The Tumor Suppressor Gene \textit{WWOX} at \textit{FRA16D} Is Involved in Pancreatic Carcinogenesis

Tamotsu Kuroki,\textsuperscript{1} Sai Yendamuri,\textsuperscript{1} Francesco Trapasso,\textsuperscript{1} Ayumi Matsuyama,\textsuperscript{1} Rami I. Aqeilan,\textsuperscript{1} Hansjuerg Alder,\textsuperscript{1} Shashi Rattan,\textsuperscript{1} Rossano Cesari,\textsuperscript{1} Maria L. Nolli,\textsuperscript{2} Noel N. Williams,\textsuperscript{3} Masaki Mori,\textsuperscript{4} Takashi Kanematsu,\textsuperscript{5} and Carlo M. Croce\textsuperscript{1}

\textsuperscript{1}Kimmel Cancer Center, Thomas Jefferson University, Philadelphia, Pennsylvania; \textsuperscript{2}Areta International srl, Gerenzano, Italy; \textsuperscript{3}Hospital of the University of Pennsylvania, Philadelphia, Pennsylvania; \textsuperscript{4}Department of Surgery, Medical Institute of Bioregulation, Kyushu University, Beppu, Japan; and \textsuperscript{5}Department of Surgery, Nagasaki University, Graduate School of Biomedical Sciences, Nagasaki, Japan

\textbf{ABSTRACT}

\textit{Purpose:} \textit{WWOX} (WW domain containing oxidoreductase) is a tumor suppressor gene that maps to the common fragile site \textit{FRA16D}. We showed previously that \textit{WWOX} is frequently altered in human lung and esophageal cancers. The purpose of this study was to delineate more precisely the role of \textit{WWOX} in pancreatic carcinogenesis.

\textit{Experimental Design:} We analyzed 15 paired pancreatic adenocarcinoma samples and 9 pancreatic cancer cell lines for \textit{WWOX} alterations. Colony assay and cell cycle analysis were also performed to evaluate the role of the \textit{WWOX} as a tumor suppressor gene.

\textit{Results:} Loss of heterozygosity at the \textit{WWOX} locus was observed in 4 primary tumors (27\%). Methylencylation analysis showed that site-specific promoter hypermethylation was detected in 2 cell lines (22\%) and treatment with the demethylating agent 5-aza-2-deoxycytidine demonstrated an increase in the expression of \textit{WWOX}. In addition, 2 primary tumor samples (13\%) showed promoter hypermethylation including the position of site-specific methylation. Transcripts missing \textit{WWOX} exons were detected in 4 cell lines (44\%) and in 2 tumor samples (13\%). Real-time reverse transcription PCR revealed a significant reduction of \textit{WWOX} expression in all of the cell lines and in 6 primary tumors (40\%). Western blot analysis showed a significant reduction of the \textit{WWOX} protein in all of the cell lines.

Furthermore, transfection with \textit{WWOX} inhibited colony formation of pancreatic cancer cell lines by triggering apoptosis.

\textit{Conclusion:} These results indicate that the \textit{WWOX} gene may play an important role in pancreatic tumor development.

\section*{INTRODUCTION}

Pancreatic cancer is one of the most common human cancers and carries a poor prognosis (1). Although complete surgical resection is the only therapeutic option for a long-term survival, the 5-year survival rate with curative resection is \textless 20\% (2). The precise molecular mechanisms of development and/or progression of pancreatic cancer remain unknown, although multiple genetic and epigenetic changes have been detected in pancreatic cancer. Therefore, additional elucidation of the molecular mechanisms involved in pancreatic cancer is urgently needed for more effective treatment.

