p53 Deficiency Accelerates Induction and Progression of Esophageal and Forestomach Tumors in Zinc-deficient Mice

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ABSTRACT

The p53 tumor suppressor protein plays a pivotal role in preventing uncontrolled cellular proliferation. By contrast, zinc deprivation enhances esophageal cell proliferation and the induction of esophageal tumors in rodents by N-nitrosomethylbenzylamine (NMBA). We investigated whether p53 deficiency rendered zinc-deficient (ZD) mice more susceptible to NMBA-induced esophageal/forestomach carcinogenesis. At 6–7 weeks of age, p53 null (−/−), heterozygous (+/−), and wild-type (+/+ ) mice were placed on ZD or zinc-sufficient (ZS) diets to form six experimental groups: ZD:p53−/−; ZD:p53+/−; ZD:p53+/+; ZS:p53−/−; ZS:p53+/−; and ZS:p53+/+. After 3 weeks, 15–23 mice in each group were treated once with NMBA (2 mg/kg body weight). Control animals were untreated. Zinc deficiency alone induced unrestrained cellular proliferation in the esophagus and forestomach of p53−/− mice. Forestomach tumors were first detected in a ZD:p53−/− mouse at 13 days. By 30 days, 100% (21 of 21) of ZD:p53−/− mice developed forestomach tumors and 38% showed esophageal tumors versus 42 and 0% in ZS:p53−/− mice (P < 0.004, esophagus; P < 0.001, forestomach). ZD:p53−/− mice showed an accelerated progression to malignancy, with 10% of esophageal tumors and 38% of forestomach tumors presenting as carcinomas. Nearly 20% of ZD:p53−/− mice developed esophageal Barrett’s metaplasia, a lesion not previously seen in NMBA-induced neoplasia. ZD:p53+/− mice had significantly higher tumor incidence than ZS:p53+/− mice. The order of tumor incidence in forestomach was as follows: naught incidence in ZS:p53+/− mice; ZD:p53−/− > ZD:p53+/− > ZS:p53−/− > ZS:p53+/−. The rapid rate of tumor induction/progression in ZD:p53−/− mice was accompanied by an increase in the rate of cell proliferation and a decrease in apoptosis. cDNA array expression analysis of known genes identified a 5-fold up-regulation of cyclin D1 and cdk4-Rb pathway that controls the G1-S-phase transition (12). Esophageal tumor reversal is also rapid in ZD rats. Replenishment with a ZS diet results in a prompt induction of apoptosis in the esophageal epithelium, an effect that is accompanied by inhibition of tumor development (13). α-Difluoromethylnitrosourea, an anticancer agent, reverses the zinc deficiency-induced esophageal cell proliferation and also inhibits NMBA-induced esophageal tumorigenesis by stimulating apoptosis (14, 15). Unlike the rat (17), NMBA-treated ZD mice show a higher tumor incidence in the forestomach than esophagus (18). The rodent forestomach is considered to be a dilation of the lower esophagus (19).

INTRODUCTION

Esophageal cancer is the sixth most common cancer worldwide (1). The marked variations in geographical distribution of ESCC indicate that environmental factors are involved in its pathogenesis. Nutritional zinc deficiency (2–4) and exposure to carcinogenic N-nitrosamines, including NMBA, are associated with an increased risk of ESCC in high incidence areas in northern China and Iran (3, 5, 6). Our NMBA-induced esophageal cancer model in ZD rats (7) mimics many aspects of ESCC. In this model, increased esophageal cell proliferation is induced by dietary deprivation of zinc (8), and tumors are induced by NMBA. Increased esophageal cell proliferation, an important mechanistic consideration in cancer development, has been reported in persons at high risk for ESCC (9), and NMBA induces esophageal lesions in rats that are morphologically similar to human ESCC (10).

Results from our studies (8, 11–15) establish that the ZD rat esophageal cancer model is well suited for investigating mechanisms underlying esophageal cancer development and its prevention. In the context of increased esophageal cell proliferation provided by zinc deprivation, a single nontumorigenic dose of NMBA (16) elicits numerous tumors in the ZD rat esophageal epithelium. Tumor initiation by NMBA is very rapid in the ZD esophagus because of early deregulation of the p16^{ink4a}-cyclin D1/Cdk4-Rb pathway that controls the G1-S-phase transition (12). Esophageal tumor reversal is also rapid in ZD rats. Replenishment with a ZS diet results in a prompt induction of apoptosis in the esophageal epithelium, an effect that is accompanied by inhibition of tumor development (13). α-Difluoromethylnitrosourea, an anticancer agent, reverses the zinc deficiency-induced esophageal cell proliferation and also inhibits NMBA-induced esophageal tumorigenesis by stimulating apoptosis (14, 15). Unlike the rat (17), NMBA-treated ZD mice show a higher tumor incidence in the forestomach than esophagus (18). The rodent forestomach is considered to be a dilation of the lower esophagus (19).

