TUMORIGENESIS AND NEOPLASTIC PROGRESSION

Specific Deletion of $p16^{INK4a}$ with Retention of $p19^{ARF}$ Enhances the Development of Invasive Oral Squamous Cell Carcinoma

Kazuhisa Ishida,^{*,†} Hiroyuki Tomita,^{*} Tomohiro Kanayama,^{*} Kei Noguchi,^{*} Ayumi Niwa,^{*} Masaya Kawaguchi,[‡] Masafumi Miyai,[§] Mikiko Matsuo,^{*} Yuko Imaizumi,^{*} Keizo Kato,[†] Yuichiro Hatano,^{*} Akihiro Hirata,[¶] Hideshi Okada,^{||} Toshiyuki Shibata,[†] and Akira Hara^{*}

From the Departments of Tumor Pathology,^{*} Oral Maxillofacial Surgery,[†] Radiology,[‡] Neuronal Surgery,[§] and Emergency and Disaster Medicine,^{||} Gifu University Graduate School of Medicine, Gifu; and the Division of Animal Experiment,[¶] Life Science Research Center, Gifu University, Gifu, Japan

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Address correspondence to
Hiroyuki Tomita, M.D., Ph.D.,
Department of Tumor Pathol-
ogy, Gifu University Graduate
School of Medicine, 1-1
Yanagido, Gifu 501-1194,
Japan. E-mail: h_tomita@gifu-u.ac.jp

The cyclin-dependent kinase inhibitor 2A (*CDKN2A*)/alternate reading frame (ARF) locus consists of two overlapping tumor suppressor genes, $p16^{INK4a}$ and $p14^{ARF}$ ($p19^{ARF}$ in mice), encoding two unrelated proteins in alternative reading frames. Previous reports suggest that $p16^{INK4a}$ and $p14^{ARF}$ alterations independently exhibit differential roles, and $p16^{INK4a}$ is more closely associated with a poor prognosis in oral cancer. However, the role of $p16^{INK4a}$ -specific loss in oral squamous cell carcinogenesis remains unclear. The authors assessed chemical carcinogen 4-nitroquinoline 1-oxide (4NQO)-induced multistep oral squamous cell carcinogenesis in mice carrying $p16^{INK4a}$ -specific loss with retention of the $p19^{ARF}$ gene ($p16^{INK4a-/-}$). 4NQO-treated $p16^{INK4a-/-}$ mice exhibited a higher incidence and multiplicity of oral squamous cell carcinoma (OSCC) development relative to 4NQO-treated wild-type mice. 4NQO-treated $p16^{INK4a-/-}$ OSCC cells exhibited higher proliferation and up-regulation of *Arf*, transcription factor E2f1, tumor protein p63 (*tp63*), and oncogenic $\Delta Np63$, an isoform *p63*, compared with observations in 4NQO-treated wild-type OSCC cells. Furthermore, the overexpression of oncogenic $\Delta Np63$ was associated with human OSCC. In conclusion, these results in mice indicate the biological significance of $p16^{INK4a}$ -specific loss with retention of $p19^{ARF}$ in oral squamous cell carcinogenesis, and $\Delta Np63$ may be a potential target for OSCC. (*Am J Pathol* 2020, 190: 1332–1342; <https://doi.org/10.1016/j.ajpath.2020.01.017>)

Head and neck cancer is the sixth most common type of cancer in humans,¹ and approximately 350,000 individuals are diagnosed with oral cancer annually worldwide.² This disease occurs in the oral cavity in 48% of cases, 90% of which are oral squamous cell carcinoma (OSCC).³ Over the last few decades, the 5-year overall survival rate of patients with OSCC has remained at approximately 50% following initial therapy.⁴ It is likely that this high mortality rate is attributed to its late diagnosis, recurrence, and resistance to therapy, whereas early detection followed by appropriate treatment can increase the cure rate to approximately 80%.^{2,5}

The development of OSCC is a multistep process involving the progression from normal tissue to dysplasia, oral intraepithelial neoplasia (OIN), and invasive squamous cell carcinoma (SCC).⁶ This progression of OSCC requires

in the accumulation of multiple genetic, epigenetic, and chromosomal alterations, which are influenced by a patient's genetic/epigenetic predisposition, and by environmental influences, including tobacco, alcohol, and chronic inflammation.^{7,8}

The $p16^{INK4a}$ and $p14^{ARF}$ ($p19^{ARF}$ in mice) (*CDKN2A*) genes are on the 9p21 region and have putative growth-suppressive activities. By investigating the functional relevance of these genes in the Rb and p53 pathways, the suppressive mechanism of malignant tumors can be elucidated.⁹ The *Ink4a/Arf* locus encodes two proteins, $p16^{INK4a}$ and $p14^{ARF}$, in overlapping reading frames.¹⁰ The *p16* gene

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with loss of *Arf* products is a component of the tumor suppressor pathway that controls cell cycle, and loss or inactivation of the *p16* gene with loss of the *Arf* gene has been reported in several malignancies, including those of the oral cavity.^{11,12}

Major inactivation of the *p16^{INK4A}/p14^{ARF}* genes results from promoter methylation, homozygous deletion, and intragenic mutation. Alterations affecting the INK4A/ARF locus can potentially disrupt the two main tumor suppression pathways, *p16^{INK4A}*-Rb and *p14^{ARF}*-p53. A previous study of OSCC in humans reported that 33% and 29% of cases examined ($N = 384$) exhibited *p16^{INK4A}* deletion and DNA methylation, respectively, whereas *p14^{ARF}* deletion and DNA methylation were observed in 12% and 18% of cases, respectively.¹³ Although *p16^{INK4A}* loss and silencing were associated with aggressiveness and poor prognosis, *p14^{ARF}* silencing was associated with a lower rate of recurrence and good clinical outcome.¹³ Furthermore, evidence supports the hypermethylation of *p16^{INK4A}* and *p14^{ARF}* to be *p16^{INK4A}* as independent events, and each appears to contribute differentially during carcinogenesis.¹⁴