The tumor suppressor gene \textit{FHIT} spans the \textit{FRA3B} fragile site, the most active common chromosomal fragile site of the human genome, and abnormal \textit{FHIT} transcripts have been detected in many human cancers, including pancreatic cancer (3–6). Additionally, our previous study demonstrated that \textit{FHIT} overexpression enhanced the susceptibility of pancreatic cancer cells to exogenous inducers of apoptosis (7). Recently, in the \textit{WWOX} (WW domain containing oxidoreductase), a candidate tumor suppressor gene, chromosome 16q23.3–24.1 was isolated. This gene maps at the location of \textit{FRA16D}, one of the most active common chromosomal fragile sites (8–11). The \textit{WWOX} gene is similar to the \textit{FHIT} gene in that both genes are \textgreater 1 Mb and encompass very active common fragile sites, both genes show frequent allelic loss and/or loss of homozygous deletion region in several human cancers, and both frequently show aberrant transcripts (5, 10, 11). Because increased risk of pancreatic cancer is associated with cigarette smoking and because the carcinogens present in tobacco smoke cause deletions at the \textit{FHIT/FRA3B} loci (5, 6), we hypothesized that the \textit{WWOX} gene may play a role in pancreatic cancer. In this report, we describe that \textit{WWOX} is altered frequently in pancreatic cancer by genetic and/or epigenetic changes and is involved in pancreatic carcinogenesis.

\section*{MATERIALS AND METHODS}

\textbf{Cell Lines and Tissues.} Human pancreatic cancer cell lines Panc1, AsPc1, CFPAC1, MiaPaca2, Capan2, BxPc3, Hs766T, and Su.86.86 were obtained from the American Type Culture Collection. PSN1 was obtained from the European Collection of Cell Cultures. All of the cell lines were maintained in RPMI 1640 with 10\% fetal bovine serum at 37°C and 5\% CO\textsubscript{2}. Tumors and corresponding noncancerous tissues were obtained from 15 patients who underwent surgery for pancreatic adenocarcinoma. The tissue samples were excised and were immediately stored at \textminus 80°C. DNA was extracted from each of

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\textbf{Note:} T. Kuroki and S. Yendamuri contributed equally to this study.

\textbf{Requests for reprints:} Carlo M. Croce, Kimmel Cancer Institute, Jefferson Medical College, Thomas Jefferson University, BLSB Room 1050, 2335 10th Street, Philadelphia, PA 19107-5799. Phone: (215) 503-4645, Fax: (215) 923-3528; E-mail: croce@calvin.jci.tju.edu.

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the 9 cell lines and from the 15 paired pancreatic tissues according to methods described previously (12). RNA was extracted with the Trizol reagent (Invitrogen Life Technologies, Inc., Carlsbad, CA) per the manufacturer’s recommendations.

**Loss of Heterozygosity (LOH) Analysis.** Allelic losses were analyzed using a PCR approach, with primers amplifying polymorphic microsatellites internal to WWOX at loci D16S3029, D16S3096, D16S504, and D16S518, as described elsewhere (13). Briefly, the primer sequences were obtained from the Genome Database and primers were labeled using 5’-fluorescein phosphorylmidate or 5’-tetrachlorofluorescein phosphorylmidate for microsatellite loci, as described by Ishii et al. (14), PCR was performed on 50 ng of DNA for each sample using the conditions described for the mutation search. PCR products were denatured in formamide for 5 min at 95°C and then loaded on a 6% denaturing gel on the Applied Biosystems 373 DNA sequencer. LOH was analyzed using the Applied Biosystems Prism Genescan and the Applied Biosystems PRISM GENETYPER ANALYSIS software (Perkin-Elmer/Applied Biosystems). Cases were defined as having LOH when an allele peak signal from tumor DNA was reduced by 50% compared with the normal counterpart.

**Mutation Screening of the WWOX Gene.** Nine pairs of primers for individual exon amplification and screening were used for PCR screening with genomic DNA. The primers for each exon are specified in GenBank (accession numbers AF325423–AF325432). The PCRs were performed with the same conditions as those described for the LOH analysis. The PCR products were purified using the QIAquick PCR purification kit (Qiagen, Inc., Valencia, CA), and sequencing reactions and analysis were performed using the Applied Biosystems Prism BigDye terminator reaction chemistry on a Perkin-Elmer Gene Amp PCR system 9600 and the Applied Biosystems Prism 377 DNA sequencing system (Applied Biosystems, Inc., Foster City, CA).