NMBA is used widely to induce esophageal cancer in rodents (17). Esophageal cytomegaly P450 enzymes bioactivate NMBA (20), a process that leads to the formation of O6-methylguanine, a promutagenic DNA adduct (21). We have reported that NMBA methylates rat esophageal DNA with a concomitant accumulation of O6-methylguanine (22). In areas associated with a high risk for ESCC incidences, human esophageal epithelial DNA showed elevated levels of O6-methyldeoxyguanosine (23). NMBA-induced rat esophageal tumors exhibit prevalent Hras oncogene (24–26) and TP53 tumor suppressor gene mutations, involving mostly G:C→A:T transitions (26, 27). By contrast, human ESCCs do not harbor activating point mutations of the ras gene (28, 29) but do show TP53 point mutations, with both transitions and transversions (30). Our earlier results (11) demonstrated that NMBA-induced ZD rat esophageal tumors acquired TP53 mutations with both transitions and transversions, a pattern more closely resembling human ESCC than esophageal tumors of nutritionally complete rats (26, 27). These mutational profiles attest to the human relevance of our ZD, NMBA esophageal cancer model in rodents.

Human ESCC typically develops through a sequence of histopathological lesions, including esophagitis, mild to severe dysplasia, carcinoma in situ, and, finally, invasive cancer. These sequential changes in the esophageal epithelium are accompanied by consistent genetic alterations, including abnormalities in the TP53 tumor suppressor gene and damage to genes that regulate pathways governing the G1-S progression and signal transduction (reviewed in Ref. 31). Over 50% of all human cancers, including ESCC and esophageal adenocarcinoma, demonstrate a loss of function of the TP53 tumor suppressor.
gene (30). Tumor suppression by p53 is a function of the transcriptional activation of genes that control cell cycle, DNA repair, and apoptosis (reviewed in Refs. 32, 33).

p53−/− null mice are viable but highly susceptible to spontaneous tumorigenesis, especially lymphoma, at an early age and heterozygous p53+/− mice at a later stage in life (34). Relative to wild-type p53+/+ mice, heterozygous p53+/− mice show increased susceptibility to a variety of chemical carcinogens, whereas p53−/− null mice are not appropriate models for most chemical carcinogenesis studies because these animals die of early spontaneous tumors before they develop the chemically induced tumors (35, 36).

p53-deficient mice have been used to study the role of apoptosis and cell proliferation in tumorigenesis. The loss of p53 per se contributed a growth advantage in a variety of spontaneous tumor types through increased tumor cell proliferation (37). Similar results were obtained using the mouse mammary tumor virus-c-ras/p53-deficient mouse (38) and Wnt-1/p53-deficient mouse (39) mammary tumor models. Another doubly recombinant model showed that loss of p53 was associated with more rapid tumor growth through decreased apoptosis (40). Lastly, p53-deficient mouse models have provided a useful tool for testing dietary interventions that may inhibit carcinogenesis. Thus, calorie restriction delayed spontaneous tumorigenesis in p53-deficient mice because of a decreased rate of cell proliferation (41, 42).

This study investigated whether loss of p53 function renders the ZD mouse more susceptible to NMBA-induced esophageal/forestomach carcinogenesis, and if so, whether the rapid tumor growth conferred by p53−/− deficiency operates through increased cell proliferation or decreased apoptosis. In addition, cDNA array expression analysis was used to determine whether zinc deficiency elicits a gene-modulating effect by comparing levels of expression of some known genes in accelerated forestomach tumorigenesis in ZD:p53−/− versus ZS: p53−/− animals.

MATERIALS AND METHODS

Chemicals and Diets. NMBA was purchased from Ash Stevens, Inc. (Detroit, MI). Custom-formulated, egg white-based ZD and ZS diets containing 1.5 and 75 ppm zinc, respectively, were prepared by Teklad (Madison, WI). ZD diet is identical to ZS diet, except for the concentration of elemental zinc, and ZS diet is nutritionally complete (8).

Animals. This study was approved by the Thomas Jefferson University Institutional Animal Care and Use Committee and conducted under NIH guidelines. Six- to seven-week-old male p53−/−, p53+/+ animals were with the same genetic background of C57BL/6 were purchased from Jackson Laboratory (Bar Harbor, ME). The animals were housed in 3–5 to a polycarbonate cage with a wire stainless steel floor. They were given 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−/−, ZD:p53+/+, ZS:p53−/−, ZS:p53+/+, and ZD:p53+/−/ZS:p53−/+. They were monitored daily for clinical signs of ill health.

Dietary Zinc Deficiency. To assess the effect of dietary zinc deficiency per se on the pathogenesis of forestomach and esophagus, ZD:p53−/−, ZS: p53−/−, ZDp53+/−, ZSp53−/−, ZD:p53+/+, ZSp53−/+, and ZD:p53+/−/ZS:p53−/+ mice (10 mice/ZD group and 5 mice/ZS group) were observed for a period of 118 days or 17 weeks, the median time to death for p53−/− mice (of similar age) because of spontaneous tumorigenesis (34). Moribund animals were sacrificed and autopsied.

NMBA-induced Tumorigenesis. To establish increased cellular proliferation in esophagus and forestomach in ZD mice, ZD:p53−/−, ZS:p53−/−, ZD:p53+/−, ZS:p53−/−, ZD:p53+/+, and ZS:p53+/+ mice were maintained on their respective diets for 3 weeks. Animals (15–23 mice/group) were then treated once with an intragastric dose of NMBA (2 mg/kg body weight). At 13 and 23 days after NMBA treatment, two moribund ZD:p53−/− mice were sacrificed. Because both animals displayed large forestomach tumors in addition to spontaneous thymic lymphoma, all p53−/− mice were sacrificed at 30 days for end point tumor incidence analysis, and p53+/+ and p53+/− mice were sacrificed at 44 days for comparison.

