Clinicopathological Significance of Fragile Histidine Triad Transcription Protein Expression in Endometrial Carcinomas

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ABSTRACT

Abnormalities in structure and expression of the fragile histidine triad transcription (FHIT) gene have been reported in a variety of cancers, including endometrial cancers. A good correlation between FHIT gene alteration and loss of Fhit expression was observed in endometrial cancers, although those are the selected cases. Therefore, we investigated the association of Fhit expression with clinicopathological features in 111 cases of endometrial cancer. Loss of Fhit expression was associated with high malignant potential, including extensive muscular invasion, advanced surgical stage, high histological grade, nonendometrioid types of adenocarcinoma, negative estrogen receptor status, and p53 overexpression. The presence of personal cancer history was also related to the loss of Fhit with a marginal significance. Survival curves determined by the Kaplan-Meier method and univariate analysis demonstrated that decreased expression of Fhit was associated with a poor outcome. However, multivariate analysis using the stepwise Cox proportional hazard model showed that whereas lymph node metastasis, advanced stage, and high tumor grade were related to poor survival rates, loss of Fhit expression was not. Consequently, loss of Fhit expression is associated with advanced surgical stage and does not appear to be an independent prognostic factor in endometrial cancers, although a still larger sample of patients will be required to assess this issue definitively.

INTRODUCTION

Recent advances in molecular biology have led to a concept that carcinomas arise from the accumulation of a series of genetic alterations involving activation of proto-oncogenes, inactivation of tumor-suppressor genes, and inactivation of DNA repair genes in a single cell. Several pieces of evidence about genetic events in carcinogenesis of the endometrium have been accumulated to our knowledge, although the pathogenesis of endometrial carcinoma is not yet fully understood (1, 2). Of proto-oncogenes, mutational activation of the ras gene has been well described. Point mutation of the ras gene was observed in 10–37% of endometrial cancers and in 10% of atypical hyperplasia. Activation of the ras gene may contribute to the initiating event in a fraction of endometrial carcinomas (3, 4). On the other hand, overexpression of C-erbB-2, Fos, Myc, and Myb is also associated with advanced clinical stage, high tumor grade, and poor prognosis and is considered a late event (5–9). Of the tumor suppressor genes, inactivation of the p53 gene has been well described in endometrial carcinomas. Alteration of p53 is observed in about 20% of endometrial adenocarcinomas and a few cases of endometrial hyperplasia, and mutations of the p53 gene are associated with high grade, advanced stage, and serious papillary adenocarcinoma of the endometrium. Therefore, alteration of p53 may occur as a late event in endometrial carcinogenesis and can be used as a biomarker of poor prognosis, although some controversial issues concerning clinical use still remain to be investigated (10–13). More recent research has shown that a putative tumor suppressor gene, called PTEN, specifically contributes to the development of endometrioid carcinomas of endometrium, especially of microsatellite instability-positive endometrial carcinomas, as an early genetic change (14).

A recently discovered novel gene, called FHIT, located at 3p14.2, has been identified as a candidate of tumor suppressor genes (15, 16). Gene abnormalities, including point mutations, lack of one or more coding exons, a homozygous deletion, and a genetic DNA rearrangement, are frequently observed in a variety of human carcinomas, such as lung, head and neck, and uterine cancers (17–22). Abnormal expression of the FHIT gene was observed in 13 of 16 (81%) uterine cervical cancers and in 4 of 7 (57%) endometrial carcinomas (20). We have reported previously that human papillomavirus-negative cervical cancers were more often associated with decreased expression of Fhit than human papillomavirus-positive cancers (22). Although the clinicopathological significance of abnormalities of the FHIT gene has been intensively investigated in lung, bladder, renal, and colon carcinomas, the significance of decreased expression of Fhit, particularly its effects on prognosis, is controversial (23–25).

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3 The abbreviations used are: FHIT, fragile histidine triad transcription; ABC, avidin-biotin complex; BSO, salpingo-oophorectomy; ER, estrogen receptor; NS, nonsignificant; PR, progesterone receptor; RT, reverse transcription.
In the present study, we investigated FHIT abnormalities in a large number of endometrial carcinomas to assess the role of FHIT gene transcript in multistep carcinogenesis and to determine whether abnormal expression of the FHIT gene is an independent prognostic marker for endometrial cancer. This is the first report describing the clinicopathological significance of abnormal Fhit expression in a large series of patients with endometrial cancer.

