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Expression of p63 and p73 in ameloblastomas

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BACKGROUND: To clarify the role of p53 homologs in oncogenesis and cytodifferentiation of odontogenic tumors, expression of p63 and p73 was analyzed in ameloblastomas as well as tooth germs.
METHODS: Tissue specimens of nine tooth germs and 48 benign and five malignant ameloblastomas were examined by immunohistochemistry and reverse transcriptase-polymerase chain reaction (RT-PCR) for the expression of p63 and p73.
RESULTS: Immunoreactivity for p63 and p73 was evident in epithelial cells neighboring the basement membrane in developing and neoplastic odontogenic tissues. p63 expression in desmoplastic ameloblastomas was significantly higher than in acanthomatous and granular cell ameloblastomas, and ameloblastic carcinomas showed higher p63 expression than metastasizing ameloblastomas. p73 expression was significantly higher in plexiform ameloblastomas than in follicular ameloblastomas, and basal cell ameloblastomas showed higher p73 expression than granular cell ameloblastomas. mRNA transcripts for ΔNp63 and TAp73 were detected in all developing and neoplastic odontogenic tissues. TAp63 mRNA was expressed in five of eight tooth germs, 16 of 34 ameloblastomas, and one of one malignant ameloblastoma, whereas ΔNp73 mRNA was recognized in one of eight tooth germs, nine of 34 ameloblastomas, and one of one malignant ameloblastoma.
CONCLUSION: The expression of p63 and p73 suggests that these p53 homologs play a role in differentiation and proliferation of odontogenic epithelial cells. Variations of predominantly expressed isoforms suggest that p63 and p73 might differentially function in odontogenic tissues.
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Introduction

Tumors arising from epithelium of the odontogenic apparatus or from its derivatives or remnants exhibit considerable histological variation and are classified into several benign and malignant entities (1–4). Ameloblastoma is the most frequently encountered tumor arising from odontogenic epithelium and is characterized by a benign but locally invasive behavior with a high risk of recurrence (1, 2, 4). Histologically, ameloblastoma shows considerable variation, including follicular, plexiform, acanthomatous, granular cell, basal cell, and desmoplastic types (1). Malignant ameloblastoma is defined as a neoplasm in which the pattern of an ameloblastoma and cytological features of malignancy are shown by the primary growth in the jaws and/or by any metastatic growth (1). Recently, malignant ameloblastoma has been subclassified into metastasizing ameloblastoma and ameloblastic carcinoma on the basis of metastatic spread and cytological malignant features (3). Several recent studies have detected genetic and cytogenetic alterations in these epithelial odontogenic tumors (5, 6); however, the detailed mechanisms of oncogenesis, cytodifferentiation, and tumor progression remain unknown.

p53 gene is a well-recognized tumor suppressor gene that is frequently altered in tumors (7, 8). Its gene product is a transcriptional factor that regulates the expression of genes involved in cell cycle arrest or apoptosis in response to genomic damage or cell stress (9). Two *p53* homolog genes, *p63* and *p73*, have been identified at loci 3q27–29 and 1p36, respectively (10–12). These genes encode multiple proteins that have a significant degree of sequence homology, particularly in the transactivation, DNA-binding, and oligomerization domains (10–13). Isoforms derived from two different promoters are named TA and ΔN isoforms. TA isoforms (TAp63 and TAp73) containing the transactivation domain are capable of transactivating p53 target genes and inducing growth arrest or cell death, whereas ΔN isoforms (ΔNp63 and ΔNp73) lacking the transactivation domain exert a dominant-negative effect on their TA isoforms and wild-type p53 by blocking their transactivation (12, 13). Recently, one of ΔNp63 isoforms has been shown to possess a second

transactivation domain in the C-terminus (14). Although these p53 homologs might have a tumor suppressor function, genetic alterations, such as mutation and loss of heterozygosity (LOH), are less frequent than those associated with *p53* (11, 13, 15, 16). In recent studies, up-regulated expression and/or activity of p63 and p73 have been demonstrated in some malignancies (17–26).

