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Targeted expression of ornithine decarboxylase antizyme prevents upper aerodigestive tract carcinogenesis in p53-deficient mice

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Upper aerodigestive tract (UADT) cancers of the oral cavity and esophagus are a significant global health burden, and there is an urgent need to develop relevant animal models to identify chemopreventive and therapeutic strategies to combat these diseases. Antizyme (AZ) is a multifunctional negative regulator of cellular polyamine levels, and here, we evaluate the susceptibility of keratin 5 (K5)-AZ transgenic mice to tumor models that combine chemical carcinogenesis with dietary and genetic risk factors known to influence human susceptibility to UADT cancer and promote UADT carcinogenesis in mice. First, p53$^{+/-}$ and K5-AZ/p53$^{+/-}$ (AZ/p53$^{+/-}$) mice were placed on a zinc-deficient (ZD) or zinc-sufficient (ZS) diet and chronically exposed to 4-nitroquinoline 1-oxide. Tongue tumor incidence, multiplicity and size were substantially reduced in both ZD and ZS AZ/p53$^{+/-}$ mice compared with p53$^{+/-}$. AZ expression also reduced progression to carcinoma *in situ* or invasive carcinoma and decreased expression of the squamous cell carcinoma biomarkers K14, cyclooxygenase-2 and metallothionein. Next, AZ-expressing p53$^{+/-}$ and p53 null mice were placed on the ZD diet and treated with a single dose of *N*-nitrosomethylbenzylamine. Regardless of p53 status, forestomach (FST) tumor incidence, multiplicity and size were greatly reduced with AZ expression, which was also associated with a significant decrease in FST epithelial thickness along with reduced proliferation marker K6 and increased differentiation marker loricrin. These studies demonstrate the powerful tumor suppressive effects of targeted AZ expression in two distinct and unique mouse models and validate the polyamine metabolic pathway as a target for chemoprevention of UADT cancers.

Introduction

Upper aerodigestive tract (UADT) cancers including those of the tongue (head and neck squamous cell carcinoma) and esophagus [esophageal squamous cell carcinoma (ESCC)] are a substantial worldwide cancer burden. Increased understanding of the biology of these diseases and the rational advancement of preventive and therapeutic agents requires the development of animal models that recapitulate the molecular and pathological progression of human disease (1). To this end, we have developed carcinogenesis models that combine nutritional, genetic and chemical factors that are relevant to human UADT cancer.

Epidemiological studies indicate that zinc (Zn) deficiency (ZD) is associated with increased risk for oral and esophageal cancer in humans (2,3) and ZD is also associated with increased DNA damage (4,5). In mice and rats, a ZD diet creates a precancerous condition in the UADT [tongue, esophagus and forestomach (FST)], by inducing cellular proliferation (6,7) and gene expression changes, including overexpression of the proinflammation gene cyclooxygenase-2 (*Cox-2*) (6,8). Mice are resistant to most oral carcinogens, but ZD p53$^{+/-}$ mice are very susceptible to 4-nitroquinoline 1-oxide (NQO)-induced tumor development in the tongue, esophagus and FST (9). NQO activation by reductases leads to DNA adduct-induced mutations and oxidative damage in mouse tongue and esophagus (10), which are similar to tobacco carcinogen-induced cellular alterations. Activating mutations in c-Ha-*Ras*, but not p53, have been detected in oral lesions of NQO-treated rats (11) and activating mutations are prevalent in NQO-treated mice (12). Long-term NQO exposure enhances p53 expression in rat tongue (13), and p53 mutation is an early event in human head and neck squamous cell carcinoma that is associated with increased progression (1).

ESCC can be modeled in mice and rats using a ZD diet to induce hyperproliferation followed by treatment with the carcinogen *N*-nitrosomethylbenzylamine (NMBA). In the mouse ZD/NMBA model, the primary region of tumor development is the FST, a stratified epithelium that is considered a dilation of the lower esophagus (7). ZD is prevalent in geographic regions with high incidence of ESCC (14,15) and environmental exposure to *N*-nitrosamines such as NMBA is also associated with increased risk for the disease (16,17). NMBA can methylate DNA following metabolic activation and NMBA-induced tumors frequently exhibit mutations in H-*ras* and p53 (18–20). Mutations in p53 are frequently detected in human squamous cell carcinoma of the esophagus (21), and p53$^{+/-}$ or p53 null animals subjected to ZD/NMBA carcinogenesis exhibit a greatly enhanced tumor induction and progression compared with wild-type mice (22).

These rodent models enable studies to interrogate the importance of particular genetic alterations and biochemical pathways in human UADT carcinogenesis, and in this study, we focus on the role of polyamine biosynthesis. The polyamines (putrescine, spermidine and spermine) are essential for normal cell growth and differentiation (23–25), yet elevated polyamine levels and ornithine decarboxylase (ODC) activity are associated with neoplastic growth (26,27). ODC catalyzes the synthesis of putrescine from ornithine, the first step of polyamine biosynthesis (reviewed in ref. (28)). ODC is strongly induced by proliferative stimuli and is clearly linked to growth promoting signals such as the proto-oncogenes *Ras* and c-*Myc*. α-Difluoromethylornithine (DFMO), which is a highly specific enzyme-activated irreversible inhibitor of ODC, has received considerable attention as a chemopreventive agent in preclinical and clinical studies (29). DFMO has been shown to inhibit tumor development by reducing cell proliferation and stimulating apoptosis in NQO-treated rodents as well as the ZD/NMBA model (30–32). The encouraging efficacy of DFMO in preclinical models (33) as well as human colorectal, non-melanoma skin and Barrett's esophagus chemoprevention trials (34–36) provides a strong rationale to advance this approach in other common epithelial malignancies.