PATIENTS AND METHODS

Patients and Samples. Endometrial carcinomas and corresponding normal endometrial tissues were obtained from 111 patients with endometrial cancers who underwent surgical resection at Kanazawa University Hospital, Osaka University Hospital, Fukui Prefectural Hospital, and Toyama Prefectural Hospital from July 1985 to December 1998 and who were not treated with neoadjuvant chemotherapy, hormonal therapy, or irradiation prior to tumor excision. The tissue samples were fixed in 10% formalin, embedded in paraffin, serially cut into 4–6-μm-thick sections, and stained by routine histopathological techniques. Histological classification and grading were performed according to the WHO typing system. Surgical stage was determined in accordance with the International Federation of Gynecology and Obstetrics staging system. The standard treatment for endometrial cancers was as follows. Patients with stage I disease were treated by total abdominal hysterectomy (TAH), bilateral salpingo-oophorectomy (BSO), and pelvic lymph node dissection. Patients with stage II disease were treated by radical hysterectomy, BSO, and pelvic lymph node dissection. In those stage I and stage II patients with pelvic lymph nodes positive for metastases and muscular invasion (more than ½ depth) and/or extensive vascular invasion, the above treatment was followed by additional irradiation of the whole pelvis with 50 Gy. Patients with stage III disease or with positive peritoneal cytology were treated by TAH and BSO followed by irradiation and combination chemotherapy, including cisplatin, doxorubicin, and cyclophosphamide.

Clinicopathological factors such as menstrual status, gravidity, parity, obesity, diabetes mellitus, hypertension, personal history of cancer, and family history of cancer were abstracted from the medical record of each patient. All patients were followed until April 1999, with follow-up times varying from 4 to 186 months.

Some tumors and corresponding normal tissues were frozen and stored at −80°C until DNA extraction, RNA extraction, and/or receptor assay.

Immunohistochemical Analysis of Fhit Protein Expression. Immunostaining for Fhit protein was performed using the ABC method in formalin-fixed, paraffin-embedded tissue samples. Sections were dewaxed in xylene, taken through a graded series of ethanol, and then microwaved in 10 mM phosphate citrate buffer (pH 6.0) at 90°C for 15 min. After incubating with 0.3% hydrogen peroxidase in methanol for 30 min and then 1% normal goat serum for 30 min, sections were treated with anti-glutathione S-transferase-Fhit antibody (rabbit; generously provided by Dr. Kay Huebner, Kimmel Cancer Center, Jefferson Medical College Philadelphia, PA) overnight at 4°C after dilution to 1:2000 in buffer (26, 27). After incubation with the primary rabbit antibody, biotinylated goat antirabbit immunoglobulin and peroxidase-conjugated streptavidin (Vector Laboratories, Burlingame, CA) were applied for each 30 min at room temperature. Sites of peroxidase activity were visualized with 0.1% 3,3-diaminobenzidine-tetrahydrochloride (Sigma Chemical Co., St. Louis, MO) containing 0.02% hydrogen peroxidase in PBS. Negative controls included sections incubated with normal-rabbit serum instead of the primary antibody. The relative number of immunoreactive cells was determined independently by two observers using a double-headed light microscope. Cases in which more than 10% of the cancer cells stained positively were defined as positive.

Overexpression of p53 was also determined immunohistochemically by the ABC method using a primary monoclonal antibody against p53 protein (Do7; Novocastera, Newcastle, United Kingdom; Ref. 28). The staining was performed with a Vectastain ABC kit (Vector Laboratories) according to the manufacturer’s recommendations. Cases in which more than 5% of the cancer cells stained positively were defined as overexpressed.

