Inactivation of the Wwox Gene Accelerates Forestomach Tumor Progression In vivo

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Abstract
The WWOX gene encodes a tumor suppressor spanning the second most common human fragile site, FRA16D. Targeted deletion of the Wwox gene in mice led to an increased incidence of spontaneous and ethyl nitrosourea–induced tumors. In humans, loss of heterozygosity and reduced or loss of WWOX expression has been reported in esophageal squamous cell cancers (SCC). In the present study, we examined whether inactivation of the Wwox gene might lead to enhanced esophageal/forestomach tumorigenesis induced by N-nitrosomethylbenzylamine. Wwox+/- and Wwox+/- mice were treated with six intragastric doses of N-nitrosomethylbenzylamine and observed for 15 subsequent weeks. Ninety-six percent (25 of 26) of Wwox+/- mice versus 29% (10 of 34) of Wwox+/- mice developed forestomach tumors (P = 1.3 × 10^-7). The number of tumors per forestomach was significantly greater in Wwox+/- than in Wwox+/- mice (3.2 ± 0.34 versus 0.47 ± 0.17; P < 0.0001). In addition, 27% of Wwox+/- mice had invasive SCC in the forestomach, as compared with 0% of wild-type controls (P = 0.002). Intriguingly, forestomachs from Wwox+/- mice displayed moderately strong Wwox protein staining in the near-normal epithelium, but weak and diffuse staining in SCC in the same tissue section, a result suggesting that Wwox was haploinsufficient for the initiation of tumor development. Our findings provide the first in vivo evidence of the tumor suppressor function of WWOX in forestomach/esophageal carcinogenesis and suggest that inactivation of one allele of WWOX accelerates the predisposition of normal cells to malignant transformation. [Cancer Res 2007;67(12):5606–10]

Introduction
The WW domain–containing oxidoreductase (WWOX) gene encodes a 46 kDa tumor suppressor. The human WWOX gene maps to the 16q23.1 chromosomal region and spans the second most common fragile site, FRA16D (1, 2). Fragile sites are genomic regions that show more frequent gaps and breaks following DNA replication stress or exposure to carcinogens (3). Intriguingly, the recombination of these genomic regions leads to altered expression of oncogenes and tumor suppressor genes at fragile sites. Indeed, WWOX expression is frequently perturbed in most human malignancies, including esophageal and gastric carcinomas (4). Loss of heterozygosity and reduction or loss of WWOX expression has been reported in both human esophageal and gastric malignancies. In particular, 39% of esophageal squamous cell carcinoma samples showed aberrant transcripts and/or loss of WWOX mRNA expression (5). Similarly, 37% of human gastric carcinoma samples exhibited loss of heterozygosity, whereas 65% of stomach cancer samples had reduced or lost WWOX protein expression, as assessed by immunohistochemistry (6). Recently, we showed that targeted deletion of the Wwox gene expression in the mouse led to an increased incidence of spontaneous and chemically induced tumor formation (7). Previously, we examined both homozygous and heterozygous mice for spontaneous tumor formation (7), although our analyses of tumors in homozygous knockouts were limited by the fact that Wwox-null mice died by 4 weeks of age. We found that inactivation of both Wwox alleles led to the formation of osteosarcoma in some juvenile Wwox-null mice, whereas inactivation of one allele increased the incidence of lung carcinomas, as compared with wild-type mice. In addition, Wwox heterozygous mice treated with the mutagen ethylnitrosourea exhibited higher tumor incidence and multiplicity of lung tumors and lymphomas in comparison with wild-type mice. These findings suggest that WWOX is a bona fide tumor suppressor and that loss of one allele of WWOX might predispose to malignant transformation (7).

Esophageal squamous cell carcinoma (SCC) is an important cause of morbidity and mortality worldwide (8). Nutritional deficiency and exposure to carcinogens, such as N-nitrosamines, are associated with an increased risk of esophageal SCC (9). The mouse forestomach has a squamous-epithelial lining analogous to the human distal esophagus. The murine squamocolumnar junction (SCJ), a zone of transition between squamous-epithelial and glandular tissue, is analogous to the human esophagogastric junction. These structures are commonly studied as model systems for the distal esophagus in human (10). N-nitrosomethylbenzylamine (NMBA), an environmental carcinogen (11), has been extensively used to induce esophageal and forestomach tumors in rodents. Following bioactivation, NMBA produces an electrophilic methylating agent that produces the mutagenic adduct O6-methylguanine in DNA. Indeed, human esophageal epithelial DNA isolated from patients in areas with a high risk for esophageal SCC showed elevated levels of O6-methyldeoxyguanosine (9, 11). To understand the role of the WWOX gene in esophageal cancer, we used this established model to investigate whether inactivation of the Wwox gene renders the mouse more susceptible to NMBA-induced esophageal/forestomach carcinogenesis (12).