Zinc Determination. The tests were removed at autopsy. Samples of tests were dried to constant weight at 90°C and ashed in a furnace. Ashed samples were dissolved in 0.1 N HCl, and zinc content was determined by atomic spectrometry (8) using a Perkin-Elmer Atomic Absorption Spectrometer Analyst 100 (Perkin-Elmer, Norwalk, CT). Zinc content was expressed as μg/g dry weight of tests.

Tumor Analysis. After anesthetization with isoflurane (Ohmeda, Inc., Madison, WI), the mice were sacrificed and subjected to complete autopsies. Whole esophagi and stomachs were excised and opened longitudinally. Tumors > 0.5 mm in diameter were mapped and counted. Whole esophagus and forestomach were fixed in buffered formalin and embedded in paraffin; 4-μm thick cross-sections were cut. Sections were either stained with H&E for histopathology or left unstained for immunohistochemical studies. Typically, there were 5–9 sections/slide for esophagus and 4–6 sections for forestomach, representing the entire organ.

Cell Proliferation Analysis. PCNA localization is used to identify cell cycle subpopulations in G1, G2, and M phases: dark staining nuclei = S-phase cells; light staining nuclei = G1-S and G2-phase cells; cells with cytoplasmic staining = usually mitoses; and nonstaining nuclei = quiescent (G0 phase) cells (43). Monoclonal mouse anti-PCNA (Santa Cruz Biotechnology, Santa Cruz, CA) was used at 1:50 dilution, followed by incubations with biotinylated goat anti-mouse antibody and streptavidin peroxidase. PCNA was localized by incubation with 3-amino-9-ethylcarbazole-substrate-chromogen system (Dako Corp., Carpenteria, CA). Cells with a red reaction product in the nucleus were considered positive for the presence of PCNA. Preliminary analysis (data not shown) found an association between the number of S-phase cells measured with PCNA and with bromodeoxyuridine (BrdU). The PCNA-labeling index, expressed as a percentage, was calculated by dividing the number of PCNA-labeled nuclei (S phase) by the total number of cells counted/cross-section in the tissue section.

Apoposis Analysis. Apoptosis was assessed by the TUNEL method and by morphological characterization of cells in H&E-stained sections.

TUNEL Assay. The 3′-OH end labeling of DNA in tissue sections was performed with an ApopTag in situ peroxidase detection kit (Intergen, Purchase, NY). Sections were deparaffinized, rehydrated in a graded alcohol series, and incubated with proteinase K (20 μg/ml, 37°C for 10 min). Endogenous peroxidase in the sections was inhibited with 3% hydrogen peroxide, and slides were incubated (37°C for 1 h) with terminal deoxynucleotidyltransferase to catalyze the addition of digoxigenin-labeled nucleotides to the 3′-OH ends of fragmented DNA. Next, slides were incubated with horseradish peroxidase-conjugated antidigoxigenin antibodies, and DNA fragmentation was detected by staining with 3,3′-diaminobenzidine tetrahydrochloride. Finally, sections were counterstained with methyl green. Sections from rat mammary gland (Intergen) in which extensive apoptosis occurs served as a positive control. Negative controls omitted terminal deoxynucleotidyltransferase.

Morphological Criteria. The morphology of apoptotic cells depends on their stage in the process. Apoptotic morphologies include diffuse cytoplasmic staining with only minimal nuclear condensation, distinct apoptotic bodies resulting from nuclear disintegration, or dense staining nuclei with normal nuclear structure. We considered all three forms to be equivalent (44). The incidence of apoptosis was measured on good-quality sections stained with H&E. The AI, expressed as a percentage, was calculated by dividing the number of apoptotic cells by the total number of cells in the tissue section.

Immunohistochemistry. To detect Bax and Bcl-2, tissues sections were incubated overnight at 37°C in a humidified chamber with a rabbit anti-Bcl-2 polyclonal antisera (Santa Cruz Biotechnology) at a 1:2000 dilution or with a rabbit anti-Bax polyclonal antisera (Santa Cruz Biotechnology) at a 1:800 dilution, followed by incubation with a biotinylated goat antirabbit antibody. To detect cytokeratin 14, sections were incubated as described above with a mouse anticytokeratin 14 monoclonal antisera (Clone LL002; Novocastra Lab., Newcastle upon Tyne, United Kingdom) at a 1:40 dilution, followed by incubation with a biotinylated goat antirabbit antibody serum. To detect cytokeratin 14, sections were incubated with an rabbit anti-Bax polyclonal antisera (Santa Cruz Biotechnology) at a 1:800 dilution, followed by incubation with a biotinylated goat antirabbit antibody.
Table 1. Rapid induction of esophageal and forestomach tumors in ZD:p53−/− mice by a single NMBA dose