RT-PCR and cDNA Sequencing of FHIT Gene Expression. To examine for abnormal transcripts of the FHIT gene in endometrial carcinomas, 13 samples of endometrial cancers were randomly selected from frozen tissues stored at −80°C. Total RNA was extracted using ULTRASPEC RNA (Biotech Laboratories, Houston, TX) according to the standard method. For RT-PCR, 1 μg of total RNA was used for RT with 1 mM dNTPs, 0.125 μM oligo dT-adaptor primer, 5 units of avian

![Figure 1](image-url) Detection of abnormal FHIT mRNA by RT-PCR. The sensitive nested PCR method revealed a single band at 614 bp in 8 of 13 cases examined (cases 1, 6, 10, 12, and 13). DNA sequencing of the alternatively spliced FHIT mRNA revealed abnormal sequences in these cases (data not shown). Fhit protein expression was immunohistochemically demonstrated in nine cases and corresponded well to normal transcription except in case 6. PC, positive control (normal endometrium); NC, negative control (no template); M, 1-kb Plus DNA ladder marker.
myeloblastosis virus-reverse transcriptase XL (Takara, Shiga, Japan), and 20 units of RNA inhibitor (Takara) in a 20-μl reaction volume. The RT reaction was carried out at 50°C for 30 min. The first round of cDNA amplification was performed in 20-μl reactions containing 1 μl of first-strand cDNA product, 200 μM dNTPs, 1 unit of Taq polymerase, and a 0.5 μM concentration of primers 5U2 (5’-ATCGTGAAGCTTCGTAT-3’) and 3D2 (5’-TCATGCTGATTCATTCGTC-3’), which cover exons 1–10 of the FHIT gene. After an initial 2-min denaturing step at 95°C, 30 PCR cycles of 30 s at 95°C, 30 s at 60°C, and 90 s at 72°C were carried out. The amplified products were diluted 10-fold with TE buffer, and 1 μl of diluted products was used in a second round of PCR amplification using the nested primers 5U1(5’-TCCGTA-GCTATCTACAT-3’) and 3D1(5’-TCATGCTGATTCCTCTCT-3’), which cover exons 3–10 of the FHIT gene, for 30 cycles under the above conditions. These nested PCR products were run on 1.5% agarose gels and visualized by ethidium bromide staining (15).

For the sequence of FHIT cDNA, normal-sized and short FHIT cDNAs were cut from the gel for automated sequence analysis (ABI Prism 377). The sequencing reactions were performed using the dye-terminator cycle sequence kit (PE Applied Biosystems, Foster City, CA) with three primers (5’-TCCGTA-GTGCTATCTACAT-3’, 5’-CAGGACATGTCCTTGTGC-3’, and 5’-GTCATGTTCTGGAGCTCT-3’) to covered the entire open reading frame of the FHIT gene (15).

**ER and PR Assays.** Tumor tissues freshly obtained at operation were assayed for cytoplasmic ER and PR according to procedures described previously (29). A single concentration assay in duplicate using a saturating concentration, 10 nm/l [3H] estradiol-17β (NEN Life Science Products, Boston, MA) or [3H]progesterone (NEN Life Science Products) was used to determine the binding capacity to ER and PR, respectively. Cases with levels of steroid receptors greater than 10 fmol/mg of protein were classified as receptor positive.

**Statistical Analysis.** Odds ratios were used to describe the univariate relationships between FHIT status and other clinicopathological factors. Statistical significance and the confidence interval of the odds ratios were calculated using the χ² test and Woolf’s method, respectively. Data were first expressed in binary or tertiary terms on the basis of the cut-off points. Age (postmenopause versus premenopause), lymph node metastasis (positive versus negative), and FHIT (positive staining versus negative staining) were treated as binary data. Surgical stage (I versus II versus III), muscular invasion (none versus 1/2 versus >1/2), and histological grade (G1, G2, and G3) were treated as tertiary data. Kaplan-Meier survival analysis was used for univariate analysis, and odds ratios were then calculated from the final survival probability of the patients in whom the factor was analyzed and from those of patients without the factor. The significance of the odds ratios was estimated using the log-rank test. For tertiary variables, odds ratios were calculated between the first and second categories and between the first and third categories. Cox’s proportional hazard method for multivariate survival analysis was used to evaluate the prognostic significance of each variable after consideration of other variables.