**Nested Real-Time Reverse Transcription PCR Analysis of the WWOX Transcripts.** cDNA was synthesized from 2 μg of total RNA and real-time reverse transcription PCR was performed as described previously (13). Glyceraldehyde-3-phosphate dehydrogenase amplification served as a control for cDNA quality. One μl of cDNA was used for the first PCR amplification with WWOX-specific primers (forward primer, 5’-AGTCCCTGAGCGATGGACC-3’; reverse primer, 5’-TTACTTTCAACAGGGCACCAC-3’) in a volume of 50 μl containing 20 pmol of each primer, 2.5 mM MgCl₂, 1.5 mM deoxynucleoside triphosphate mix, 1X PCR buffer, and 2 units of AmpliTaq Gold (Perkin-Elmer). PCR cycles included one cycle at 95°C for 5 min followed by 30 cycles at 94°C for 30 s, 57°C for 30 s, and 72°C for 1 min, with a final extension step at 72°C for 5 min in a Perkin-Elmer Gene Amp PCR system 9600. One μl of the first PCR amplification product was used for a second PCR amplification with WWOX-nested primers (forward primer, 5’-AGTCCCTGAGCGATGGACC-3’; reverse primer, 5’-TTACTTTCAACAGGGCACCAC-3’) under the same conditions as those of the first PCR. The amplified products were analyzed by electrophoresis on a 1.5% agarose gel. DNA bands corresponding to the normal- and abnormal-sized WWOX transcripts were excised from the gel, purified using the QIAquick gel extraction kit (Qiagen), and sequenced on the Applied Biosystems Prism 377 DNA sequencing system. Two primer sets were designed to amplify the entire open reading frame.

**Methylation Analysis.** Genomic DNA was treated with sodium bisulfite according to methods described previously (15). Briefly, 1 μg of the genomic DNA was denatured by 2 M NaOH, and then incubated in 3 μM sodium bisulfite and 10 mM hydroquinone for 17 h at 55°C. Bisulfite-treated DNA was extracted using a genomic DNA clean-up kit (Promega, Madison, WI). Modified DNA was amplified using nested PCR that amplify the putative promoter region, including a CpG-rich area surrounding the translation start codon (11). Primers for the nested PCR amplification were 5’-TGGTTTATTATATATTAGTTTTTATTAT-3’ (forward primer) and 5’-ACTCATCCTACTACCATCATCAT-3’ (reverse primer) for the first PCR, and 5’-AGTTTTATTATATTAGTTTTTATTAT-3’ (forward primer) and 5’-CATCATTACCATCACCCCCAC-3’ (reverse primer) for the second PCR. PCR was performed in 50-μl reaction volumes containing 1× PCR buffer II (Perkin-Elmer, Branchburg, NJ), 2 mM MgCl₂, 0.2 mM of each deoxynucleoside triphosphate, 10 pmol of each primer, 1 unit of AmpliTaq Gold polymerase (Perkin-Elmer), and 50 ng bisulfite-modified DNA. PCR cycling conditions were 10 min at 95°C, 35 cycles of a 30-s denaturation at 94°C, a 30-s annealing at 55°C, a 60-s extension at 72°C, and a final extension step of 5 min at 72°C. PCR products were subcloned into the pGEM-T Easy vector (Promega), and six clones were sequenced.

**Real-Time Reverse Transcription PCR.** Real-time reverse transcription PCR was performed using primers designed to span intron 6. The forward primer on exon 6 was 5’-CTGTCAGCAGTATTGTGCTGAAG-3’ and the reverse primer on exon 7 was 5’-AGTTCTGGTGTTGACACA-3’. The probe sequence 5’-AGGCCAAGAATGTTGCTCTATATGC-3’ was labeled with the reporter dye VIC. 6-Carboxyfluorescein was used as the quencher. β-Actin was used as an internal control. Thirty-five cycles were run with an annealing temperature of 62°C and an elongation time of 30 s. All of the reactions were performed in an ABI PRISM 7000 Sequence Detection System (Applied Biosystems). Each sample was performed in triplicate. After normalization of the samples with the respective β-actin control, a t test was used to compare the quantitation of WWOX expression. All of the analyses were performed with the Statistical Package for the Social Sciences for Windows (SPSS, Inc., Release 10.0.7, 2000). The t tests were considered statistically significant if P < 0.05.

