**Advances in Brief**

**Genetic Alterations of the Tumor Suppressor Gene WWOX in Esophageal Squamous Cell Carcinoma**

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**Abstract**

The WWOX (WW domain containing oxidoreductase) gene was recently identified as a candidate tumor suppressor gene at 16q23.3-24.1, a chromosome region that spans the common fragile site FRA16D. To evaluate the potential role of the WWOX gene in esophageal squamous cell carcinomas, we examined 36 tumors for genetic alterations of the WWOX gene. Loss of heterozygosity (LOH) at the WWOX locus was observed in 14 (39%) tumors. A tumor-specific missense mutation was found in one tumor, and LOH analysis had shown that the other allele was missing. Furthermore, we detected aberrant WWOX gene transcripts with absence of exons 6–8 in two tumors, and complete absence of transcript in one tumor. These results indicate that alteration and inactivation of the WWOX gene may play a role in esophageal squamous cell carcinogenesis.

**Introduction**

The genesis of human cancers is generally a multistep process reflecting cumulative genetic alterations that include activation of oncogenes or inactivation of tumor suppressor genes. Although frequent allelic deletions and other genetic abnormalities have been detected in esophageal squamous cell carcinoma (1–3), the precise molecular mechanism of development and/or progression of esophageal squamous cell carcinoma still remains unclear. WWOX gene, a candidate tumor suppressor gene, was recently detected in chromosome 16q23.3-24.1 (4–7). Chromosome 16q was implicated as the chromosome region that spans the common fragile site FRA16D (8–10). Furthermore, we have reported that FRA16D locus in esophageal carcinoma was susceptible to damage by environmental carcinogens, such as Aflatoxin B1 were observed (13). Furthermore, we have reported that the FHIT/FRA3B locus in esophageal carcinoma (14). Thus we hypothesized that the WWOX gene may be a tumor suppressor gene in esophageal squamous cell carcinoma. In this report, we describe the occurrence of genomic alterations and aberrant transcripts of WWOX gene in human esophageal squamous cell carcinoma.

**Materials and Methods**

**Tissues.** Tumors and corresponding noncancerous tissues were obtained from 36 Japanese patients who underwent surgery for esophageal squamous cell carcinoma (31 male, 5 female; median age, 59 years; range, 48–74 years). Four were stage I, 5 were stage II, 25 were stage III, and 2 were stage IV, according to the Tumor-Node-Metastasis classification. Tumors and corresponding normal tissue samples were obtained for each patient.

**DNA and RNA Extraction.** The tissue samples were excised and immediately stored at −80°C. DNA and RNA were extracted from each sample according to methods described previously (15).

**LOH Analysis.** Allelic losses were analyzed by a PCR approach with primers amplifying polymorphic microsatellites internal to WWOX at loci D16S3029, D16S3096, D16SS04, and D16SS18. The primer sequences were obtained from the Genome database, and primers were labeled using 5′-fluorescein phosphoramidite or 5′-tetrachlorofluorescein phosphoramidite for microsatellite loci, as described by Ishii et al. (16). PCR was performed on the genomic DNA samples using the following conditions: 50 ng of genomic DNA template, 10 pmol of each primer, 2.5 mM MgCl₂, 1.5 mM dNTP mix, 1× PCR buffer, and 0.5 unit of AmpliTaq Gold (Perkin-Elmer, Branchburg, NJ) in a 20-μl final volume. PCR cycles included one cycle of 95°C for 12 min followed by 35 cycles consisting of 10 cycles at 94°C for 15 s, 55°C for 15 s, and 72°C for 30 sec and by 25 cycles at 89°C for 15 s, 55°C for 15 s, and 72°C for 30 s, followed by 72°C for 10 min in a Perkin-Elmer Gene Amp PCR system 9600. 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 by using the Applied Biosystems Prism Genescan and the Applied Biosystems PRISM GENETYPER ANALYSIS software (Perkin-Elmer/Applied Biosystems). Cases were defined as 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 with genomic DNA. The primers for each exon are specified in GenBank (accession nos. AF325423–AF325432). The PCR products were performed with the same condition 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 done 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 Biosystem, Inc., Foster City, CA).