The effect of loss of *p16^{INK4a}*, but retention of *p19^{ARF}*, is unclear in squamous cell carcinogenesis. Mice lacking *p19^{ARF}*, but not *p16^{INK4a}*, were observed to develop spontaneous tumors and to enhance tumor response to carcinogen. In addition, exposure to carcinogens, including DMBA, X-rays, and irradiation, rendered *Arf*-knockout (KO) mice more prone to tumors.¹⁵ However, Sharpless et al¹⁶ reported that mice with *p16^{INK4a}* with retention of *Arf* had a significantly higher risk of developing tumors than

wild-type (WT) mice following carcinogen treatment, but were less prone than *p53^{-/-}* or *p19^{ARF}* animals. In addition, mice with *p16^{INK4a}* and retention of *Arf* did not spontaneously develop malignancy. Although the number of malignant lesions was increased in mice with *p16^{INK4a}* with retention of *Arf*, the tumor grade did not differ significantly from that in WT mice. However, the mechanisms underlying squamous cell carcinogenesis in mice lacking *p16^{INK4a}* (*p16^{INK4a}* but retaining *p19^{ARF}* remain unclear.

The function of p63 is also important for maintaining normal tissue and has been studied widely. p63 is essential for normal formation of the epidermis. In SCC of the head and neck, the expression of p63 has been reported to indicate a poor prognosis,¹⁷ and the overexpression of p63 is associated with poor radiation response and shorter survival rates in OSCC.^{18,19} The protein expression of p63 originates from two promoters, giving rise to transactivating (TAp63) and nontransactivating (Δ Np63) isoforms.²⁰ The TAp63 isoforms act synergistically and/or subsequently to control differentiation of upper epidermal layers; TAp63 isoforms contain a p53-homologous N-terminal transactivation (TA) domain and have tumor suppressor properties similar to those of p53 in inducing growth arrest, apoptosis, and senescence. By contrast, Δ Np63 controls the expansion of progenitor cells in the basal layer; Δ Np63 isoforms lacking this TA domain are thought to possess oncogenic activities through the inhibition of TAp63, TAp73, and p53.^{18,21} Although it has been reported that the balance of TAp63 and Δ Np63 is disrupted in various types of cancer,^{9,15,22} the

Table 1 List of Primers Used for Real Time RT-PCR

Mouse gene	Forward primer	Reverse primer
<i>p16^{INK4a}</i>	5'-GTCGCAGGTTCTTGGTCACT-3'	5'-GATCGCACGAACTTCACCAA-3'
<i>p19^{ARF}</i>	5'-GAGCGGGGACATCAAGACAT-3'	5'-AAGCTATGCCCCGTCGGTCT-3'
p16 suppressor pathway		
<i>p21</i>	5'-CGCTGTCTTGCACCTCTGGT-3'	5'-CGTTTTCGGCCCTGAGATGTT-3'
<i>Cdk4</i>	5'-AAGGTCACCCAGTAGTGTGAGC-3'	5'-CCGCTTAGAACTGACGCATTAG-3'
<i>Cdk6</i>	5'-GGCGTACCCACAGAAACCATA-3'	5'-AGGTAAGGGCCATCTGAAAAC-3'
<i>pRb</i>	5'-TGCATCTTTATCGCAGCAGTT-3'	5'-GTTACACGTCGGTCTAATTTG-3'
<i>Arf</i>	5'-CGCAGGTTCTTGGTCACTGT-3'	5'-TGTTACGAAAGCCAGAGCG-3'
<i>p27</i>	5'-TCAAACGTGAGAGTGTCTAACG-3'	5'-CCGGGCCGAAGAGATTTCTG-3'
<i>Ccnd1</i>	5'-GCGTACCCGACACCAATCTC-3'	5'-CTCCTCTTCGCACTTCTGCTC-3'
<i>E2F1</i>	5'-GAGAAGTCACGCTATGAAACCTC-3'	5'-CCCAGTTCAAGTCAACGACAC-3'
<i>Trp53</i>	5'-CTCTCCCCCGCAAAGAAAAA-3'	5'-CGGAACATCTCGAAGCGTTTA-3'
p53-independent pathway		
<i>Trp63</i> (Δ Np63)	5'-CTGGAACAATGCCAGAC-3'	5'-GAGGAGCCGTCTGAATCTG-3'
<i>Hif1α</i>	5'-GATGACGGCGACATGGTTTAC-3'	5'-CTCACTGGGCCATTTCTGTGT-3'
<i>Foxm1</i>	5'-ATCACGGAGACGTTGGGAC-3'	5'-CCACTGGATATTGGTTAAGCTGT-3'
<i>Myc</i>	5'-GGAGGACCAAGCTCTGTATTCTG-3'	5'-GTAGCCAACACCCAGGGAT-3'
<i>Mdm2</i>	5'-GGATCTTGACGATGGCGTAAG-3'	5'-AGGCTGTAATCTTCCGAGTCC-3'
<i>Ctbp1</i>	5'-ATCATTTGGACTAGGTCGTGTGG-3'	5'-CATAGAAGAGGACGTTGAAGCC-3'
<i>Ctbp2</i>	5'-TCGGTAGTGGCTACGACAAC-3'	5'-CGCCGATACAGATTGAGAATGT-3'
<i>Wtn</i>	5'-AAGGATTCTTGGTAGAGTTCCC-3'	5'-AGACTGCAACTGGCTTCTCC-3'
<i>Miz1</i>	5'-TTCCCTTATTCAGTTGATCCCC-3'	5'-CCACTGCTGGTTATGACCCC-3'
<i>Bckdha</i>	5'-CTCCTGTTGGGACGATCTGG-3'	5'-CATTGGGCTGGATGAAGTCAA-3'

