Increased ΔNp63 expression is predictive of malignant transformation in oral epithelial dysplasia and poor prognosis in oral squamous cell carcinoma

RYOTA MATSUBARA, SHINTARO KAWANO, TAKAHIRO KIYOSUE, YUICHI GOTO, MITSUHIRO HIRANO, TEPPEI JINNO, TAKESHI TOYOSHIMA, RYOJI KITAMURA, KAZUNARI OOBU and SEIJI NAKAMURA

Section of Oral and Maxillofacial Oncology, Division of Maxillofacial Diagnostic and Surgical Sciences, Faculty of Dental Science, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan

Received May 30, 2011; Accepted June 28, 2011

DOI: 10.3892/ijo.2011.1151

Abstract. This study examined immunohistochemical expression of ΔNp63, a keratinocyte stem cell marker, in oral leukoplakia (OL) and oral squamous cell carcinoma (OSCC) and then to elucidate usefulness of ΔNp63 as a marker for diagnosis and prognosis. One-hundred and twelve cases of OL and 81 cases of OSCC were analyzed by immunohistochemical staining for ΔNp63, Ki-67, and cytokeratin 14. These labeling indices (LIs) were calculated, and the association of these LIs with clinicopathologic characteristics in the OL and OSCC was evaluated. In the OL, these LIs increased significantly according to the severity of epithelial dysplasia ($p<0.0001$). ΔNp63-LI in the OL with malignant transformation was significantly higher than that in the OL without (49.3 vs. 34.2%; $p<0.01$). In the OSCC, the LIs increased significantly in association with the histologic grade ($p<0.0001$). A significant difference between the high and low ΔNp63-LI groups was found in the incidence of cervical lymph node and distant metastasis ($p<0.05$). The prognosis of the high ΔNp63-LI (mean value >73.8%) group is poorer than that of the low ΔNp63-LI (mean value ≤73.8%) group ($p<0.05$). These results suggested that increased ΔNp63 expression is involved in malignant transformation in epithelial dysplasia and poor prognosis in OSCC.

Correspondence to: Dr Shintaro Kawano, Section of Oral and Maxillofacial Oncology, Division of Maxillofacial Diagnostic and Surgical Sciences, Faculty of Dental Science, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan
E-mail: skawano@dent.kyushu-u.ac.jp

Abbreviations: CK, cytokeratin; CSCs, cancer stem cells; HYP, hyperplasia; LI, labeling index; MLD, mild dysplasia; MOD, moderate dysplasia; NOE, normal oral epithelium; OL, oral leukoplakia; OSCC, oral squamous cell carcinoma; SED, severe dysplasia

Key words: ΔNp63, oral leukoplakia, oral squamous cell carcinoma, immunohistochemistry, malignant transformation

Introduction

p63 gene is a homolog of *p53*, it is located on chromosome 3q27-29 and encodes multiple isotypes with divergent abilities in a variety of organs (1-4). *p63* has two different promoter domains that generate two protein isoforms, TAp63 and ΔNp63; each isoform yields three isotypes (α, β, γ) generated by alternative splicing of the p63 COOH terminus; and TAp63 includes an NH_2-terminal transactivation domain, which is absent in ΔNp63 (1-4,6,7). TAp63 transactivates p53 target genes to induce apoptosis by inhibiting cell proliferation in response to exposure of cells to DNA-damaging agents such as ultraviolet irradiation. In contrast, ΔNp63 exerts dominant-negative activities against TAp63 and p53, and ΔNp63 is thus considered as an oncoprotein (1,5-14).

Recent studies have shown that p63 is essential for craniofacial development during morphogenesis, because *p63*$^{-/-}$ mice represented craniofacial abnormalities including hypoplasia of jaws and defects of the teeth, hair follicles, lachrymal glands, and salivary glands (1,15). Furthermore, *p63*$^{-/-}$ mice showed lack of squamous stratification in the epidermis and oral epithelium, suggesting that p63 plays critical roles in the epithelial development. Pellegrini *et al* also demonstrated that p63 was expressed in keratinocyte stem cells and involved in the proliferation and maintenance of this cell population (16). Thus, p63 has been characterized as a marker of keratinocyte stem cells.

