Introduction

Upper aerodigestive tract (UADT) cancer, including oral and esophageal cancer, is an important cause of cancer deaths worldwide. Patients with UADT cancer are frequently zinc deficient (ZD) and show a loss of function of the pivotal tumor suppressor gene p53. The present study examined whether zinc deficiency in collaboration with p53 insufficiency (p53+/−) promotes lingual and esophageal tumorigenesis in mice exposed to low doses of the carcinogen 4-nitroquinoline 1-oxide. In wild-type mice, ZD significantly increased the incidence of lingual and esophageal tumors from 0% in zinc sufficient (ZS) ZS:p53+/+ mice to ~40%. On the p53+/− background, ZD:p53+/− mice had significantly greater tumor incidence and multiplicity than ZS:p53+/− and ZD:p53+/+ mice, with a high frequency of progression to malignancy. Sixty-nine and 31% of ZD:p53+/− lingual and esophageal tumors, respectively, were squamous cell carcinoma versus 19 and 0% of ZS:p53+/− tumors (tongue, P = 0.003; esophagus, P = 0.005). Immunohistochemical analysis revealed that the increased cellular proliferation observed in preneoplastic lingual and esophageal lesions, as well as invasive carcinomas, was accompanied by overexpression of cytokeratin 14, cyclooxygenase-2 and metallothionein-1. In summary, a new UADT cancer model is developed in ZD:p53+/− mice that recapitulates aspects of the human cancer and provides opportunities to probe the genetic changes intrinsic to UADT carcinogenesis and to test strategies for prevention and reversal of this deadly cancer.

Zinc deficiency potentiates induction and progression of lingual and esophageal tumors in p53-deficient mice

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Upper aerodigestive tract (UADT) cancer, including oral and esophageal cancer, is an important cause of cancer deaths worldwide. Patients with UADT cancer are frequently zinc deficient (ZD) and show a loss of function of the pivotal tumor suppressor gene p53. The present study examined whether zinc deficiency in collaboration with p53 insufficiency (p53+/−) promotes lingual and esophageal tumorigenesis in mice exposed to low doses of the carcinogen 4-nitroquinoline 1-oxide. In wild-type mice, ZD significantly increased the incidence of lingual and esophageal tumors from 0% in zinc sufficient (ZS) ZS:p53+/+ mice to ~40%. On the p53+/− background, ZD:p53+/− mice had significantly greater tumor incidence and multiplicity than ZS:p53+/− and ZD:p53+/+ mice, with a high frequency of progression to malignancy. Sixty-nine and 31% of ZD:p53+/− lingual and esophageal tumors, respectively, were squamous cell carcinoma versus 19 and 0% of ZS:p53+/− tumors (tongue, P = 0.003; esophagus, P = 0.005). Immunohistochemical analysis revealed that the increased cellular proliferation observed in preneoplastic lingual and esophageal lesions, as well as invasive carcinomas, was accompanied by overexpression of cytokeratin 14, cyclooxygenase-2 and metallothionein-1. In summary, a new UADT cancer model is developed in ZD:p53+/− mice that recapitulates aspects of the human cancer and provides opportunities to probe the genetic changes intrinsic to UADT carcinogenesis and to test strategies for prevention and reversal of this deadly cancer.

Introduction

Upper aerodigestive tract (UADT) cancer, including esophageal and tongue tumors, is an important cause of morbidity and mortality worldwide (1). The incidence of UADT cancer is increasing worldwide, including that in young adults and those without the known risk factors of tobacco and alcohol use (2). The prognosis of esophageal cancer is poor, with a 5-year survival rate of only 10%. The survival with oral cancer, the major site being the tongue, is equally dismal. Patients with oral cancer have a high mortality rate, because of field carcinization effects that result in second primary tumors, particularly in the esophagus (3,4). In addition, patients with oral cancer are frequently zinc deficient (ZD) (5,6), a condition associated with an increased risk for esophageal squamous cell cancer (ESCC) (7,8). Abnet et al. (9) established a direct connection between zinc deficiency and human ESCC, by using X-ray fluorescence spectroscopy to measure zinc, copper, iron, nickel and sulfur in esophageal biopsy samples obtained from residents in a high ESCC incidence area in China. Subjects were matched on baseline histology and followed for 16 years: 90% of subjects in the highest zinc quartile versus 65% in the lowest quartile were cancer-free for 16 years. No associations with ESCC cancer risk were found for any of the other elements studied. These findings in humans are consistent with our conclusion from rodent model studies that zinc deficiency promotes esophageal cancer (10–12).

