

Short Communication

p53 Haploinsufficiency Profoundly Accelerates the Onset of Tongue Tumors in Mice Lacking the Xeroderma Pigmentosum Group A Gene

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Mice lacking the xeroderma pigmentosum group A gene (*XPA*^{-/-} mice), which have a complete deficiency in nucleotide excision repair (NER), are highly predisposed to tongue squamous cell carcinoma (SCC) when exposed to 4-nitroquinoline 1-oxide (4NQO). To explore the effects of the interaction of the NER machinery with *p53* in oral tumorigenesis, we generated an *XPA*^{-/-} mouse strain carrying mutant alleles for *p53*. This mouse model of 4NQO carcinogenesis demonstrated that despite the same tumor frequency, *XPA*^{-/-}*p53*^{+/-} mice reached 100% SCC incidence at 25 weeks compared with 50 weeks for *XPA*^{-/-}*p53*^{+/+} littermates. *XPA*^{-/-}*p53*^{-/-} mice succumbed to spontaneous thymic lymphomas before the development of tongue tumors (before 13 weeks of age). SCC originated in *XPA*^{-/-}*p53*^{+/-} mice maintained the *p53*^{+/-} genotype and the retained wild-type *p53* allele appeared to be structurally intact. Only one of 20 *XPA*^{-/-}*p53*^{+/+} SCC showed a missense mutation of *p53*. Collectively, the accelerated tongue tumor growth may be a consequence of haploinsufficiency but not of mutation of *p53* in the context of NER deficiency. (*Am J Pathol* 2003, 163:1729–1733)

p53 plays a complex and critical role in cellular proof-reading in response to many stress signals including potentially oncogenic DNA damage.^{1–3} Abrogation of *p53* function results in increased genomic instability, de-

creased growth arrest, deregulated cell proliferation, reduced apoptosis and promoted angiogenesis. Consequently, loss of *p53*, through clonal expansion of cells in which unrepaired DNA damage would lead to mutation, potentiates tumor development in both humans and mice. The *p53* heterozygous (*p53*^{+/-}) mouse, an animal model for human Li-Fraumeni syndrome,⁴ contains a single normal allele of *p53* and provides the first opportunity to study the function of *p53* in the carcinogenesis initiation, promotion, and progression steps *in vivo*.^{5–7} There are contradictory data concerning the status of the functional *p53* allele in tumors derived from *p53*^{+/-} mice;⁶ however, a growing body of evidence to date indicated that haploid loss of *p53* is sufficient for driving tumorigenesis^{6–14} and may have different potential in different tissues.^{7,15,16} Despite the importance of *p53* in oral carcinogenesis,¹⁷ the lack of an appropriate genetically based mouse model has left this issue in considerable doubt.

We reported recently that the application of 4-nitroquinoline 1-oxide (4NQO) in the drinking water selectively induced a very high frequency of tongue squamous cell carcinoma (SCC) in the xeroderma pigmentosum group A gene-deficient (*XPA*^{-/-}) mice.¹⁸ At 50 weeks of exposure, the tumor incidence exceeded 90% for *XPA*^{-/-} mice, with no tumors occurring in *XPA*^{+/-} and *XPA*^{+/+} littermates. Similar to human oral SCC, a sequence of histopathological lesions develops: mild to severe dysplasia, conversion to carcinoma *in situ*, and eventually, invasive SCC. Unlike the mouse skin carcinogenesis, papilloma formation, papilloma growth, and progression to SCC are an uncommon event. Thus, this is a unique multistep model of oral cancer.¹⁸ In the present study, we asked whether the introduction of *p53* deficiency renders *XPA*^{-/-} mice more susceptible to 4NQO-induced tongue carcinogenesis.