In the tumorigenesis as well as morphogenesis, the existence of a stem-like cell population with a self-renewal potential, capacity of development into multiple lineages, extensive proliferation activity, and high migration capability in the tumor tissue was revealed. This cell population has been termed cancer stem cells (CSCs) or tumor-initiating cells. CSCs have been considered to be generated from normal stem cells, so that most of them have been isolated from tumor cells based on the expression of markers that characterize the stem cells of the original normal tissues (17,18). Moreover, it has been suggested that CSCs generate heterogeneity of the tumor tissue and are possibly involved in tumorigenesis (18). Consequently, it is important to examine the expression of p63 as a marker of

Table I. The characteristics of patients with OL.

Characteristics	Cases (%)
Gender	
Male	72 (64.3)
Female	40 (35.7)
Lesion site	
Gingiva	48 (42.9)
Tongue	41 (36.6)
Palate	12 (10.7)
Buccal mucosa	10 (8.9)
Lip	1 (0.9)
Degree of epithelial dysplasia	
Hyperplasia	76 (67.9)
Mild dysplasia	22 (19.6)
Moderate dysplasia	8 (7.1)
Severe dysplasia	6 (5.4)

Table II. The characteristics of patients with OSCC.

Characteristics	Cases (%)
Gender	
Male	58 (71.6)
Female	23 (28.4)
Primary site	
Tongue	42 (51.9)
Gingiva	27 (33.3)
Floor of mouth	9 (11.1)
Buccal mucosa	3 (3.7)
Clinical growth pattern	
Superfacial type	8 (9.9)
Exophytic type	15 (18.5)
Endophytic type	58 (71.6)
Clinical stage	
I	15 (18.5)
II	28 (34.6)
III	12 (14.8)
IV	26 (32.1)
Histologic grade	
Grade 1	62 (76.5)
Grade 2	14 (17.3)
Grade 3	5 (6.2)
Mode of tumor invasion (Yamamoto-Kohama's criterion)	
Grade 1	2 (2.5)
Grade 2	17 (21.0)
Grade 3	43 (53.1)
Grade 4C	15 (18.5)
Grade 4D	4 (4.9)

keratinocyte stem cell for revealing the mechanism of tumorigenesis in oral squamous cell carcinoma (OSCC).

Oral leukoplakia (OL) is defined as an oral white lesion that can not be rubbed off or characterized as any other definable lesion, and generally considered to be a most frequent precancerous lesion of OSCC. OL is histopathologically classified into hyperplasia (HYP) which shows no cellular atypia and dysplasia which is accompanied by cytologic atypia (19). Furthermore, leukoplakia displaying epithelial dysplasia is classified as mild (MLD), moderate (MOD), or severe (SED). In a previous study, Burkhardt demonstrated that OL progressed to OSCC in 5% of mild and in 43% of severe dysplasia (20). Schepman *et al* also showed that 12% of OL developed into OSCC (21). Therefore, investigating the mechanisms by which OL becomes malignant is important for understanding the processes of tumorigenesis and the development of OSCC.

Recently, some studies have demonstrated that ΔNp63 is expressed more highly in certain tumor tissues (22-28). However, little is known about the expression of ΔNp63 in OL and OSCC. In the present study, we thus examined the expression of ΔNp63 in the OL and OSCC immunohistochemically, and then evaluated the association of the positive rates with clinicopathologic characteristics of the patients with OL or OSCC. Moreover, this study also elucidated whether ΔNp63 was useful as a marker for diagnosis and prognosis in OL and OSCC.

Patients and methods

Patients. One-hundred and twelve patients with primary OL (mean age, 61.9±13.6 years; range 12-91 years) and 81 patients with primary OSCC (mean age 62.6±15.1 years; range 19-88 years), who were diagnosed at the Department of Oral and Maxillofacial Surgery, Kyushu University Hospital, from January 2004 to December 2008, were enrolled in this study. Ten control cases (normal oral epithelium, NOE) were also evaluated. Following the initial biopsy, all specimens were fixed in 4% buffered formalin solution and embedded in paraffin blocks. Subsequently, the paraffin-embedded specimens were processed to 5 μm thick sections, stained with hematoxylin-eosin (HE), and examined by three experienced oral pathologists to confirm the diagnoses and histologic grade. The degree of epithelial dysplasia in the OL and the histologic grade in the OSCC were assessed according to the World Health Organization classification (29,30). The tumor extent was evaluated according to the TNM classification established by the American Joint Committee on Cancer and the International Union Against Cancer (UICC) (31). The mode of tumor invasion was also determined on the H&E stained specimens according to Yamamoto-Kohama's criteria, as follows: Grade 1, well-defined borderline; Grade 2, cords, less-marked borderline; Grade 3, groups of cells, no distinct borderline; Grade 4, diffuse invasion; 4C, cord-like type; 4D, widespread type (32). Medical records were reviewed to collect the information concerning the clinical characteristics. The detail clinical data describing the OL and OSCC patients are presented in Tables I and II, respectively.

