Abnormal Fhit Expression in Malignant and Premalignant Lesions of the Cervix

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ABSTRACT

Genetic analysis of cervical cancer has demonstrated frequent allelic loss in the 3p chromosomal region. The newly described gene FHIT is located at chromosome region 3p14.2, and its expression has been demonstrated previously by reverse transcription-PCR to be abnormal in a majority of cervical cancer cell lines. In this study, 98 different lesions of the cervix were examined for Fhit expression by immunohistochemical staining. Whereas normal cervical epithelium demonstrated diffuse, moderate to intense cytoplasmic staining, many pathological lesions of the cervix displayed reduced or absent Fhit expression. Sixty-one percent of squamous carcinomas and 40% of adenocarcinomas of the cervix had abnormal Fhit expression. Sixty-five preneoplastic lesions of the cervix were examined. Eleven of 33 high-grade squamous intraepithelial lesions and 1 of 12 low-grade squamous intraepithelial lesions had abnormal Fhit expression. In summary, Fhit expression is frequently abnormal in both glandular and squamous cervical cancers, with a higher frequency of Fhit alterations observed in squamous lesions. In addition, abnormal Fhit expression can be detected in some preneoplastic lesions of the ectocervix. Alterations in Fhit expression may be an important marker of early progression in the development of cancers of the cervix.

INTRODUCTION

In 1998, 13,700 new cases of carcinoma of the uterine cervix were predicted to occur, resulting in 4,900 deaths in the United States (1). Cervical cancer represents an even larger problem worldwide, where it remains one of the most common causes for cancer-related mortality and morbidity for women (2). Despite these statistics, little is known about the molecular etiology of this disease. HPV3 is suspected to play an important initiating role in cervical cancer (2). However, it is well known that many preneoplastic (HPV-induced) lesions of the cervix do not progress to cancer, suggesting that additional genetic events are required for full transformation of the cervical epithelium. Recent studies focusing on genetic changes occurring during cervical cancer development have demonstrated clonal abnormalities in different chromosomal regions (3–5). One region that has been demonstrated to have a particularly high frequency of allelic loss is chromosome 3p (6–8). Detailed analysis of this region has demonstrated that heterozygous/homozygous deletions occur early in the development of cervical neoplasias (9, 10). An intense effort has been focused on identifying and characterizing genes from this region, anticipating the identification of critical tumor suppressor gene(s).

The Fragile histidine triad gene (FHIT), located at 3p14.2, is a newly described gene that is a putative tumor suppressor gene (11). It has been extensively characterized in several epithelial cancers such as lung, head and neck, Merkel cell, and colon carcinomas, where its expression is abnormal in approximately 50% of tumors (11–15). The majority of these abnormalities include aberrant mRNA transcripts with the absence of one or more exons within the mRNA. Genomic analysis demonstrates frequent allelic loss and homozygous deletions (12, 16, 17). More recent studies have confirmed abnormalities in FHIT gene expression by demonstrating decreased or absent Fhit expression by Western blot analysis (16). We have reported previously that a high percentage of cervical cancer cell lines express aberrant FHIT transcripts (18). These transcripts are missing one or more exonic sequences, which predicts for the lack of a functional Fhit being synthesized. A recent study confirmed this observation, demonstrating a high incidence (76%) of altered FHIT expression in primary squamous cervical carcinomas (19). We now extend these results to primary cervical tumors (both squamous carcinomas and adenocarcinomas) and their precursor lesions. We demonstrate that Fhit is frequently abnormally expressed in primary cervical cancers, and this abnormal expression appears to occur as an early event in a subset of tumors, because it can be seen in some preneoplastic lesions of the ectocervix.

MATERIALS AND METHODS

Tissue Specimens. We evaluated 110 paraffin blocks representing 85 different cases selected from the surgical pathology files of the Walter Reed Army Medical Center (Washington, D.C.) and from the Walter Reed Army Medical Center (Bethesda, MD) using institutional review board-approved protocols. All cases were reviewed by the study pathologist (J. G.), and all lesions were placed in the following pathological categories: (a) condyoma (HPV effect without basal dysplasia); (b) LGSIL (cervical intraepithelial neoplasia I); (c) HGSIL (CIN II and III); (d) invasive squamous cell carcinoma; (e) glandular dysplasia; (f) adenocarcinoma in situ; and (g) invasive adenocarcinoma. As negative controls, we used paraffin-embedded cell pellets from one cervical and two lung cancer cell lines that had previously been shown to be Fhit negative (20). As positive controls, we used similarly processed cell pellets of a Fhit-positive lung cancer cell line and a cervical cancer cell line transfected with the FHIT gene. From each paraffin block, 4-μm sections were cut onto gelatin-coated slides.