<table>
<thead>
<tr>
<th>Diet:genotype</th>
<th>Days after NMBA treatment</th>
<th>Testis zinc content (μg/g)</th>
<th>Tumor incidence (%)a</th>
<th>Foremostach tumor size % of mice</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Esophagus</td>
<td>Forestomach</td>
</tr>
<tr>
<td>ZD:p53−/−</td>
<td></td>
<td></td>
<td>30</td>
<td>146 ± 10</td>
</tr>
<tr>
<td>ZS:p53−/−</td>
<td></td>
<td></td>
<td>30</td>
<td>179 ± 8</td>
</tr>
<tr>
<td>ZD:p53+/−</td>
<td></td>
<td></td>
<td>44</td>
<td>147 ± 10</td>
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<td>ZS:p53+/−</td>
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<td>179 ± 10</td>
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<td>137 ± 8</td>
</tr>
<tr>
<td>ZS:p53+/+</td>
<td></td>
<td></td>
<td>44</td>
<td>187 ± 14</td>
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</table>


b Tumor incidence (number of mice with tumors/total number of mice) was analyzed by Fisher’s exact test, two-tailed.

cDNA probes for array hybridization were synthesized with the Atlas cDNA probes for array hybridization were synthesized with the Atlas cDNA probe synthesis kit (Clontech, Palo Alto, CA), using primers designed from the individual gene sequences to be tested; details of primers are available from the authors. Primers and TaqMan probe were designed from the individual gene sequences to be tested; details of primers are available from the authors.

RESULTS

Effect of Dietary Zinc Deficiency. After 118 days on a deficient diet (zinc = 1.5 ppm), ZD mice, regardless of p53 genotype, did not exhibit the overt signs of zinc deficiency that include retarded growth, loss of hair, and foci of alopecia (8). However, ZD mice had 18–26% lower tests zinc content than ZS mice (P < 0.01, Table 1, data not shown for NMBA-untreated groups). It should be noted that lymphocyte levels that are only 12% less than normal are considered evidence for zinc deficiency in human head and neck cancer patients (47). Thus our model would seem to represent a degree of zinc deficiency not much greater than that reported in humans. At autopsy, ZD mice generally displayed a thickened forestomach epithelium compared with ZS animals, with ZD:p53−/− mice showing the most thickened forestomach, often with tumor-like outgrowth at the SCJ (Fig. 1A).

Lymphomas appeared earlier in ZD:p53−/− mice than in their ZS:p53−/− counterparts. Four of 10 ZD:p53−/− mice developed splenic and thymic lymphomas between 44 and 64 days after the start of a deficient diet, and another 3 mice exhibited these lymphomas at the end of the observation period (118 days). By contrast, 5 of 5 ZS:p53−/− mice were apparently healthy throughout the experiment, with no mice showing thymic lymphoma at 118 days. Only 1 of 10 ZD:p53+/− mice had thymic lymphoma, whereas the ZS:p53+/− counterparts, mild-type ZD:p53+/+, and ZS:p53+/+ mice were all tumor-free at 118 days.

Histopathological examination of NMBA-untreated esophageal and forestomach sections revealed that the level of cell proliferation was highest in ZD:p53−/− and lowest in ZS:p53+/+ tissues in the following order: ZD:p53−/− > ZD:p53+/− > ZD:p53−/+ ≥ ZD:p53+/+ > ZS:p53−/− ≥ ZS:p53+/+. Because zinc deficiency produced the most dramatic effect in ZD:p53−/− tissues, photomicrographs (Fig. 2, a–l) are presented of these tissues and their ZS:p53−/− counterparts, including H&E and PCNA immunohistochemistry and apoptosis by TUNEL assay. Fig. 2, a–c, are near serial sections of ZS:p53−/− esophagus no. 11 and show a mildly proliferative epithelium (Fig. 2a), with many PCNA-positive nuclei confined to the basal cells, occasional focal hyperplasia (Fig. 2b), and several TUNEL-positive apoptotic cells in the basal layer (Fig. 2c). By contrast, near serial sections of ZD:p53−/− esophagus no. 4 (Fig. 2, d–f) show an extremely hyperplastic epithelium (Fig. 2d), with abundant PCNA-positive cells in almost all cell layers (Fig. 2e) and a few TUNEL-positive apoptotic nuclei in the outermost cell layer (Fig. 2f). ZS:p53−/−/esophagus no. 13 shows mild basal cell proliferation (Fig. 2g) with PCNA-positive nuclei restricted to basal cells (Fig.
2h) and a few apoptotic cells in the epithelium (Fig. 2i). ZD:p53−/−/forestomach no. 4 (Fig. j–l) shows a hyperplastic SCJ (Fig. 2j) with numerous PCNA-positive cells in several cell layers (Fig. 2k) and isolated apoptotic cells in the outermost cell layer but absent in the proliferative areas of the epithelium (Fig. 2f).

