Expression of FRA16D/WWOX and FRA3B/FHIT Genes in Hematopoietic Malignancies

Hideshi Ishii,1,3 Andrea Vecchione,3 Yutaka Furukawa,1 Krittaya Sutheesophon,1 Shuang-Yin Han,3 Teresa Druck,3 Tamotsu Kuroki,3 Francesco Trapasso,3 Miki Nishimura,2 Yasushi Salto,2 Keiya Ozawa,1 Carlo M. Croce,3 Kay Huebner,3 and Yusuke Furukawa1

1Center for Molecular Medicine, Jichi Medical School, Tochigi, Japan; 2Department of Clinical Cell Biology, Graduate School of Medicine, Chiba University, Chiba, Japan; and 3Kimmel Cancer Center, Thomas Jefferson University, Philadelphia, PA

Abstract
The WW domain containing oxidoreductase (WWOX) gene was recently identified as a candidate tumor suppressor gene at a common fragile site, FRA16D. Because the fragile histidine triad (FHIT) gene, a tumor suppressor gene encompassing the most active, common fragile site FRA3B, is frequently deleted in various cancers, we evaluated the expression of WWOX and FHIT in 74 cases of primary hematopoietic neoplasias and 20 leukemia cell lines. Aberration or absence of WWOX transcripts was detected in 51% of the primary cases and 55% of cell lines, and three WWOX nucleotide variants were detected among the leukemia cell lines. FHIT expression was absent or altered in 36% of the primary cases and 15% of cell lines. The occurrence of aberrant FHIT reverse transcription-PCR products correlated significantly with the occurrence of WWOX alterations. Wild-type transcripts of both genes were expressed in normal hematopoiesis along with a small fraction of short transcripts. A DNA blot study showed that WWOX and FHIT genes were deleted in 2 of 18 cases with primary acute leukemias; both genes were not expressed in the 2 cases. Furthermore, treatment of cells with a demethylating or histone acetylating agent in culture resulted in increased expression of WWOX and FHIT mRNA in leukemia cell lines. Conclusions are that WWOX expression is frequently altered or absent in hematopoietic disorders, often in association with FHIT alterations, and that alterations of these fragile genes may result not only from genomic deletions but also from epigenetic modifications associated with expression of fragility.

Introduction
The observation that common chromosomal fragile sites, expressed as gaps or constrictions at specific cytogenetic bands under the condition of mild DNA replication stress, apparently coincide with chromosomal bands that are frequently altered in cancer (1) led to the hypothesis that they may contribute to cancer development. Although the exact number of common fragile sites is a matter of interpretation, more than 70 aphisidicolin-induced common fragile sites are listed in GenBank. Gaps and breaks at just 20 fragile sites, however, represent over 80% of all fragile lesions observed in lymphocytes following treatment with low doses of aphisidicolin (2). FRA3B at 3p14.2 stands out as the most active fragile site in the human genome, followed by 16q23 (FRA16D), 6q26 (FHIT), 7q31.2 (FRA7G), and Xp22.3 (FRAXB). A recent study suggests that FRA3B and four other fragile sites (FRA3B, FRA7G, FRA7H, FRA16D), and several associated genes are unstable in some cancer cells (3).

In 1996, the fragile histidine triad (FHIT) gene was isolated from the region encompassing the FRA3B locus (4). Regions within the large FHIT genomic locus are deleted in a large fraction of tumors (4, 5). FHIT protein expression is absent in overall 60% (1162 of 1948 cases) of tumors, including lung, breast, esophagus, stomach, and bladder cancers (reviewed in Ref. 6), due to genomic deletions (5) or epigenetic changes, such as DNA methylation (7, 8). Fhit protein expression is absent in lymphocytes following treatment with low doses of aphisidicolin (2). FRA3B at 3p14.2 stands out as the most active fragile site in the human genome, followed by 16q23 (FRA16D), 6q26 (FHIT), 7q31.2 (FRA7G), and Xp22.3 (FRAXB). A recent study suggests that FRA3B and four other fragile sites (FRA3B, FRA7G, FRA7H, FRA16D), and several associated genes are unstable in some cancer cells (3).

