate tumor suppressor gene, FHIT, maps to chromosome band 3p14.2 (15). The FHIT gene contains at least 10 exons and spans approximately 1 Mb of genomic DNA. The open reading frame, beginning in exon 5 and ending in exon 9, encodes a 16.8-kDa protein that has been shown to function as a 5′,5″-P1,P3-triphosphate (Ap 3 A) hydrolase in vitro (16). Other studies have provided evidence supporting a role for FHIT in the regulation of apoptosis and the cell cycle (17, 18). Several lines of investigation have led to the proposal that FHIT is a candidate tumor suppressor gene targeted by 3p14 allelic losses in several epithelial cancers (reviewed in Refs. 19–21). Findings that support the candidacy of FHIT as a tumor suppressor gene include: (a) inclusion of the t(3;8) (p14.2;q24) translocation breakpoint in a familial renal carcinoma kindred within the FHIT locus; (b) homozygous deletions of FHIT (some of which encompass exons) in several cancer cell lines and primary tumors; and (c) aberrant FHIT transcripts and absent or reduced Fhit protein expression in several types of epithelial cancers.

Preliminary studies have revealed marked reduction or absence of FHIT mRNA and protein expression in over half of cervical carcinomas and in a substantial percentage of HSILs (encompassing lesions previously referred to as moderate and severe dysplasias and carcinoma in situ), but not in LSILs (22–24). In light of these findings, we were interested in analyzing a large group of preneoplastic cervical lesions (LSILs and HSILs) and invasive squamous carcinomas to determine whether loss of expression of Fhit is a relatively early or late event in cervical carcinogenesis. Of particular interest in this study was the comparison of Fhit protein expression in HSILs with associated invasive carcinoma versus HSILs without invasive carcinoma, in an attempt to evaluate whether the loss of Fhit protein might be a clinically useful marker of lesions more likely to progress to invasive disease.

MATERIALS AND METHODS

Tissue Specimens. Formalin-fixed, paraffin-embedded tissue specimens from a total of 212 individual patients were evaluated. Of these 212 cases, 5 normal cervix specimens and 33 invasive cancers have been reported previously (23). Three normal cervix specimens were obtained from the Surgical Pathology archives of the University of Michigan Hospital. Twenty-seven invasive cancers (25 squamous cell carcinomas, 1 clear cell carcinoma, and 1 adenocarcinoma), 38 HSILs, 24 LSILs, 4 HSILs associated with invasive carcinoma, and 19 normal cervix specimens were obtained from the Surgical Pathology archives of the Johns Hopkins Hospital. Sixty-eight invasive cancers (66 squamous cell carcinomas and 2 adenocarcinomas) and 25 HSILs with adjacent invasive carcinoma were obtained from previous studies conducted in Spain, Colombia, and the Philippines (25–27). Four cases of HSIL with adjacent invasive cancer were obtained from the University of Auckland (Auckland, New Zealand). The histopathological diagnosis of all specimens was confirmed by a gynecological pathologist (K. R. C.). Formalin-fixed, paraffin-embedded specimens of previously reported tumor xenografts of the cervical cancer cell line C33a (negative for Fhit protein expression) and C33a transfected with the FHIT gene (positive for Fhit protein expression) were used as negative and positive controls, respectively, for immunohistochemical staining (28).

Immunohistochemistry. Immunohistochemical detection of Fhit protein has been described previously (23, 28). Briefly, 5-μm sections from representative tissue blocks were cut and mounted on Probe-On Plus or Superfrost Plus slides (Fisher Scientific, Itasca, IL), deparaffinized in xylene, and then rehydrated into distilled H2O through graded alcohols. Antigen retrieval was enhanced by microwaving slides in citrate buffer (pH 6.0, Biogenex, San Ramon, CA) for 10 min. Endogenous peroxidase activity was quenched by incubation with 6% hydrogen peroxide in methanol. Slides were then incubated with primary rabbit polyclonal glutathione S-transferase-Fhit antiserum (generously provided by Dr. Kay Heubner, Kimmel Cancer Center, Thomas Jefferson University, Philadelphia, PA) at a dilution of 1:4000 overnight at 4°C (29). Slides were washed three times in PBS and then incubated with a biotinylated goat antirabbit secondary antibody for 30 min at room temperature. Antigen-antibody complexes were detected with the avidin-biotin-peroxidase method using diaminobenzidine as a chromogenic substrate (Vectastain ABC kit, Vector Laboratories, Burlingame, CA) per the manufacturer’s protocol. Tissue sections were lightly counter-stained with hematoxylin and then examined by light microscopy.

Immunostained samples were scored on a three-tiered scale for both intensity (absent or weak, 1; moderate, 2; strong, 3) and extent (percentage of positive cells: <10%, 1; 10–50%, 2; >50%, 3), as we have described previously (23). The intensity and extent scores were multiplied to give a composite score of 1–9 for each specimen. Composite scores of 1–3 were defined as having absent or reduced Fhit protein expression, and scores of 4–9 were considered to be positive for Fhit protein expression. Slides were scored independently by two investigators (D. C. C. and K. R. C.). The rare cases with discordant scores were re-evaluated and scored on the basis of consensus opinion.