Our previous studies confirmed p53 and its related factors, such as $p21^{WAF1/Cip1}$, Bax, MDM2, and $p14^{ARF}$, in tooth germs and ameloblastomas, suggesting that the p53 signaling cascade has an important role in oncogenesis or cytodifferentiation of odontogenic epithelium (27–30). Recently, several syndromes associated with *p63* gene mutations have shown to include various tooth abnormalities of both the primary and permanent dentition (31, 32). In the present study, expression of p63 and p73 in benign and malignant ameloblastomas as well as in tooth germs was examined by immunohistochemistry and reverse transcriptase-polymerase chain reaction (RT-PCR) to clarify the possible role of these p53 homologs in epithelial odontogenic tumors.

Materials and methods

Tissue preparation

Specimens were surgically removed from 53 patients with epithelial odontogenic tumors at the Department of Oral and Maxillofacial Surgery, Tohoku University Dental Hospital, and affiliated hospitals. The specimens were fixed in 10% buffered formalin for one to several days and were embedded in paraffin. The tissue blocks were sliced into 3-μm thick sections for routine histological and subsequent immunohistochemical examinations. Tissue sections were stained with hematoxylin and eosin for histological diagnosis according to the WHO histological typing of odontogenic tumors (1). The tumors comprised 48 ameloblastomas and five malignant ameloblastomas. Ameloblastomas were divided into 31 follicular and 17 plexiform types, including 17 acanthomatous, six granular cell, three basal cell, and four desmoplastic subtypes. Malignant ameloblastomas were classified into two metastasizing ameloblastomas and three ameloblastic carcinomas according to the criteria of Eversole (3). For RT-PCR analysis, tumor tissues were immediately frozen on dry ice and stored at −80°C. Specimens of nine tooth germs of the mandibular third molars, enucleated for orthodontic reasons at the initial stage of crown mineralization, were similarly prepared and compared with the epithelial odontogenic tumors.

Immunohistochemistry

The tissue sections were deparaffinized, immersed in methanol with 0.3% hydrogen peroxide, and heated in 0.01 M citrate buffer (pH 6.0) for 10 min by autoclave (121°C, 2 atm). After treatment with normal serum for 30 min, the sections were incubated with primary antibodies at 4°C overnight. The applied antibodies were mouse anti-p63 monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA; subclass IgG2a; diluted at 1:100) and rabbit anti-p73 polyclonal antibody (Chemicon, Temecula, CA, USA; diluted at 1:500). The standard streptavidin-biotin-peroxidase complex method was performed to bind the primary antibodies with the use of Histofine SAB-PO Kits (Nichirei, Tokyo, Japan). Reaction products were visualized by immersing the sections in 0.03% diaminobenzidine solution containing 2 mM hydrogen peroxide for 1–3 min. Nuclei were lightly counterstained with methylgreen. For control studies of the antibodies, the serial sections were treated with phosphate-buffered saline, mouse anti-L26 (CD20) monoclonal antibody (Nichirei; subclass IgG2a), and normal rabbit IgG instead of the primary antibodies and were confirmed to be unstained.

Immunohistochemical reactivity for p63 and p73 was evaluated and classified into three groups: (+) positive in peripheral epithelial cells, (++) positive in peripheral and some central epithelial cells, and (+++) positive in most epithelial cells.

RT-PCR

Total RNA was extracted from frozen tissue samples of eight tooth germs, 34 ameloblastomas, and one malignant ameloblastoma using an RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. First-stranded complementary DNA (cDNA) was synthesized from 1 μg of RNA using an Omniscript RT Kit (Qiagen) with oligo-$(dT)_{15}$ primer (Roche Diagnostics, Mannheim, Germany) as outlined by the manufacturer. The cDNA samples were amplified using a HotstarTaq Master Mix Kit (Qiagen) with specific primers in a DNA thermal cycler (Eppendorf, Hamburg, Germany). Primers used in this study were as follows: 5′-GTCCCAGAGCACACAGACAA-3′ (forward) and 5′-GAGGAGCCGTTCTGAATCTG-3′ (reverse) for TAp63, yielding a 267-bp product, 5′-GAA AACAATGCCCAGACTCAA-3′ (forward) and 5′-TGC GCGTGGTCTGTGTTA-3′ (reverse) for ΔNp63, yielding a 125-bp product, 5′-CGGGACGGACGCCGA TG-3′ (forward) and 5′-GGTGGAAGACGTCC ATGCTGG-3′ (reverse) for TAp73, yielding a 199-bp product, and 5′-ACAAACGGCCCGCATGTT-3′ (forward) and 5′-GCGACATGGTGTCGAAGGTGG AGC-3′ (reverse) for ΔNp73, yielding a 367-bp product. A housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), was used as an internal control for the examination of human gene expression, and the primer sequences were 5′-GGAGTCAACGG ATTTGGT-3′ (forward) and 5′-GTGATGGGATTTC CATTGAT-3′ (reverse), yielding a 206-bp product. PCR was performed in a total volume of 50 μl, containing 1 μg of template cDNA and 0.5 μM of each specific primer set. The procedure for amplification included 40 cycles of denaturation at 94°C for 45 s, annealing at 57°C (for TAp63 and ΔNp63) or 55°C (for TAp73 and ΔNp73) for 45 s, and elongation at 72°C for 60 s with heat starting at 95°C for 15 min and final elongation at 72°C for 10 min. The PCR products were electrophoresed on 2% agarose gel at 100 V for 30 min and visualized with ethidium bromide.