As cellular polyamine levels increase, they stimulate a +1 frame shifting event in the translation of the ODC antizyme (AZ) messenger RNA (mRNA) thereby increasing the expression of full-length functional AZ protein. AZ is a negative regulator of cellular polyamine content that inhibits ODC activity, enhances ubiquitin-independent ODC degradation by the 26S proteasome, promotes polyamine excretion and suppresses polyamine uptake (reviewed in ref. (37)). Due to these multiple biochemical activities, forced AZ expression is expected to deplete cellular polyamine levels more effectively than DFMO. We have previously generated transgenic mice with K5- or K6-driven AZ expression to test the ability of AZ to suppress tumor development in epithelial tissues where these promoter elements are active. The studies clearly demonstrate that transgenic mice with targeted overexpression of AZ exhibit a cancer resistance phenotype when challenged with chemical, genetic and physical tumor-inducing

Abbreviations: AZ, antizyme; COX-2, cyclooxygenase-2; DFMO, α-difluoro methylornithine; ESCC, esophageal squamous cell carcinoma; FST, forestomach; K5, keratin 5; MT, metallothionein; NMBA, *N*-nitrosomethylbenzylamine; NQO, 4-nitroquinoline 1-oxide; ODC, ornithine decarboxylase; PCNA, proliferating cell nuclear antigen; SCJ, squamocolumnar junction; UADT, upper aerodigestive tract; ZD, zinc deficient; Zn, zinc; ZS, zinc sufficient.

stimuli (38–41). The K5 promoter also drives expression in the tongue, esophagus and FST. K5-AZ transgenic mice exhibit substantial resistance to NMBA-induced FST carcinogenesis in both ZD and Zn-sufficient (ZS) animals, and this protective effect was associated with reduced cell proliferation and increased apoptosis (42). In addition to the tumor resistance phenotype of AZ-expressing transgenic mice, an increasing body of evidence suggests that AZ loss or amplification of its antagonist, AZ inhibitor, are common alterations in multiple human cancers (43).

The NQO and NMBA models described above incorporate DNA-damaging and oxidative stress-inducing carcinogens with dietary (ZD) and genetic (p53 deficiency) risk factors that are relevant to human head and neck squamous cell carcinoma and ESCC. Therefore, they provide a platform to evaluate the importance of polyamine biosynthesis to UADT carcinogenesis. In this study, we first investigated whether targeted AZ expression is capable of preventing tumor development in the novel ZD/NQO model of tongue carcinogenesis in $p53^{+/-}$ mice. Second, we examined the ability of AZ to prevent NMBA-induced FST tumor development in $p53^{+/-}$ as well as p53 null mice. AZ expression reduced tumor incidence, multiplicity and size in both models, even in the absence of p53.

Materials and methods

Chemicals, reagents and diets

Chemicals were purchased from Sigma Chemical Company (St Louis, MO) and Fisher Scientific (Pittsburgh, PA) unless noted. NQO was purchased from Wako Chemicals (Richmond, VA) and NMBA was purchased from Ash Stevens (Detroit, MI). Oligonucleotide primers were synthesized and purified in the Macromolecular Core Facility (Pennsylvania State University College of Medicine). Custom-formulated egg white-based diets were from Harlan Teklad (Madison, WI). ZD and ZS diets were identical except for the Zn content, which was 1.5 and 70 p.p.m., respectively.

Breeding and identification of transgenic and knockout mice

Transgenic mice with K5 promoter-driven expression of AZ were identified by PCR as described previously (39). This transgenic line was backcrossed to the C57BL/6J (Jackson Labs, Bar Harbor, ME) inbred strain for at least eight generations prior to the initiation of our experiments. The AZ transgene sequence contains a single nucleotide deletion (T205), which permits AZ expression without the need for polyamine-stimulated frameshifting during translation.

Mice heterozygous for a targeted disruption of p53 ($p53^{+/-}$ strain 01BM4) were received from the National Cancer Institute Mouse Models of Human Cancers Consortium and permission for their use was obtained from Taconic (Germantown, NY). K5-AZ mice were crossed with this line to generate K5-AZ/$p53^{+/-}$ animals that were then bred to mice harboring a homozygous disruption of p53 (p53 null strain B6.129S2-*Trp53*^{tm1Tyj}/*J*; Jackson Labs). All mouse lines were maintained on the C57BL/6 background. These studies were designed and conducted based on the expectation that mice from both the Mouse Models of Human Cancers Consortium and Jackson Labs had the exact same targeted disruption of the p53 gene, as originally described by Jacks *et al.* (44). However, it was later determined that the Mouse Models of Human Cancers Consortium mice utilized a different targeting construct to disrupt the p53 gene, as originally described by Donehower *et al.* (45). There is no production of functional p53 protein in either mouse model; therefore, the ability to evaluate the effect of AZ on carcinogenesis in a p53-deficient background was not compromised and the studies were continued.