We have developed ZD rodent cancer models and found that zinc deficiency creates a precancerous condition in the rat UADT by causing unrestrained cell proliferation (11–13) and extensive changes in gene expression, including upregulated expression of cyclooxygenase-2 (COX-2), keratin 14 (KRT14) and metallothionein-1 (MT-1) (13,14). Overexpression of COX-2 has been reported in a variety of human premalignant and malignant lesions, including UADT cancer (15–17). MT protein is overexpressed in human squamous cell carcinoma (SCC) of the esophagus (18) and tongue (19). KRT14 is a biomarker of human and rodent esophageal carcinogenesis (20,21). A ZD diet in rats accelerates carcinogenesis in the esophagus and forestomach that results from a single exposure to the carcinogen N-nitrosomethylbenzylamine (NMBA) (22,23) and at multiple sites in the UADT with continuous exposure to the carcinogen 4-nitroquinoline 1-oxide (NQO) (13). On the other hand, zinc replenishment rapidly reverses cell proliferation, stimulates apoptosis, corrects abnormal gene expression in esophageal epithelium and inhibits tumorigenesis (13,14,24).

The p53 tumor suppressor protein plays a pivotal role in preventing uncontrolled cellular proliferation. A loss of function of the TP53 tumor suppressor gene is demonstrated in over 50% of all human cancers, including oral and esophageal cancer (25). Our previous work shows that zinc deficiency modulates the enhanced genetic susceptibility to esophageal cancer by NMBA in p53-deficient mice (21).

The mouse tongue is not sensitive to chemical carcinogenesis. Mice exposed to 10 p.p.m. NQO in the drinking water for 50 weeks (26) or 20 p.p.m. for 8 weeks and killed at week 24 (27) did not develop any lesions in the tongue. NQO at high concentrations of 50 and 100 p.p.m. in the drinking water, however, induced both esophageal and tongue tumors in mice, with progression to malignancy (27). In the present
study, we tested the hypothesis that zinc deficiency increases cellular proliferation in the murine tongue, as it does in the rat tongue (13), and that zinc deficiency in collaboration with p53 insufficiency (p53\(^{-/-}\)) promotes lingual and esophageal tumorgenesis in ZD:p53\(^{-/-}\) mice exposed to low doses of NQO in the drinking water (26,27). In addition, we examined whether an association exists between increased cellular proliferation and overexpression of the tumor markers KRT14, COX-2 and MT in premalignant and malignant lesions during UADT carcinogenesis.

Materials and methods

**Chemicals and diets**

NQO was from Wako Chemicals, USA (Richmond, VA). Custom-formulated, egg white-based ZD and zinc sufficient (ZS) diets containing 1.5 and 70 p.p.m. zinc, respectively, were prepared by Teklad (Madison, WI). The deficient diet is nutritionally complete and is identical to ZS diet except for the concentration of elemental zinc (21).

**P53-deficient mice**

Breeder pairs, homozygous C57BL/6J-Trp53 males and heterozygous C57BL/6J-Trp53 females that have been backcrossed for 11 generations, were purchased from The Jackson Laboratory (Bar Harbor, ME) to generate p53\(^{-/-}\) and p53\(^{-/-}\) mice for this study. The p53\(^{-/-}\) and p53\(^{-/-}\) offsprings were differentiated by genotyping of tail DNA using a PCR-based method (28). Wild-type C57BL/6J controls were obtained from The Jackson Laboratory.

**Experimental design**

This study was approved by the Institutional Laboratory Animal Care of and Use Committee of the Thomas Jefferson University, Philadelphia, PA and conducted under National Institutes of Health guidelines. The experiment was conducted in three batches when the animals became available from our breeding colonies. Each batch contained all treatment groups listed below. Four-week-old mice were housed 3–5 to a polycarbonate cage with a stainless-steel wire floor. The animals had free access to deionized drinking water. The mice were randomized into two dietary groups and were fed ad libitum a ZD or control ZS diet, forming six experimental groups: ZD:p53\(^{-/-}\) (n = 15), ZS:p53\(^{-/-}\) (n = 14), ZD:p53\(^{+/+}\) (n = 16), ZS:p53\(^{+/+}\) (n = 26), ZD:p53\(^{+/+}\)/C0 (n = 14) and ZS:p53\(^{+/+}\)/C0 (n = 12). After 3 weeks, all animals were switched to drinking water containing 20–10 p.p.m. NQO. NQO was from Wako Chemicals, USA (Richmond, VA). Custom-formulated, egg white-based ZD and zinc sufficient (ZS) diets containing 1.5 and 70 p.p.m. zinc, respectively, were prepared by Teklad (Madison, WI). The deficient diet is nutritionally complete and is identical to ZS diet except for the concentration of elemental zinc (21).