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Materials and Methods

Mouse Model

XPA^{-/-} mice used in this study were developed and maintained on a C3H/HeN background as described previously.¹⁸⁻²¹ They were crossed with *p53*^{+/-} mice on a mixed C57BL/6 (25%) and CBA (75%) genetic background^{13,22,23} to generate the following cohorts of mice: *XPA*^{-/-}*p53*^{-/-} (*n* = 20), *XPA*^{-/-}*p53*^{+/-} (*n* = 50) and *XPA*^{-/-}*p53*^{+/+} (*n* = 40). Littermate controls included *p53*^{-/-} (*n* = 35) and *p53*^{+/-} (*n* = 50) mice. On entry into the carcinogenicity experiment, about half of the mice were used for the aging study.

4NQO-Induced Carcinogenesis and Tumor Analyses

Six-week-old mice had free access to drinking water containing 10 ppm 4NQO (Iwai Chemical, Tokyo, Japan) as detailed in our previous report.¹⁸ At necropsy, each tumor was divided into halves; one was fixed in 10% phosphate-buffered formalin for histological and immunohistochemical studies, and the other flash-frozen in liquid nitrogen and stored at -80°C for later extraction of DNA. Tumors and autopsied tissues were prepared for routine examination of hematoxylin and eosin (H&E)-stained sections. *p53* expression in tongue tumors was investigated with a polyclonal antibody (NCL-*p53*-CM5p, Novocastra, Newcastle-on-Tyne, UK). Specific immune complexes were detected by the streptavidin-biotin peroxidase complex method.^{13,18} Data for male and female mice were combined, as there was no significant difference in both mortality and tumorigenicity data between the sexes (preliminary observations).

PCR-Based and Southern Blot-Based Genotyping Analyses

Loss of the wild-type *p53* allele in *XPA*^{-/-}*p53*^{+/-} tumors (*n* = 20) was detected by polymerase chain reaction (PCR)-based genotyping analysis as detailed previously.^{13,22,23} These results were also confirmed by Southern blot-based genetic analysis.²² Briefly, DNA was digested with *Sst*I, separated by electrophoresis in 1% agarose gels and transferred to nitrocellulose. Hybridization was performed in 50% formamide/10X Denhardt's reagent/4X SSC containing 20 µg per ml of denatured salmon sperm DNA at 42°C followed by washing in 0.2X SSC/0.1% SDS at 50°C. The probes used were the random-primed *Sst*I-*Xmn*I fragment.²²

Mutational Analysis of *p53*

Total genomic DNA extracted from tongue tumors of *XPA*^{-/-}*p53*^{+/-} (*n* = 20) and *XPA*^{-/-}*p53*^{+/+} (*n* = 20) mice was subjected to PCR-based cold single-strand conformation polymorphism (SSCP) analysis of exons 5-8 of the mouse *p53* as described elsewhere.^{14,16} Briefly, exon-specific PCR products were denatured at

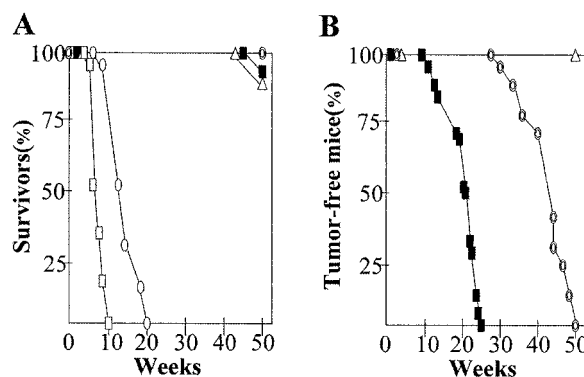


Figure 1. A: Survival curves in 10 *XPA*^{-/-}*p53*^{-/-} (□), 28 *XPA*^{-/-}*p53*^{+/-} (■), 20 *XPA*^{-/-}*p53*^{+/+} (○), 20 *p53*^{-/-} (○), and 30 *p53*^{+/-} (△) mice. B: Tongue tumor incidence in 22 *XPA*^{-/-}*p53*^{+/-} (■), 20 *XPA*^{-/-}*p53*^{+/+} (○), and 20 *p53*^{+/-} (△) mice. Points represent mice that had tongue tumors visible to the naked eye.