Recently, a WW domain containing oxidoreductase (WWOX) gene was identified from chromosome region 16q23.3–24.1 at common fragile site FRA16D (10). WWOX expression has been examined in tumors and cancer-derived cell lines by reverse transcription-PCR analysis and aberrant products observed in many tumors, including breast (10), ovary (11), esophageal (12), and lung cancers (13). Wwox protein inhibitor tumor cell growth (14) perhaps through c-Jun NH2-terminal kinase interaction (15). The two fragile genes, FHIT and WWOX, have common features; both encompass a genomic locus of more than 1 Mb with an associated fragile region, show frequent altered expression in cancers, and are suspected tumor suppressor genes (12).

Because conditions of replication stress affect expression of all common fragile regions and a number of cancer cell lines...
exhibited homozygous deletions in two or more common fragile regions, we have examined the coordinate expression of the WWOX and FHIT genes in hematopoietic disorders. Recent studies have identified new modifications, such as DNA demethylation and histone acetylation and functional links to epigenetic change and RNA interference pathways (16). The present study provides evidence that loss or alteration of expression of the two genes can occur concordantly in hematopoietic tumors through mechanisms that result in aberrant reverse transcription-PCR products and/or epigenetic modification at these fragile loci.

Results
Characteristics of Hematopoietic Disorders

Seventy-four individual cases, preneoplastic or neoplastic, were studied for expression of WWOX and FHIT mRNA by reverse transcription-PCR analysis: 42 acute myelogenous leukemias (AMLs; 9 M1, 9 M2, 6 M3, 11 M4, 5 M5, and 2 unclassified AML), 17 acute lymphoblastic leukemias (ALLs), 9 chronic myelogenous leukemias (CMLs), 2 cases with myelodysplastic syndrome (MDS), 1 chronic lymphoblastic leukemia (CLL), and 3 multiple myelomas (MMs; Table 1). Twenty leukemia-derived cell lines, as listed in “Materials and Methods,” were similarly analyzed, and selected cell lines were examined for FHIT and WWOX expression after treatment with CpG demethylation and histone acetylation agents and for the protein expression by immunoblot analysis.

Expression of WWOX and FHIT

Because the level of WWOX and FHIT mRNA expression is too low to detect routinely by RNA blot analysis from small samples, reverse transcription-PCR amplification was performed to detect WWOX and FHIT expression, as previously described for other tumors (4, 12, 13). As summarized in Table 1 and shown in Fig. 1A, 38 of 74 cases (51%) showed altered or absent expression of WWOX. Eleven of 20 leukemia cell lines (55%) showed absence or aberrant WWOX expression. Sequence analysis of the reverse transcription-PCR amplified products showed WWOX transcripts missing exons 2–6 and a part of exon 7, resulting in a frame-shifted sequence in a primary ALL-L2 case (Fig. 2A). In addition, WWOX reverse transcription-PCR products missing exons 2–9 and 1–8 were amplified from tumor cells of an AML-M4 and an ALL-L1 case, respectively. A cell line, KCL22, expressed WWOX products missing exons 6–8 and a part of exon 9, which if translated would affect the peptide coding region of the WWOX gene.

Similarly, FHIT RNA was altered or absent in 27 of 74 cases (36%; Table 1). Three of 20 leukemic cell lines (15%) showed absence or aberrant FHIT expression. Sequencing revealed FHIT transcripts missing exons 5–7 and incorrect exon-intron boundaries in an AML-M1 case (Fig. 2B). An AML-M2 case showed absence of exons 4–6 (Fig. 2B). Alterations of the FHIT locus were correlated with WWOX alterations (P < 0.001); the 27 cases and 3 leukemic cell lines having FHIT alteration also showed WWOX alteration.