**RESULTS**

**Molecular Analysis of the FHIT Gene Is Well Correlated with the Immunohistochemical Analysis.** We examined the correlation between abnormal transcript of FHIT gene and Fhit protein expression. Normal-sized FHIT transcripts were observed in all samples of 13 cancer tissues by a nested PCR method. (Fig. 1). An aberrant smaller-sized transcript was...
detected in addition to the normal-sized one in five cases. These PCR products were excised from the agarose gel and subsequently sequenced. Although all of the normal-sized 708-bp fragments had normal sequences, the abnormal transcripts detected by nested PCR exhibited deletion of exons 4–7 (317 bp) in case 1, exons 4–6 (347 bp) in case 6, exons 4–8 (248 bp) in case 10, exons 4–7 (317 bp) in case 12, and exons 4–6 (347) in case 13. No point mutations or homozygous deletions were observed in these samples. The tumor sections obtained from the same cases were immunohistochemically analyzed for Fhit expression. Staining was observed in all eight cases with normal FHIT gene transcript. No staining was observed in four cases with abnormal transcripts. However, one case with abnormal transcript (case 6) was defined as positive for staining. This might be explained by the finding that Fhit-negative cells with the aberrant transcript occupied the larger areas in cancer nests. In the present analysis, the finding of more than 10% of cancer cells with staining was considered positive. Thus, immunohistochemical analysis revealed that the status of Fhit expression was correlated well with the results of RT-PCR analysis.

**Fhit Expression Is Associated with Clinicopathological Parameters.** The loss of Fhit protein expression was immunohistochemically detected in 41 (37%) of 111 patients with endometrial cancers (Fig. 2). The correlations between Fhit expression and pathological parameters are summarized in Table 1. Loss of Fhit protein expression was significantly associated with poor survival, muscular invasion (more than ½ depth of uterine muscle), advanced surgical stage, high histological grade, nonendometrioid type of adenocarcinoma, and negative ER status. Interestingly, p53 mutation (p53 overexpression) was also associated with the loss of Fhit protein expression, although it had only a marginal significance.

**Loss of Fhit Expression Was Associated with Poor Survival.** Table 3 summarizes the results of univariate analysis of the prognostic factors. The loss of Fhit protein expression, nonendometrioid type of histology, surgical stage III/IV, muscular invasion (more than ½ depth of uterine muscle), lymph node metastasis, and histological grade 3 were each significantly associated with a poor prognosis.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Expression of Fhit protein</th>
<th>Negative</th>
<th>Positive</th>
<th>Negative rate (%)</th>
<th>Odds ratio</th>
<th>95% confidence interval</th>
<th>P</th>
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<td>19</td>
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<td>2</td>
<td>12</td>
<td>14.3</td>
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<td>&lt;1/2</td>
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<td>15</td>
<td>41</td>
<td>26.8</td>
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<td>≥1/2</td>
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<td>22</td>
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<td>56.4</td>
<td>7.76</td>
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<td>4</td>
<td>78.9</td>
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<td>0.00–0.19</td>
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<td>11</td>
<td>50.0</td>
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<td>41.7</td>
<td>0.71</td>
<td>0.17–2.95</td>
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<td>14</td>
<td>9</td>
<td>60.9</td>
<td>3.0</td>
<td>1.13–7.88</td>
<td>0.02</td>
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*NS, no significance.
2/3 (P = 0.008), endometrioid type (P < 0.001), and no lymph node metastases (P < 0.001), loss of Fhit expression was significantly associated with a poor survival rate (Fig. 3, B–D), but loss of Fhit did not affect the outcome in patients with any fractions of muscular invasion (no invasion, NS; invasion <1/2, P = 0.09; invasion ≥1/2, NS), surgical stage (stage 1, NS; stages 2–4, NS), nonendometrioid type (NS), or lymph node metastasis (NS). The loss of Fhit expression did not affect the prognosis in any subgroup when data were analyzed in terms of the status of steroid hormone receptor and p53 overexpression (data not shown).

Table 2 summarizes the results of multivariate analysis. Stepwise Cox proportional hazard analysis showed that lymph node metastasis, high histological grade, and advanced surgical stage were significantly related to poor survival rate. However, status of Fhit expression, histological type, and muscular invasion were not related to survival rate. Multivariate analysis for thus showed that loss of Fhit expression was not related to the survival rate after consideration of other factors, although it was significantly related to survival rate in the univariate analysis. Loss of Fhit expression appears to affect the outcome in the only multivariate analysis for five covariates, excluding surgical stage, indicating that the loss of Fhit expression depends on surgical stage.