Statistical analysis

The statistical significance of differences in the percentages of cases with different immunohistochemical reactivity levels or with mRNA/cDNA expression was analyzed by the Mann–Whitney *U*-test for differences between two groups or the Kruskal–Wallis test for differences among three or more groups. *P*-values of less than 0.05 were considered to indicate statistical significance.

Results

Immunohistochemical reactivity for p63 and p73

Immunohistochemical reactivity for p63 and p73 was detected in the nuclei of normal and neoplastic odontogenic epithelial cells; mesenchymal cells in tooth germs and stromal cells in benign and malignant ameloblastomas were faintly reactive with anti-p73 antibody (Figs 1 and 2; Table 1). In tooth germs, expression of p63 and p73 was found in most cells of inner and outer enamel epithelium and dental lamina and in fewer cells of stratum intermedium and stellate reticulum (Figa 1a and 2a). p63-positive cells were more numerous than p73-positive cells in stratum intermedium and stellate reticulum.

Ameloblastomas showed p63 and p73 reactivity in most peripheral columnar or cuboidal cells and in fewer central polyhedral cells (Figs 1b and 2b). Central polyhedral cells in ameloblastomas were far more positive for p63 than for p73. p73 expression in plexiform ameloblastomas was significantly higher than that in follicular ameloblastomas ($P < 0.001$). Keratinizing cells and granular cells showed markedly decreased reactivity for p63 and p73 in acanthomatous and granular cell ameloblastomas (Fig 1c). Basal cell and desmoplastic ameloblastomas exhibited p63 and p73 reactivity in most neoplastic cells (Fig 2c), and staining intensity in desmoplastic ameloblastomas was low. Basal cell ameloblastomas showed significantly higher p73 expression than granular cell ameloblastomas ($P < 0.05$), and desmoplastic ameloblastomas demonstrated significantly higher p63 expression than acanthomatous and granular cell ameloblastomas ($P < 0.05$).

Metastasizing ameloblastomas showed p63 and p73 expression patterns similar to those of the benign ameloblastomas, while ameloblastic carcinomas demonstrated p63 and p73 reactivity in most neoplastic cells (Figs 1d and 2d). p63 expression in ameloblastic