**5-Aza-2’-Deoxycytidine (5-AZAC) Treatment.** To determine whether treatment of a methylated cell line with a demethylating agent induced WWOX expression, we treated the pancreatic cancer cell line Hs766T with 5-AZAC. Cells were plated and incubated for 3 days with 1 μM of 5-AZAC. The medium and the drug were replaced every 24 h. RNA obtained from wild-type and 5-AZAC-treated cells was used to make cDNA for real-time reverse transcription PCR analysis. Primers and probe spanning intron 8 were used as described elsewhere (11). Analysis of data was performed as described previously.

**Western Blot.** Proteins were extracted from all 9 of the pancreatic cancer cell lines by lysing cells in a Triton extraction buffer containing 10 mM Tris-HCl (pH7.4), 5 mM NaCl, 5 mM EDTA, 10% glycerol, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml, and 1 mM sodium orthovanadate.
Concentration of proteins in the lysates was measured using the Bio-Rad protein assay reagent (Bio-Rad Inc., Richmond, CA) according to the manufacturer’s protocol. Normal human pancreas tissue lysate was obtained from ProSci, Inc. (Poway, CA). Equal amounts of protein (20 μg) were boiled in Laemmli sample buffer, separated in a 4–20% SDS-PAGE gel (Ready Gels; Bio-Rad, Inc.), and transferred to nitrocellulose membranes (Bio-Rad, Inc.).

Table 1  Summary of alteration to WWOX

<table>
<thead>
<tr>
<th>Cell line or case no.</th>
<th>Allelic loss</th>
<th>Methylation*</th>
<th>Reverse transcription-PCR</th>
<th>Aberrant productα</th>
</tr>
</thead>
<tbody>
<tr>
<td>Panc1</td>
<td>Exon 7-8 homozygous deletion</td>
<td>−37</td>
<td>Absent</td>
<td>—</td>
</tr>
<tr>
<td>MiaPaca2</td>
<td>—</td>
<td>None</td>
<td>Aberrant</td>
<td>Exon 1*-6* Deletion</td>
</tr>
<tr>
<td>Capan2</td>
<td>—</td>
<td>None</td>
<td>Aberrant + N</td>
<td>Exon 1*-8* Deletion</td>
</tr>
<tr>
<td>Hs766T</td>
<td>—</td>
<td>−37</td>
<td>Aberrant</td>
<td>Exon 1*-8* Deletion</td>
</tr>
</tbody>
</table>

1 Retention           | −37, −43, −53                 | Normal       | —                         |
2 Retention           | None                          | Absence      | —                         |
3 LOH                 | None                          | Normal       | —                         |
5 LOH                 | −37 to −148                   | Normal       | —                         |
6 Retention           | None                          | Aberrant + normal | Exon 2-8 deletion       |
7 LOH                 | None                          | Normal       | —                         |
14 LOH                | None                          | Normal       | —                         |

α  Cytosine position of methylated CpG site relative to the ATG start codon.
β  Represents a partial deletion of the exon.
γ  LOH, loss of heterozygosity.

RESULTS

LOH and Mutation Analysis. We searched for LOH using four microsatellite markers within WWOX. The loci D16S3029, D16S3096, and D16S304 are located in intron 8, and D16S518 is located in exon 1 (13). All 15 of the cases were informative for at least one of the loci examined, and in 4 cases (27%) we detected LOH (Table 1). Representative results of LOH analysis are shown in Fig. 1A. To determine whether the WWOX gene was the target of functional inactivation in pancreatic adenocarcinoma, we screened for somatic mutations in the WWOX gene by direct sequencing of PCR products of all of the WWOX exons in tumor cells, in normal counterparts for each cancer, and in 9 human pancreatic cancer cell lines. In the Panc1 cancer cell line, exons 7 and 8 were not amplified, indicating a homozygous deletion. We confirmed this homozygous deletion of exons 7 and 8 in Panc1 by duplex PCR (data not shown). All of the exons were amplified in the other 8 cell lines and in 15 paired samples. No somatic point mutations were identified in the cell lines and clinical samples.