Immunohistochemistry. The paraffin sections were reacted with rabbit polyclonal anti-Fhit antibody (generously provided by Dr. Kay Huebner, Kimmel Cancer Center, Philadelphia, PA) that had been raised against a glutathione S-transferase-Fhit fusion protein (16), without a preceding antigen retrieval step. The antibody was used at a 1:2500 dilution for 2 h at room temperature. The detection reaction followed the Vectastain Elite ABC kit protocol (Vector Laboratories, Burlingame, CA). Diaminobenzidine was used as chromogen, and hematoxylin was used as counterstain.

Interpretation of Stains. Diffuse, moderate to strong cytoplasmic staining characterized Fhit-positive cells, including squamous epithelia (score, 2+/3+). Fhit-negative lesions were devoid of any cytoplasmic reactivity (score, 0), with preserved staining in adjacent normal cells. A subset of lesions showed positive reactivity at a level that was markedly diminished relative to positive internal and external controls (score, 1+/2+), and these lesions were scored as reduced.

Statistical Analysis. χ2 tests were performed to determine the statistical significance of differences (see Table 1). Values of P < 0.05 were interpreted as being statistically significant.

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3 The abbreviations used are: HPV, human papilloma virus; LGSIL, low-grade squamous intraepithelial lesion; HGSIL, high-grade squamous intraepithelial lesion.

4 Unpublished data.

5 D. Hendricks and M. J. Birrer, unpublished data.
RESULTS

Fhit Expression in Primary Cervical Cancers. Pathological review revealed 98 different cervical lesions in 110 paraffin blocks representing 85 different cases. The relative frequencies of the various squamous and glandular lesions are summarized in Table 1. Multiple cases contained more than one pathological lesion, and many contained normal cervical epithelium. Immunohistochemical staining of normal cervical epithelial cells revealed intense cytoplasmic staining, consistent with the subcellular localization of Fhit (Fig. 1, A and B). Immunohistochemical analysis of the 18 primary invasive squamous cell cancers for Fhit expression revealed abnormal expression in 11 of 18 (61%) of the specimens, with 7 of 18 specimens showing reduced expression, and 4 of 18 specimens having no staining (Table 1; Fig. 1, C–E). Strong staining similar to that seen in normal cells was observed in 7 of 18 cases. Reduced or absent Fhit staining was determined by comparing the intensity of the staining of the malignant

<table>
<thead>
<tr>
<th>Pathology</th>
<th>Reduced$^{a}$</th>
<th>Absent</th>
<th>Total abnormal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Squamous lesions</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Invasive</td>
<td>7/18 (39)$^{c}$</td>
<td>4/18 (22)</td>
<td>11/18 (61)$^{d}$</td>
</tr>
<tr>
<td>HGSIL</td>
<td>6/33 (18)</td>
<td>5/33 (15)</td>
<td>11/33 (33)</td>
</tr>
<tr>
<td>LGSIL</td>
<td>1/12 (8)</td>
<td>0/12</td>
<td>1/12 (8)</td>
</tr>
<tr>
<td>Condyloma</td>
<td>0/7</td>
<td>0/7</td>
<td>0/7</td>
</tr>
<tr>
<td>Endocervical lesions</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Invasive</td>
<td>1/15 (7)$^{e}$</td>
<td>5/15 (30)</td>
<td>6/15 (40)$^{f}$</td>
</tr>
<tr>
<td>AIS$^{g}$</td>
<td>0/3</td>
<td>0/3</td>
<td>0/3</td>
</tr>
<tr>
<td>Dysplasia</td>
<td>0/10</td>
<td>0/10</td>
<td>0/10</td>
</tr>
</tbody>
</table>

$^{a}$ Staining scale: absent, 0; reduced, 1++; normal, 2++;.