Figure 1 *p16^{INK4a}*-specific deletion in the tongue of *p16^{INK4a}^{-/-}* mice. **A:** Alternative transcripts and products encoded by the cyclin-dependent kinase inhibitor 2A (*CDKN2A*) locus. Alternative splicing occurs as indicated to give rise to two transcripts: exons that splice to encode p16 are shown above and those that encode p14^{ARF} (p19^{ARF} in mice) are shown below. The sizes and composition of the respective mouse and human proteins are indicated. **B:** RNA expression levels of *p16^{INK4a}* and *p19^{ARF}* in tongue tissues of *p16^{INK4a}^{-/-}* knockout (KO) and wild-type (WT) mice by real-time RT-PCR analysis. Data are expressed as means $\pm$ SD. $n = 5$ mice each condition (**B**); $n = 3$ experiments each condition (**B**). * $P < 0.05$ (*U*-test).

status in OSCC remains to be elucidated. *p16^{INK4a}* stimulates *p14^{ARF}* by mediating pRb to activate E2F, and activation leads to increased tumor apoptosis.²³ This phenomenon is caused by a decline in the function of tumor suppressor genes, including *TAp63* and *p53*, by the activation of Δ Np63.

In the present study, the authors investigated and identified the biological significance of *p16^{INK4a}^{-/-}* retaining *p19^{ARF}* and Δ p63 in mouse tongue carcinogenesis induced by 4NQO.

Materials and Methods

Mice

p16^{INK4a}^{-/-} (FVB.129-*Cdkn2atm2.1Rdp*/Nci; strain no. 01XE4) mice were obtained from the Animal Production Area, National Cancer Institute-Frederick Cancer Research and Development Center (NCI-FCRDC, Frederick, MD). The mice were backcrossed to *C57/BL6J* mice for at least five generations. All mice were maintained under specific pathogen-free conditions with isolated ventilation cages in an air-conditioned room with a 12:12 light:dark cycle. The mice were bred and maintained on a basal diet, CE-2 (CLEA Japan, Tokyo, Japan) until termination of the study. Genotypes were identified by PCR analysis of tail DNA using allele-specific primers. All animal experiments and breeding were performed under the Regulations for Animal Experiments in Gifu University.

4NQO Exposure

The 4NQO carcinogen was dissolved in ethyl alcohol (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) as a stock solution (20 mg/mL), stored at 4°C. The mice were administered drinking water containing 4NQO at a concentration of 0.1 mg/mL using light-shielded bottles.

Human Samples

Human tissue sections were obtained from patients who underwent surgical resection or biopsy at Gifu University Hospital, Gifu, Japan. All study protocols were approved by the ethics committees of Gifu University, and written informed consent was obtained from all patients.

Tissue Preparation and Histologic Examination

The mice were sacrificed under anesthesia. From each animal, the tongue was removed and cut longitudinally. All tissues were fixed for 24 hours in neutral-buffered 10% formalin. The fixed samples were processed using standard methods, embedded in paraffin, sectioned at 3 μ m, and stained with hematoxylin and eosin.

Immunohistochemistry and Immunofluorescence

The avidin–biotin–peroxidase complex technique was used for immunohistochemical analysis. The sections (3- μ m thick) were cut, deparaffinized, rehydrated in phosphate-buffered saline, and placed in 10 mmol/L citrate buffer (pH 6.0), and the Decloaking Chamber NxGen (Biocare Medical, Pacheco, CA) was used (110°C, 2.5 minutes) for heat-induced antigen retrieval. The endogenous peroxidase activity was blocked by incubation for 10 minutes in 0.3% H₂O₂. Following washing three times with phosphate-buffered saline, nonspecific antibody binding was blocked with normal blocking serum for 40 minutes at room temperature. The sections were then incubated with the following primary antibodies overnight at 4°C: Ki-67 (1:200, rabbit-monoclonal, ab16667; Abcam, Cambridge, UK), cleaved caspase-3 (1:200, rabbit-polyclonal, #9661S; Cell Signaling Technology, Danvers, MA), PCNA (1:1000, mouse monoclonal, PC10; DakoCytomation, Glostrup, Denmark), total p63 (4A4) (1:400, mouse-monoclonal,

ab735; Abcam), and TAp63 (1:200, rabbit-polyclonal, #618902; BioLegend, San Diego, CA). Subsequently, the sections were incubated with biotinylated secondary antibodies (Vectastain ABC kit; Vector Laboratories, Burlingame, CA) for 30 minutes at room temperature, followed by incubation with avidin-coupled peroxidase (Vector Laboratories) for 30 minutes at room temperature. The sections were developed with 3,3'-diaminobenzidine (DAB) using the DAKO Liquid DAB Substrate-Chromogen system (DakoCytomation) and then counterstained with hematoxylin. No specific staining was observed in the negative control slides prepared without primary antibody. Human lung SCC and prostate tissues were used as positive controls for total p63 and TAP63 antibodies, respectively (Supplemental Figure S1).