To characterize WWOX and FHIT expression in freshly isolated nonneural cells, RNA from pooled cell fractions of CD34+, CD34−, monocytes, granulocytes, T cells, and erythroblasts as well as peripheral lymphocytes from four healthy volunteers were analyzed by reverse transcription-PCR amplification. The results showed that these primary, noncancerous cells expressed abundant, intact WWOX and FHIT transcripts, although faint short forms of WWOX and FHIT were detected in differentiated hematopoietic cells (Fig. 1B). Nonne leukemic 293 cells expressed wild-type WWOX RNA and abundant, intact FHIT RNA (Fig. 3A).

We performed reverse transcription-PCR and immunoblot analyses to study transcript and protein expression (Fig. 1C). The absence of wild-type Wox and Fhit proteins was correlated with the absence of wild-type transcripts of those genes. The immunoblot study detected absent, reduced, or smaller-sized fragments of Wox in hematopoietic malignancies; although the immunoblot study detected absence and reduction of Fhit, smaller-sized Fhit proteins were undetectable, and normal lymphocyte expressed wild-type Wox and Fhit proteins (Fig. 1D).

Table 1. Summary of Reverse Transcription-PCR Analysis of WWOX and FHIT Expression in Hematopoietic Disorders

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>Number of Cases</th>
<th>WWOX</th>
<th>FHIT</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Intact</td>
<td>Altereda</td>
</tr>
<tr>
<td>AML</td>
<td></td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>M1</td>
<td></td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>M2</td>
<td></td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>M3</td>
<td></td>
<td>11</td>
<td></td>
</tr>
<tr>
<td>M4</td>
<td></td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>M5</td>
<td></td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Unclassified</td>
<td></td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>ALL</td>
<td></td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>L1</td>
<td></td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>L2</td>
<td></td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>Others</td>
<td></td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>CML</td>
<td></td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>Chronic phase</td>
<td></td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Blastic crisis</td>
<td></td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>MDS</td>
<td></td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>CLL</td>
<td></td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>MM</td>
<td></td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>74</td>
<td></td>
</tr>
</tbody>
</table>

*aIncluding cases with aberrant and normal-sized products.
Sequence Variants and Study of FHIT and WWOX Gene

The search for mutations in the coding exons of WWOX and FHIT was performed by direct sequencing of the PCR amplification products of each exon DNA. No point mutations or large deletions were detected in the primary tumors. Among the cultured cells, sequence analysis revealed four cases of nucleotide variations in the WWOX gene (Fig. 4A). The KG1 cDNA showed a G insertion at nucleotide 706, which would result in a frame shift in the amino acid sequence. A C-to-T transition at nucleotide 357, which would result in an Arg-to-Trp variation, was found in U937 cDNA. The third variation was in HEL cells, an A-to-T substitution at nucleotide 805. Because there are no normal counterparts of these cell lines, whether the variations represent rare polymorphisms or point mutation is not known. The fourth variation involved a C-to-G transition at nucleotide 844, resulting in a Pro-to-Ala transition in cultured cells from peripheral lymphocytes of a healthy volunteer (data not shown), a previously reported polymorphism (17). No variations or deletions, except known polymorphisms, were detected in FHIT exons.

DNA blot analysis was performed to examine genomic deletions in 18 cases (13 cases with AML and 5 cases with ALL) with altered or absent WWOX and FHIT expression.

