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Advances in Brief

Genetic Alterations of the Tumor Suppressor Gene *WWOX* in Esophageal Squamous Cell Carcinoma[1]

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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*[3] gene, a candidate tumor suppressor gene, was recently detected in chromosome 16q23.3-24.1 (4–7). Chromosome 16q was implicated as the site of tumor suppressor genes in a large spectrum of tissues because a high frequency of LOH[3] of microsatellite markers at this chromosomal arm had been detected in multiple tumor types (8–10). Furthermore, the *WWOX* gene is located within the common fragile site *FRA16D* (4–7). *FRA3B* and *FRA16D* have been observed to be the most frequently expressed of the common chromosomal fragile site loci. The tumor suppressor gene, *fragile histidine triad* (*FHIT*) gene, was found to span the *FRA3B* fragile site (11), and abnormal *FHIT* transcripts were detected in various human cancers, including esophageal squamous cell carcinoma (12). Recently, genetic alterations at *FRA16D* associated with exposure to environmental carcinogens such as Aflatoxin B1 were observed (13). Furthermore, we have reported that the *FHIT*/*FRA3B* locus in esophageal carcinoma was susceptible to damage by environmental carcinogens, such as smoking or alcohol consumption (12). Smoking and alcohol consumption are the major risk factors for esophageal carcinoma (14). We thus 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 abnormal 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*, *D16S504*, and *D16S518*. 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 $MgCl_2$, 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 PCRs 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′-AGTTCCTGAGCGAGTGGACC-3′; reverse primer, 5′-TTACTTTCAAACAGGCCACCAC-3′) in a volume of 50 μl containing 20 pmol of each primer, 2.5 mM $MgCl_2$, 1.5 mM dNTP mix, 1× PCR buffer, and

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[3] The abbreviations used are: WWOX, WW domain containing oxidoreductase; LOH, loss of heterozygosity; RT, reverse transcription; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; FHIT, fragile histidine triad.

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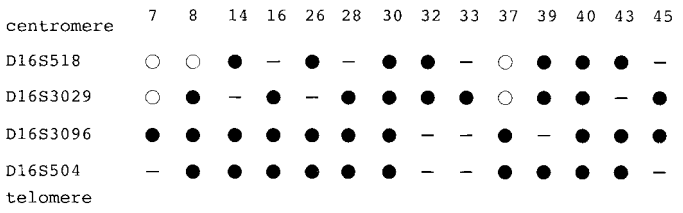

Fig. 1. LOH analysis of *WWOX* gene locus in esophageal squamous cell carcinoma. All of the patients with allelic loss at least at one locus are shown. ●, loss of an allele; ○, retention of both alleles; —, not informative (homozygosity).

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′-AGGTGCCTCCACAGTC-3′; reverse primer, 5′-GTGTGTGC-CCATCCGCTCT-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 *D16S3029*, *D16S3096*, and *D16S504* 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 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* 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. 2*B*). This case also showed allelic loss at the *WWOX* locus (Fig. 2*A*).

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. 2*C*), 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*

Sample	Exon	Codon	Mutation	LOH
26	8	291	CTG(Leu) to CCG(Pro)	+
Sample		**RT-PCR**	**Aberrant product**	
12		Normal + Aberrant	Exon 6–8 deletion	
31		Absent		
51		Normal + Aberrant	Exon 6–8 deletion	

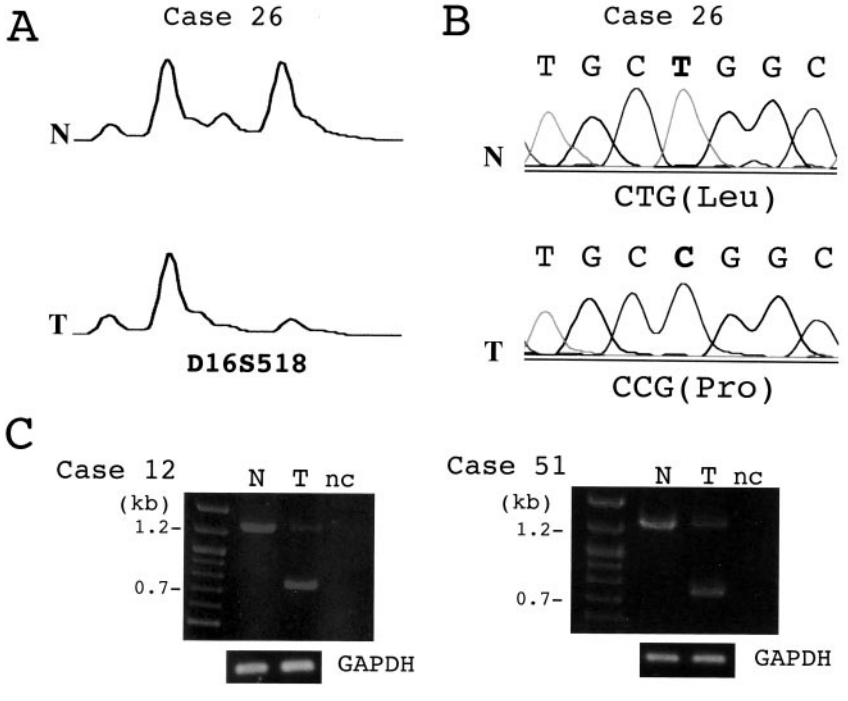

Fig. 2. Analysis of *WWOX* gene in esophageal squamous cell carcinoma. *A*, representative LOH analysis of case 26 at the *D16S518* locus. Microsatellite locus is identified below chromatogram. *T*, tumor; *N*, corresponding normal tissue. Case 26 shows LOH at *D16S518*. *B*, case 26 represents a somatic 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.

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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, *FRA3B* (11, 18). Interestingly, the *WWOX* 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.

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