Enhanced Cell Proliferation in NMBA-untreated ZD:p53−/− Esophagus/Forestomach. Consistent with previous reports (8, 18), nutritional zinc deficiency increases cellular proliferation in esophagus and forestomach, independent of p53 status (Fig. 3A). The PCNA-labeling index of S-phase cells, a measure of cellular proliferation, was significantly greater in ZD:p53−/− versus ZS:p53−/− tissues (esophagus, forestomach, P < 0.001), in ZD:p53−/− versus ZS: p53+/− tissues (esophagus, P < 0.01; forestomach, P < 0.05), and in ZD:p53+/+ versus ZS:p53+/+ tissues (esophagus, P < 0.01; forestomach, P < 0.05). In addition, ZD:p53−/− mice showed a substantially higher labeling index than ZD:p53+/+ mice (P < 0.001 for both esophagus and forestomach) and a greater labeling index than ZD:p53+/− mice (P < 0.001 for forestomach and <0.01 for esophagus). The effect of loss of p53 alleles on the PCNA-labeling index was less pronounced in ZS groups. Only ZS:p53−/− esophagus showed a significantly higher labeling index versus ZS:p53+/−/esophagus (P < 0.05). Taken together, these data indicate that combining zinc deprivation with the loss of p53 increased cell proliferation in the esophagus and forestomach to a greater extent than occurred with either condition alone.

Reduced Apoptosis in NMBA-untreated ZD:p53−/− Esophagus/Forestomach. AIs (percentage) were determined in the H&E-stained sections of esophagi and forestomachs of ZD and ZS mice of the three p53 genotypes, −/−, +/+ , and +/+ (Fig. 3B). Three conclusions were drawn. (a) Zinc deficiency significantly reduced the AI in the esophagus of p53−/− mice (E4 versus E5, P < 0.01), in p53+/− mice (E4 versus E5, P < 0.01), and in the forestomach of p53+/− mice (F2 versus F3, P < 0.01) and p53+/+ mice (F2 versus F1, P < 0.05). (b) Loss of TP53 alleles was associated with a lower AI. AI was significantly lower in the esophagus and forestomach of p53−/− mice than in wild-type p53+/+ tissues (E5 versus E5, P < 0.001; E4 versus E5, P < 0.05; and F5 versus F5, P < 0.01), independently of zinc status. In addition, AI was significantly lower in the tissues of heterozygous p53+/− mice than in wild-type p53+/+ tissues, (E4 versus E5, P < 0.001, F4 versus F5, P < 0.05, and E5 versus E5, P < 0.05), again regardless of zinc status. (c) Within the same dietary group, esophagi generally displayed a higher AI than forestomach, independent of genotype. For example, among p53−/− mice, a significant difference was found between E4 and F4 (P < 0.001) and between E5 and F5 (P < 0.05); among p53+/− mice, between E5 and F5 (P < 0.001), between E4 and F4 (P < 0.01), and among p53+/+ mice, between E4 and F1, and between E2 and F2 (P < 0.001).

Rapid Induction of Tumors in ZD:p53−/− Mice. Tumor induction by NMBA was extremely rapid in nullizygous mice fed a ZD diet. Thirteen days after a single NMBA treatment, a moribund ZD: p53−/− mouse with spontaneous thymic lymphoma showed profound thickening in the forestomach and SCJ (Fig. 1B), which on histopathological examination revealed the occurrence of papillomas (results not shown). At 23 days, a second mouse with thymic lymphoma displayed large fused tumors in the forestomach and SCJ (Fig. 1B). By 30 days, 21 of 21 (100%) ZD:p53−/− mice had developed tumors in forestomach and SCJ (Table 1), 76% of which were fused and occupied almost the entire forestomach (Fig. 1B). Importantly, ZD:p53−/− mice had very edematous esophagi, and 38% (8 of 21) exhibited 1–3 small tumors/esophagus at 30 days. On the other hand, all of 19 ZS:p53−/− mice remained healthy at 30 days. None had esophageal tumors. Although 42 and 74% of ZS:p53−/− mice displayed tumors in the forestomach and SCJ, respectively, the tumors were mostly solitary and small, and the tumor incidence at all three locations was significantly lower than that in ZD:p53−/− mice (esophagus, P = 0.004; forestomach, P < 0.001 and SCJ, P = 0.018; Table 1).
ZS:p53+/− mice exhibited lymphomas of the spleen and thymus, although the difference in incidence was not significant (P = 0.29).

Likewise, dietary zinc deficiency in heterozygous p53+/− mice resulted in a higher incidence of forestomach/SCJ tumors with larger tumor size than their counterparts on a ZS diet (ZD:p53+/− versus ZS:p53+/−: forestomach [69 versus 6%]; SCJ [88 versus 19%], P < 0.001; Table 1). Zinc deficiency, however, did not promote esophageal carcinogenesis in heterozygous mice. The difference in esophageal tumor incidence between ZD:p53+/− and ZD:p53+/− was significant at P = 0.004. These results indicate that both zinc deficiency and the absence of p53 are necessary for esophageal tumor induction by a single NMBA dose.

In wild-type ZS:p53+/+ mice, a single NMBA dose did not elicit a tumorigenic response in the target organs, but it did generate 1–3 very small tumors (<1 mm)/forestomach in 13% of ZD:p53+/+ mice. However, the difference in tumor incidence was not significant.