**Nested RT-PCR Analysis of the WWOX Transcript.** cDNA was synthesized from 2 μg of total RNA, and RT was performed as described previously (12). GAPDH amplification served as a control for cDNA quality. One μl of cDNA was used for the first PCR amplification with primers (forward primer, 5′-AGTTCTGAGCGAGTGGACCC-3′; reverse primer, 5′-TTATCTTCCAAACAGGGCACCAC-3′) in a volume of 50 μl containing 20 pmol of each primer, 2.5 mM MgCl₂, 1.5 mM dNTP mix, 1× PCR buffer, and 0.5 unit of AmpliTaq Gold (Perkin-Elmer/Applied Biosystems). The nested RT-PCR products were purified using the QIAquick PCR purification kit (QIAGEN, Valencia, CA), and sequencing reactions and analysis were done 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 Biosystem, Inc., Foster City, CA).
the of LOH analysis in these 14 cases. Case 8 retained heterozygosity at D16S3096, D16S3029 and the D16S3029 locus but showed LOH in the region distal to D16S3096.

**Mutation Analysis.** To determine whether the WWOX gene was the target of functional inactivation in esophageal squamous cell carcinoma, we screened for somatic mutations of the WWOX gene by direct sequencing of PCR products of all WWOX exons in tumor and normal counterpart for each cancer. One somatic missense mutation was detected in 36 cases (Table 1). In case 26, the tumor DNA showed a T to C transition at the second nucleotide of codon 291 and resulted in a Leucine to Proline substitution (Fig. 2B). This case also showed allelic loss at the WWOX locus (Fig. 2A).

**Aberrant WWOX Gene Transcripts.** We observed that WWOX mRNA expression was undetectable in our samples by Northern blot analysis (data not shown). Therefore, we performed RT-PCR analysis to detect the WWOX expression. Two cases (cases 12 and 51) showed aberrant transcript plus normal-sized WWOX RT-PCR product (Fig. 2C), and sequence analysis of aberrant transcript showed deletion of exons 6–8 in both cases (Table 1). In one case (case 31), no WWOX transcript was detected (Table 1). The remaining cases showed only normal-sized transcript.

**Discussion**

In the present study, mutation analysis revealed one missense mutation among DNAs from 36 esophageal squamous cell carcinomas. Although infrequent, our findings represent the first report of a WWOX somatic missense mutation in esophageal squamous cell carcinoma. In this tumor in which we identified somatic missense mu-

### Table 1 Summary of alteration to WWOX

<table>
<thead>
<tr>
<th>Sample</th>
<th>Exon</th>
<th>Codon</th>
<th>Mutation</th>
<th>LOH</th>
</tr>
</thead>
<tbody>
<tr>
<td>26</td>
<td>8</td>
<td>291</td>
<td>CTG(Leu) to CCG(Pro)</td>
<td>+</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Sample</th>
<th>RT-PCR</th>
<th>Aberrant product</th>
</tr>
</thead>
<tbody>
<tr>
<td>12</td>
<td>Normal</td>
<td>Aberrant</td>
</tr>
<tr>
<td>31</td>
<td>Absent</td>
<td>Exon 6–8 deletion</td>
</tr>
<tr>
<td>51</td>
<td>Normal</td>
<td>Aberrant</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Exon 6–8 deletion</td>
</tr>
</tbody>
</table>

2 units of AmpliTaq Gold (Perkin-Elmer). PCR cycles included one cycle of 95°C for 8 min followed by 35 cycles of 94°C for 30 sec, 57°C for 30 sec, and 72°C for 1 min, and a final extension step of 72°C for 5 min in a Perkin-Elmer Gene Amp PCR system 9600. A sample of 1 μl of the first PCR amplification product was used for a second PCR amplification with primers (forward primer, 5’-AGGTGCTTCACAGTCTC-3’; reverse primer, 5’-GTGTGTGTC-CCATCGCCCTCT-3’) under the same condition as the first PCR. The amplified products were analyzed by electrophoresis on a 1.5% agarose gel. DNA bands corresponding to the normal and abnormal size 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 whole open reading frame.