K5-AZ/$p53^{+/-}$ mice were crossed with p53 null animals to generate $p53^{+/-}$, K5-AZ/$p53^{+/-}$, p53 null and K5-AZ/p53 null experimental groups. All groups of p53-deficient mice, regardless of K5-AZ transgene status, had identical p53 alleles such that $p53^{+/-}$ mice harbor the Jacks *et al.* (44) targeted allele and p53 null mice harbor one Jacks *et al.* targeted allele and one Donehower *et al.* (45) targeted allele. Genotyping for p53 status was done by PCR using two primer sets. The first primer pair amplified an 840 bp product from the wild-type p53 allele (forward 5′-GCTATTCTGCCAGCTGGCGAAGACG-3′ and reverse 5′-ATAGGTCGGCGGTTCAT-3′), which is a region from exon 5 to 7 of the p53 gene that is deleted in both strains of p53-targeted mice. The second primer pair amplified a 280 bp product from the neomycin-resistance gene (forward 5′-CTTGGGTGGAGAGGCTATTC-3′ and reverse 5′-AGGTGAGATGACAGGAGATC-3′), which is inserted by the targeting construct in both strains of p53-targeted mice. The genotype of all animals in the carcinogenesis studies was confirmed at killing using a second tissue biopsy.

NMBA and NQO carcinogenesis

The tumor studies were conducted according to approved institutional animal protocols. For NQO carcinogenesis, animals were placed on nutritionally equivalent ZS or ZD diets at 4 weeks of age. Chronic NQO treatment (10 p.p.m. in the drinking water) began 4 weeks later and was continued along with dietary manipulation until killing 160 days later. For NMBA carcinogenesis, ZS diet was not employed because a single NMBA treatment does not enhance FST carcinogenesis in ZS $p53^{+/-}$ mice (22). Animals were placed on a ZD diet at 4 weeks of age. After 25 days, mice were treated with a single intragastric dose of NMBA (2 mg/kg body weight) and then killed 44 days later (22). Lesions 0.5 mm in diameter or larger were scored by gross examination of the tongue, esophagus and FST in both experiments.

Biochemical analysis, immunohistochemistry and histopathology

FST polyamine content and ODC activity were determined as described previously (38,42). Samples for histology and immunohistochemistry were fixed in neutral-buffered formalin. Immunohistochemical staining for COX-2 (161126; Cayman Chemical, Ann Arbor, MI), metallothionein (MT) (M0639; Dako, Carpinteria, CA), proliferating cell nuclear antigen (PCNA) (sc-56; Santa Cruz Biotechnology, Santa Cruz, CA), loricrin, K6, K5 and K14 (PRB-145P, PRB-169P, PRB-160P and PRB-155P, respectively; Covance Research Products, Denver, PA) and AZ was conducted as described previously (9,39,42). FST epithelial thickness was measured (12–18 measurements per mouse; $n = 3$ mice per group) using a Nikon DS-5M camera and control unit DS-L1.

Hair Zn measurement

At autopsy, hair was removed from mice (one mouse per cage of five ZD or ZS mice). Hair samples were washed, dried and ashed in a furnace. Ashed samples were dissolved in 0.1 N HCl and their Zn content was determined by atomic absorption spectroscopy (9). Consistent with our previous data (9,22,42), hair Zn content (μg Zn/g dry weight) was significantly lower in ZD than ZS mice, regardless of genotype. As examples, at 5 weeks of experimental diet, hair Zn content (mean ± standard error of the mean) was significantly lower in ZD AZ/$p53^{+/-}$ versus ZS AZ/$p53^{+/-}$ mice (117 ± 3.8 versus 149 ± 4.7 μg/g, $P = 0.003$, $n = 4$ mice per dietary group) and in ZD $p53^{+/-}$ versus ZS $p53^{+/-}$ mice (119 ± 1.9 versus 146 ± 5.1 μg/g, $P = 0.016$, $n = 4$ mice per dietary group).

Statistical analysis

Tumor incidence values were compared using Fisher's exact test and tumor multiplicity values were compared using Student's unpaired *t*-test. Statistical tests were two-sided and were considered significant at $P < 0.05$.