**DISCUSSION**

It is very difficult to detect the status of the Fhit gene locus in cancer cells by molecular analyses, because the Fhit gene spans over 1 Mb in the FRAB common fragile site at 3p14 (15, 16). Recent studies have reported that alteration in the Fhit locus detected by DNA and/or RT-PCR analysis is well correlated with loss of Fhit protein expression in tumors (23). The alternative transcripts of Fhit have been identified in a few normal tissues by the method of highly sensitive nested PCR of

2/3 (P = 0.008), endometrioid type (P < 0.001), and no lymph node metastases (P < 0.001), loss of Fhit expression was significantly associated with a poor survival rate (Fig. 3, B–D), but loss of Fhit did not affect the outcome in patients with any fractions of muscular invasion (no invasion, NS; invasion <1/2, P = 0.09; invasion ≥1/2, NS), surgical stage (stage 1, NS; stages 2–4, NS), nonendometrioid type (NS), or lymph node metastasis (NS). The loss of Fhit expression did not affect the prognosis in any subgroup when data were analyzed in terms of the status of steroid hormone receptor and p53 overexpression (data not shown).

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the RT products, demonstrating polymorphism of transcription of the FHIT gene (20, 30). DNA/RNA analysis also has the disadvantage of yielding false-positive or false-negative results that are attributable to contamination by noncancerous tissue in tumor samples. Recent reports have noted that FHIT gene alterations could be simply detected by immunohistochemical analysis of tumor specimens (23). We therefore investigated the relationship between abnormal FHIT transcription and the expression of Fhit protein in selected samples of patients. We found that Fhit protein expression status was correlated well with transcription of the FHIT gene. All of five cases with aberrant transcripts detected by the nested PCR method, and subsequent sequencing exhibited the loss of Fhit protein expression. Positive expression of Fhit protein was observed in all eight cases with normal FHIT gene transcript. One case (case 6) with an abnormal transcript was positive for Fhit expression. This case might be explained by the finding that most cancer cells harboring abnormal transcript are negative for staining, but

Table 4  Prognostic factors for survival by multivariate analysis

<table>
<thead>
<tr>
<th>Covariates</th>
<th>Six covariates</th>
<th>Five covariates excluding stage</th>
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<tbody>
<tr>
<td>Loss of Fhit expression (vs. Fhit expression)</td>
<td>RR = 5.49, 95% CI = 1.94–15.53, P = 0.001</td>
<td>RR = 6.57, 95% CI = 3.98–23.0, P = 0.001</td>
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<tr>
<td>Nonendometrioid (vs. endometrioid)</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Stage III/IV (vs. stage I)</td>
<td>RR = 8.39, 95% CI = 2.43–28.9, P &lt; 0.001</td>
<td>RR = 3.90, 95% CI = 1.52–10.0, P = 0.005</td>
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<td>Grade 3 (vs. grade 1)</td>
<td>RR = 5.25, 95% CI = 2.05–13.46, P &lt; 0.001</td>
<td>RR = 3.90, 95% CI = 1.52–10.0, P = 0.005</td>
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<td>LN metastasis + (vs. LN metastasis −)</td>
<td>RR = 3.59, 95% CI = 1.38–9.31, P = 0.009</td>
<td>RR = 6.57, 95% CI = 3.98–23.0, P &lt; 0.001</td>
</tr>
<tr>
<td>Muscular invasion ≥1/2 (vs. no invasion)</td>
<td>NS</td>
<td>NS</td>
</tr>
</tbody>
</table>

*RR, relative risk; CI, confidential interval; NS, no significance; LN, lymph node.
other cancer cells with normal transcript occupying around 20% of cancer nests are positive for staining. Immunohistochemical analysis is thus a simple, convenient, and reliable way to screen for the presence of FHIT alterations in cancer tissues. We therefore performed immunohistochemical analysis of Fhit protein expression in formalin-fixed, paraffin-embedded endometrial cancer specimens using the polyclonal antibody.