95°C and then subjected to rapid chilling on ice. This mixture was loaded onto a 20% TBE gel and electrophoresed under exon-specific optimized conditions at 300V. After electrophoresis, gels were stained with 0.5 µg/ml ethidium bromide (1X TBE) and visualized using a 340 nm UVR. Samples with a mobility shift in PCR-SSCP analysis were confirmed by sequencing after subcloning as described previously.¹⁸ Briefly, DNA fragments from the SSCP gel were subcloned into the *Eco*RV site of the T-tailed pBluescript II SK(-) (Stratagene, La Jolla, CA). Sequences of inserted DNA from more than 50 recombinant mixed colonies were determined for both of the strands by the dideoxyribonucleotide chain termination method, using a T7 sequence kit (Pharmacia, Uppsala, Sweden). Each analysis was repeated at least twice for confirmation.

Results

Life Expectancy

Viability of *XPA*^{-/-}*p53*^{-/-} (*n* = 10), *XPA*^{-/-}*p53*^{+/-} (*n* = 28), *XPA*^{-/-}*p53*^{+/+} (*n* = 20), *p53*^{-/-} (*n* = 20), and *p53*^{+/-} (*n* = 30) mice was closely monitored. As shown in Kaplan-Meier survival plots (Figure 1A), *XPA*^{-/-}*p53*^{-/-} and *p53*^{-/-} mice died from spontaneous lymphomas of the thymus and spleen or unknown causes within 13 or 20 weeks of age and also about 10% of mice with *p53*^{+/-} genotype succumbed to lymphoma by 50 weeks. Consistent with our previous reports,^{13,23} nullizygoty for *p53* significantly reduced mouse survival. This confounded the present chronic carcinogenicity regimen.

Tongue Tumor Incidence, Latency, and Pathology

The incidence and latency times of tongue tumor development are summarized in Figure 1B. There was no difference in total intake of 4NQO per mouse among mice examined (data not shown). All tongue tumors, regard-