Methylation Analysis of the WWOX Gene Promoter Region. To evaluate the methylation status of the promoter region of WWOX in pancreatic adenocarcinoma, we used a sodium bisulfite sequence approach. Two of 9 pancreatic cell
lines (22%; Panc1 and Hs766T) showed methylated CpG site only at the cytosine \(-37\) bp relative to the ATG start codon of 55 CpG sites (Table 1). The result of bisulfite sequence analysis of Hs766T is shown in Fig. 1B. In the PCR product from the specific primers for bisulfite-treated DNA, methylated cytosine appears as a G signal in the complementary strand at the CpG dinucleotide only at the \(-37\) position. Other unmethylated cytosine nucleotides, including those in the CpG dinucleotides, changed to thymines because of bisulfite modification. This site-specific methylation of a single CpG site of Panc1 and Hs766T was detected in six of six and five of six clones, respectively. In primary tumors, case 1 showed methylation at cytosines \(-37, -43, \) and \(-53\) in six of six clones. Case 5 showed methylation at all of the cytosines from \(-148\) to \(-37\) in six of six clones (Table 1).

**5-AZAC Treatment.** After treatment with 5-AZAC, Hs766T cell lines demonstrated an increase in expression of WWOX by real-time reverse transcription-PCR. The WWOX:actin ratio increased from a mean of 0.34 to 0.44. This difference was statistically different and represents a 30\% increase in expression.

**Analysis of the WWOX Transcripts.** The mRNA expression of WWOX was analyzed in 9 pancreatic cancer cell lines and in 15 clinical samples by reverse transcription-PCR. One cell line (Panc1) showed no WWOX transcript. Three cell lines (MiaPaca2, Capan2, and Hs766T) exhibited aberrant

![Figure 1](https://example.com/figure1.png)

**Fig. 1** Analysis of WWOX gene in pancreatic cancer. A, representative loss of heterozygosity analysis of case 3 at the D16S518 locus. The microsatellite locus is identified below the chromatogram. T, tumor; N, corresponding normal tissue. Arrow indicates loss of heterozygosity at D16S518. B, direct sequencing chromatogram of PCR products from Hs766T. The CpG sites are indicated by a bar and the complementary strand at methylated cytosine are indicated by an asterisk. C, reverse transcription PCR analysis of WWOX in pancreatic cancer cell lines. Arrow indicates location of normal transcripts and arrowheads indicate location of aberrant transcripts. Glyceraldehyde-3-phosphate dehydrogenase amplification served as a control for cDNA quality. NC, negative control. D, representative reverse transcription PCR analysis from two cases, 2 and 6. Case 2 shows the absence of WWOX transcript in tumor sample. Case 6 shows the aberrant transcript with normal transcript of WWOX in tumor sample.

![Figure 2](https://example.com/figure2.png)

**Fig. 2** Real-time reverse transcription-PCR analysis of WWOX expression relative to \(\beta\text{-actin}\) in human pancreatic cancer cell lines. Analysis was performed with the Statistical Package for the Social Sciences for Windows (SPSS, Inc, Release 10.0.7, 2000). The \(t\) tests were considered to be statistically significant if \(P < 0.05\).
transcripts, and Capan2 showed an aberrant band with a normal-sized transcript (Fig. 1C). Sequence analysis of aberrant transcripts showed frameshifts in the open reading frame (Table 1). Of 15 primary tumors, 1 case (case 6) showed an aberrant transcript plus a normal-sized WWOX reverse transcription-PCR product (Fig. 1D), and sequence analysis of the aberrant transcript showed deletion of exons 2–8. In 1 case (case 2), no WWOX transcript was detected (Table 1). The remaining primary tumors showed only a normal-sized transcript.