Table I. NQO-induced tongue and FST/SCJ tumors in AZ/$p53^{+/-}$ mice at 160 days

Diet	No. of mice	Genotype	Tumor incidence (%)		Tongue tumors			FST/SCJ tumors		
			Tongue	FST/SCJ	No. of tumors/site	Incidence (%)		No. of tumors/site	Incidence (%)	
						<1 mm	≥1 mm		<1.5 mm	≥1.5 mm
ZS	19	$p53^{+/-}$	16/19 (84)	16/19 (84)	2.4 ± 0.4	9/19 (47)	7/19 (37)	2.5 ± 0.5	7/19 (37)	9/19 (47)
	19	AZ/$p53^{+/-}$	12/19 (56)	9/19 (47)	0.9 ± 0.2	10/19 (53)	2/19 (10)	0.7 ± 0.2	9/19 (47)	0/19 (0)
			NS	$P = 0.038$	$P = 0.001$	NS	NS	$P = 0.001$	NS	$P = 0.001$
ZD	28	$p53^{+/-}$	26/28 (93)	21/28 (75)	3.5 ± 0.4	11/28 (39)	15/28 (54)	2.9 ± 0.5	10/28 (36)	11/28 (39)
	21	AZ/$p53^{+/-}$	14/21 (67)	8/21 (38)	1.4 ± 0.3	9/21 (43)	5/21 (24)	0.7 ± 0.2	4/21 (19)	4/21 (19)
			$P = 0.028$	$P = 0.018$	$P = 0.0001$	NS	$P = 0.045$	$P = 0.0005$	NS	NS

NS, not significant.

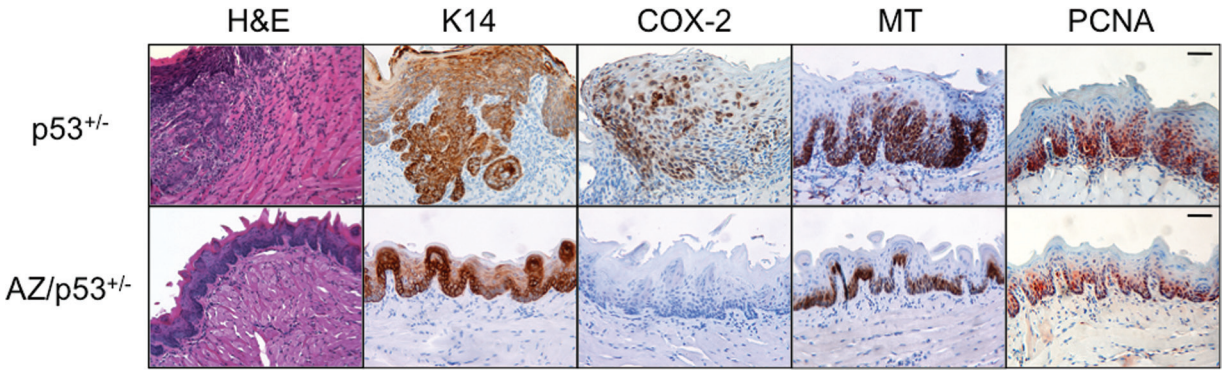

Fig. 1. AZ restricts tumor progression in tongue lesions. Tongue sections taken from ZD mice after 160 days of NQO exposure were stained with hematoxylin and eosin (H&E) and examined for malignant progression. Additional slides were immunostained for the epithelial marker K14 (brown), which is overexpressed in carcinomas and allows clear visualization of invasive epithelial cells within the underlying muscle. Staining for squamous cell carcinoma biomarkers demonstrated areas of strong COX-2 (brown) expression and MT (brown) overexpression more frequently in $p53^{+/-}$ than $AZ/p53^{+/-}$ mice. Reduced expression of the proliferation marker PCNA (red) was also detected in $AZ/p53^{+/-}$ mice. Bars at the right edge of each row represent 50 µm.

Results

NQO-induced UADT carcinogenesis in p53-deficient mice

Our initial study was designed to evaluate the importance of polyamine biosynthesis in UADT carcinogenesis in a mouse model that incorporates genetic, dietary and chemical manipulations that are relevant to human cancer. We bred K5-AZ transgenic mice, which exhibit targeted expression of AZ in basal epithelial cells of the UADT, with mice harboring a p53 null allele. AZ is a multifunctional negative regulator that will suppress both polyamine synthesis and uptake in the relevant cell population. $p53^{+/-}$ and K5-$AZ/p53^{+/-}$ (designated $AZ/p53^{+/-}$) mice were placed on either ZD or ZS diets for 4 weeks before treatment with NQO (10 p.p.m. in the drinking water) for 160 days.

All ZD and ZS mice survived 160 days of NQO treatment. Consistent with our previous report (9), ZD $p53^{+/-}$ mice showed higher tongue tumor incidence and multiplicity compared with ZS $p53^{+/-}$ mice (Table I). The difference, however, was not statistically significant probably because of a lower level of NQO used in this study. AZ expression reduced the susceptibility of $p53^{+/-}$ animals to NQO carcinogenesis of the tongue regardless of dietary Zn intake (Table I). Most remarkably, in ZD animals, tongue tumor incidence was significantly reduced in $AZ/p53^{+/-}$ mice (67%) compared with $p53^{+/-}$ mice (93%), and there was also a significant reduction in tumor multiplicity (1.4 for $AZ/p53^{+/-}$ versus 3.5 for $p53^{+/-}$) and large tumor incidence (24% for $AZ/p53^{+/-}$ versus 54% for $p53^{+/-}$). A strong reduction in tumor susceptibility was also observed in the FST/squamocolumnar junction (SCJ) region of AZ-expressing $p53^{+/-}$ mice, which exhibited significantly reduced tumor incidence and a >70% reduction in tumor multiplicity regardless of dietary Zn intake (Table I). ZD animals killed after 120 days of NQO treatment exhibited a lesser tumor burden but the protective effect of AZ expression was maintained (Supplementary Table I, available at *Carcinogenesis* Online). Because of the low level of NQO employed (10 p.p.m.), the incidence of lesions in the esophagus was very low (<35%) in all treatment groups, which made it difficult to determine whether AZ expression affected esophageal carcinogenesis.