$^{b}$ Includes one adenosquamous carcinoma.

$^{c}$ The number in parentheses represents the percentage of abnormal expression.

$^{d}$ Invasive versus noninvasive, $P = 0.008$.

$^{e}$ One case exhibited areas of reduced and absent staining.

$^{f}$ Invasive versus noninvasive, $P = 0.0135$.

$^{g}$ AIS, adenocarcinoma in situ.

Fig. 1. Immunohistochemical staining of Fhit in normal and cancerous cervical epithelium. Immunohistochemical staining was performed in formalin-fixed, paraffin-embedded specimens as described in "Materials and Methods." Data shown are: A, Fhit staining in the normal cervix, ×400; B, normal cervical epithelium (N) with absent Fhit staining in an underlying invasive squamous cell cervical cancer (T), ×200; C, normal Fhit staining in an invasive squamous cell cervical cancer, ×400; D, reduced Fhit staining in an invasive squamous cell carcinoma of the cervix, ×400; E, absent Fhit staining in an invasive squamous cell carcinoma of the cervix, ×400; F, absent Fhit staining in an invasive adenocarcinoma of the cervix, ×400.
cervical epithelial cells to that of stromal cells or normal cervical epithelial cells found within the same specimen (Fig. 1B).

Fifteen cases of invasive adenocarcinoma of the cervix were also analyzed. Forty percent of the specimens displayed abnormal expression, with 5 of 15 (30%) having no expression, and 1 case revealing areas of both reduced expression and absent expression (Table 1; Fig. 1F).

**Fhit Expression in Premalignant Lesions of the Cervix.** The finding of abnormal Fhit expression in a majority of primary cervical cancers raised the question as to the timing of the alteration in Fhit expression during the development of cervical cancer. To address this issue, we analyzed 65 preneoplastic lesions of the cervix for Fhit expression. Of 33 HGSILs, 11 (33%) displayed abnormal staining, with 5 having no staining, and 6 revealing reduced staining (Fig. 2, A–C). LGSILs revealed a smaller percentage of abnormalities. Only 1 of 12 (8%) LGSILs revealed reduced staining. Finally, none of the condylomas demonstrated abnormal Fhit expression. Overall, 12 of 52 (23%) of the premalignant squamous lesions demonstrated abnormal Fhit expression that is lower than that found in the invasive squamous cell cancers (11 of 18; *P* = 0.008).

No abnormal Fhit expression was detected in the 13 preneoplastic endocervical lesions studied (invasive lesions *versus* noninvasive, *P* = 0.0135).

**Fhit Expression in Synchronous Lesions.** Among the cases with purely squamous neoplastic lesions, 10 cases had two topographically distinct lesions of varying severity (Table 2). In six of these cases (including four with invasive carcinoma), both the more severe and the less severe lesions showed a normal Fhit expression pattern. For three cases, the invasive squamous cell carcinoma showed significantly less reactivity than adjacent HGSIL (cases 2, 3, and 10). In two of these three cases (cases 2 and 3), the invasive tumor was Fhit negative, whereas the preneoplastic lesion was Fhit positive (Fig. 2, E and F) or showed reduced Fhit expression (Fig. 2, B and D), respectively; and in the third case (case 10), the precursor lesion was Fhit positive, whereas the invasive carcinoma showed a reduced level of Fhit expression. Finally, a Fhit-positive LGSIL coexisted with...
reduced Fhit expression in a HGSIL (case 6). These findings provide further evidence that progression at various stages of squamous neoplasia of the cervix can be associated with down-regulation of Fhit.