**Results**

**UADT tumorgenesis in ZD:p53\(^{-/-}\) mice**

In order to determine whether the combined deficiency of zinc and p53 promotes UADT carcinogenesis, p53\(^{-/-}\), p53\(^{+/+}\) and p53\(^{+/+}\) mice on ZD or ZS diet were given for 21 weeks drinking water containing 20 to 10 p.p.m. NQO. While all heterozygous p53\(^{+/+}\) and wild-type p53\(^{+/+}\) mice survived for 21 weeks, only 2 of 15 (13%) nullizygous ZD:p53\(^{-/-}\) mice and 10 of 14 (71%) ZS:p53\(^{-/-}\) mice were alive after 15 weeks (Table I). Among the heterozygous and wild-type mice, only 2 of 16 ZD:p53\(^{+/+}\) mice had spontaneous (non-UADT) splenic lymphomas at week 21. In contrast, ZD:p53\(^{-/-}\) mice succumbed to the rapid development of spontaneous tumors (29), in addition to induced lingual and esophageal lesions (Table I). These NQO-induced lesions appeared to occur earlier in ZD:p53\(^{-/-}\) than in ZS:p53\(^{-/-}\) mice, a finding in agreement with our previous studies with NMBA-induced forestomach tumors in ZD:p53\(^{-/-}\) mice (21).

Leukoplakia, the most common oral intraepithelial neoplasia in humans and a precursor of SCC, was consistently found on the ZD:p53\(^{-/-}\) tongue between 2 and 8 weeks after NQO exposure. By week 13, ZD:p53\(^{-/-}\) mice typically harbored tumors at multiple sites in the UADT. For example, ZD:p53\(^{-/-}\) mice no. 15 showed multiple tumors in tongue, esophagus, forestomach and hard palate at week 21 (Figure 1A, lower panel, forestomach tumor data not shown). Histopathological examination revealed progression to malignancy in ZD:p53\(^{-/-}\) tongue and esophagus at week 11 (Figure 2B and D) but mostly hyperplasia in ZS:p53\(^{-/-}\) tongue and esophagus at week 12 (Figure 2A and C). By 20 weeks, however, malignant changes in tongues became evident in ZS:p53\(^{-/-}\) mice (data not shown). These data demonstrate that in the absence of both p53 alleles, zinc deficiency greatly accelerates the induction and progression of UADT tumors in mice.
Wild-type ZS mice did not develop lingual or esophageal lesions (0%) after exposure for 21 weeks to low levels of NQO, a result consistent with previous reports (26,27). Zinc deficiency, however, significantly enhanced the development of tumors at multiple sites in wild-type mice, with a tumor incidence of 50, 36 and 43% in tongue, esophagus and forestomach, respectively (Figure 1B; ZD:p53+/– mice (tongue, P = 0.003; esophagus, P = 0.005; Figure 1C). These results demonstrate that in wild-type mice, zinc deficiency increases the incidence of NQO-induced UADT tumors as it does in rats (13).

Haploinsufficiency for p53 alone enhanced the induction of lingual and esophageal tumors in ZS mice, resulting in a significantly higher incidence of lingual (62%) and esophageal (31%) compared with 23% of lingual and 0% of esophageal tumors from ZS:p53+/– mice (tongue, P = 0.003; esophagus, P = 0.005; Figure 1C). These results demonstrate that zinc deficiency or p53 haploinsufficiency acting alone increases UADT tumors induction by low levels of NQO and acting together greatly enhances tumor induction with rapid progression to malignancy.