Fhit protein expression was markedly reduced in 41 (36.9%) of 111 endometrial cancers. This reduction was significantly associated with a potential for high malignancy, including advanced surgical stage, poor differentiation, nonendometrioid type of tumor, deep muscular invasion, and ER-negative status. p53 mutation and personal cancer history were also associated with loss of Fhit expression, although with marginal significance. Thus, alteration of the FHIT gene seems to be a late event in carcinogenesis of the endometrium. This hypothesis has been described previously for non-small cell carcinoma of the lung and breast cancers (23, 31). In contrast, some studies have supported a role for FHIT in the early step of tumorigenesis, because deletions at 3p14.2 within the FHIT locus are detected in the precursor lesions of the cervix and early phase renal cell cancers and lung cancers (24–25, 32, 33). The present immunohistochemical analysis may support the former hypothesis because all samples of atypical hyperplasia as well as normal endometrium were strongly positive for Fhit protein (data not shown). The exact role of FHIT gene alteration in carcinogenesis is unclear, because the abnormal transcripts of the FHIT are observed in some noncancerous tissues, and the existence of some other genes located close to the FHIT gene has been considered as another possibility.

In addition to identifying the phase during which FHIT gene abnormality occurs in carcinogenesis, it is very important for practical medical purposes to clarify whether loss of Fhit expression will really prove to be a prognostic indicator for endometrial cancers. We therefore examined the correlation between the status of Fhit expression and patient clinicopathological factors. Decreased expression of Fhit was not associated with clinical parameters such as obesity, hypertension, diabetes mellitus, gravidity, parity, menopausal status, or family history of cancers. It was associated with the presence of personal cancer history with marginal significance. Recent studies have revealed that loss of Fhit expression was associated with smoking in lung cancer (23). The present study does not clarify the association of smoking with endometrial cancers because fewer Japanese women smoke than women in Western countries. Interestingly, FHIT alterations were associated with ER-negative status, p53 accumulation, and nonendometrioid type of tumor, which are characteristics of estrogen-independent, so-called type II cancers. Estrogen-dependent endometrial carcinomas, so-called type I, composing of the large fraction of cancers, have been demonstrated to frequently harbor alterations of the PTEN gene at chromosome 10p. These differences in molecular events between two types of endometrial cancer suggest the possibility of differences in cell lineage. It is possible that FHIT may play a role in estrogen-independent carcinogenesis of the endometrium.

Notably, reduction of Fhit expression was significantly associated with poor survival of patients with endometrial cancers. The endometrial cancer-specific survival curves determined by the Kaplan–Meier method showed that the outcome in patients with loss of Fhit expression was poor overall. This poor survival was significantly associated with loss of Fhit expression in subgroups of patients with histological grade 1, histological grade 2/3, endometrioid types of adenocarcinoma, and negative lymph node metastasis. Our univariate analysis also showed that loss of Fhit expression is associated with poor outcome, as well as other poor prognostic parameters, such as nonendometrioid type of tumor, advanced surgical stage, high histological grade, lymph node metastasis, and deep muscular invasion. The loss of Fhit expression thus appears to be a reliable prognostic biomarker. However, multivariate analysis using the stepwise Cox proportional hazard model demonstrated that reduced Fhit expression was not related to poor survival after consideration of other prognostic factors. The important prognostic factors are lymph node metastasis, histological grade, and postoperative surgical stage. Multivariate analysis excluding each prognostic parameter revealed that loss of Fhit expression depended on tumor stage.

In conclusion, loss of Fhit expression seems to occur in a late stage of tumorigenesis and did not itself appear to be an independent prognostic factor for endometrial cancer on the multivariate analysis. The loss of Fhit protein expression was associated with tumor stage. Because a striking difference in survival was associated with absence versus presence of Fhit protein expression in the patients with favorable prognostic factors, it is still interesting to speculate that the Fhit protein may be useful in the treatment of endometrial cancers as a decision-making biomarker for aggressive treatment after operation. Larger amounts of material from patients are still required to definitively determine the biological significance of Fhit protein.

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Clinicopathological Significance of Fragile Histidine Triad Transcription Protein Expression in Endometrial Carcinomas

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