Materials and Methods
Mice. C57BL/6J-129/SvJ mixed heterozygous mice were crossed with C57BL/6J mice (The Jackson Laboratory) for four generations (N4). The Wwox offspring were differentiated by genotyping of tail DNA using a PCR-based method (7).
Figure 1. Macroscopic appearance, histopathology, and expression of PCNA, KRT14, and Wwox protein in Wwox$^{+/+}$ and Wwox$^{+/-}$ forestomachs at 15 wks after NMBA treatment. A, macroscopic appearance. Wwox$^{+/+}$ mouse no. 131 (a) and mouse no. 103 (b) showed a thickened forestomach and SCJ with small tumors. Wwox$^{+/-}$ mouse no. 134 (c) displayed large fused tumors in the forestomach and SCJ, and mouse no. 102 had large fused tumors in the SCJ with tumors (>1 mm) in the forestomach (d). B, histopathology. H&E-stained sections of Wwox$^{+/-}$ forestomach from mouse no. 112 showed a thickened epithelium with basal cell hyperplasia (a); Wwox$^{+/-}$ forestomach from mouse nos. 123 and 102 showed invasive SCC (d and g). PCNA-positive nuclei were mostly restricted to the basal cell layer in Wwox$^{+/+}$ forestomach epithelium (b), but was abundant in Wwox$^{+/-}$ SCC mice nos. 123 and 102 (e and h; near serial sections of d and g). KRT14 expression showed basal cell staining in Wwox$^{+/-}$ forestomach from mouse no. 112 (c, near serial section of a), but intense and abundant staining in invasive SCC from Wwox$^{+/-}$ forestomach from mouse nos. 123 and 102 (f and i, near serial sections of d and g). C, Wwox protein expression was strong in Wwox$^{+/-}$ forestomach epithelium (a). In Wwox$^{+/-}$ forestomach from mouse no. 102, Wwox protein expression was moderately strong in areas of the epithelium showing mild hyperplasia (b), weak and diffuse in dysplasia (c), and very weak in SCC (d, near serial sections of B, g). Bars, 5 mm (A); 100 μm (B); and 25 μm (C).
Table 1. Incidence of forestomach tumors in Wwox+/- and Wwox+/+ mice after 15 wks of treatment with NMBA

<table>
<thead>
<tr>
<th>Genotype</th>
<th>No. of mice</th>
<th>Tumor incidence (%)</th>
<th>Foregastroch tumors</th>
<th>No. of tumors per forestomach</th>
<th>Incidence (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Forestomach</td>
<td>SCJ</td>
<td>&lt;1 mm</td>
</tr>
<tr>
<td>Wwox+/-</td>
<td>26</td>
<td>25/26 (96)</td>
<td>19/26 (73)</td>
<td>3.2 ± 0.34</td>
<td>6/26 (23)</td>
</tr>
<tr>
<td>Wwox+/+</td>
<td>34</td>
<td>10/34 (29)</td>
<td>13/34 (38)</td>
<td>0.47 ± 0.17</td>
<td>5/34 (14.7)</td>
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<tr>
<td>P</td>
<td></td>
<td>1.3 × 10^-2</td>
<td>0.01</td>
<td>&lt;0.0001</td>
<td>0.5</td>
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</tbody>
</table>

*Tumor incidence (number of mice with tumors / total number of mice) in Wwox+/- and Wwox+/+ mice was compared by two-tailed Fisher exact test. The number of tumors per forestomach was compared by one-way ANOVA. All statistical tests were two-sided. SCJ between forestomach and glandular stomach.

NMBA-induced forestomach carcinogenesis study. This study was approved by the Ohio State University Animal Facility Institutional Animal Care and Use Committee and conducted according to NIH guidelines.

Thirty-four Wwox+/- mice and 26 Wwox+/+ mice (10–12 weeks old) were given six intragastric doses of NMBA (Ash Stevens) for a period of 3 weeks, at 2 mg/kg body weight, twice weekly. After 15 weeks, all animals were sacrificed and analyzed for tumor incidence. Whole esophagi and stomachs were excised and opened longitudinally. The number of animals bearing tumors (≥0.5 mm in diameter) in the forestomach and SCJ with the glandular stomach was scored. Tissues were fixed in 10% buffered formalin and embedded in paraffin. Sections (4 μm) were either stained with H&E for histopathology or left unstained for immunohistochemical studies.