For fluorescent double-labeling, after deparaffinization, antigen retrieval, and blocking of the endogenous peroxidase activity and nonspecific antibody, the sections were incubated with the following primary cocktail antibodies overnight at 4°C: p63 (1:200, mouse-monoclonal, ab735; Abcam) and TAp63 (1:100, rabbit-polyclonal, #618902; BioLegend). The following day, the sections were rinsed in

phosphate-buffered saline (3×5 minutes), followed by incubation with secondary antibody for 30 minutes at room temperature: goat anti-mouse IgG (1:200, Alexa Fluor 488, ab150117; Abcam, Cambridge, UK) and goat anti-rabbit IgG (1:200, Alexa Fluor 594, ab150084; Abcam) for double staining with the same species primary antibodies. The tissue sections were then incubated for 1 minute with DAPI (Molecular Probes, Eugene, OR) to reveal the nuclei. Finally, coverslips were mounted using Shandon Immumount (Thermo Fisher Scientific, San Jose, CA).

RNA Extraction and the Reverse Transcription Reaction

Tissues were removed and then immediately immersed into the liquid nitrogen for freezing and stocked at -80°C . The frozen tissues were homogenized using Bullet Blender (Next Advance, Averill Park, NY), and total RNA was extracted using an RNeasy Mini kit (Qiagen, Valencia, CA). cDNA was synthesized from 0.4 μg of RNA, using ReverTra Ace qPCR RT Master Mix (TOYOBO, Osaka, Japan) according to the manufacturer's instructions.

Figure 2 Tongue tumors induced by carcinogen 4-nitroquinoline 1-oxide (4NQO). **A:** Experimental design of the 4NQO model. **B:** Macroscopic images of the tongue in water-treated *p16^{INK4a}*^{-/-} mice, 4NQO-treated wild-type (WT) mice, and 4NQO-treated *p16^{INK4a}*^{-/-} mice at 15 weeks. **Arrows** indicate macroscopic polypoid tumors. **C:** Representative hematoxylin and eosin staining of dysplasia, oral intraepithelial neoplasia (OIN), and oral squamous cell carcinoma (OSCC) in 4NQO-treated *p16^{INK4a}*^{-/-} and WT mice at 15 weeks. Scale bars: 3 mm (**B**); 100 μm (**C**).

Table 2 Incidence and Multiplicities of Microscopic Tumors of the Tongue in 4NQO-Treated Mice

	Genotype	Tongue		
		Dysplasia	OIN	OSCC
Incidence, %	WT	100	48	20
	<i>p16^{INK4a}-/-</i>	100	38.1	52.4
Multiplicity, means $\pm$ SD	WT	3.32 $\pm$ 1.26	0.48 $\pm$ 0.50	0.20 $\pm$ 0.40
	<i>p16^{INK4a}-/-</i>	3.14 $\pm$ 1.49	0.52 $\pm$ 0.73	0.71 $\pm$ 0.76*

* $P < 0.05$ versus OSCC of WT.

4NQO, 4-nitroquinoline 1-oxide; OIN, oral intraepithelial neoplasia; OSCC, squamous cell carcinoma; WT, wild type.

Real-Time RT-PCR

Real-time RT-PCR was performed using Fast SYBR Green Master Mix (Applied Biosystems, USA) on a Step One Plus Real-Time PCR system (Applied Biosystems, Foster City, CA). The expression levels of mRNAs were normalized to the expression of β -actin using the $2^{-\Delta\Delta C_t}$ method. All primers are listed in Table 1.

Cell Counting

The scoring of positive cells was performed independently by two pathologists (H.T. and A.Ha.). Ki-67, cleaved caspase-3, PCNA, 4A4, and TAp63 were counted in five randomly selected high-power fields at 40 $\times$ magnification, and the counts were averaged.

Statistical Analysis

Continuous data are presented as means $\pm$ SD. Statistical differences between two groups were analyzed with the *t*-test or *U*-test according to the circumstances. $P < 0.05$ was considered to indicate a statistically significant difference. Statistical analysis was performed using MedCalc software version 12.4.0 (MedCalc Software, Ostend, Belgium) and StatMate V version 1.0 for Win&Mac Hybrid (ATMS Co., Ltd., Tokyo, Japan).

Results

p16^{INK4a}-Specific Loss Enhances the Development of OSCC in Mice

p16^{INK4a}/p14^{ARF} (*p16^{INK4a}/p19^{ARF}* in mice) is among the most commonly mutated loci in human cancer, encoding two different tumor suppressors translated from alternatively spliced mRNAs (Figure 1A). *p16^{INK4a}* is composed of exons 1a, 2, and 3, and is designated here as INK4A (inhibitor of cyclin-dependent kinase 4). *p14^{ARF}* is encoded by exon 1b and ARFs of *CDKN2A/ARF* exons 2 and 3, herein referred to as ARF. KO mice with targeted deletion of specific *Cdkn2A/Arf* exons have disrupted *p16^{INK4a}* or *p19^{ARF}* genes, or both,^{24–27} and develop a different spectrum of spontaneous tumors.

To examine the functional role of *p16^{INK4a}*-specific loss in oral squamous cell carcinogenesis in this study, the RNA

expression level of tongue tissues was first confirmed in *p16^{INK4a}*-specific homozygous KO (*p16^{INK4a}-/-*) mice, which retain *p19^{ARF}*, and wild-type (WT) mice by real-time RT-PCR. The results showed that the RNA expression level of *p16^{INK4a}* was lower than WT mice and that of *p19^{ARF}* was retained in the tongue tissues of *p16^{INK4a}-/-* mice (Figure 1B).