Statistical Analysis. The analysis used dichotomized composite immunostaining scores for Fhit expression; scores of 1–3 were considered negative and scores of 4–9 were considered positive. Comparisons between groups were analyzed in a pairwise fashion and were tested using Fisher’s exact test.

RESULTS

Fhit Expression in Normal Cervical Epithelium and Squamous Intraepithelial Cervical Lesions Unassociated with Invasive Carcinoma. All tissue specimens were evaluated for expression of Fhit protein by immunohistochemical detection, and the resultant data are summarized in Table 1. The
The specificity of the antiglutathione S-transferase-Fhit polyclonal antiserum and its utility for immunohistochemical detection of Fhit protein have been evaluated and reported previously (23). All 22 normal cervix specimens and 24 LSILs showed diffuse immunoreactivity of moderate to strong intensity (Fig. 1, A and B). Of the 38 HSILs without associated invasive carcinoma, 8 (21%) had weak or absent FHTT immunoreactivity (composite score, ≤3; Fig. 1C), which was significantly different from the uniform retention of Fhit expression observed in normal cervix (P = 0.022) and LSILs (P = 0.019). The remaining 30 HSILs without associated invasive carcinoma (79%) had a composite score of ≥4 and were considered to be positive for Fhit expression (Fig. 1D).

**Table 1** Fhit protein expression in normal cervix, preneoplastic lesions, and cervical cancers

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>No. of cases</th>
<th>Reduced or absent (%)</th>
<th>Positive (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>22</td>
<td>0 (100)</td>
<td>22 (100)</td>
</tr>
<tr>
<td>LSIL</td>
<td>24</td>
<td>0 (100)</td>
<td>24 (100)</td>
</tr>
<tr>
<td>HSIL</td>
<td>38</td>
<td>8 (21)</td>
<td>30 (79)</td>
</tr>
<tr>
<td>HSIL + cancer</td>
<td>33</td>
<td>17 (52)</td>
<td>16 (48)</td>
</tr>
<tr>
<td>Cancer</td>
<td>95</td>
<td>67 (71)</td>
<td>28 (29)</td>
</tr>
</tbody>
</table>

*a Composite score, intensity × extent (see “Materials and Methods”).

*b The number in parentheses indicates the percentage of cases with the indicated level of Fhit expression.

**Fhit Protein Expression in Primary Cervical Cancers.** A total of 95 primary cervical carcinomas were evaluated for Fhit protein expression by immunohistochemistry. Of these, 91 were squamous cell carcinomas, 2 were adenosquamous carcinomas, 1 was an adenocarcinoma, and 1 was a clear cell carcinoma. Results of the Fhit immunostaining are summarized in Table 1 and revealed that 67 of 95 cancers (71%) had a composite score of ≤3, indicating reduced or absent FHTT protein expression (Fig. 1G). The remaining 28 (29%) demonstrated diffuse immunoreactivity of moderate to strong intensity (Fig. 1H). All three of the adenocarcinomas and adenosquamous carcinomas and the single clear cell carcinoma were found to have reduced or absent Fhit protein expression (composite score, ≤3). Loss of Fhit expression was observed significantly more frequently in invasive carcinomas than in HSILs with unknown risk of progression to invasive cancer (P < 0.001). Loss of Fhit was also observed more frequently in invasive carcinomas than in HSILs associated with invasive carcinoma, but this difference did not achieve statistical significance (P = 0.057).

**DISCUSSION**

Although infection with HPVs has been established as an important initiating event in the development of preinvasive lesions of the cervix, relatively few will progress to invasive carcinoma if left untreated (2, 3). Because of a lack of morphological criteria and/or molecular markers known to be associated with progression of intraepithelial lesions to invasive cancer, there is currently no reliable means of distinguishing between patients who would benefit from aggressive treatment of intraepithelial disease and those who can consider more conservative management. Because ablative treatment modalities employing electrocautery, cryotherapy, or laser are not without consequence (e.g., increased risk of cervical stenosis and cervical incompetence), identifying HSILs with little potential for progression to invasive cancer would have a significant clinical impact. Unfortunately, prospective studies evaluating the utility of candidate molecular markers to predict progression of high-grade intraepithelial lesions are not feasible, given the unacceptable risks associated with a “watch and wait” type of approach.