For a subset of ZD animals, tongue sections were scored for signs of malignant progression including microinvasion, carcinoma *in situ* or invasive carcinoma (Figure 1; hematoxylin and eosin). Less malignant progression was detected in sections from ZD $AZ/p53^{+/-}$ mice compared with $p53^{+/-}$ animals (1/12 versus 9/17, respectively; $P = 0.019$). Immunohistochemistry of the squamous cell carcinoma biomarker K14 confirmed the presence of invasive epithelial cells within the underlying muscle tissue (Figure 1; K14). Consistent with our previous report (9), ZD $p53^{+/-}$ tongue showed a highly inflammatory and proliferative cancer phenotype with abundant expression of the human oral tumor markers COX-2 (46) and MT (47), and cell proliferation marker PCNA (Figure 1). In contrast, $AZ/p53^{+/-}$ sections exhibited reduced expression of all three markers.

p53 null mice were included in the original experimental design, but very few of these animals survived to the endpoints due to their propensity to develop spontaneous thymic lymphomas. A small number of surviving animals within the 160d ZS cohort revealed a protective effect of AZ expression even in the absence of p53, with AZ/p53 null mice ($n = 3$) developing fewer lesions than p53 null mice ($n = 3$) in both the tongue (1.0 ± 0.0 versus 4.3 ± 1.2, respectively; $P = 0.050$) and FST/SCJ (1.7 ± 0.7 versus 4.0 ± 2.0, respectively; not significant).

NMBA-induced FST carcinogenesis in p53-deficient mice

Following the novel observation of reduced lingual carcinogenesis in AZ-expressing $p53^{+/-}$ mice, we utilized our well-characterized FST carcinogenesis model to more clearly determine the tumor protective effects of AZ in the complete absence of functional p53. AZ/

Table II. NMBA-induced FST and SCJ tumors in Zn-deficient $AZ/p53^{+/-}$ and AZ/p53 null mice

Genotype (*n*)	FST				SCJ	
	Tumor multiplicity	Tumor incidence (%)	Tumor size, % of mice		Tumor multiplicity	Tumor incidence (%)
			Small (<1.5 mm)	Large (≥1.5 mm)		
$p53^{+/-}$ (37)	3.3 ± 0.5	26/37 (70%)	46%	24%	2.5 ± 0.3	30/37 (81%)
$AZ/p53^{+/-}$ (28)	0.4 ± 0.2	6/28 (21%)	21%	0%	0.6 ± 0.2	11/28 (39%)
	$P = 0.00001$	$P = 0.0001$	NS	$P = 0.008$	$P = 0.000004$	$P = 0.0007$
p53 null(17)	11.7 ± 1.2	17/17 (100%)	12%	88%	4.4 ± 0.5	17/17 (100%)
AZ/p53 null(17)	3.1 ± 1.0	11/17 (65%)	41%	24%	2.4 ± 0.5	13/17 (76%)
	$P = 0.000007$	$P = 0.018$	NS	$P = 0.0004$	$P = 0.014$	NS

NS, not significant.

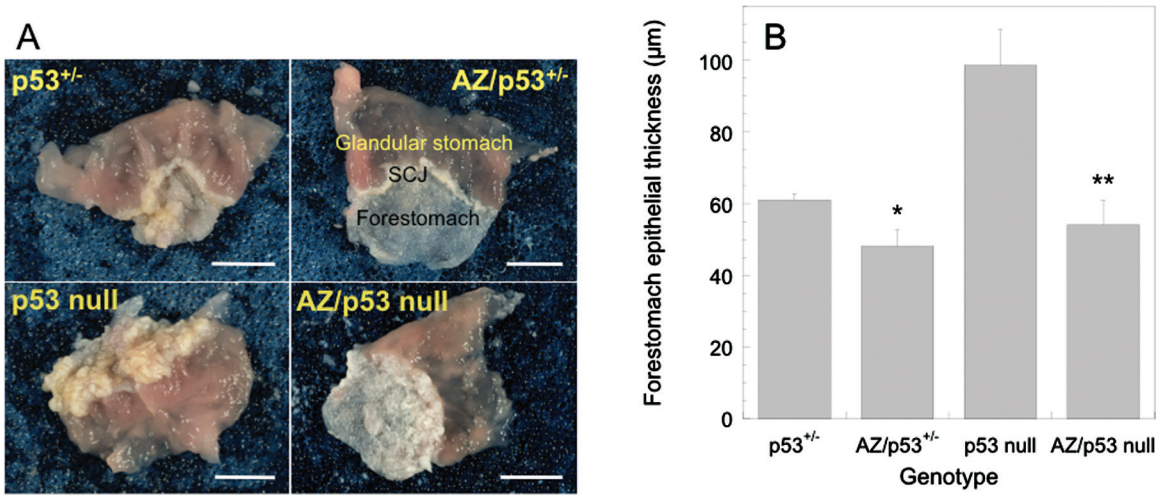

Fig. 2. FST epithelial thickness is reduced in AZ-expressing mice. (**A**) Gross morphology of representative stomach samples from ZD mice killed 44 days after NMBA treatment. Scale bars represent 5 mm. (**B**) Thickness of the epithelium was measured in non-tumor-bearing regions of ZD/NMBA-treated FST from animals described in Table II. Bars represent mean ± SD and $n = 3$ mice per group. $^{*}P < 0.05$ AZ/p53$^{+/-}$ versus p53$^{+/-}$; $^{**}P < 0.005$ AZ/p53 null versus p53 null.