Cell proliferation and marker gene expression in tongue and esophagus of NQO-treated wild-type mice

Histopathological examination revealed that esophagus and tongue from wild-type ZS:p53+/+ mice at week 21 showed a moderately thickened epithelium, with PCNA-positive nuclei in dysplastic areas (Figure 3B), with abundant PCNA-positive nuclei in dysplastic areas (Figure 3F). In contrast, similarly treated ZD:p53+/+ esophagi were highly proliferative, with PCNA-positive nuclei in many high proliferative esophagus from wild-type ZS:p53+/– mice (tongue, Figure 3C and G) and in focal hyperplastic lesions (tongue, Figure 3A and E). In contrast, similarly treated ZD:p53+/+ esophagi were highly proliferative, with PCNA-positive nuclei in many cell layers and in focal hyperplastic lesions (Figure 3D and H). ZD:p53+/+ lingual epithelia were typically hyperplastic, showing dysplastic changes or early carcinoma in situ (Figure 3B), with abundant PCNA-positive nuclei in dysplastic areas (Figure 3F). Immunohistochemistry was then used to determine whether these early precancerous lesions in the ZD:p53+/+ mice are accompanied by overexpression of the tumor markers KRT14, COX-2 and MT. ZS:p53+/+ esophagus and tongue displayed

### Table I. Tumor types in p53+/– mice on ZD or control ZS diet after continuous exposure to 20–10 p.p.m. NQO in the drinking water

<table>
<thead>
<tr>
<th>Case</th>
<th>Sex</th>
<th>TTS (weeks)</th>
<th>Age (weeks)</th>
<th>Gross anatomy</th>
</tr>
</thead>
<tbody>
<tr>
<td>ZD</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>F</td>
<td>2.1</td>
<td>8</td>
<td>Leukoplakia, Thick, Thick, Spleen</td>
</tr>
<tr>
<td>2</td>
<td>M</td>
<td>2.6</td>
<td>9</td>
<td>Leukoplakia, Thick, Thick, –</td>
</tr>
<tr>
<td>3</td>
<td>M</td>
<td>4.0</td>
<td>10</td>
<td>Leukoplakia, Thick, Fused Ts, 6, –</td>
</tr>
<tr>
<td>4</td>
<td>M</td>
<td>4.1</td>
<td>10</td>
<td>Leukoplakia, Thick, 6 Ts, 0.5–2, –</td>
</tr>
<tr>
<td>5</td>
<td>F</td>
<td>4.7</td>
<td>11</td>
<td>2 Ts, 0.5 mm, Thick, Thick, –</td>
</tr>
<tr>
<td>6</td>
<td>M</td>
<td>5.9</td>
<td>12</td>
<td>Leukoplakia, Thick, 2 Ts, 1 mm, –</td>
</tr>
<tr>
<td>7</td>
<td>M</td>
<td>7.4</td>
<td>13</td>
<td>Leukoplakia, Thick, Fused Ts, 3 mm, –</td>
</tr>
<tr>
<td>8</td>
<td>M</td>
<td>7.6</td>
<td>14</td>
<td>Leukoplakia, Thick, Thick, –</td>
</tr>
<tr>
<td>9</td>
<td>M</td>
<td>10.9</td>
<td>17</td>
<td>3 Ts, 0.5 mm, Thick, Thick, Spleen</td>
</tr>
<tr>
<td>10</td>
<td>M</td>
<td>10.9</td>
<td>17</td>
<td>3 Ts, 0.5–1 mm, Thick, Thick, Thymus, kidney</td>
</tr>
<tr>
<td>11</td>
<td>F</td>
<td>10.9</td>
<td>17</td>
<td>2 Ts, 1 mm, Thick, Thick, Thymus</td>
</tr>
<tr>
<td>12</td>
<td>M</td>
<td>13.1</td>
<td>19</td>
<td>4 Ts, 0.5–1 mm, 1 T, 1 mm, Thick, Spleen</td>
</tr>
<tr>
<td>13</td>
<td>F</td>
<td>14.7</td>
<td>22</td>
<td>2 Ts, 2 and 1 mm, Very thick, Fused tumors, Spleen</td>
</tr>
<tr>
<td>14</td>
<td>M</td>
<td>20.9</td>
<td>27</td>
<td>5 Ts, 1–1.5 mm, 3 Ts, 1–2.5 mm, Fused tumors, –</td>
</tr>
<tr>
<td>15</td>
<td>M</td>
<td>20.9</td>
<td>27</td>
<td>6 Ts, 1–3 mm, 9 Ts, 1–3 mm, 1 T, 2.5 mm, –</td>
</tr>
<tr>
<td>ZS</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>F</td>
<td>6.9</td>
<td>13</td>
<td>–, –, –, Thymus</td>
</tr>
<tr>
<td>17</td>
<td>F</td>
<td>12</td>
<td>18</td>
<td>–, –, –, Thymus</td>
</tr>
<tr>
<td>18</td>
<td>M</td>
<td>12.1</td>
<td>18</td>
<td>Leukoplakia, –, –, Thymus</td>
</tr>
<tr>
<td>19</td>
<td>M</td>
<td>12.1</td>
<td>18</td>
<td>Leukoplakia, –, –, –</td>
</tr>
<tr>
<td>20</td>
<td>F</td>
<td>15.0</td>
<td>21</td>
<td>1 fused T, 7 × 3 mm, 1 T, 1.5 mm, –, –</td>
</tr>
<tr>
<td>21</td>
<td>M</td>
<td>15.7</td>
<td>22</td>
<td>7 Ts, 0.5–1 mm, 3 Ts, all 1 mm, –, –</td>
</tr>
<tr>
<td>22</td>
<td>M</td>
<td>15.9</td>
<td>22</td>
<td>2 Ts, 1 mm, 6 Ts, 1–2 mm, –, Spleen, thymus</td>
</tr>
<tr>
<td>23</td>
<td>M</td>
<td>17.9</td>
<td>24</td>
<td>5 Ts, 0.5–1.5 mm, 5 Ts, 0.5–1 mm, –, Spleen</td>
</tr>
<tr>
<td>24</td>
<td>F</td>
<td>18.1</td>
<td>24</td>
<td>3 Ts, 0.5 mm, 1 T, 1 mm, –, –</td>
</tr>
<tr>
<td>25</td>
<td>F</td>
<td>18.9</td>
<td>25</td>
<td>1 T, 3 mm, 1 lg, T, 4 mm, –, Spleen</td>
</tr>
<tr>
<td>26</td>
<td>F</td>
<td>19.4</td>
<td>25</td>
<td>1 T, 3 × 2 mm, 1 T, 1 mm, –, Kidney</td>
</tr>
<tr>
<td>27</td>
<td>M</td>
<td>19.7</td>
<td>26</td>
<td>2 Ts, 1–2 mm, 1 T, 1 mm, –, Subcutis, liver</td>
</tr>
<tr>
<td>28</td>
<td>M</td>
<td>21.0</td>
<td>27</td>
<td>4 Ts, 0.5–1.5 mm, 8 Ts, 0.5–1.5 mm, –, Spleen</td>
</tr>
<tr>
<td>29</td>
<td>M</td>
<td>21.0</td>
<td>27</td>
<td>2 Ts, 1 and 3 mm, 6 Ts, 1–2 mm, 4 Ts, 0.5–1 mm, Thymus</td>
</tr>
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</table>