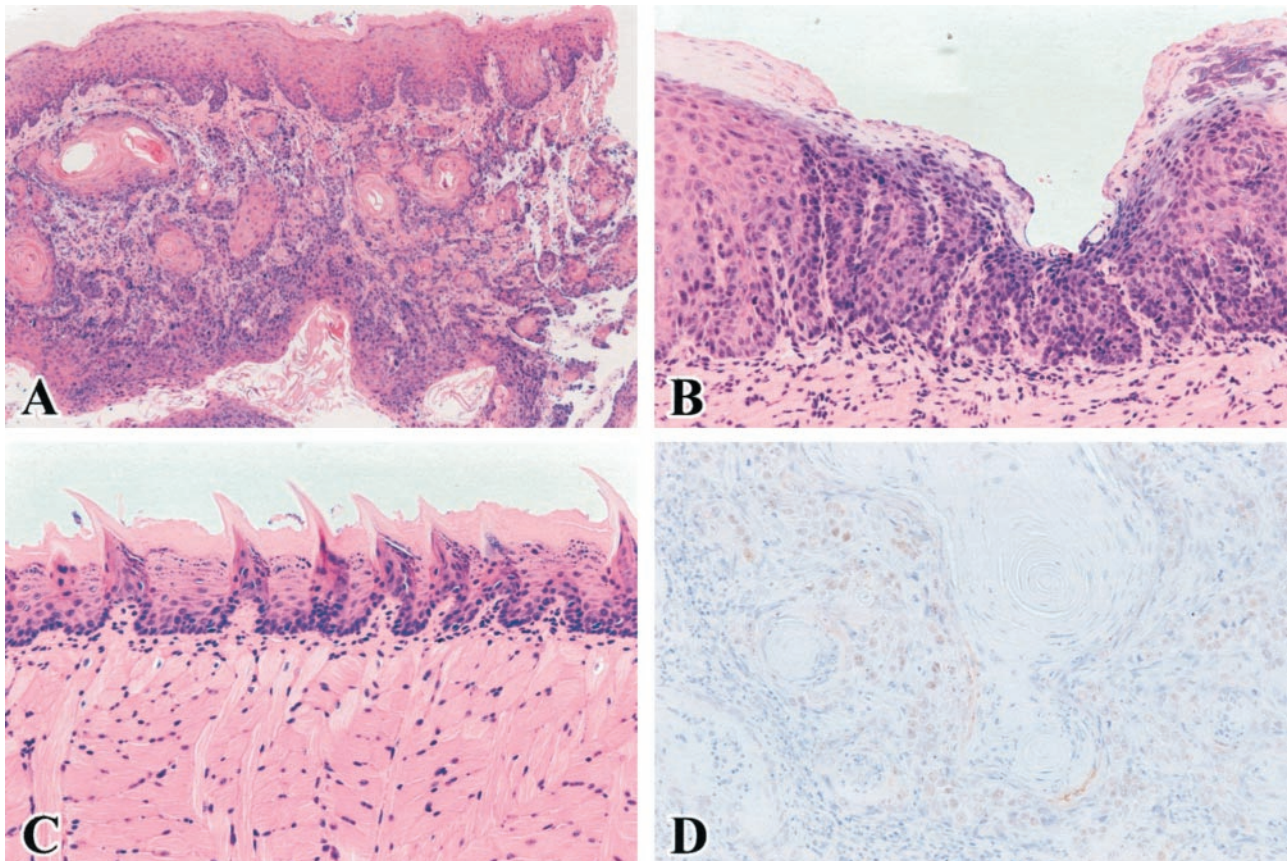


Figure 2. **A:** Tongue SCC of *XPA*^{-/-}*p53*^{+/-} mice. **B:** Moderate epithelial dysplasia of tongue in *XPA*^{-/-}*p53*^{-/-} mice. **C:** No evidence of neoplastic changes in *p53*^{+/-} tongue. H&E staining. **D:** *p53* immunohistochemistry in tongue SCC of *XPA*^{-/-}*p53*^{+/-} mice. Hematoxylin counterstain. Original magnifications: ×200 (**A**), ×100 (**B**), ×40 (**C**), and ×200 (**D**).

less of the genotype, were diagnosed as invasive, well-differentiated SCC (Figure 2A). Tumors were first detected in a *XPA*^{-/-}*p53*^{+/-} mouse line at 16 weeks. Of 22 *XPA*^{-/-}*p53*^{+/-} mice, two died before the development of tongue tumor and by 25 weeks, 100% (20 of 20) of mice bore tumors. Interestingly, half of *XPA*^{-/-}*p53*^{+/-} mice had multiple SCC in the upper aerodigestive tract. In *XPA*^{-/-}*p53*^{+/+} mice, tumors began to appear at 31 weeks and by the end of 50 weeks, 100% (20 of 20) of mice developed tumors. All of 10 *XPA*^{-/-}*p53*^{-/-} mice died until 7 weeks of 4NQO administration, with no tumor detected by gross examination (data not shown). Although adequate necropsy could not be performed because of either a long delay in identification or cagemate cannibalism, at least three moribund *XPA*^{-/-}*p53*^{-/-} mice showed histological evidence of dysplastic changes in the tongue (Figure 2B). *p53*^{+/-} and *p53*^{-/-} littermates were all tumor-free when followed for >1.5 years or 20 weeks, the time of death for *p53*^{-/-} mice (Figure 2C). Nuclear accumulation of p53 protein was detected in all of *XPA*^{-/-}*p53*^{+/-} SCC (Figure 2D) and the pattern was largely heterogeneous, and ranged from slight (a few focal areas) to diffuse (a wide portion of the tumor). In *XPA*^{-/-}*p53*^{+/+} mice, 10 of 20 SCC contained clusters of p53-positive cells in variable proportions.

p53 Status in *XPA*^{-/-}*p53*^{+/-} Tumors

A combination of PCR and Southern blotting procedures revealed that tongue SCC of *XPA*^{-/-}*p53*^{+/-} mice maintained the *p53*^{+/-} genotype, as both the wild-type and mutant *p53* alleles were identified (Figure 3, A and B).