**Rapid Progression of Tumors in ZD:p53−/− Mice.** ZD:p53−/− mice demonstrated an accelerated progression to malignancy...
at 30 days. Histopathological examination revealed the occurrence of ESCCs in 10% (2 of 21) of mice (Fig. 2m) and forestomach squamous cell carcinomas, often accompanied by inflammation and the presence of focal ulceration in 38% (8 of 21) of animals (Fig. 2n). Forestomach carcinomas were also identified in 5% (1 of 19) of ZS:p53−/− mice and 13% (2 of 16) of heterozygous ZD:p53+/− mice (data not shown). The distribution of forestomach carcinomas differed significantly between ZD and ZS nullizygous mice (*P* = 0.021) but not between ZD and ZS heterozygous mice (*P* = 0.14). Importantly, Barrett’s metaplasia, a preneoplastic condition of esophageal adenocarcinoma, which had not previously been reported with NMBA-induced lesions, was identified in 19% (4 of 21) of ZD:p53−/−-esophagi (Fig. 2p) but not in ZS:p53−/−-esophagi. The difference in the incidence of esophageal metaplasia between ZD and ZS nullizygous mice was not significant but suggestive (*P* = 0.108).

Near serial sections of ESCC (Fig. 2, m–o), esophageal glandular metaplasia (Fig. 2, p–r), and forestomach squamous cell carcinoma (Fig. 2, s–u) demonstrated the presence of abundant PCNA-positive nuclei and the virtual absence of apoptotic cells. Apoptotic activities were detected in locations of the esophageal or forestomach lesions that were not highly proliferative. These results indicate that together with zinc deficiency-induced cell proliferation, the absence of p53 results in a rapid induction and progression of esophageal tumors.

**Bax and Bcl-2 Expression.** Immunohistochemical staining for Bcl-2, an antiapoptotic protein, and Bax, a proapoptotic protein, in NMBA-treated ZD:p53−/−-esophagi revealed strong staining for the former and weak or absent for the latter. Three examples, a hyperplastic esophagus no. 80 (Fig. 4, a and b), an ESCC no. 79 (Fig. 4, c and d), and an esophageal glandular metaplasia (Fig. 4, e and f), are shown in Fig. 4. Bcl-2 was strongly expressed in the hyperplastic epithelium (Fig. 4a) and in the glandular metaplasia (Fig. 4c) and was absent or weakly expressed in the corresponding areas of these lesions (Fig. 4, b–d).

**Differentially Expressed Genes in ZD:p53−/− Mice Fore- stomach.** We used four array filters containing a total of 823 mouse genes to compare expression levels of known genes during accelerated forestomach tumorigenesis in ZD:p53−/− versus ZS:p53−/−-animals (Table 2). Labeled cDNA probes prepared from mRNA of pooled forestomachs at 30 days after NMBA treatment were hybridized to one or more of the four mouse cDNA filter arrays. After analysis of the hybridization results, a number of candidate genes for up- or down-regulation in ZD versus ZS forestomachs were selected for additional analysis.

Criteria for selection of candidate genes to be verified by real-time PCR amplification included: (a) indication by array analysis of >2-fold up- or down-modulation of expression in ZD:p53−/− versus ZS:p53−/−-foremostachs; (b) availability of at least 300 bp of nucleotide sequence for design of oligonucleotide primers and TaqMan probes, as well as preliminary data showing that the designed primers resulted in amplification of the expected sequence; and (c) the sequence included a potential protein coding region. The selected candidates were then tested by real-time PCR to determine whether up- or down-modulation detected by array hybridization could be confirmed by quantitative real-time PCR amplification assay.

Fifteen up-regulated gene candidates with >2-fold increase in expression and two down-regulated gene candidates with >2-fold decrease in expression were identified in ZD:p53−/− mouse forestomach. Table 2 indicates that expression level differences were confirmed by the quantitative real-time PCR amplification analysis. Increased expression of keratin complex 1, acidic gene 14 (cytokeratin 14), was identified on two arrays (Mouse cDNA expression array filter and Mouse 1.2 array filter) harboring this mouse gene. Similarly, keratin complex 2, basic gene 1, was detected on the two arrays (Mouse cDNA expression array filter and Mouse 1.2 array II) containing this gene clone. Tumor necrosis factor receptor and ligand superfamily member genes were up-modulated 3–4-fold by zinc deprivation, as were a serine protease inhibitor gene, a few cell...
Immunohistochemical Analysis of Cytokeratin 14. Cytokeratin 14 is an important biomarker in human esophageal cancer development (48). To assess its relevance in mouse esophageal/forestomach tumorigenesis and to confirm the up-modulation of this gene in the ZD:p53−/− forestomach, we performed immunohistochemical analysis of this protein on NMBA-untreated and -treated mouse tissues.

Table 2. Altered gene expression in NMBA-induced ZD:p53−/− forestomach tumors versus ZS:p53−/− forestomach

<table>
<thead>
<tr>
<th>Gene Description</th>
<th>ZD versus ZS expression ratio</th>
<th>GenBank accession No.</th>
<th>Sequence ID</th>
<th>Array filter</th>
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<td>Up-regulated</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Keratin complex 1, acidic, gene 1</td>
<td>+5.2, 5.0</td>
<td>M13806</td>
<td>NM101663</td>
<td>A, B</td>
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<td>Keratin complex 2, basic, gene 1</td>
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<td>NM008473</td>
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<tr>
<td>Keratin complex 1, acidic, gene 10</td>
<td>+3.8</td>
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<tr>
<td>Serine protease inhibitor, Kaval type 3</td>
<td>+3.9</td>
<td>X06342</td>
<td>NM009258</td>
<td>D</td>
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<td>U25416</td>
<td>NM009401</td>
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<td>A001373</td>
<td>NM008403</td>
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<td>L12687</td>
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<td>U45977</td>
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<td>Praja 1, RING-H2 motif containing</td>
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<td>U06944</td>
<td>NM008853</td>
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<td>NM100015</td>
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<td>DEAD (aspartate-glutamate-alanine-aspartate) box polypeptide 6</td>
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<td>D50494</td>
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<td>J04179</td>
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<td>Down-regulated</td>
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*Genes, showing >2-fold increase (+) or decrease (−) in gene expression in ZD:p53−/− versus ZS:p53−/− forestomach were verified by real-time PCR.