To elucidate whether deletion of the *p16^{INK4a}* gene with retention of *p19^{ARF}* is associated with squamous cell carcinogenesis in the oral cavity, the 4NQO model²⁸ was used for the development of invasive SCC in *p16^{INK4a}-/-* and WT mice. A total of 25 *p16^{INK4a}-/-* and 25 WT mice at 6 to 8 weeks of age were provided with drinking water containing 100 μ g/mL 4NQO for 15 weeks (Figure 2A). Ten *p16^{INK4a}-/-* and WT mice were provided with plain drinking water from birth until termination of the experiment, and there were no obvious abnormalities in any mice. There were no significant differences in the body weights of the 4NQO-treated *p16^{INK4a}-/-* and WT mice at the end of experiment or any apparent differences in the liver, kidney, lung, or heart between the 4NQO-treated *p16^{INK4a}-/-* and WT mice.

Following 15 weeks of 4NQO administration, macroscopically, nodular, and polypoid tumors were observed in the dorsum and tip of the tongue in the 4NQO-treated *p16^{INK4a}-/-* and WT mice (Figure 2B). Microscopically, those tongue tumors were identified as dysplasia, oral intraepithelial neoplasia (OIN), and OSCC (Figure 2C). However, there were no histopathologic differences between the 4NQO-treated *p16^{INK4a}-/-* and WT mice. Histologic assessment of the squamous lesions in the tongue is summarized in Table 2. There were no significant differences in the incidence or multiplicity of early lesions, that is, dysplasia and OIN, between the *p16^{INK4a}-/-* and WT mice. The incidence and multiplicity of OSCC in the *p16^{INK4a}-/-* mice were significantly higher than in the WT mice (Table 1).

The above results indicate that *p16^{INK4a}*-specific loss with retention of *p19^{ARF}* enhanced the development of OSCC in mice.

Increased Cell Proliferation in 4NQO-Treated *p16^{INK4a}-/-* Mice Contributes to the Accelerated Development of OSCC

To investigate the mechanisms underlying the progression of OSCC carrying the depleted *p16^{INK4a}* gene, the

Figure 3 Increased cell proliferation contributes to the progression of oral squamous cell carcinoma (OSCC) in $p16^{INK4a-/-}$ mice treated with carcinogen 4-nitroquinoline 1-oxide (4NQO). Immunohistochemistry for marker of proliferation Ki-67 (*Mki67*) (A), proliferating cell nuclear antigen (PCNA) (C), and cleaved caspase 3 (E) antibodies in OSCC cells of 4NQO-treated $p16^{INK4a-/-}$ and WT mice Ki-67- (B), PCNA- (D), and cleaved caspase 3-positive (F) cell ratios in OSCC cells of 4NQO-treated $p16^{INK4a-/-}$ and wild-type (WT) mice. Data are expressed as means $\pm$ SD. $n = 5$ mice each condition. * $P < 0.05$, ** $P < 0.01$ (*U*-test). Scale bars: 50 μ m.

proliferation activity and apoptosis of tumor cells from 4NQO-treated mice were assessed. Immunohistochemical analyses for Ki-67, PCNA, and cleaved caspase-3 were conducted in the OSCC cells of 4NQO-treated $p16^{INK4a-/-}$ and WT mice (Figure 3). Ki-67 and PCNA are reliable immunohistochemical markers for evaluating cell proliferation in oral tumors.²⁹ Cleaved caspase-3 is an immunohistochemical marker of the cell's entry point into the apoptotic signaling pathway.³⁰

The Ki-67-positive cell ratio in OSCC cells of the 4NQO-treated $p16^{INK4a-/-}$ mice was significantly higher than that of the cells of the 4NQO-treated WT mice (Figure 3, A and B). Further, the PCNA-positive cell ratio in OSCC cells of the 4NQO-treated $p16^{INK4a-/-}$ mice was also significantly higher than 4NQO-treated WT mice (Figure 3, C and D). The cleaved caspase-3-positive cell ratio in OSCC cells of the 4NQO-treated $p16^{INK4a-/-}$ mice did not differ significantly from that of cells of the 4NQO-treated WT mice (Figure 3, E and F). Further, the $p16^{INK4a}$ RNA expression level in invasive SCC of the 4NQO-treated $p16^{INK4a-/-}$ mice was significantly lower than that in the

WT mice (Supplemental Figure S2). These data indicate that the proliferation of cancer cells carrying depletion of the $p16^{INK4a}$ gene was associated with the progression of OSCC in mice.

The mouse model initiated with 4-NQO is useful for investigating field cancerization in the oral cavity.³¹ The concept of field cancerization was conceived by Slaughter almost a decade prior to introducing the term in 1953.³² Field cancerization has been established in oral cavity carcinogenesis. In carcinogenesis, the early genetic and/or epigenetic events might lead to clonal expansion of pre-neoplastic daughter cells in a particular tumor field. Subsequent genomic and/or epigenetic changes in some of these cells drive them toward the malignant phenotype.³³ To evaluate whether the normal-looking squamous epithelial cells have high proliferative activity without any histologic changes after 4NQO exposure, cell proliferation in the normal-looking epithelia adjacent to SCC of the 4NQO-treated $p16^{INK4a-/-}$ and WT mice was assessed. There was no significant difference in the Ki-67-positive cell ratio in the normal-looking epithelia between the

Figure 4 Oncogenic p63 (*Trp63*) is up-regulated in $p16^{INK4a-/-}$ oral squamous cell carcinoma (OSCC) induced by carcinogen 4-nitroquinoline 1-oxide (4NQO) treatment. Quantitative real-time RT-PCR analysis of genes related to the $p16^{INK4a}/p19^{ARF}$ suppressor pathway (A) and p53-independent pathway (B), and expression of $\Delta Np63$ in OSCC cells (C) of the 4NQO-treated $p16^{INK4a-/-}$ and wild-type (WT) mice. Data are expressed as means $\pm$ SD. $n = 5$ mice each condition; $n = 3$ experiments each condition. * $P < 0.05$, ** $P < 0.01$ (t-test).