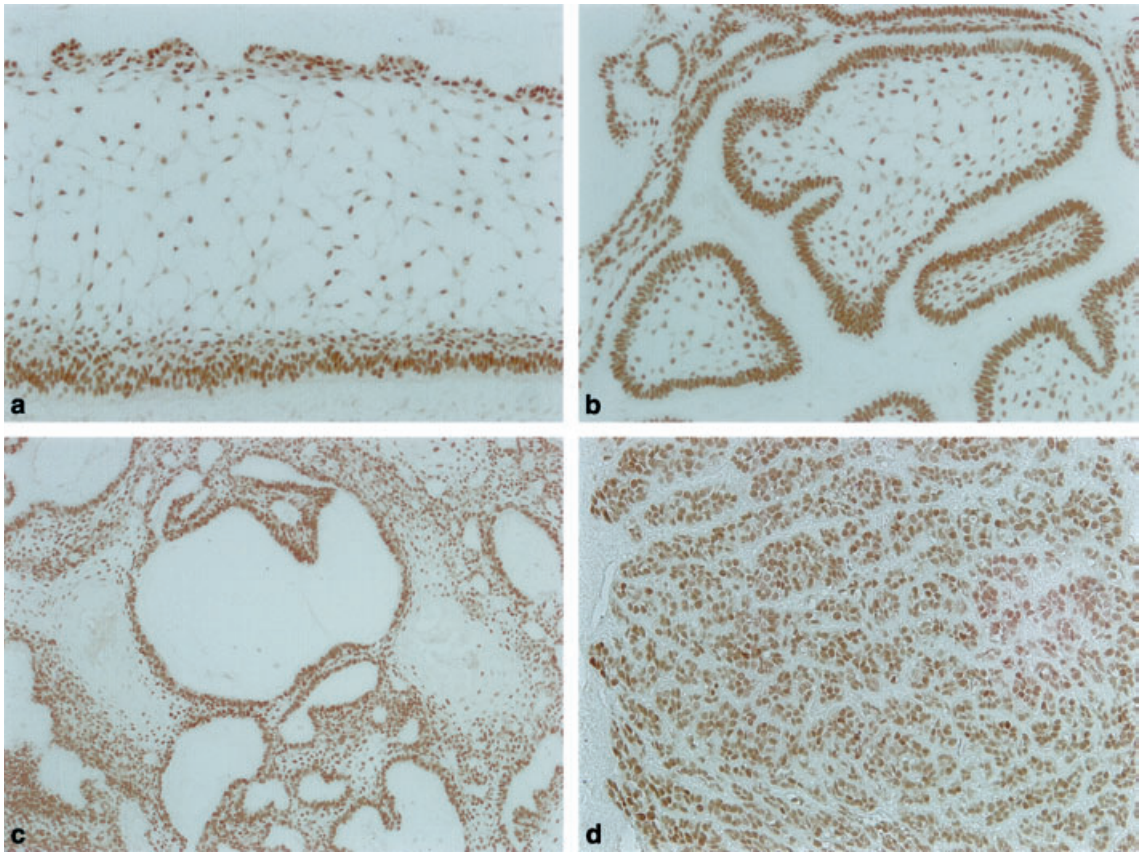

Figure 1 Immunohistochemical reactivity for p63. (a) Tooth germ showing reactivity in most cells of inner and outer enamel epithelium and stratum intermedium and in slightly fewer cells of stellate reticulum (×125). (b) Follicular ameloblastoma showing reactivity in most peripheral columnar cells and slightly fewer central polyhedral cells (×115). (c) Acanthomatous ameloblastoma showing decreased reactivity in keratinizing cells (×70). (d) Ameloblastic carcinoma showing reactivity in most neoplastic cells (×125).