*Denotes an increase (+) or a decrease (−) of gene expression in ZD:p53−/− versus ZS:p53−/− forestomach, as determined by real-time PCR amplification.

*Array filter A, B, C, and D, respectively, denotes Mouse cDNA expression array, Mouse 1.2 array, Mouse 1.2 array II, and Mouse cancer 1.2 array (BD Bioscience Clontech).
in the basal cell layer of untreated ZS:p53+/+ esophagus (Fig. 5a). ZD:p53+/+ esophagus showed moderately strong cytoplasmic staining in locations with hyperplasia (Fig. 5b). By contrast, the highly proliferative ZD:p53−/− esophagus no. 4 displayed very strong expression of cytokeratin 14 in all cell layers of the hyperplastic epithelium (Fig. 5d). ZS:p53−/− esophagus no. 11 showed expression only in the basal cell layer (c), compared with ZD:p53−/− esophagus no. 4 showing very strong staining throughout the hyperplastic epithelium (a). e–h, (+NMBA). ZS:p53−/− esophagus no. 67 shows moderately strong staining of cytokeratin 14 in basal cells but weak expression in suprabasal cells (e), as compared with ESCC from ZD:p53−/− mouse no. 79 showing strong expression in tumor areas and hyperplastic epithelium (f). ZS:p53−/− forestomach no. 59 showing moderately strong expression in upward and downward focal hyperplasia (g), as compared with ZD:p53−/− forestomach squamous cell carcinoma no. 84 that exhibits very strong expression in tumor areas and moderately strong staining in the proliferative epithelium (h). Counterstaining with hematoxylin. Scale bar = 25 μm.

**DISCUSSION**

The analysis of NMBA-initiated tumorigenesis in p53-deficient mice has yielded several interesting insights into the interaction between the loss of p53 and nutritional zinc deprivation in esophageal/forestomach carcinogenesis. Zinc-deprived p53−/− mice demonstrated extraordinarily rapid forestomach tumor development after a single NMBA treatment. In addition, tumors and glandular metaplasia appeared in the esophagus. Tumor incidence in all locations, forestomach/SCJ and esophagus, was significantly higher in ZD:p53−/− than in ZD:p53+/+ or ZD:p53+/+ mice (Table 1). Likewise, nutritionally complete ZS:p53−/− mice had significantly more tumors in forestomach/SCJ than ZS:p53+/− mice, whereas ZS:p53+/+ animals had no tumors. Thus, independent of zinc status, mice with two TP53 alleles missing were more sensitive to the
carcinogenic effects of NMBA than were mice with one allele missing or mice with two intact alleles. These results agree with reports of glandular stomach carcinogenesis in p53-deficient mice exposed to multiple doses of N-methyl-N-nitrosourea, a direct-acting N-nitroso compound (50, 51). Independent of p53 status, ZD:p53−/− and ZD:p53+/+ mice had significantly higher forestomach/SCJ tumor incidence than their ZS counterparts. These findings clearly demonstrate that the general susceptibility to cancer in mice that accompanies the loss of the p53 tumor suppressor protein is additionally enhanced by nutritional zinc deprivation (8, 18).

The mouse esophagus is less sensitive than the forestomach to the action of a variety of carcinogens, although both are squamous cell epithelia and the forestomach is a dilated lower esophagus (19). In this regard, the esophagus of the C57BL/6 mouse is much less susceptible to tumor induction by NMBA than the forestomach (18). Cyclin D1 transgenic mice (C57BL/6 strains), exposed to multiple NMBA doses, showed only increased dysplasia in the esophageal epithelium after 12 months (52). In the current study, a single NMBA dose elicited a vigorous tumorigenic response from ZD:p53−/− esophagus with progression to malignancy in just 30 days. These data suggest that collaboration between a genetic alteration (a loss of p53) and the microenvironment of increased cell proliferation (zinc deficiency) created conditions favorable for esophageal carcinogenesis after a single carcinogenic stimulus.

Uncontrolled cell proliferation occurs in the esophagi and forestomachs of ZD:p53−/− mice in the absence of NMBA treatment, thus setting the stage for tumor initiation when a carcinogenic stimulus is applied. The nutritionally complete ZS:p53−/− mouse esophagus and forestomach showed mostly basal cell proliferation and the presence of apoptotic cells, more numerous in esophagus than forestomach (esophagus, Fig. 2, a–c; forestomach, Fig. 2, g–i), a result indicating that neither apoptosis is abrogated nor cell proliferation uncontrolled in these tissues. By contrast, the ZD:p53−/− mouse had a highly proliferative esophagus (Fig. 2, d–f) and SCJ (Fig. 2, j–l), with numerous PCNA-positive nuclei. At the same time, TUNEL-positive apoptotic cells were nearly absent in the many cell layers of the epithelia. Thus, cell proliferation has become unchecked and apoptosis severely curtailed as a consequence of zinc deprivation.