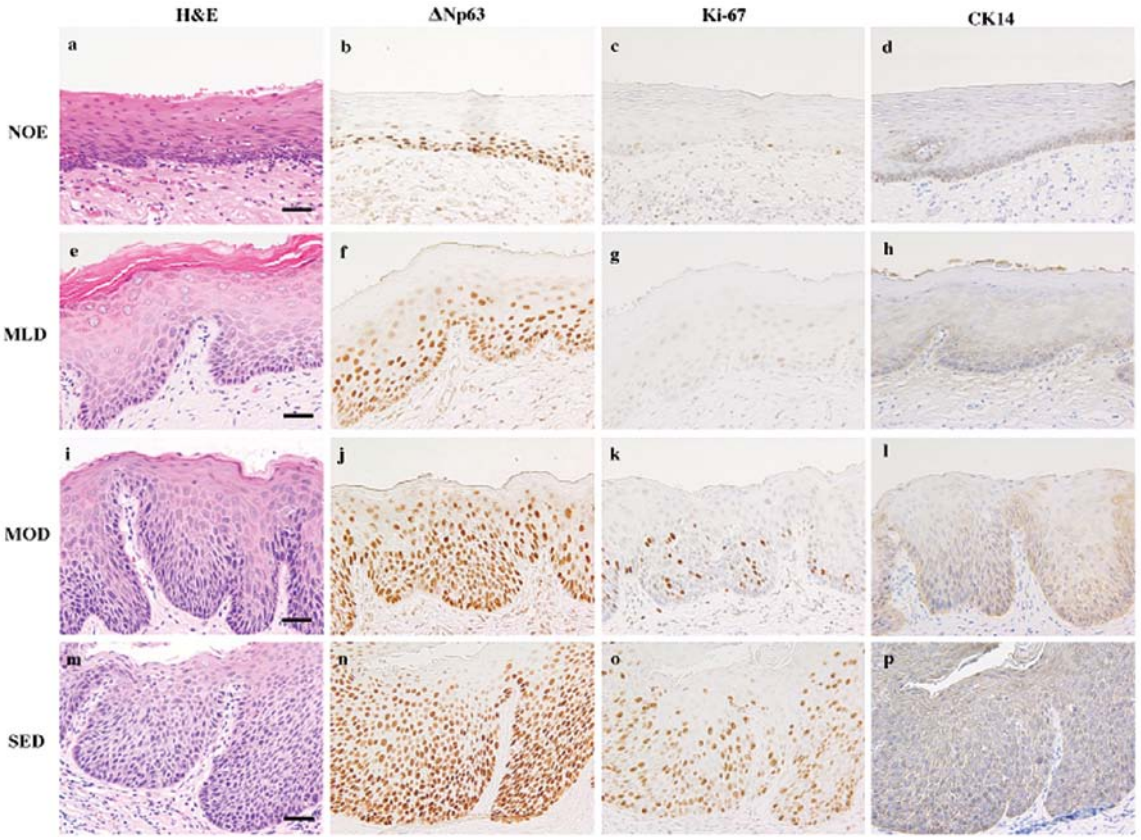

Figure 1. Immunohistochemical detection of ΔNp63, Ki-67, and CK14 in the NOE and OL. Each section was stained with H&E (a, e, i, and m), anti-ΔNp63 (b, f, j, and n), anti-Ki-67 (c, g, k, and o), and anti-CK14 (d, h, l, and p) monoclonal antibodies. In the NOE, ΔNp63 is detected in the basal and parabasal layers (b). Ki-67 is predominantly detected in the parabasal layer rather than the basal layer (c), and CK14 is also localized in the basal layer (d). In the OL with the MLD, ΔNp63 is expressed in the basal and parabasal layer, whereas it is detected almost all layers in the MOD and SED (f, j, and n). The expression patterns of Ki-67 and CK14 are also similar to that of ΔNp63 in the OL (g, h, k, l, o, and p). (a-d) NOE; (e-h) MLD; (i-l) MOD; (m-p) SED; scale bars, 100 μm.