**Real-Time Reverse Transcription-PCR Analysis.** Fig. 2 summarizes the results of the comparison of WWOX expression in the 9 pancreatic cancer cell lines and in the normal pancreatic tissue control. Although Panc1 showed no expression of the WWOX gene, the remaining 8 cell lines showed a reduction in expression. Subsequently, we assessed the expression of WWOX in 15 primary pancreatic adenocarcinomas. Six cases (cases 2, 6, 9, 10, 13, and 14; 40%) showed a reduction in expression of WWOX.

**Western Blot.** Fig. 3 shows the results of a Western blot analysis of the pancreatic cancer cell lines. Although Panc1 showed no expression of WWOX, the other 8 cell lines showed a clear reduction in WWOX expression compared with normal pancreas tissue lysate.

**Fig. 3** Western blot demonstrating the decreased expression of WWOX in human pancreatic cancer cell lines. Protein loading normalized with β-actin; bars, ±SD.

**Fig. 4** *A,* colony assay demonstrating decreased colony formation after WWOX transfection. *B,* quantification of decreased colony formation by colony count; bars, ±SD.
Alterations in Pancreatic Carcinoma

**Fluorescence-Activated Cell Sorter Analysis.** Significantly greater fractions of transfected cells were found in the sub-G1 phase by fluorescence-activated cell sorter analysis after transfection with pMV-7/WWOX compared with the empty pMV-7 vector (Fig. 5), strongly suggesting a role for WWOX in triggering apoptosis of malignant pancreatic cells.

**DISCUSSION**

In this study, we demonstrated that: (a) WWOX is altered by deletion and/or aberrant expression in 4 of 9 pancreatic cancer cell lines (44%) and 6 of 15 primary pancreatic adenocarcinomas (40%); (b) promoter hypermethylation of WWOX, including −37 position site-specific methylation, is detected in 2 cell lines (22%) and in 2 samples (13%), and treatment with the demethylating agent 5-AZAC elevated significantly WWOX expression in Hs766T; (c) all of the cell lines showed low levels of WWOX expression using real-time reverse transcription-PCR and Western blot, and 6 primary cases (40%) showed a statistically significant reduction in WWOX expression; and (d) transfection with WWOX induced apoptosis and suppressed colony formation in cell lines. The WWOX gene was identified recently as a tumor suppressor gene at 16q23.3–24.1, a chromosome region that spans the common fragile site FRA16D (8–11). Several studies have revealed alterations of WWOX in several types of human cancers (8–11, 14). The finding of aberrant WWOX transcripts is consistent with the pattern of WWOX alterations described in recent studies (10, 13). We found that most aberrant transcripts in esophageal and lung cancers lacked exons 5, 6, 7, and 8 (13, 18). These exons encode the oxidoreductase domain of the WWOX protein (19), and their absence may cause loss of function of WWOX. In this study, all of the aberrant transcripts showed absence of these exons. Recent studies have reported that promoter hypermethylation of several tumor suppressor genes may be an important mechanism for gene inactivation in several types of cancer (15, 20). Interestingly, we detected −37 position site-specific methylation in pancreatic cancer cell lines. In 2 cell lines, the position of the methylated site was the same as the CpG island. Furthermore, treatment of Hs766T cells with the demethylating agent 5-AZAC elevated WWOX expression significantly. In clinical samples, 2 cases showed promoter hypermethylation within the promoter region including site-specific methylation at −37 position. Pogribny et al. (21) demonstrated that site-specific methylation within the p53 promoter region was associated with transcriptional inactivation of the p53. These findings suggest that site-specific methylation of the WWOX may be involved in pancreatic carcinogenesis.

Transfection of WWOX into pancreatic cancer cell lines resulted in apoptosis. Chang et al. (22) reported that murine WWOX (WOX1) is a mitochondrial apoptotic protein and an essential partner of p53 in apoptosis. Furthermore, to evaluate the potential of WWOX as a tumor suppressor gene, we performed a colony-forming assay using tumor-derived cell lines. WWOX inhibited the colony formation of pancreatic cancer cell lines. Thus, we conclude that WWOX plays a role in pancreatic carcinogenesis by affecting apoptosis. WWOX may represent a new target for the development of a gene therapy approach to the treatment of pancreatic cancer.

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