4NQO-treated $p16^{INK4a-/-}$ and WT mice (Supplemental Figure S3).

Up-Regulation of Oncogenic $\Delta p63$ Is Associated with the Progression of OSCC in 4NQO-Treated $p16^{INK4a-/-}$ Mice

To analyze the impact of genes related to the p16-cyclinD/CDK4-pRb pathway in cell proliferation in the development of OSCC, the expression levels of genes, including *p21*, *Cdk4*, *Cdk6*, *pRb*, *Arf*, *p17*, *Ccnd1*, *E2f1*, and *Trp53*,²³ were measured in tongue tissues from the 4NQO-treated mice by real-time RT-PCR analysis. RNA samples were obtained from noncancerous normal-looking epithelial tissues close to tongue tumors in 4NQO-treated $p16^{INK4a-/-}$ and WT mice.

The RNA expression levels of *Arf* in $p16^{INK4a-/-}$ and WT mice without 4NQO exposure in tongue tissues were similar (Figure 1B). The RNA levels of *Arf* and *E2f1* were significantly up-regulated in OSCC of the 4NQO-treated $p16^{INK4a-/-}$ mice compared with those of the 4NQO-treated WT mice (Figure 4A). However, the RNA levels of *Trp53*, *p21*^{WAF1/CIP1}, *Ccnd1*, *Cdk4*, *cdk6*, *pRb*, and *p27* did not

differ significantly between the 4NQO-treated $p16^{INK4a-/-}$ and WT mice (Figure 4A).

Subsequently, to clarify the role of the transcriptional up-regulation of *Arf* in OSCC in the 4NQO-treated $p16^{INK4a-/-}$ mice, the expression of genes, including *p63*, *Hif1a*, *Foxm1*, *Myc*, *Mdm2*, *Ctbp1*, *Ctbp2*, *Wrn*, *Miz1*, and *Bckdha*, which are located downstream of the p53-dependent and -independent pathways,¹⁵ was assessed by real-time RT-PCR analysis (Figure 4B). Among these genes, the RNA expression of *p63* was significantly up-regulated in OSCC in the 4NQO-treated $p16^{INK4a-/-}$ mice compared with that in the 4NQO-treated WT mice (Figure 4B).

To determine whether $\Delta Np63$, the oncogenic isoform of p63, is up-regulated or not, the RNA expression levels of $\Delta Np63$ in OSCC by real-time RT-PCR analysis was measured. The results showed that the RNA expression level of $\Delta Np63$ was significantly up-regulated in OSCC of the 4NQO-treated $p16^{INK4a-/-}$ mice compared with that in the 4NQO-treated WT mice (Figure 4C).

The above results suggest that deletion of $p16^{INK4a}$ with retention of *Arf* enhanced $\Delta Np63$ expression. This might lead to the promotion of OSCC in the 4NQO OSCC model.

Figure 6 Schematic representation of the mutations in cyclin-dependent kinase inhibitor 2A [*CDKN2A*; inhibitors of cyclin-dependent kinase 4 (INK4a)/alternate reading frame (ARF)] locus and the role of *p16^{INK4a}*-specific loss in the progression of oral squamous cell carcinoma (OSCC).

the normal-looking epithelium and OIN, whereas Δ Np63-positive cells were spread through the tumor nest in OSCC (Figure 5C).

Finally, the total p63-positive, TAp63-positive, and Δ Np63-positive cell ratios were analyzed. The total p63-positive cell ratio in OSCC was significantly higher than that in the normal-looking epithelium and OIN (Figure 5D). The Δ Np63-positive cell ratio was significantly higher in OIN compared with that in the normal-looking epithelium and OSCC. The Δ Np63-positive cell ratio was significantly higher in OSCC compared with that in the normal-looking epithelium (Figure 5E).

Taken together, the overexpression of oncogenic Δ Np63 was associated with the progression of OSCC in human oral squamous cell carcinogenesis.

Discussion

In this study, the depletion of *p16^{INK4A}* with retention of *p19^{ARF}* enhanced the progression of OSCC in oral squamous cell carcinogenesis. This was caused by the proliferation of cancer cells mediated by the up-regulation of oncogenic Δ Np63. Overexpression of Δ Np63 was observed in the progression of human oral squamous cell carcinogenesis (Figure 6).

Deletion of *p16^{INK4A}* with Retention of *p19^{ARF}* Is Associated with the Progression of OSCC via Cell Cycle Acceleration

The relative roles of *p16^{INK4A}* and *p19^{ARF}* in the genesis of human oral tumors were examined. The chemical carcinogenesis experiments in the present study demonstrated that

the *p16^{INK4a}* tumor suppressor pathway is likely to be associated with increased malignant transformation to OSCC, although field cancerization on the proliferation of 4NQO-injured normal epithelial cells was not observed. This was similar to a study in humans in which *p16^{INK4A}* deletion (low expression of *p16^{INK4A}* and high *p16^{INK4A}* promoter methylation) was associated with aggressive oral tumors.¹³

However, the tumor suppressive function of *p19^{ARF}* is p53-dependent, *p19^{ARF}/p53^{-/-}* mice have a tumor incidence similar to *p53^{-/-}* mice.¹⁸ *p19^{ARF}* also inhibits the activities of various transcriptional factors, including *E2f1*, *Hif1a*, *Foxm1*, and *Myc*, through direct interaction, thus, preventing cell proliferation.³⁵ In the current experiment, activating the *E2F* gene with the loss of *p16^{INK4a}* may lead to activating the cell cycle. Usually, p53, which serves a major role in the suppressor pathway, induces atypical cell apoptosis, but there was no such change in this experiment. However, whether *p16^{INK4A}* deletion with retention of *p19^{ARF}* induced the progression of OSCC remains unclear.