Immunohistochemistry. Immunohistochemical staining was performed on 5 μm thick sections sliced serially from paraffin-embedded blocks after formalin fixation of the excised specimens. The sections were deparaffinized in xylene and rehydrated in a graded series of ethanol/water concentrations (100, 95, 90, 85 and 75%). For antigen retrieval, the sections were immersed in Dako Target Retrieval Solution (Dako Cytomation, Denmark) and autoclaved at 120˚C for 5 min. The endogeneous peroxide activity was then eliminated with 1% hydrogen peroxide for 30 min, and the sections were rinsed twice for 10 min with phosphate-buffered saline (PBS) at pH 7.4. Non-specific protein binding was blocked by incubation for 1 h with 10% goat serum, and then the sections were incubated with each primary antibody for 3 h at room temperature. The following antibodies were used: anti-human monoclonal ΔNp63 antibody (clone 4A4, Dako Cytomation; diluted 1:200), anti-human monoclonal Ki-67 antibody (clone MIB-1, Dako Cytomation; diluted 1:100) as a marker of cell proliferation activity, and anti-human monoclonal cytokeratin (CK) 14 antibody (clone LL002, Chemicon, USA; diluted 1:300) as a marker of basal cells. The sections were rinsed twice for 10 min with PBS and incubated with secondary antibodies conjugated with peroxidase-labeled amino acid polymer for 1 h at room temperature. After rinsing with PBS twice for 10 min, the immunoreactivity was visualized by immersing the sections in 3, 3'-diaminobenzidine and 0.6% hydrogen peroxide (DAB substrate kit, Nichirei, Japan). Subsequently, the sections were counterstained with Mayer's hematoxylin, dehydrated in graded ethanol (75, 85, 90, 95 and 100%), cleared with xylene, and finally mounted with permanent mounting medium (Mount-Quick, Daido Sangyo, Japan). Negative controls were prepared by substituting PBS for each primary antibody. To evaluate the expression of ΔNp63, Ki-67, and CK14 in the OL and OSCC, positively stained cells were counted in at least three randomly selected areas at magnifications x200, and then each percentage of these positive cells was calculated as a labeling index (LI). The LI was computed by dividing the number of the positively stained cells by that of the epithelial-derived cells excluding the salivary epithelial cells. The patients with OSCC were divided into two groups based on mean value of ΔNp63-LI: the low ΔNp63-LI group, and the high ΔNp63-LI group.

Statistical analyses. All statistical analyses in the present study were performed with JMP software version 8 (SAS Institute, Japan). χ^2 test, Kruskal-Wallis test, and Mann-Whitney *U* test were used to assess the significant differences between each group. Survival rates were calculated and evaluated by the Kaplan-Meier method and the log-rank test, respectively. A $p<0.05$ was considered statistically significant.

Results

Expression of ΔNp63 proteins in normal oral epithelium and oral leukoplakia. In the NOE and OL, the immunoreactivities for ΔNp63, Ki-67, and CK14 were detected in all specimens.

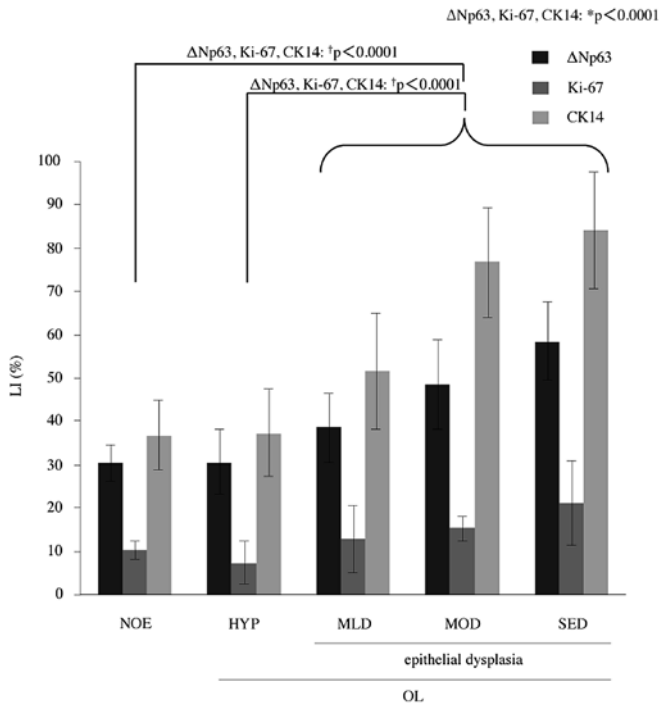

Figure 2. Association of the LIs of ΔNp63, Ki-67, and CK14 with the degree of epithelial dysplasia in the OL. Each LI of these dysplasia is significantly higher in the epithelium, compared with the NOE and HYP (†p<0.0001). Furthermore, significant increase of each LI according to the severity of epithelial dysplasia is found (*p<0.0001). The bars show the standard deviations of the means. Statistical analyses were performed by *Kruskal-Wallis test; and †Mann-Whitney *U* test.