FIGURE 1. Expression of WWOX and FHIT genes. Reverse transcription-PCR amplification was performed using RNA from primary tumor cells of ALL, AML, and CML cases (A) and from peripheral lymphocytes from healthy volunteers (V1, V2, V3, and V4) and pools of fractionated CD34+, CD34−, monocytes, granulocytes, T cells, and erythrocytes (B). After nested PCR amplification, the products were separated by 1.5% agarose gel and stained by ethidium bromide. Amplification of GAPDH cDNA served as a control for quantity and quality of RNA preparations. C. Representative results of five cases (lanes 1, 2, 3, 4, and 5) and two cell lines analyzed by immunoblot analysis with anti-Wwox, anti-Fhit, or anti-actin antibody and by reverse transcription-PCR for WWOX, FHIT, or GAPDH transcript. A faint band in FHIT reverse transcription-PCR in lane 2 is due to a small fraction of contaminated noncancerous lymphocytes. D. Immunoblot analysis of Wwox and Fhit protein. Normal lymphocyte (lane 1) and primary leukemia (lanes 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, and 12). Film was exposed longer to detect short aberrant proteins. Shorter, various sized bands of Wwox were detected; no apparent shorter fragments of Fhit were detected.
Result showed that \textit{WWOX} and \textit{FHIT} genes were deleted in two cases with AML-M4 and AML-M5, in which those genes were not expressed (representative DNA blot data shown in Fig. 4B), suggesting that not only large genomic losses but also small deletions or other epigenetic modifications may be involved in altered fragility expression.

\textbf{Expression of Truncated Forms}

As shown in Fig. 1B, the nonleukemic hematopoietic cells expressed not only wild-type transcript but also small amounts of shorter \textit{WWOX} and \textit{FHIT} cDNA amplification products. To determine whether chromatin modification may have a role in control of expression of the fragile genes, \textit{WWOX} and \textit{FHIT}, we cultured control 293 kidney cells, which express intact \textit{FHIT} and \textit{WWOX}, and K562 leukemia cells, which express a low level of \textit{WWOX} and \textit{FHIT}, with inhibitors of methylation and deacetylation. We detected increased expression of the wild-type and short form transcripts of \textit{WWOX} and \textit{FHIT} after culturing K562 cells in medium with 5-aza-2'-deoxycytidine (5-azaCdR) or depsipeptide (Fig. 3A). The exposure of 293 cells, which express only wild-type \textit{FHIT}, to depsipeptide resulted in the production of short forms, although this effect was not observed with 5-azaCdR. We found that both Lys9 of histone 3 and Lys8 of histone 4 were acetylated after exposure to depsipeptide (Fig. 3B). After exposure to 5-azaCdR or depsipeptide, no apparent effect on \textit{WWOX} expression was observed in 293 cells, which express the endogenous, intact \textit{WWOX} (Fig. 3A).

\textbf{Discussion}

Numerous studies have reported loss or reduction of \textit{FHIT} in many types of cancers, including lung (5), esophagus (4), bladder (18), and stomach (19), and in some types of cancer, \textit{FHIT} is altered in an early stage of cancer or precancer development (6). More recently, the \textit{WWOX} gene, at a second common fragile site, has been reported to be deleted and its expression is altered in various cancers, including breast (10), ovary (11), esophagus (12), and lung (13). Because common fragile sites are susceptible to activation by the same agents, including carcinogens, we were interested in determining if some cancers might involve inactivation of multiple fragile genes. Thus, we have studied expression of genes at two of the most active fragile regions in hematopoietic neoplasias and preneoplastic conditions. As the best of our knowledge, very few, large-scale studies have been reported regarding \textit{WWOX} involvement in hematopoietic disorders. The present study showed that the expression of the \textit{WWOX} and \textit{FHIT} genes was frequently altered concordantly in hematopoietic disorders, including AML, ALL, CML, and MM, and the frequency of \textit{WWOX} alteration, as determined by reverse transcription-PCR amplification of mRNA, was higher than that of \textit{FHIT} (51\% versus 36\%). In previous studies, the reported frequency of \textit{FHIT} alterations in hematopoietic disorders has varied; the expression of \textit{FHIT} mRNA and/or protein was reported to be altered in 30–60\% of AML (20, 21), 20–70\% of ALL (21–23), and 4\% of CML (24). In our study, the cases with \textit{FHIT}...
alteration also showed WWOX alteration, suggesting that WWOX and FHIT genes are concordantly affected in the progression of hematopoietic disorders. The reasons for the differing frequencies of alteration of FHIT and WWOX expression are not yet known. However, it is likely to reflect the selective advantage conferred by WWOX and FHIT inactivation versus alteration of either tumor suppressor alone. The DNA blot study detected homozygous deletions only in 2 of 18 cases with primary leukemias, and WWOX and FHIT expression was undetectable in the 2 cases, showing that genomic deletions contribute to the absence of the genes expression and suggesting that the other mechanisms, such as small deletions or epigenetic modifications, also may be involved in altered fragility expression. We speculate that possible causes of concordant expression of aberrant transcripts of WWOX and FHIT may be not only due to genomic alterations but also at least in part due to the nature of splicing machinery in rapidly growing tumor cells; unrestricted proliferation of tumor cells likely will affect huge unspliced RNA in those fragile site genes, encompassing a genomic locus of more than 1 Mb with an associated fragile region. The size of wild-type FHIT product is smaller than that of wild-type WWOX, and aberrantly sized Wwox proteins were detected while aberrantly sized Fhit was hardly detected, suggesting that a different nature of translational or post-translational regulation might be involved between two genes.