Immunohistochemistry. Tissue sections were dewaxed, rehydrated, and pretreated with H2O2 followed by citrate buffer (pH 6.0) and Target Retrieval Solution (Dako) for a total of 25 min in a pressure cooker. To detect proliferating cell nuclear antigen (PCNA), cytokeratin 14 (KRT14), and Wwox protein expression, tissues were incubated overnight in a humidified chamber at room temperature with a monoclonal mouse anti-PCNA (Neomarkers) at 1:100 dilution with hematoxylin, dehydrated, and coverslipped. For PCNA visualization, slides were counterstained with hematoxylin, dehydrated, and coverslipped. For PCNA visualization, slides were incubated with 3-amino-9-ethylcarbazole substrate chromogen system (Dako). Cells with a red reaction product in the nucleus were defined as positive for PCNA, an endogenous marker for cell proliferation.

Statistical analysis. Tumor incidence differences were analyzed by two-tailed Fisher's exact test (Biostate). Multiplicity (tumor/mice) was analyzed using one-way ANOVA.

Results

NMBA-induced gastric tumors. A total of 60 C57BL/6J (N4/F1-F2) hybrid mice (34 Wwox+/-, and 26 Wwox+/+) were given six intragastric doses of NMBA and sacrificed 15 weeks later. Macroscopically, wild-type mice typically showed a slightly thickened forestomach mucosa and SCJ (Fig. 1A, a and b). By contrast, heterozygous mice mostly displayed a thickened forestomach mucosa and SCJ, with an array of solitary and fused tumors (Fig. 1A, c and d).

In particular, 96% (25 out of 26) of Wwox+/- mice developed forestomach tumors compared with 29% (10 out of 34) of wild-type controls, thus showing a 3-fold difference in tumor incidence (P = 1.3 × 10^-2; Table 1). In addition, the number of tumors per forestomach was ~7-fold higher in Wwox+/- mice than in wild-type mice (3.2 ± 0.34 versus 0.47 ± 0.17; P < 0.0001). Furthermore, Wwox+/- mice showed a higher incidence of large tumors (≥1 mm) than wild-type controls. The incidence of large tumors among Wwox+/- mice was ~5-fold higher than in wild-type mice (P = 1 × 10^-5) and the number of these tumors per mouse was ~8-fold higher than in wild-type littermates (Fig. 2; P < 0.0001).

These data suggest that inactivation of Wwox led to the formation of more aggressive tumors as compared with wild-type mice.

Both tumor incidence (Table 1) and tumor multiplicity (data not shown) were also higher in SCJ scored in Wwox+/- mice compared with Wwox+/+ mice. Tumors in SCJ were fused and variable in size, and no statistical significance was observed associated with size of tumors in SCJ and mice genotype (data not shown).

Progression to malignancy in Wwox+/- mice. Histopathologic examination of forestomach tumors revealed that 27% (7 of 26) of Wwox+/- mice had invasive SCC in the forestomach (Fig. 1B, Table 1).

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Figure 2. Tumor multiplicity in Wwox+/- and Wwox+/+ forestomach. The number of forestomach tumors per mouse was analyzed by one-way ANOVA (columns, mean; bars, SE). The number of small tumors (<1 mm) per mouse in Wwox+/- mice was ~6-fold higher than in Wwox+/+ mice (**, P < 0.0001). The number of large tumors (≥1 mm) per mouse was ~8-fold higher in Wwox+/- mice than in wild-type littermates (**, P < 0.0001). All statistical tests were two-sided.
d and g), as compared with none of the Wwox+/− mice (Table 1; P = 0.0002). In addition, forestomachs from Wwox−/− mice showed an array of deep downgrowths, dysplasia, squamous papillomas, focal ulcerated lesions, and glandular metaplasia (data not shown). By contrast, forestomachs from Wwox+/− mice typically displayed a thickened epithelium with basal cell hyperplasia (Fig. 1B, a) and occasional presence of squamous papillomas (data not shown). These data show that inactivation of the Wwox gene leads to tumor progression towards malignancy.

Cell proliferation and expression of tumor marker KRT14.
To study proliferation in NMBA-induced forestomach tumors, we used PCNA (an endogenous cell proliferation marker) immunohistochemistry. In wild-type mice, PCNA-positive nuclei were largely found in the basal cell layers of the forestomach (Fig. 1B, b) and in suprabasal cells in areas with mild hyperplasia (data not shown). In general, Wwox−/− forestomachs were proliferative with frequent deep downgrowths that showed abundant PCNA-positive nuclei in areas of focal hyperplastic lesions and dysplasia (data not shown). Wwox+/− forestomach SCCs that were highly proliferative exhibited numerous PCNA-positive cells in invasive tumor areas (Fig. 1B, e and h). These data showed that inactivation of Wwox led to a highly proliferative phenotype.