In the NOE and HYP patients, ΔNp63 was localized in the cell nuclei of the basal and parabasal layers (Fig. 1b). Ki-67 was found more frequently in the cell nuclei of the parabasal layer rather than the basal layer, and the expression of CK14 was localized in the cytoplasm of the basal layer (Fig. 1c and d). Meanwhile, in the OL with epithelial dysplasia, the distributions of ΔNp63, Ki-67, and CK14 more widely extended from the basal layer toward the outer layer of the oral epithelium (Fig. 1f-h, j-l, and n-p). In the MLD, the expression of ΔNp63 was detected in the basal and suprabasal layers, while it was expressed in almost all layers in the MOD and SED. The expression patterns of Ki-67 and CK14 in the OL were also similar to that of ΔNp63.

To quantify the expression of ΔNp63, Ki-67, and CK14 in the NOE and OL, each LI was calculated. The ΔNp63-LI in the OL with epithelial dysplasia was significantly higher than that in each of the NOE and HYP (Mann-Whitney *U* test, †p<0.0001; Fig. 2). Furthermore, the ΔNp63-LI was significantly increased in the OL in association with the severity of epithelial dysplasia (Kruskal-Wallis test, *p<0.0001; Fig. 2). However, significant associations of the ΔNp63-LI with clinical findings including gender, age, and lesion sites, were not found (data not shown). The LIs of Ki-67 and CK14 in the OL were also significantly increased with the severity of epithelial dysplasia as well as the ΔNp63-LI (Kruskal-Wallis test, *p<0.0001; Fig. 2).

The association of the ΔNp63-LI with the malignant transformation of the OL was further examined. The frequency of the malignant transformation into OSCC was 6 (5.4%) of 112 patients with the OL, and the ΔNp63-LI of the OL with