**DISCUSSION**

We have demonstrated previously that the FHIT gene is abnormally expressed in five of eight (63%) cervical cancer cell lines (18). Independent studies have confirmed that a high proportion of cervical cancer cell lines express abnormal FHIT transcripts (19, 21). To extend these results and to determine whether the FHIT abnormalities occur during cervical carcinogenesis, as opposed to occurring as an artifact of cell line establishment, we analyzed primary cervical cancers. Unfortunately, because epithelial cancers are frequently contaminated with normal stromal cells, reverse transcription-PCR analysis of the FHIT transcript, as performed previously, becomes difficult to interpret. In addition, the lack of inactivating mutations suggested that analysis on the level of protein expression would be most helpful. Thus, we analyzed our tumor specimens by immunohistochemical staining using a sensitive polyclonal antibody directed against the Fhit protein (16). Our data demonstrate that the majority of invasive squamous cell cervical cancers display reduced or absent Fhit expression. The percentage of specimens with abnormal staining is similar to the percentage of cell lines we determined to have abnormal transcripts. The percentage of specimens with abnormal staining is similar to squamous cell cervical cancers display reduced or absent Fhit expression using a sensitive polyclonal antibody directed against the Fhit gene. Thus, we analyzed our tumor specimens by immunohistochemical staining. This presumably reflects the abnormal expression of one allele (missing exons) in the presence of a remaining normal allele or allelic loss (both of which have been described for this locus; Refs. 12 and 22). Whereas gene dosage effects are generally not thought to be important for tumor suppressor genes, recent reports for p27 demonstrate that reduced expression is sufficient to predispose for tumor formation (23).

Although squamous cell carcinomas account for the vast majority of cervical cancers, there has been a noted increase in adenocarcinoma of the cervix (especially in women under the age of 35 years). Adenocarcinomas now account for 15–20% of all cervical cancers (2). Given this increase and studies suggesting different molecular origins for adenocarcinomas of the cervix, it was important to analyze them along with their precursor lesions (24). Our results show a lower frequency of FHIT alterations in invasive adenocarcinomas (40%) compared to invasive squamous cell carcinomas (61%). It also appears that a higher frequency of FHIT alterations occurs in the preneoplastic squamous lesions than in the preneoplastic adenocarcinoma lesions, suggesting a difference in timing of the acquisition of Fhit abnormalities in these two types of cervical cancers. However, the sample size in this study precludes a conclusive statement on this issue. Interestingly, the difference in FHIT expression between squamous cell carcinomas and adenocarcinomas is not confined to cervical cancer. Recent studies in lung cancer also report a higher incidence of FHIT alterations in squamous cell carcinomas than in adenocarcinomas (25, 26). These results suggest a correlation between the frequency of FHIT gene alterations and the histological origin of the cells involved in the tumorigenic process.

To our knowledge, this is the first study examining the expression of Fhit in premalignant lesions of the cervix. It is very clear from our results that in a subset of cases, Fhit expression is lost/reduced before the development of invasive cancer (HGSIL and LGSIL). However, reduction of Fhit expression does not appear to be an initiating event, because in the earliest lesion of the cervix, such as HPV-induced lesions (condyloma), Fhit expression appears normal. Furthermore, we demonstrated the presence of HPV-16 or -18 in 6 of 17 preneoplastic lesions (LGSIL and HGSIL) with normal FHIT expression (data not shown), suggesting that the presence of HPV alone does not seem to affect Fhit expression. These results correlate with multiple studies determining loss of heterozygosity on 3p in cervical cancers and their precursor lesions (9, 10). Furthermore, the presence of reduced staining in LGSIL versus reduced and absent staining in HGSIL may represent the progression from allelic loss with a dose reduction of normal Fhit expression to the complete absence of normal transcripts and protein. Finally, it is well known that only a percentage of preneoplastic lesions of the cervix progress to higher degrees of dysplasia and eventually carcinoma. It is generally accepted that approximately 16% of LGSILs will progress to HGSILs, whereas 22% of HGSILs will progress to carcinoma in situ (2). It is interesting to note that those percentages are similar to the percentage of preneoplastic lesions that demonstrate abnormal Fhit expression. This raises the speculation that alterations in the FHIT gene may play a role in the development of cervical cancer or serve as a marker of cervical lesions of clinical importance.

**ACKNOWLEDGMENTS**

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**Table 2** Fhit expression in synchronous ectocervical lesions

<table>
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<th>Case</th>
<th>Condyloma</th>
<th>Ectocervical lesion</th>
<th>Squamous carcinoma</th>
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<tbody>
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<td>1</td>
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<td>10</td>
<td>+</td>
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</tbody>
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*+, positive Fhit staining; R, reduced Fhit staining; –, no cytoplasmic staining.


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