p53$^{+/-}$ mice were crossed with p53 null animals and we evaluated the susceptibility of the progeny to NMBA-induced FST carcinogenesis. p53$^{+/-}$, AZ/p53$^{+/-}$, p53 null and AZ/p53 null mice were placed on a ZD diet at 4 weeks of age and treated once with NMBA (2 mg/kg body weight, intragastric gavage) 25 days later. Elevated levels of AZ protein were detected in the FST of mice with the K5-AZ transgene, and there was no significant change in AZ content with ZD (Supplementary Figure 1, available at *Carcinogenesis* Online). p53 null mice were included due to the shorter time frame of the ZD/NMBA protocol relative to the NQO studies. These animals had not developed spontaneous lymphomas based on examination of the thymus and spleen at necropsy, with the exception of a single animal in the AZ/p53 null group.

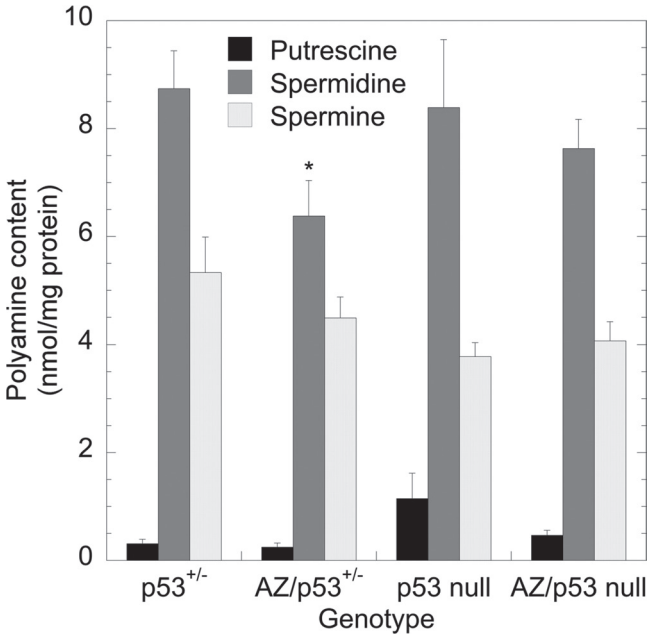

Fig. 3. Effects of AZ on polyamine content in NMBA-treated FST. Polyamine content was determined in FST samples from ZD mice 44 days after NMBA treatment as described in Table II. AZ expression is associated with reduced polyamine content, especially spermidine. Bars represent mean ± standard error of the mean and $n = 4$–5 mice per group. $^{*}P < 0.05$ AZ/p53$^{+/-}$ versus p53$^{+/-}$.

The gross appearance and number of lesions in the FST and SCJ were scored 44 days after NMBA treatment (Table II). AZ expression greatly decreased tumor incidence, diminished tumor size and dramatically reduced tumor multiplicity by 55–88% in the p53$^{+/-}$ or p53 null background. The FST of p53$^{+/-}$ mice had a greatly thickened appearance, whereas AZ/p53$^{+/-}$ FSTs were very thin. Most p53 null mice had numerous large tumors that occupied >50% of the FST, but AZ/p53 null mice exhibited mainly thickened patches and isolated tumors (Figure 2A). Histopathological analysis was also completed on ZD/NMBA FST sections. FST epithelial thickness was significantly reduced in AZ-expressing animals on both p53$^{+/-}$ and p53 null backgrounds (Figure 2B). This result correlated with increased staining for the apoptosis marker cleaved caspase 3 in AZ-expressing tissues that failed to reach statistical significance (data not shown).

Polyamine content and ODC activity were analyzed in FST samples taken 44 days after NMBA treatment. Both parameters were highly variable within all groups due to the varying tumor burden of individual mice and the resulting differences in amount of neoplastic tissue within each FST specimen. The extreme variability in ODC activity values, which exhibited standard deviations of >60% in all groups ($n = 5$ samples per group), precluded any determination of AZ effects on this parameter. In the p53$^{+/-}$ background, AZ expression did lead to a significant reduction in FST spermidine content (Figure 3), which is consistent with our previous studies (42). AZ reduced spermidine and putrescine levels in the p53 null background, but these differences were not statistically significant.