The present study showed that the K562 cell line, with barely detectable FHIT and very little WWOX mRNA, expressed increased amounts of normal and aberrant transcripts after exposure to 5-azaCdr or depsipeptide; culturing cells with depsipeptide causes acetylation of Lys9 of histone 3 and Lys8 of histone 4. The 293 noncancerous kidney cell line, which expressed solely wild-type FHIT, produced short transcripts in addition to wild-type transcript after exposure to depsipeptide,

FIGURE 3. Effect of acetylation and CpG methylation on WWOX and FHIT expression. K562 and 293 cells were cultured in medium with depsipeptide (FK) or 5-azaCdr (AZA). A. RNA was extracted and subjected to semiquantitative PCR amplification. After 24 cycles of the first round PCR, 1/20 volume of purified product was subjected to the second PCR round for 20, 25, 30, and 35 cycles. B. Acetylation in K562 cells was studied by immunoblot analysis with antisera against acetylated Lys9 of histone 3 (H3K9) and antiacetylated Lys8 of histone 4 (H4K8).
suggesting the possibility that changes in chromatin configuration may affect not only the level of transcription, although we cannot exclude the possibility that 293 cells have some FHIT genes with deletions that were not expressed in the deacetylated form. Recent studies have shown that methylation of the FHIT regulatory region is involved in 14–16% of esophageal, prostate, and bladder cancer (8, 25, 26) and 28–37% of oral, lung, and breast carcinoma (7, 27). Some early studies of FHIT expression had reported detection of aberrant FHIT transcripts in normal hematopoietic cells (28, 29), while absence of wild-type FHIT was restricted to leukemia (28), which is compatible with the present results. The present study suggests that the observation of faint aberrant transcripts in normal hematopoietic cells may be associated with epigenetic modifications. We speculate that an epigenetic modification precedes the initiating events, such as inactivation of tumor suppressor genes, proto-oncogene activation, and/or oncogenic chromosome translocations in hematopoietic malignancies. Although previous observations suggest that FHIT promoter methylation contributes to inactivation of FHIT in an early stage of environmental carcinogen-induced cancer, the methylation status of the WWOX promoter in an early stage of hematopoietic malignancies is not fully understood. A large-scale study of premalignant stage, such as MDS, would be necessary to understand an early epigenetic event of leukemic hematopoiesis. 5-azaCdr and depsipeptide increased the expression of WWOX and FHIT, suggesting a rationale for further research of combined usage of demethylating and anti-deacetylating agents as reactivating agents for these tumor suppressor genes perhaps in preclinical cancer prevention models.