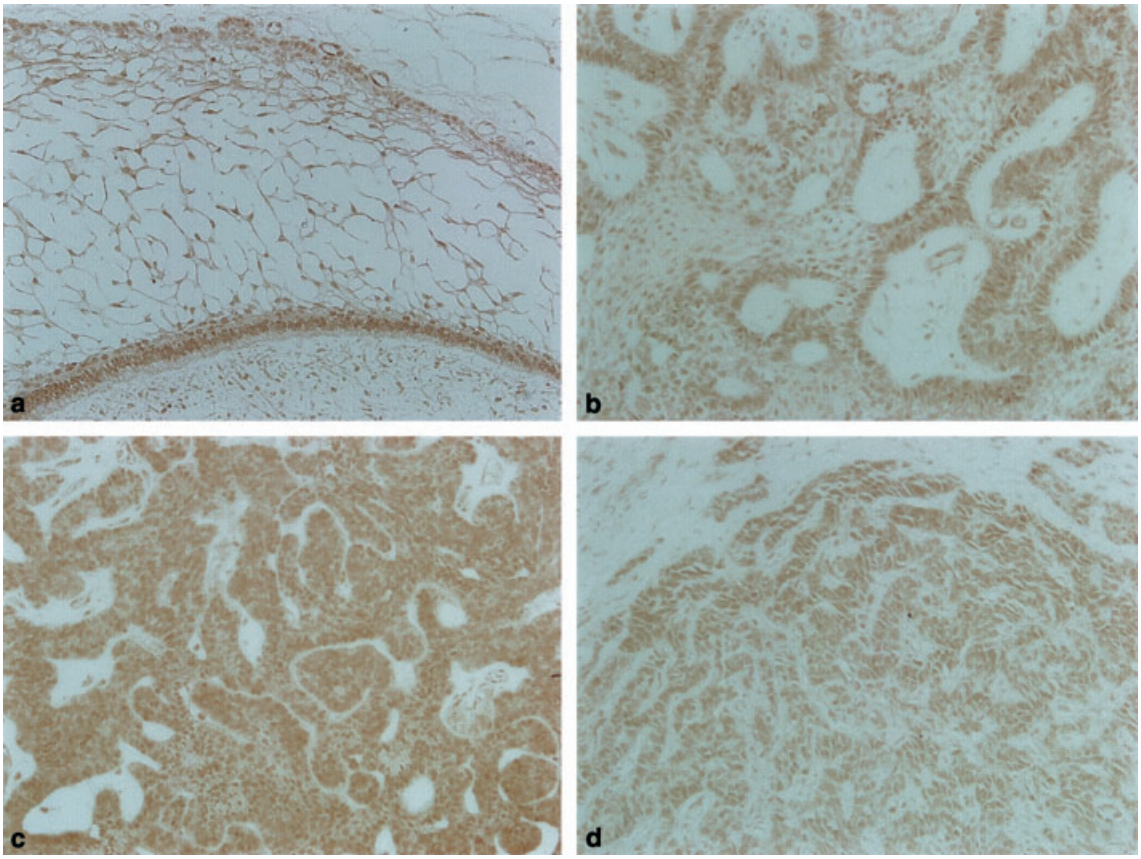

Figure 2 Immunohistochemical reactivity for p73. (a) Tooth germ showing reactivity in most cells of inner and outer enamel epithelium and in some cells of stratum intermedium and stellate reticulum (×100). (b) Plexiform ameloblastoma showing reactivity in many peripheral cuboidal cells and some central polyhedral cells (×115). (c) Basal cell ameloblastoma showing reactivity in most neoplastic cells (×100). (d) Ameloblastic carcinoma showing reactivity in most neoplastic cells (×125).

carcinomas was significantly higher than that in metastasizing ameloblastomas ($P < 0.05$).

mRNA expression of p63 and p73 isoforms

To elucidate p63 and p73 isoforms expressed in odontogenic tissues, RT-PCR was carried out using isoform-specific primers. mRNA transcripts for ΔNp63 and TAp73 were detected in all 34 ameloblastomas and the one malignant ameloblastoma as well as in the eight tooth germ tissues, whereas expression of TAp63 and ΔNp73 mRNA was limited in tooth germs and ameloblastomas (Fig. 3; Table 1). TAp63 mRNA was

Table 1 Expression of p63 and p73 in tooth germs and ameloblastomas

	Immunohistochemical reactivity [n (%)]							*RT–PCR profile [n (%)]*				
		p63			*p73*				*p63*		*p73*	
	n	+	+ +	+ + +	+	+ +	+ + +	*n*	*TAp63*	*ΔNp63*	*TAp73*	*ΔNp73*
Tooth germ	9	0 (0)	8 (89)	1 (11)	0 (0)	9 (100)	0 (0)	8	5 (63)	8 (100)	8 (100)	1 (13)
Ameloblastoma	48	0 (0)	38 (79)	10 (21)	6 (13)	37 (77)	5 (10)	34	16 (47)	34 (100)	34 (100)	9 (26)
Follicular type	31	0 (0)	25 (81)	6 (19)	6 (19)	25 (81)	0 (0) ***	20	9 (45)	20 (100)	20 (100)	6 (30)
Plexiform type	17	0 (0)	13 (76)	4 (24)	0 (0)	12 (71)	5 (29) ***	14	7 (50)	14 (100)	14 (100)	3 (21)
Acanthomatous subtype	17	0 (0)	17 (100)	0 (0) *	3 (18)	14 (82)	0 (0)	14	6 (43)	14 (100)	14 (100)	4 (29)
Granular subtype	6	0 (0)	6 (100)	0 (0) *	2 (33)	4 (67)	0 (0) *	4	1 (25)	4 (100)	4 (100)	0 (0)
Basal cell subtype	3	0 (0)	1 (33)	2 (67) *	0 (0)	1 (33)	2 (67) *	1	0 (0)	1 (100)	1 (100)	1 (100)
Desmoplastic subtype	4	0 (0)	1 (25)	3 (75) *	1 (25)	3 (75)	0 (0)	1	0 (0)	1 (100)	1 (100)	0 (0)
Non-cellular variation	18	0 (0)	13 (7)	5 (28)	0 (0)	15 (83)	3 (17)	14	9 (64)	14 (100)	14 (100)	4 (29)
Malignant ameloblastoma	5	0 (0)	2 (40)	3 (60)	0 (0)	3 (60)	2 (40)	1	1 (100)	1 (100)	1 (100)	1 (100)
Metastasizing ameloblastoma	2	0 (0)	2 (100)	0 (0) *	0 (0)	2 (100)	0 (0)	1	1 (100)	1 (100)	1 (100)	1 (100)
Ameloblastic carcinoma	3	0 (0)	0 (0)	3 (100) *	0 (0)	1 (33)	2 (67)	0	–	–	–	–