In addition, microarray analysis was done on mRNA harvested from a limited number of available FST epithelial samples in order to generate hypotheses to explain the ability of AZ to suppress tumor development. These studies suggested that AZ expression results in increased expression of the differentiation marker loricrin and decreased expression of the proliferation marker K6. Subsequent immunohistochemical staining provided clear confirmation of these suggestions (Figure 4). In the presence of AZ, the epithelial differentiation pattern was readily apparent compared with large regions of hyperproliferative, non-loricrin expressing cells in the absence of AZ. Similarly, the hyperproliferative marker K6 was rarely observed in the presence of AZ, whereas large regions of intense K6 staining were detected in the absence of AZ. The expansion of the proliferative basal cell compartment (labeled by K5 and K14) was also readily detected in tissues from mice not expressing AZ. Therefore, AZ expression restrained expansion of the proliferative compartment and increased the differentiation program within the FST epithelium.

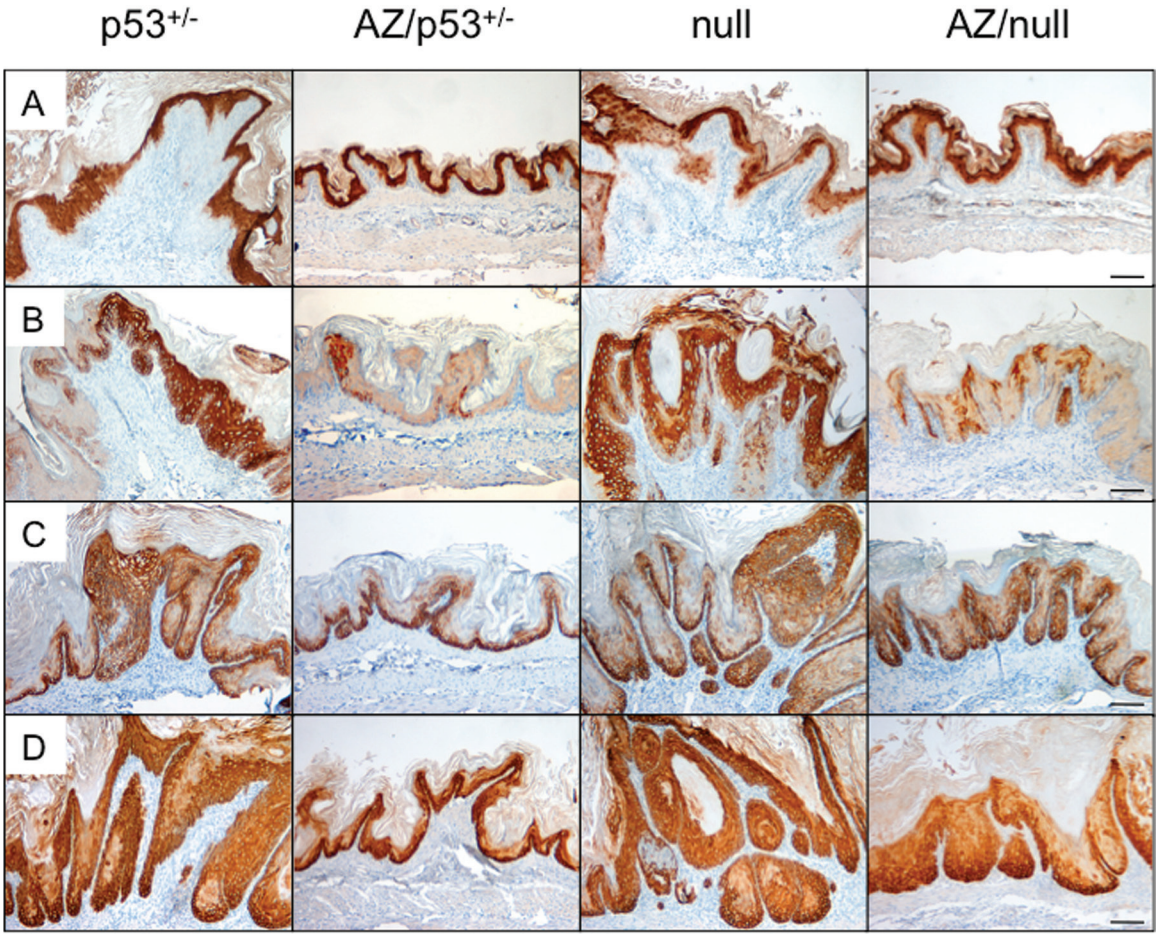

Fig. 4. AZ restricts FST hyperproliferation and promotes differentiation. Sections of ZD/NMBA-treated FST from animals described in Table II were stained for the differentiation marker loricrin (**A**), the hyperproliferation marker K6 (**B**), or the basal cell markers K5 (**C**) and K14 (**D**). Bars at the right edge of each row represent 100 μm.