Materials and Methods

Cell Culture and Primary Tumor Samples

Twenty cell lines, KG-1 (AML-M1), ML-1 (AML), KCL22 (CML in crisis), HL60 (AML-M2), THP-1 (AML-M5), U937 (histiocytic lymphoma), JOSK-I (AML-M4), KU812 (CML in basophilic crisis), K562 (CML in erythroid crisis), HEL (erythroid cell from AML-M6), UT-7 (AML-M7), HPB-ALL (ALL), MOLT3 (T-cell ALL), Jurkat (T cell from non-Hodgkin’s lymphoma), REH (B-cell ALL), NALM-6 (B-cell ALL), Daudi (Burkitt), U-266 (MM), KS-1 (MM), and M-O7e (AML-M7), were maintained as described (30, 31). Human embryonic kidney 293 cells served as control nonleukemic cells in some experiments. Cells were cultured in growth medium with or without 2-µM depsipeptide, FR901228, a cyclic peptide inhibitor of histone deacetylase, or 1-µM 5-azaCdr (Sigma Chemical Co., St. Louis, MO). Uncultured, primary hematopoietic cells and neoplasias were categorized according to the French-American-British classification and were obtained from patients at the Jichi Medical School and the Chiba Graduate School of Medicine, Japan, according to the institutional ethical agreements.
Expression Analyses

RNAs were extracted with the Isogen kit (Nippongene, Toyama, Japan). cDNAs were synthesized from 1 μg of total RNA with Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA) and were subjected to PCR amplification with ExTaq DNA polymerase (Takara, Shiga, Japan) using nested primer sets for FHIT (4) and WWOX (12, 13), which were previously characterized. PCR amplifies coding exons 1–9 of WWOX and coding exons 5–9 of FHIT. Reactions were performed as follows: 94°C for 1 min and 35 cycles at 94°C for 10 s, 58°C for 10 s, and 72°C for 1 min using Applied Biosystems 7400 (Applied Biosystems, Tokyo, Japan). Reaction products were purified and 1/20 volume was subjected to a second PCR amplification round with nested primers. For semiquantitative evaluation of expression, the first reaction cycle was for 24 cycles, where the control experiment showed linear amplification; 1/20 volume of the purified, first round PCR reaction products was used in the second PCR amplification round for 20, 25, 30, or 35 cycles. Products were fractionated on 1.5% agarose gels. Amplified fragments were cut from the gel and purified with Qiagen gel extraction kit (Qiagen, Valencia, CA). DNA sequencing was performed using the RISA 384 capillary DNA sequencing system (Shimadzu, Kyoto, Japan). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA was amplified as control, as described previously (32).

Immunoblot analysis was performed using antisera against acetylated Lys9 of histone 3 (H3K9), Lys8 of histone 4 (H4K8; Upstate Biotechnology, Inc., Lake Placid, NY), Fhit (Zymed, San Francisco, CA), Wwox (K. Huebner and S-Y. Han, Thomas Jefferson University, Philadelphia, PA), and actin (ICN Radiochemicals, Irvine CA), detected with the secondary antisera, followed by enhanced chemiluminescence detection (Amersham, Tokyo, Japan).

DNA Blot Hybridization

DNA (5 μg) from primary tumors were digested with EcoRI, fractionated by 0.8% agarose gel electrophoresis, transferred to Nylon membrane (Amersham), and cross-linked under UV light. The following probes were used: cDNAs of peptide coding region of FHIT and WWOX, which were amplified by reverse transcription-PCR, were subcloned and sequenced. The hybridization probes were prepared by random primer extension. Unincorporated nucleotides were removed by spin filtration in G-50 column. Heat-denatured probes were added to the prehybridized filter in the Perfect Hybridization buffer (Sigma Chemical) at 5 × 10⁶ to 6 × 10⁶ counts/min/ml. After incubation for 16 h at 65°C, the filter was washed in the high stringent condition (33) and exposed to X-ray film.

References

24. Kantarjian, H. M., Talpaz, M., O’Brien, S., Manshouri, T., Cortes, J., Giles,


Expression of \textit{FRA16D/WWOX} and \textit{FRA3B/FHIT} Genes in Hematopoietic Malignancies

In part by the Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan, by the research funds from the Kato Memorial Bioscience Foundation, the Cell Science Research Foundation, and the Takeda Science Foundation, Japan, and by the USPHS grants PO1, CA77738 from National Cancer Institute.

Hideshi Ishii, Andrea Vecchione, Yutaka Furukawa, et al.