Δ Np63 Is Associated with the Development of OSCC

Δ Np63 and TAp63 belong to the p53/p63/p73 family and differ in the N-terminal transactivation domain. Δ Np63 is overexpressed and promotes cell survival in head and neck SCC, but the extent to which it regulates gene expression that contributes to the malignant phenotype of head and neck SCC is not well defined. In the current experiment, Δ Np63 was up-regulated in 4NQO-treated *p16^{INK4a}-/-* mice and human OSCC, whereas the expression of p53 did not differ significantly between the 4NQO-treated *p16^{INK4a}-/-* and WT mice. This was considered to be caused by a decline in the function of tumor suppressor

genes, including *TAp63* and *p53*, by the activation of $\Delta Np63$. *TAp63* acts synergistically and/or subsequently to control differentiation of the upper epidermal layers. *TAp63* isoforms contain a p53-homologous N-terminal TA domain and have similar tumor suppressor properties to *p53* in inducing growth arrest, apoptosis, and senescence. By contrast, $\Delta Np63$ controls the expansion of progenitor cells in the basal layer, and $\Delta Np63$ isoforms lacking this TA domain are reported to possess oncogenic activities through the inhibition of *TAp63*, *TAp73*, and *p53*.^{9,17} Therefore, the altered expression of $\Delta Np63$ may be responsible for the increased tumor susceptibility, suggesting that $\Delta Np63$ may be a potential target of OSCC therapy.

In summary, the loss of *p16^{INK4a}* with retention of the expression of *p19^{ARF}* in the oral cavity leads to the progression of OSCC from the proliferation of cancer cells via the up-regulation of $\Delta p63$ in mice. Although the status of p16 and its isoforms in human tissues is unknown in this study, similar events may be associated with human oral squamous cell carcinogenesis. Further analysis on the $\Delta p63$ signaling pathway will contribute to target therapy of OSCC.

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Supplemental Data

Supplemental material for this article can be found at <http://doi.org/10.1016/j.ajpath.2020.01.017>.