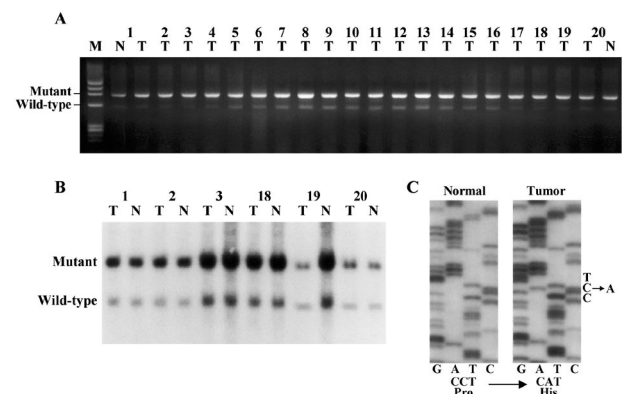


Figure 3. **A and B:** Genotyping analysis of 20 *XPA*^{-/-}*p53*^{+/-} tongue SCC by PCR (**A**) and Southern blot (**B**) showing no loss of the wild-type *p53* allele. M, molecular weight standards; N, normal tissue; T, tumor tissue. **C:** Sequence analysis of *p53* in a *XPA*^{-/-}*p53*^{+/-} tongue SCC showing a CCT-to-CAT mutation at codon 139 of exon 5.

There were no polymorphisms (mutated-shifted bands) in tumors that retained the *p53* wild-type allele by PCR-SSCP analysis (data not shown).

p53 Mutation in *XPA*^{-/-}*p53*^{+/+} Tumors

Only 1 of 20 *XPA*^{-/-}*p53*^{+/+} tongue SCC showed a mobility-shifted band in PCR-SSCP analysis (data not shown). By sequencing after subcloning, *p53* missense mutation at codon139, CCT to CAT, resulting in an amino acid substitution of proline for histidine was detected (Figure 3C).

Discussion

SCC of the oral cavity is common worldwide, being among the third most common cancer in developing nations and the sixth most common in the general population and responsible for significant mortality and morbidity.^{17,24,25} Substantial efforts have been made to explore the pivotal role of *p53* in human oral cancer and there are several classic hamster and rat models to induce oral SCC; however, these studies have limitations.²⁵ To date, no conclusive evidence exists to link *p53* deficiency to oral cancer development. In this study, we used a well-characterized cancer-prone *XPA* mouse model to examine the effects of loss of *p53* on oral carcinogenesis.¹⁸ We found that the onset of tongue SCC was drastically accelerated in *XPA*^{-/-}*p53*^{+/-} mice and that the structurally intact wild-type *p53* allele continued to be expressed in tumors. Taken together, haploinsufficiency of *p53* by itself has a profound effect on nucleotide excision repair (NER)-deficient *XPA* mouse carcinogenesis with gene dosage effect in the tongue.

Tumorigenesis is a multistep process that involves a series of genetic and epigenetic alterations.²⁶ During tumor development, the disruption of *p53* signaling is deemed critical.¹⁻³ Loss of *p53* occurs at various stages and its effect is organ/tissue-specific.^{3,7,15,16} Since *p53* mutations can be detected in premalignant dysplastic lesions, *p53* might be expected to play a role in the early event in oral carcinogenesis.^{17,18} Contrary to this expectation, it was surprising that none of *p53*-deficient mice harbored tongue tumors in the present experimental setup. As described above, both *p53*^{+/-} (followed for >1.5 years) and *p53*^{-/-} (all less than 20 weeks of age) mice orally exposed to 4NQO showed no histological evidence of a tumorigenic response in the tongue. A simple explanation may be that the oral mucosa of mice is far less susceptible to chemical carcinogenesis than that of hamsters and rats.¹⁸ In support of this notion, these mice were also resistant to tongue carcinogenesis by 7,12-dimethylbenz[a]anthracene, one of the most powerful and widely used carcinogen to induce oral SCC in rodents (unpublished results).