**Results**

**LOH Analysis.** To determine whether the WWOX locus undergoes allelic loss in esophageal cancers, we searched for LOH using four polymorphic microsatellite markers within WWOX. The loci D16S5029, D16S3096, and D16S3029 are located in intron 8, and D16S518 is exon 1. All of the 36 cases were informative for at least one of the loci examined, and in 14 cases (39%) we detected LOH; 11 cases showed LOH at all of the informative loci examined and 3 cases showed LOH at one of the loci examined, and in 14 cases (39%) we detected LOH; 11 cases showed LOH at all of the informative loci examined and 3 cases revealed partial deletions of the WWOX locus. Fig. 1 shows the results of LOH analysis in these 14 cases. Case 8 retained heterozygosity at the D16S518 locus, but showed LOH in the region distal to D16S3029. Cases 7 and 37 retained heterozygosity at the D16S518 locus, but showed LOH in the region distal to D16S3096.

![Fig. 2. Analysis of WWOX gene in esophageal squamous cell carcinoma. A, representative LOH analysis of case 26 at the D16S518 locus. Microsatellite locus is identified below chromatogram. T, tumor; N, corresponding normal tissue. Case 26 shows LOH at D16S518. B, case 26 represents a somatic missense mutation of T to C transition at codon 291, resulting in a change from Leu to Pro. N, normal tissue; T, tumor. C, representative RT-PCR analysis from two cases, cases 12 and 51. GAPDH amplification served as a control for cDNA quality. N, normal tissue; T, tumor; nc, negative control.](image-url)
tation, we demonstrated that both alleles of the WWOX gene were inactivated as a combination of tumor-specific mutation and LOH of WWOX gene locus. These results suggest that WWOX gene alterations may play a role in the esophageal squamous cell carcinogenesis via a two-hits mechanism, as proposed by Knudson (17). Several studies have suggested that 16q23-24 contains a candidate tumor suppressor gene involved in various tumor types (8–10). The WWOX gene, a recently cloned novel candidate tumor suppressor gene in this region, is also at a common fragile site, FRA16D (4–7). Our studies showed a high frequency of LOH at the WWOX locus in esophageal squamous cell carcinomas. In our three cases, microsatellite markers within intron 8 of WWOX showed LOH at least at one locus with retention of two alleles at the locus exon 1 (D16S518). Interestingly, Bednarek et al. (4) reported that the intron 8 region appears to be an area prone to breakage within FRA16D. Tumor suppressor genes are inactivated by genetic or epigenetic changes such as point mutations, deletions, promoter methylation, aberrant splicing, deregulation of imprinting, and haploinsufficiency.

Recently, Bednarek et al. (7) have reported the expression of abnormal WWOX gene transcripts missing exon 6–8 in more than 30% of breast cancer. We observed such aberrant WWOX transcripts in two esophageal squamous cell carcinoma cases. The WWOX somatic mutation in our study was detected in exon 8. Interestingly, exons 6–8 of WWOX gene encode the major portion of the enzymatic WWOX domain (4).

**FHIT** is a tumor suppressor gene that encompasses the most common fragile site, FRA1B (11, 18). Interestingly, the FHIT gene shows some similarities with the FHIT gene; for example: (a) both genes are larger than 1 Mb and encompass fragile sites; (b) both genes show frequent allelic loss region in various human cancers; (c) both span a region of homozygous deletion in multiple cancers; and (d) both frequently show aberrant transcripts (6, 7, 18). Our data indicate that the WWOX gene could act as a tumor suppressor in esophageal squamous cell carcinoma. In some cases, the WWOX gene is inactivated by two-hits, including allelic loss and point mutation, and the normal function of WWOX gene may be altered by different genetic or epigenetic mechanisms.

**References**


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