References

- Pai SI, Westra WH: Molecular pathology of head and neck cancer: implications for diagnosis, prognosis, and treatment. *Annu Rev Pathol* 2009, 4:49–70
- Li J, Liang F, Yu D, Qing H, Yang Y: Development of a 4-nitroquinoline-1-oxide model of lymph node metastasis in oral squamous cell carcinoma. *Oral Oncol* 2013, 49:299–305
- Jemal A, Siegel R, Ward E, Hao Y, Xu J, Murray T, Thun MJ: Cancer statistics, 2008. *CA Cancer J Clin* 2008, 58:71–96
- Leemans CR, Braakhuis BJM, Brakenhoff RH: The molecular biology of head and neck cancer. *Nat Rev Cancer* 2011, 11:9–22
- Kao Y-Y, Tu H-F, Kao S-Y, Chang K-W, Lin S-C: The increase of oncogenic miRNA expression in tongue carcinogenesis of a mouse model. *Oral Oncol* 2015, 51:1103–1112
- Tanaka T, Ishigamori R: Understanding carcinogenesis for fighting oral cancer. *J Oncol* 2011, 2011:603740
- Choi S, Myers JN: Molecular pathogenesis of oral squamous cell carcinoma: implications for therapy. *J Dent Res* 2008, 87:14–32
- Gasche JA, Goel A: Epigenetic mechanisms in oral carcinogenesis. *Future Oncol* 2012, 8:1407–1425
- Lopez F, Sampedro T, Llorente JL, Hermesen M, Alvarez-Marcos C: Alterations of p14 (ARF), p15 (INK4b), and p16 (INK4a) genes in primary laryngeal squamous cell carcinoma. *Pathol Oncol Res* 2017, 23:63–71
- Lowe SW, Sherr CJ: Tumor suppression by Ink4a-Arf: progress and puzzles. *Curr Opin Genet Dev* 2003, 13:77–83
- Nemes JA, Deli L, Nemes Z, Marton IJ: Expression of p16(INK4A), p53, and Rb proteins are independent from the presence of human papillomavirus genes in oral squamous cell carcinoma. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod* 2006, 102:344–352
- Ribeiro DA, Fracalossi AC, Uatari SA, Oshima CT, Salvadori DM: Imbalance of tumor suppression genes expression following rat tongue carcinogenesis induced by 4-nitroquinoline 1-oxide. *In Vivo* 2009, 23:937–942
- Sailasree R, Abhilash A, Sathyan KM, Nalinakumari KR, Thomas S, Kannan S: Differential roles of p16INK4A and p14ARF genes in prognosis of oral carcinoma. *Cancer Epidemiol Biomarkers Prev* 2008, 17:414–420
- Esteller M, Tortola S, Toyota M, Capella G, Peinado MA, Baylin SB, Herman JG: Hypermethylation-associated inactivation of p14(ARF) is independent of p16(INK4a) methylation and p53 mutational status. *Cancer Res* 2000, 60:129–133
- Ko A, Han SY, Song J: Dynamics of ARF regulation that control senescence and cancer. *BMB Rep* 2016, 49:598–606
- Sharpless NE, Ramsey MR, Balasubramanian P, Castrillon DH, DePinho RA: The differential impact of p16(INK4a) or p19(ARF) deficiency on cell growth and tumorigenesis. *Oncogene* 2004, 23:379–385
- Rekhtman N, Ang DC, Sima CS, Travis WD, Moreira AL: Immunohistochemical algorithm for differentiation of lung adenocarcinoma and squamous cell carcinoma based on large series of whole-tissue sections with validation in small specimens. *Mod Pathol* 2011, 24:1348–1359
- Candi E, Rufini A, Terrinoni A, Dinsdale D, Ranalli M, Paradisi A, De Laurenzi V, Spagnoli LG, Catani MV, Ramadan S, Knight RA, Melino G: Differential roles of p63 isoforms in epidermal development: selective genetic complementation in p63 null mice. *Cell Death Differ* 2006, 13:1037–1047
- Carvalho JC, Thomas DG, McHugh JB, Shah RB, Kunju LP: p63, CK7, PAX8 and INI-1: an optimal immunohistochemical panel to distinguish poorly differentiated urothelial cell carcinoma from high-grade tumours of the renal collecting system. *Histopathology* 2012, 60:597–608
- Mukhopadhyay S, Katzenstein AL: Subclassification of non-small cell lung carcinomas lacking morphologic differentiation on biopsy specimens: utility of an immunohistochemical panel containing TTF-1, napsin A, p63, and CK5/6. *Am J Surg Pathol* 2011, 35:15–25
- Nekulova M, Holcakova J, Nenutil R, Stratmann R, Bouchalova P, Muller P, Moukova L, Coates PJ, Vojtesek B: Characterization of specific p63 and p63-N-terminal isoform antibodies and their application for immunohistochemistry. *Virchows Arch* 2013, 463:415–425
- Moore L, Venkatachalam S, Vogel H, Watt JC, Wu CL, Steinman H, Jones SN, Donehower LA: Cooperativity of p19ARF, Mdm2, and p53 in murine tumorigenesis. *Oncogene* 2003, 22:7831–7837
- Campisi J, d'Adda di Fagnola F: Cellular senescence: when bad things happen to good cells. *Nat Rev Mol Cell Biol* 2007, 8:729–740
- Serrano M, Lee H, Chin L, Cordon-Cardo C, Beach D, DePinho RA: Role of the INK4a locus in tumor suppression and cell mortality. *Cell* 1996, 85:27–37
- Kamijo T, Zindy F, Roussel MF, Quelle DE, Downing JR, Ashmun RA, Grosfeld G, Sherr CJ: Tumor suppression at the mouse INK4a locus mediated by the alternative reading frame product p19ARF. *Cell* 1997, 91:649–659
- Kamijo T, van de Kamp E, Chong MJ, Zindy F, Diehl JA, Sherr CJ, McKinnon PJ: Loss of the ARF tumor suppressor reverses premature replicative arrest but not radiation hypersensitivity arising from disabled atm function. *Cancer Res* 1999, 59:2464–2469

27. Sharpless NE, Bardeesy N, Lee KH, Carrasco D, Castrillon DH, Aguirre AJ, Wu EA, Horner JW, DePinho RA: Loss of p16Ink4a with retention of p19Arf predisposes mice to tumorigenesis. *Nature* 2001, 413:86–91
28. Baba S, Yamada Y, Hatano Y, Miyazaki Y, Mori H, Shibata T, Hara A: Global DNA hypomethylation suppresses squamous carcinogenesis in the tongue and esophagus. *Cancer Sci* 2009, 100:1186–1191
29. Bologna-Molina R, Mosqueda-Taylor A, Molina-Frechero N, Mori-Estevez AD, Sanchez-Acuna G: Comparison of the value of PCNA and Ki-67 as markers of cell proliferation in ameloblastic tumors. *Med Oral Patol Oral Cir Bucal* 2013, 18:e174–e179
30. Nicholson DW, Ali A, Thornberry NA, Vaillancourt JP, Ding CK, Gallant M, Gareau Y, Griffin PR, Labelle M, Lazebnik YA, Munday NA: Identification and inhibition of the ICE/CED-3 protease necessary for mammalian apoptosis. *Nature* 1995, 376:37–43
31. Miyamoto S, Yasui Y, Kim M, Sugie S, Murakami A, Ishigamori-Suzuki R, Tanaka T: A novel rasH2 mouse carcinogenesis model that is highly susceptible to 4-NQO-induced tongue and esophageal carcinogenesis is useful for preclinical chemoprevention studies. *Carcinogenesis* 2008, 29:418–426
32. Slaughter DP, Southwick HW, Smejkal W: Field cancerization in oral stratified squamous epithelium; clinical implications of multicentric origin. *Cancer* 1953, 6:963–968
33. Dakubo GD, Jakupciak JP, Birch-Machin MA, Parr RL: Clinical implications and utility of field cancerization. *Cancer Cell Int* 2007, 7:2
34. Karni-Schmidt O, Castillo-Martin M, Shen TH, Gladoun N, Domingo-Domenech J, Sanchez-Carbayo M, Li Y, Lowe S, Prives C, Cordon-Cardo C: Distinct expression profiles of p63 variants during urothelial development and bladder cancer progression. *Am J Pathol* 2011, 178:1350–1360
35. Wang Y, Dong Q-Z, Fu L, Stoecker M, Wang E, Wang E-H: Overexpression of CRKL correlates with poor prognosis and cell proliferation in non-small cell lung cancer. *Mol Carcinog* 2013, 52: 890–899