Four-week-old mice were given ZD or ZS diet and deionized water for 2 weeks before switching to drinking water containing 20–10 p.p.m. NQO, 20 p.p.m. for 3 weeks and 10 p.p.m. for the remaining 18 weeks. T, tumors; TTS, time to sacrifice (weeks) after exposure to NQO; moribund animals were killed.
moderate immunostaining of KRT14, mostly confined to the basal cell layers (KRT14; esophagus, Figure 3K, tongue, Figure 3L; MT; esophagus, Figure 3S, tongue, Figure 3Q). Invariably, COX-2 staining was weak and diffuse in both tissues of these mice (esophagus, Figure 3O, tongue, Figure 3P). Together, these results show an association between the cell proliferation marker PCNA and tumor markers KRT14, COX-2 and MT, in particular, are highly expressed in hyperplastic esophagus and tongue showing dysplasia with microinvasion is presented in Figure 4A. In combination, p53 insufficiency and zinc deficiency led to high incidence of SCC in both tongue and esophagus in ZD:p53+/− mice (Figure 1D). Examples of invasive lingual SCC and ESCC are shown in Figure 4F and K. ZD:p53+/− carcinomas were highly proliferative with abundant PCNA-positive nuclei in the tumors and in adjacent hyperplastic epithelia (lingual SCC, Figure 4G; ESCC, Figure 4L). A high correlation was evident between the spatial localization of KRT14, COX-2 or MT and that of PCNA in serial lingual sections of early cancerous lesions of ZS:p53+/− and in lingual SCC and ESCC of ZD:p53+/− mice (lingual early cancerous lesion, compare Figure 4C–E with Figure 4B; lingual SCC, compare Figure 4H–J with Figure 4G; ESCC, compare Figure 4M–O with Figure 4L).