Efforts to characterize the genetic alterations that occur in cervical carcinogenesis have revealed several chromosomal regions that have a high frequency of allelic loss in cervical cancers (10, 11, 30). Chromosomal region 3p14.2 has been shown by numerous investigators to be commonly deleted in both invasive cervical carcinomas and in intraepithelial precursor lesions (12–14). The identification of the FHTT gene, a candidate tumor suppressor gene that overlaps a common fragile region (FRA3B) on chromosome 3p14.2, led several groups to investigate its potential role in cervical carcinogenesis. We and others have demonstrated that a high percentage of cervical cancer cell lines and primary cervical carcinomas have both altered FHT gene transcripts and reduced or absent Fhit protein expression (22–24, 31, 32). In addition, recent analyses of premalignant lesions of the cervix showed that 33% of HSILs...
and 8% of LSILs have reduced or absent Fhit protein expression (24). Collectively, investigations of the role of the FHIT gene in cervical carcinoma suggest that the loss of FHIT mRNA and protein expression is likely to occur in greater than 50% of primary cervical carcinomas and in a significant percentage of HSILs, but only rarely in LSILs.

In this study, we analyzed a large group of preneoplastic lesions (24 LSILs and 71 HSILs) and 95 invasive cancers to expand these initial observations and to determine whether loss of Fhit expression was an early or late event in cervical carcinogenesis. Of specific interest was the comparison of Fhit protein expression in 33 HSILs with associated invasive carcinoma versus 38 HSILs without invasive carcinoma to evaluate whether loss of FHIT protein expression might be a molecular marker of lesions more likely to progress to invasive disease. Of the total of 71 HSILs in this study, 25 (35%) exhibited reduced or absent Fhit protein expression. Similarly, Birrer et al. (24) reported that 11 of 33 (33%) HSILs had reduced or absent Fhit protein expression. The HSILs without associated invasive carcinoma showed loss of Fhit protein expression in 8 of 38 (21%) of the cases, whereas 17 of 33 (52%) of the HSILs associated with invasive carcinoma showed reduced or absent Fhit protein expression ($P = 0.012$). These results indicate that loss of Fhit protein expression is a relatively late event in cervical carcinogenesis and, perhaps more significantly, that loss of Fhit protein expression in HSILs may be clinically useful molecular marker.

**Fig. 1** Representative immunostaining of Fhit protein in cervical tissue samples encompassing a spectrum of cervical epithelial lesions. 

- A, normal cervix showing diffuse immunoreactivity of the differentiating squamous epithelial cells; 
- B, LSIL with detectable Fhit protein in most of the epithelial cells; 
- C, HSIL lacking Fhit protein; 
- D, HSIL showing diffuse expression of Fhit; 
- E, HSIL and underlying invasive carcinoma, both with absence of Fhit expression; 
- F, HSIL and underlying invasive carcinoma, both with retention of Fhit expression; 
- G, immunonegative invasive squamous carcinoma; 
- H, invasive squamous carcinoma with retention of Fhit expression. ×200.
of preneoplastic lesions with a greater likelihood of progression to invasive cervical carcinoma.

Another recent study has attempted to define clinically useful biomarkers predictive of premalignant cervical lesions more likely to progress to invasive disease (33). Allelic losses and microsatellite alterations at 3p14.2 and other chromosomal loci were evaluated in recurrent and nonrecurrent cases of cervical dysplasia. Lin et al. (33) found that both LOH and microsatellite alterations at FHIT occurred at significantly higher frequencies in the dysplastic lesions known to recur compared to the nonrecurrent lesions ($P = 0.005$ and $P = 0.0025$, respectively). LOH at FHIT occurred in 7 of 12 (58%) informative recurrent lesions and in only 1 of 4 (7%) of the nonrecurrent lesions. Notably, the frequency of LOH in recurrent lesions (58%) is virtually the same as the loss of Fhit protein expression in HSILs associated with invasive cancers (52%) observed in the present study. Significantly higher frequencies of microsatellite alterations at both 3p14.2 (FHIT) and 17p13 (p53) were also observed in recurrent versus nonrecurrent lesions (33). This increased frequency of microsatellite alterations suggests that genomic instability is associated with lesions more likely to progress to invasive disease.

After the initial identification of FHIT in 1996, numerous investigators studying a variety of epithelial tumor types demonstrated aberrant FHIT transcripts and frequent deletions at the FHIT locus in cancer-derived cell lines and primary tumors. Subsequent analyses have shown that Fhit protein expression is frequently lost or reduced in many of these tumors. Previous studies analyzing Fhit protein expression by immunohistochemistry have shown that loss of Fhit protein expression occurs in a subset of premalignant lesions of the lung (34), esophagus (35, 36), cervix (24), breast (37), and colon (38). It is possible that loss of Fhit protein expression may prove to be a clinically useful biomarker of progression risk in a number of epithelial neoplasms, in addition to those of the uterine cervix. Prospective studies to test this hypothesis may be more feasible for premalignant lesions, such as bronchial dysplasias or Barrett’s metaplasia of the esophagus, in which close clinical follow-up rather than ablation remains an acceptable management choice. Clearly, larger studies are warranted to confirm our initial observations in the cervix and in other sites as well.

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REFERENCES


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