The antiapoptotic protein Bcl-2 was overexpressed in the highly proliferative esophageal epithelium, esophageal carcinoma, and esophageal glandular metaplasia of ZD:p53−/− mice (Fig. 4). By contrast, the proapoptotic protein Bax was either absent or very selectively expressed in these lesions (Fig. 4). Such a clear inverse relationship was not evident in the esophagus or forestomach of ZS:p53−/− mice (data not shown). These data are consistent with the reduced apoptosis observed in ZD:p53−/− tissues. Because ZD:p53−/− mice lack p53, zinc must modulate events independent of or downstream of p53. Recently, we showed that 24 h after replenishing NMBA-treated ZD rats with a ZS diet, a surge in Bax and reduction in Bcl-2 expression accompanied apoptosis in the still proliferative esophageal epithelium, thereby stopping the development of cancer at an early stage (13). Thus, the Bcl-2/Bax apoptosis pathway plays a critical role in esophageal carcinogenesis modulated by zinc. Calorie restriction and dehydroepiandrosterone, a chemopreventive steroid, have been reported to suppress spontaneous thymic lymphoma development in p53−/− mice (41, 42). Both agents decrease the thymocyte proliferative rate (42). However, the apoptosis-inducing effect of dehydroepiandrosterone was mediated by decreased Bcl-2 gene expression and that of calorie restriction was independent of the Bcl-2/Bax apoptotic regulatory pathway (42). Our data demonstrate that in p53−/− mice, deprivation of zinc produces an increase in cell proliferation together with a decrease in apoptosis, thereby accelerating the induction and progression of esophageal cancer. Finally, within the same dietary group and independently of p53 status, mouse esophagi, in general, displayed a higher AI than forestomachs (Fig. 3B). This observation may explain why the mouse esophagus is less sensitive to tumor induction by chemicals than the forestomach.

There are two major mechanisms by which zinc can modulate gene expression (53). Zinc may alter the activity of transcription factors that bind to DNA via specific zinc-finger regions and, in conjunction with other families of transcription factors, control cell proliferation, differentiation, and cell death. Alternatively, zinc may modulate gene expression through indirect interactions, whereby secondary mediators respond to the supply of this nutrient in the diet. In this regard, the expression of several known genes was altered in the early stages of rodent zinc deficiency, including several intestinal genes that influence signaling pathways, growth, transcription, redox, and energy utilization (54).

In our study, three keratin complex genes were up-regulated ~4- and 5-fold. One of these, cytokeratin 14, is a known marker of ESCC (48). Overexpression of several keratins is thus consistent with the extreme susceptibility of the ZD forestomach to tumor formation (Fig. 5). Other genes that are up-regulated in the ZD:p53−/− forestomachs include several secreted or surface specific genes such as members of the tumor necrosis receptor superfamily and a serine protease inhibitor. The TNFRSF8 gene, also known as CD30 and Ki-1, is expressed on Hodgkin’s disease lymphoma cells (55). The RNASEL gene, with 2.5-fold higher mRNA level in ZD:p53−/− forestomach, is involved in the IFN-regulated antiproliferative pathway (56). The apoptosis-related antiapoptotic genes DAD1 (57) and the DEAD box protein 6 gene were both overexpressed in zinc-deficient cells. These genes may participate in pathways leading to decreased apoptosis and showed consistent evidence of increased expression in ZD forestomach. Only two down-regulated genes were detected in the zinc-deprived condition, the WNT10B gene and the FEN1 gene. The WNT10B gene behaved as an oncogene when overexpressed in mammary epithelial cells (58), so down-modulation here seems counterintuitive. The FEN1 gene is involved in DNA repair and its absence could lead to mutations.

In conclusion, this study showed that in zinc-deprived p53−/− mice, the balance of cell proliferation and apoptosis, two critical factors that influence the course of carcinogenesis, is tipped toward cell proliferation, thereby unleashing a cascade of events that accelerate cancer development when a carcinogenic stimulus is applied. Our results also suggest that zinc deficiency can modulate the enhanced genetic susceptibility to cancer because of inactivation of the TP53 tumor suppressor gene, including the p53-mediated apoptotic Bcl-2/Bax regulatory pathway and the expression of cytokeratin 14. It deserves emphasis that mutations in TP53 are well known in both of the human esophageal carcinomas, ESCC, and the adenocarcinoma that arises in Barrett’s esophagus (30). Thus, it is possible that the genetic changes that occur in the appearance of cancers in the zinc- and p53-deficient mice in just 30 days may recapitulate those that occur in similar human cancers. Accordingly, the animal model developed in this study may prove ideal for the study of genetic changes intrinsic to esophageal cancer development, prevention, and reversal.

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p53 Deficiency Accelerates Induction and Progression of Esophageal and Forestomach Tumors in Zinc-deficient Mice

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