After 21 weeks, ZS:p53+/− esophagus and tongue showed mostly basal cell hyperplasia, focal cell hyperplasia and papillomas, with a very low incidence of tumor progression only in the tongue (Figure 1D). An example of a ZS:p53+/− tongue showing dysplasia with microinvasion is presented in Figure 4A. In combination, p53 insufficiency and zinc deficiency led to high incidence of SCC in both tongue and esophagus in ZD:p53+/− mice (Figure 1D). Examples of invasive lingual SCC and ESCC are shown in Figure 4F and K. ZD:p53+/− carcinomas were highly proliferative with abundant PCNA-positive nuclei in the tumors and in adjacent hyperplastic epithelia (lingual SCC, Figure 4G; ESCC, Figure 4L). A high correlation was evident between the spatial localization of KRT14, COX-2 or MT and that of PCNA in serial lingual sections of early cancerous lesions of ZS:p53+/− and in lingual SCC and ESCC of ZD:p53+/− mice (lingual early cancerous lesion, compare Figure 4C–E with Figure 4B; lingual SCC, compare Figure 4H–J with Figure 4G; ESCC, compare Figure 4M–O with Figure 4L).

Together, these results show an association between the cell proliferation marker PCNA and tumor markers KRT14, COX-2 or MT in hyperplasia (Figure 3), dysplasia (Figure 4) and invasive carcinomas (Figure 4). They provide evidence that all three markers, MT, in particular, are highly...
relevant intermediate and endpoint biomarkers in UADT carcinogenesis.

Discussion

The data presented above show that with combined deficiency of zinc and p53, the mouse tongue and esophagus become exquisitely sensitive to the carcinogenicity of low concentrations of NQO (Figure 1). In addition, the results document that zinc deficiency and haploinsufficiency acting alone increases the yield of NQO-induced UADT benign tumors, whereas the combined action drives tumor progression to malignancy. Although UADT tumor induction and progression occurred earlier in nullizygous ZD:p53+/C0/C0 mice than heterozygous ZD:p53+/+/- C0/C0 mice, the nullizygous mouse is not a feasible cancer model for UADT cancer because it succumbs to induced and spontaneous tumorigenesis at an early age (Table I). In contrast, Ide et al. (31) reported that p53+/+/- C0/C0 and p53+/-/ C0/C0 mice, on a mixed genetic background of C3H/HeN, C57BL/6 and CBA, were tumor-free with exposure to drinking water containing 10 p.p.m. NQO for 50 and 20 weeks, respectively. It is possible that p53-deficient mice on a C57BL/6 background, as in the case of the present study, are more sensitive to the tumorigenic activity of NQO than those on a mixed genetic background.

Complex genetic mouse models provide a useful framework for understanding the interrelationship of p53 deficiency and specific genes in oral–esophageal cancer development, for example, the collaboration of p53 deficiency and overexpression of cyclin D1 (30), or p53 deficiency and xeroderma pigmentosum group A (XPA) gene deficiency (31). The L2D1+/p53+/- model that overexpresses cyclin D1 in the oral–esophageal epithelium of p53-deficient mice develops invasive oral–esophageal cancer, whereas control cyclin D1 L2D1+ mice exhibit only oral–esophageal dysplasia (30). XPA+/-p53+/+/- mice generated by crossing XPA+/- mice with p53+/+/- mice demonstrate that p53 haploinsufficiency greatly accelerates the onset of lingual tumors in XPA+/- mice with continuous exposure to 10 p.p.m. NQO in the drinking water (31). In either model, the remaining p53 allele was not lost during tumor formation (30–33) and p53 deficiency acting alone did not bring about progression to malignancy. These genetic models support the concept that p53 plays an important role in oral–esophageal epithelial carcinogenesis.

The growing list of genes in human cancers with aberrant expression, however, points toward a more complex view of the carcinogenesis process. In this regard, our UADT cancer model in ZD:p53+/+/- C0/C0 mouse has obvious benefits. By rendering the p53+/+/- mice nutritionally deficient in zinc, this model mimics aspects of human UADT cancers. Both zinc deficiency (5–9) and a loss of function of the tumor suppressor gene TP53 (25) are associated with human UADT carcinogenesis. Zinc deficiency causes substantial cell proliferation in the squamous epithelium of the UADT, as well as extensive changes in gene expression (13,14). On the other hand, loss of p53 function results in genetic instability and increased cell cycle progression (34,35). Thus, the grave biological consequences of combining zinc deficiency and p53 deficiency are rapid UADT tumor induction and progression to malignancy (Figure 1D, Table I).