Immunohistochemical reactivity: +, positive in peripheral epithelial cells; + +, positive in peripheral and some central epithelial cells; + + +, positive in most epithelial cells.
Statistical significance: *$P < 0.05$, ***$P < 0.001$.

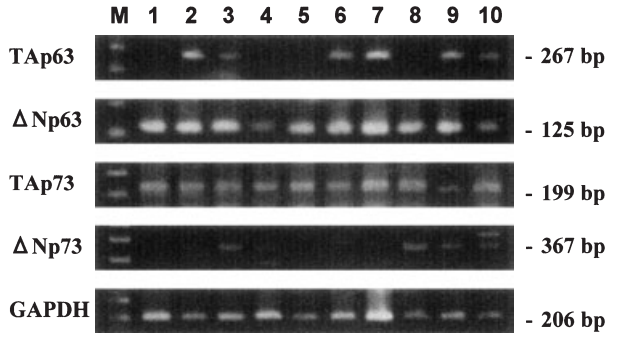

Figure 3 Reverse transcriptase-polymerase chain reaction analysis of p63 and p73 mRNA expression in tooth germs and ameloblastomas. (M: molecular-weight standard, 1–3: tooth germs, 4–9: ameloblastomas, 10: malignant ameloblastoma) ΔNp63 and TAp73 mRNA expression was seen in all samples, and differential mRNA expression of TAp63 and ΔNp73 was detected. GAPDH was run as a control to ascertain the integrity of mRNA/cDNA. The sizes of TAp63, ΔNp63, TAp73, ΔNp73, and GAPDH PCR products were 267, 125, 199, 367, and 206 bp, respectively.

expressed in five of eight tooth germs, 16 of 34 ameloblastomas, and one of one malignant ameloblastomas, while ΔNp73 mRNA was recognized in one of eight tooth germs, nine of 34 ameloblastomas, and one of one malignant ameloblastomas. There was no distinct difference in TAp63 and ΔNp73 mRNA expression among the different types or subtypes of ameloblastomas. Differences in the RT-PCR profiles of p63 and p73 isoforms did not reach statistical significance.

Discussion

While p53 is ubiquitously expressed, p63 expression is restricted to certain tissues, including the skin, bladder, prostate, uterus, mammary gland, and skeletal muscle (11, 12). Mice lacking *p63* gene have defects in their limb, craniofacial, and epidermal development, suggesting that p63 is essential for various aspects of ectodermal differentiation (33, 34). Mutations of *p63* gene have recently been shown to cause several inherited human syndromes with abnormal limb development and/or ectodermal dysplasia, such as ectrodactyly, ectodermal dysplasia, facial cleft (EEC) syndrome, ankyloblepharon, ectodermal dysplasia, and clefting (AEC) syndrome, acro-dermato-ungual-lacrimal-tooth (ADULT) syndrome, and limb-mammary syndrome (LMS), which often accompany abnormal tooth development ranging from enamel dysplasia to a tooth loss (31, 32). High expression of p73 is detected in the brain and skin (13). *p73*-deficient mice have neurological, pheromonal, and inflammatory defects, suggesting that the biological function of p73 is to regulate development of nervous system, sexual maturation, and epithelial barrier function (35). The ability to generate mice lacking *p53* implies that p53 is not required for embryonic development (9), and our previous study revealed that p53 expression in tooth germs is much lower than expression of its upstream regulators, MDM2 and $p14^{ARF}$ (30). In the present study, tooth germs immunohistochemically showed p63 and p73 expression, chiefly in epithelial components, suggesting that these p53 homologs are involved in epithelial differentiation during tooth development. p63 mRNA was predominantly expressed in its ΔN isoform, while p73 mRNA was mostly expressed in its TA isoform. These results in tooth germs are similar to those in human keratinocytes (36), suggesting that p63 and p73 play differential roles in developing odontogenic epithelium.