Discussion

Relevant animal models are necessary in order to increase our understanding of UADT carcinogenesis and to identify targets to prevent and treat these diseases. The mouse models utilized in this study to examine the role of polyamine metabolism in UADT carcinogenesis exhibit numerous significant advantages including (i) reproducible tumor induction within a reasonable time frame, (ii) tumor induction in the typically refractory lingual compartment, (iii) manipulation of polyamine levels within the relevant cell population and (iv) analysis of AZ effects in the background of reduced or absent p53 gene dosage. Therefore, there is tremendous utility of these models as *in vivo* systems to evaluate the impact of genetic, pharmacologic or dietary manipulations on epithelial carcinogenesis. The significance and relevance of our studies is supported by substantial evidence indicating a role for both ZD and p53 mutation in human UADT susceptibility and pathogenesis. In addition, recent successes in clinical trials of polyamine-targeted chemoprevention with DFMO (34–36) validate the pathway as a target in epithelial carcinogenesis to be further explored in the UADT. Our results provide additional evidence of the pronounced tumor resistance associated with targeted AZ expression, as detected previously in mouse models of cancer (38–42). They also demonstrate for the first time that AZ-mediated targeting of cellular polyamine content inhibits lingual carcinogenesis, and they represent the first *in vivo* evidence that the tumor suppressive effects of AZ in UADT carcinogenesis persist in the background of reduced or absent expression of the powerful tumor suppressor p53.

K5-AZ mice are resistant to NQO-induced lingual and FST carcinogenesis in a $p53^{+/-}$ background as well as NMBA-induced FST carcinogenesis in both the $p53^{+/-}$ and p53 null backgrounds. In both models, AZ expression was associated with more tumor-free mice, fewer tumors per mouse and smaller tumor size. These tumor suppressive effects were also observed in mice on a ZS diet (Table I), which indicates that AZ also blocks tumor growth in the absence of ZD-induced hyperplasia. In the tongue, AZ inhibited the progression of lesions to more advanced carcinoma *in situ* and invasive carcinoma. Importantly, AZ expression is associated with a less inflammatory and proliferative tongue tumor phenotype as evidenced by the reduced expression of the cancer-associated inflammation and proliferation markers COX-2, MT and PCNA (Figure 1). In the ZD/NMBA FST, AZ expression was associated with suppressed polyamine content, reduced epithelial thickness, decreased levels of the hyperproliferative marker K6 as well as enhanced expression of the differentiation marker loricrin. Remarkably, tumor incidence and multiplicity data (Table II) demonstrate that the addition of the K5-AZ transgene to the mouse genome (AZ/p53 null mice) protects against NMBA-induced FST carcinogenesis to the same extent as the addition of a single copy of the p53 tumor suppressor gene ($p53^{+/-}$ mice).

A number of studies have reported induction of p53 in response to polyamine depletion, which is mediated primarily through posttranscriptional mechanisms including mRNA and protein stabilization (reviewed in refs (48) and (49)). It is important to note that most of these experiments were performed in cultured cells and involved massive polyamine depletion following growth in the presence of DFMO for several days. In contrast, our results in

the ZD/NMBA model clearly indicate that p53 is dispensable for AZ-mediated inhibition of tumor development. Others have also reported growth suppression or arrest upon polyamine depletion in p53 mutant or deleted cancer cell lines typically due to p21 or p27 induction (48–50); however, immunohistochemical staining of AZ-expressing tongue and FST sections failed to detect induction of either protein (data not shown).

The molecular mechanisms that mediate tumor resistance in AZ-expressing animals remain unclear and are a critical direction for future experimental efforts. Potential mechanisms may be specific to AZ or common to both AZ expression and DFMO-mediated polyamine depletion. These include altered DNA and chromatin structure, changes in mRNA stability and translation, increased differentiation associated with altered DNA methylation, induced cell death and modified transcription including reduced oncogene expression (reviewed in refs (23,24,26,37,48)). It has been postulated that polyamine depletion may selectively inhibit the proliferation of cells containing activated Ras and/or mutant p53 (51), and further studies are required to elucidate the mechanisms that are responsible for such selectivity. Microarray analysis of ZD/NMBA FST epithelia suggested that AZ exerts a pro-differentiation and anti-proliferative effect, which was confirmed by immunohistochemistry (Figure 4). Future studies will pursue the mechanisms that mediate these responses as well as additional molecular alterations induced in the presence of AZ that were detected by mRNA microarray analysis. The effects are probably extensive and far-reaching because numerous transcription factors were found to be up- or downregulated.

The strong staining for K6 in hyperproliferative and neoplastic lesions within the ZD/NMBA FST suggests that transgenic animals with a K6 promoter-driven AZ transgene (39) may exhibit an even more dramatic tumor resistance phenotype due to increased AZ transgene expression within hyperproliferative cells. In addition, a recently developed transgenic mouse model with regulated AZ expression (D.J.Feith, in preparation) will permit an assessment of the ability of AZ to induce the regression of established neoplastic lesions.

Our studies provide a strong rationale to advance DFMO chemoprevention in human UADT cancers, especially when considered together with the recent success of DFMO as a single agent in Barrett's metaplasia (36), non-melanoma skin cancer (34) and when used in combination with sulindac in colorectal cancer (35). In addition, the models described in this study provide ideal preclinical platforms to test the efficacy of combination therapies that target polyamine biosynthesis along with other biochemical pathways in order to identify the best regimens to advance to human studies. Candidates that exhibited strong potential in UADT preclinical models include rapamycin (52), metformin (53), celecoxib (6,54), 5-aza-2′-deoxycytidine and *all-trans* retinoic acid (55).

Supplementary material

Supplementary Table I and Figure 1 can be found at http://carcin.oxfordjournals.org/

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