The sequence of histopathologic events of human oral and ESCC is well characterized and proceeds from hyperplasia to focal hyperplastic lesions, dysplasia and finally invasive carcinoma. Although many molecular alterations have been reported in UADT carcinogenesis, intermediate biomarkers that accurately predict the risk of developing cancer, prognosis and effect of therapeutic treatment have not been clearly

![Fig. 2. Histopathology of tongue and esophagus from ZD:p53+/- and ZS:p53+/- mice. The mice were treated with NQO in the drinking water at 20 p.p.m. for 3 weeks and then 10 p.p.m. for another 18 weeks. Representative H&E-stained sections are shown. At week 12, ZS:p53+/- tongue and esophagus showed a highly hyperplastic epithelium (A, tongue; C, esophagus). At week 11, a ZD:p53+/- tongue showed evidence of early carcinoma in situ (B) and a ZD:p53+/- esophagus, SCC (D). Scale bars: 50 μm (A and C); 25 μm (B and D).](https://academic.oup.com/carcin/article/27/7/1489/2391047)
defined. In human cancers, MT overexpression is often positively correlated with the metastatic and proliferative activities of ESCC (18) and a worse prognosis for oral SCC (36). KRT14 is an indicator of tumor progression in human ESCC (20) and oral dysplasia-SCC sequence (37,38). COX-2 expression is correlated with the proliferation activity in esophageal dysplasia (39) and with early stage carcinogenesis in the oral dysplasia-carcinoma sequence (40).

Recent data from our laboratory (13,14) show that (i) the hyperplastic esophagus and tongue of ZD rats overexpressed COX-2 protein and mRNA; (ii) the zinc sensitive gene MT-I and tumor marker KRT14 are upregulated >6-fold in hyperplastic ZD rat esophagus; and (iii) upon zinc replenishment, the overexpression of all three markers, as well as the hyperplastic phenotype of the esophagus were rapidly reduced. The present study examined whether the sequence of histopathologic events in UADT carcinogenesis after 21 weeks of NQO treatment is accompanied by overexpression of these three biomarkers, KRT14, COX-2 and MT. Our results demonstrate in a mouse UADT cancer model that expression of biomarkers KRT14, MT and COX-2 in lingual and esophageal carcinogenesis is correlated with cell proliferation and the histopathologic hyperplasia-dysplasia-carcinoma sequence (Figures 3 and 4).

In summary, we have developed an in vivo UADT cancer model in ZD:p53+/− mouse that reproduces features of human oral–esophageal cancer. This model will be useful to study the molecular mechanisms of UADT carcinogenesis and to test novel strategies for prevention and reversal of this deadly cancer.

Fig. 3. Spatial localization of PCNA, KRT14, COX-2 and MT in premalignant lingual and esophageal lesions in ZD wild-type mice. The mice were treated with 20 p.p.m. of NQO for 3 weeks and 10 p.p.m. for another 18 weeks. Representative serial H&E and immunohistochemically-stained sections from ZD and ZS wild-type mice are shown. H&E-stained sections showed a moderately thickened lingual (A) and esophageal (C) epithelium from ZS mice and a hyperplastic tongue with dysplasia (B and inset) and a proliferative esophageal epithelium with focal hyperplastic lesions from ZD mice. PCNA, E–H; KRT14, I–L; COX-2, M–P; and MT, Q–T. Scale bars: 50 μm (A, B); 25 μm (C–T).
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**References**


**Fig. 4.** Spatial localization of KRT14, COX-2, MT and PCNA in ZS:p53+/− lingual dysplasia showing microinvasion and ZD:p53+/− lingual SCC and ESCC. The mice were treated with 20 p.p.m. of NQO for 3 weeks and then 10 p.p.m. for another 18 weeks. Representative serial H&E and immunohistochemically-stained sections are shown: (A–E) ZS:p53+/− lingual dysplasia, (F–J) ZD:p53+/− lingual SCC, and (K–O) ZD:p53+/− ESCC. H&E-stained sections showed lingual dysplasia (A), invasive lingual SCC (F) and ESCC (K). Immunohistochemical staining for PCNA (B, G and L), KRT14 (C, H and M), COX-2 (D, I and N) and MT (E, J and O). Scale bars: 50 μm.