While *p53* gene is mutated in more than 50% of human cancers, mutations of its homolog *p63* gene are infrequent (11, 15, 21). Recently, ΔNp63 amplification and/or overexpression has been identified in human malignancies, including nasopharyngeal, bladder, prostate, skin, and esophageal carcinomas (20, 21, 24–26). In transformation assays using Rat 1a cells, overexpression of ΔNp63 results in a p53-independent pathway of cell transformation (23). These features support the notion that ΔNp63 plays an oncogenic role in human tumors. In the present study, immunoreactivity for p63 in epithelial odontogenic tumors was higher in peripheral neoplastic cells than in central neoplastic cells, and ΔNp63 mRNA was the major isoform of p63 expressed in the epithelial odontogenic tumors. The expression of p63 isoforms in ameloblastomas are similar to that in squamous cell carcinomas of the head and neck (37, 38). Proliferative activity in ameloblastomas is known to be higher in peripheral neoplastic cells than in central neoplastic cells (27, 29, 39). These features suggest that transcriptionally inactive ΔNp63 expression is associated with proliferation of neoplastic odontogenic epithelial cells. In this study, immunohistochemical expression of p63 tended to increase in epithelial odontogenic tumors, especially in ameloblastic carcinomas, as compared with tooth germs, suggesting that p63 might be involved in oncogenesis or malignant transformation of odontogenic epithelium. Basal cell and desmoplastic ameloblastomas show higher expression of some apoptosis inhibitory factors, including bcl-2, bcl-x, and survivin, than other subtypes, and these ameloblastoma variants are considered to possess a high potential for cell survival (28, 40, 41). In the present study, p63 immunoreactivity was slightly higher in basal cell and desmoplastic ameloblastomas than in other subtypes. These features suggest that ΔNp63 expression in ameloblastomas might suppress the function of TAp63, acting with the bcl-2 and IAP family members to inhibit apoptosis induction.

Although 1p36 abnormalities have been reported in a variety of human malignancies, including neuroblastoma, colorectal cancer, and breast cancer, *p73* gene mutations are quite rare (10, 13, 16). In recent studies, some tumors, including lung, bladder, and breast carcinomas, have been shown to express higher levels of p73 than the tissue from which they arose does (17–19). In the present study, immunoreactivity for p73 in epithelial odontogenic tumors was found evidently in neoplastic cells neighboring the basement membrane. TAp73 mRNA expression was detected in all epithelial odontogenic tumors, whereas ΔNp73 was expressed in a limited number of neoplastic tissues. These results suggest that p73 plays a role in differentiation and/or

proliferation of ameloblastoma cells. A recent study has demonstrated ΔNp73 accumulation in neuroblastic tumors (42). In this study, ameloblastomas had slightly higher expression of ΔNp73 mRNA than did tooth germs. These features suggest that ΔN isoform of p73 might play an oncogenic role not only in neuronal tissue but also in odontogenic epithelium. TAp73 has been proven to be induced by DNA damage, similar to wild-type p53 (43). In this study, immunoreactivity for p73 was significantly higher in plexiform ameloblastomas than in follicular ameloblastomas. Our previous study has revealed that expression of p53, MDM2, and $p14^{ARF}$ is higher in plexiform ameloblastomas than in follicular ameloblastomas (30). These features suggest that genomic damage or cell stress is greater in plexiform ameloblastomas than in follicular ameloblastomas, being involved in tissue structuring of ameloblastomas. In the present study, p73 reactivity in acanthomatous and granular cell ameloblastomas was markedly decreased in keratinizing cells and granular cells. Our previous studies have detected increased apoptotic cell death in keratinizing cells and granular cells of these ameloblastoma subtypes (27, 44). These features suggest that induction of apoptosis by TAp73 is minimal during terminal differentiation of neoplastic cells in acanthomatous and granular cell ameloblastomas.

In conclusion, expression of p63 and p73 in tooth germs and benign and malignant ameloblastomas suggests that these p53 homologs play a role in differentiation and proliferation of odontogenic epithelial cells. In addition, variations of expressed p63 and p73 isoforms suggest that these p53 homologs might differentially function in both developing and neoplastic odontogenic tissues.

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