We next used KRT14 immunohistochemistry to examine tumor progression in Wwox−/− mice. KRT14, a tumor marker for human and rodent esophageal carcinogenesis (14, 15), has been found to be useful in the diagnosis of basaloïd squamous carcinoma, an aggressive variant of SCC (16). In control Wwox+/− mouse epithelium, KRT14 was found exclusively in the basal cell layer of the forestomach (Fig. 1B, c). By contrast, Wwox−/− mouse forestomach tumors showed strong KRT14 expression in dysplastic epithelia and in invasive SCC (Fig. 1B, f and i). These results show the aggressive nature of the tumors formed in Wwox+/− mouse forestomach.

Wwox haploinsufficiency is cancer-predisposing.
To examine whether NMBA treatment resulted in the inactivation of the remaining Wwox allele in Wwox−/− forestomach tumors, we analyzed Wwox protein expression by immunohistochemistry. Wwox expression is strong in Wwox+/− mice forestomach epithelium (Fig. 1C, a). In heterozygous mice, normal forestomach epithelial cells showed moderately strong Wwox expression (Fig. 1C, b). Interestingly, Wwox staining in the same forestomach section was weak and diffuse in epithelial cells showing dysplasia (Fig. 1C, c), and very weak in the invasive tumor areas of SCC in Wwox+/− mice (Fig. 1C, d). This finding suggests that Wwox is haploinsufficient for tumor initiation in forestomach tissue.

Discussion
Our present data show that inactivation of Wwox enhances forestomach carcinogenesis, accelerates tumor progression, and produces an aggressive tumor phenotype. This conclusion is supported by (a) a significantly higher tumor incidence and multiplicity observed in Wwox−/− mice versus Wwox+/− mice (Table 1; Fig. 2); (b) progression to malignancy in Wwox−/− tumors but not Wwox−/− tumors (Fig. 1B; Table 1); and (c) strong KRT14 immunostaining in Wwox−/− SCC, indicative of an aggressive tumor phenotype (16). These data provide direct in vivo evidence that Wwox plays a critical role in murine forestomach/esophageal carcinogenesis because the rodent forestomach is considered as a dilation of the lower esophagus.

Intriguingly, weak Wwox protein expression was detected in Wwox−/− forestomach carcinomas (Fig. 1C), a condition that resembles loss of heterozygosity in human tumors due to the fragility of the WWOX locus in human. In our present study, the only genetic difference between Wwox−/− and wild-type mice is the disrupted Wwox allele in Wwox+/− mice, suggesting that the second Wwox allele may have anti-growth properties in cancer transformation. It is quite possible that in more advanced malignant stages, the second allele of WWOX is lost and tumors become more invasive and probably metastatic. Indeed, our immunohistochemical analysis indicated that Wwox expression is reduced in invasive SCC cases when compared with normal epithelium of the same mouse section (Fig. 1C, b–d). Together, our findings suggest that WWOX is a single hit tumor suppressor gene and that WWOX is haploinsufficient for tumor suppression and significant reduction in Wwox levels is selected during tumor progression. This observation of WWOX haploinsufficiency is in agreement with early findings showing that some tumors retain an intact copy of the WWOX gene and express reduced levels of the WWOX protein (4, 17).

The WWOX gene shares a number of similarities with the FHIT gene, which is located at 3p14.2 and spans the FRA3B common fragile site (reviewed in ref. 18). Decrease or loss of both WWOX and FHIT expression was reported in a number of common cancers (reviewed in refs. 4, 17). Interestingly, WWOX and FHIT protein expression is coordinately altered in gastric adenocarcinoma (6). Likewise, FHIT−/− and Fhit−/− mice exhibit increased NMBA tumor incidence (10, 18). This data suggests that the mechanism of WWOX and FHIT inactivation could be similarly contributing to cancer development.

In summary, our data provide the first direct in vivo evidence that the WWOX gene plays a critical role in mouse forestomach, and thus, human esophageal cancer development and progression. Therefore, the Wwox-mutant mouse is a useful model for designing experiments to investigate the role of the WWOX gene in human cancers, including esophageal cancer, and to develop novel strategies to prevent and treat these cancers.

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References
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