It is becoming increasingly clear that *p53* mutations are infrequent in chemically induced epithelial tumors of *p53*^{+/-} mice.^{7,8,10,14} Since CM5 used in this experiment recognizes both wild-type and mutant *p53*, *p53*-positive immunohistochemical results in *XPA*^{-/-}*p53*^{+/-} tumors

are only suggestive of an increased stability or half-life of *p53*, which might be due to either up-regulation of *MDM2* or *p19^{ARF}* dysregulation and not mutation. Indeed, the PCR, Southern blot, and SSCP data suggested that *p53* allele is structurally intact. Similarly, our previous analysis indicated that the mutational frequency of *p53* was low in 4NQO tongue carcinogenesis.¹⁸ In conjunction with the absence of *p53* mutation in 4NQO-induced rat tongue SCC,²⁷ *p53* is not a primary target for gene alteration in oral tumorigenesis. Conceivably, additional genetic alterations such as activation of oncogenes and/or inactivation of other tumor-suppressor genes are essential for neoplastic transformation of *p53*-deficient cells. The lack of obvious tongue tumor formation in *XPA*^{-/-}*p53*^{-/-} mice might be best explained by the very limited time window (<7 weeks of 4NQO exposure); however, we could not exclude the possibility that a reduction of *p53* levels has a more dramatic effect than the complete absence of *p53*. We are now trying to establish cell lines derived from tongue epithelium of *XPA*^{-/-}*p53*^{-/-} mice for further investigation.

XPA^{-/-} mice used in this study are characterized by a complete deficiency in both global genome repair (GGR) and transcription-coupled repair (TCR) of NER.¹⁹ Since DNA repair is not successively accomplished in these mice, functional *p53* may promote death of cells with unrepaired DNA damage by triggering apoptosis. To clarify the synergism of *p53* and NER in tumorigenesis, we and others have generated a *XPA/p53*-deficient mouse strain.²⁸⁻³⁰ It has been suggested that *p53* play a role in regulating NER, especially the GGR subpathway.³¹⁻³⁶ As shown in our experiment, a heterozygous loss of *p53* appears to act synergistically to a NER defect in tongue tumor induction. The bulky DNA lesions elicited by 4NQO are known to substrate for GGR and 4NQO-DNA damage *per se* provokes up-regulation of *p53*.^{37,38} The hypersensitivity of human and mouse *XPA* cells to killing by 4NQO may be associated with increased activity of *p53*, leading to augmented *p53*-mediated apoptosis.³⁷ Although 4NQO-induced NER is *p53*-dependent,^{37,38} it remains ambiguous that the modulating effect of *p53* on NER is gene-dosage-dependent.

The precise mechanism of *p53* loss in tumors is uncertain but is likely to occur by a two-step process. Our *XPA*^{-/-}*p53*^{+/-} mice have already undergone a first step of the process, the inactivation of one *p53* allele. The recent investigations indicated that loss of the remaining *p53* allele is not a prerequisite for tumor formation.^{6,7,11,16} The present data also provide a strong argument that *p53* haploinsufficiency creates a condition that drive tongue carcinogenesis. Further functional assays including the activation of *p21^{WAF1/CIP1}* and *MDM2* expression and inducibility of apoptosis are underway to identify whether structurally intact wild-type *p53* protein in *XPA*^{-/-}*p53*^{+/-} tumors is functioning normally.

In summary, we believe that *XPA/p53*-deficient mice provide an attractive model for studying the histopathological and genetic changes and molecular mechanisms underlying oral SCC development and also allow for the investigation of the relationship between DNA damage, cell cycle regulation, apoptosis, and cancer *in vivo*.

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