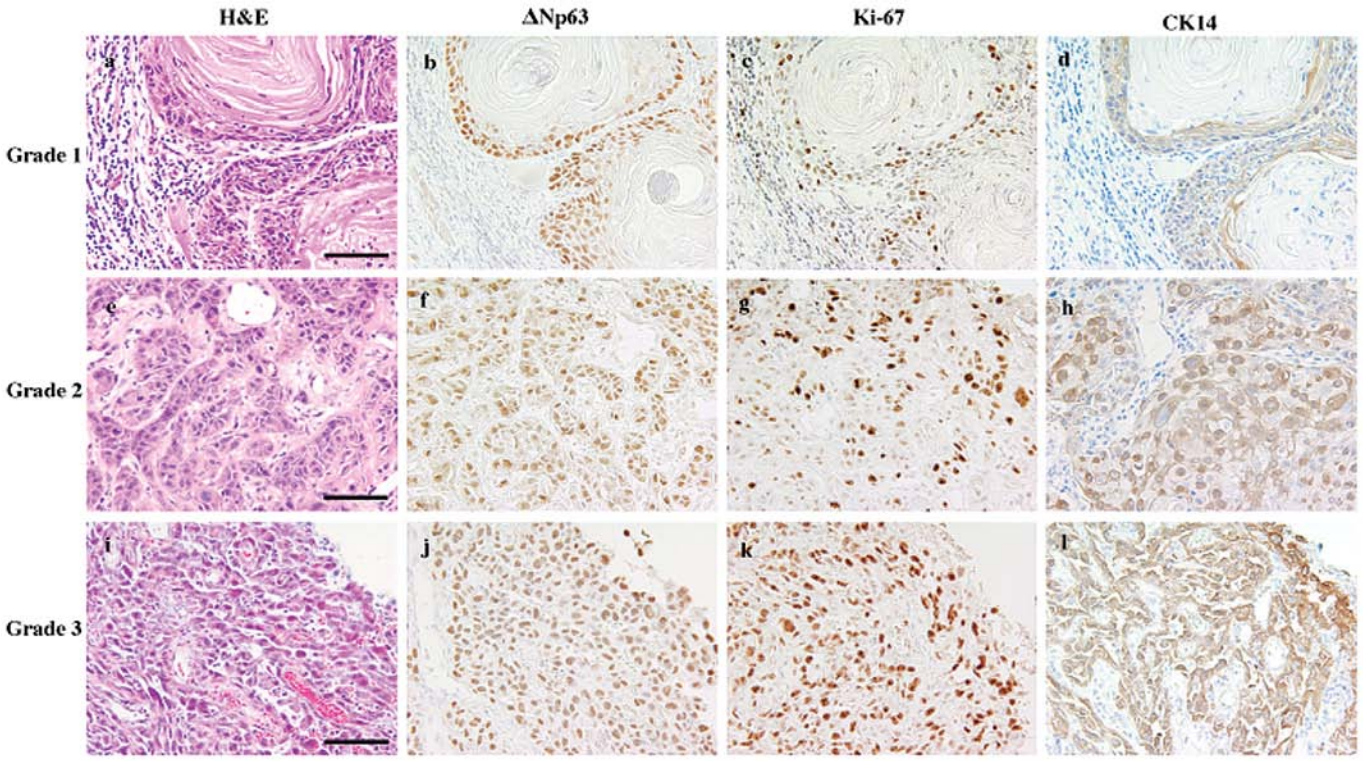

Figure 3. Immunohistochemical detection of ΔNp63, Ki-67, and CK14 in the OSCC. Each section was stained with H&E (a, e, and i), anti-ΔNp63 (b, f, and j), anti-Ki-67 (c, g, and k), and anti-CK14 (d, h, and l) monoclonal antibodies. In the well differentiated OSCC, the ΔNp63 expression is localized only in the outer edge of the cancer nest (b). Meanwhile, in the moderately and poorly differentiated OSCC, it is detected in almost all cancer cells (f and j). The expression patterns of Ki-67 and CK14 also resemble that of ΔNp63 (c, d, g, h, k, and l). (a-d) Grade 1 (well differentiated OSCC); (e-h) Grade 2 (moderately differentiated OSCC); (i-l) Grade 3 (poorly differentiated OSCC); scale bars, 100 μm.

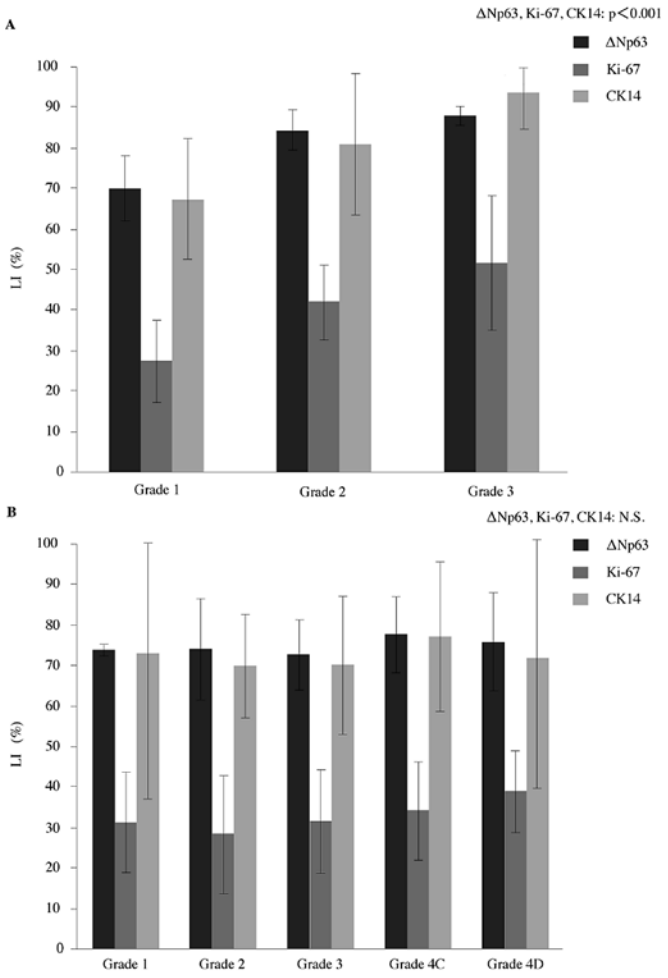

Figure 4. Association of the LIs of ΔNp63, Ki-67, and CK14 with the histopathologic findings (A, histologic grade; B, mode of tumor invasion) in the OSCC. (A) Each LI increases significantly in association with the histologic grade (p<0.0001). However, no association of each LI with the mode of tumor invasion is found as shown in (B). The bars show the standard deviations of the means. Statistical analyses were performed by Kruskal-Wallis test.

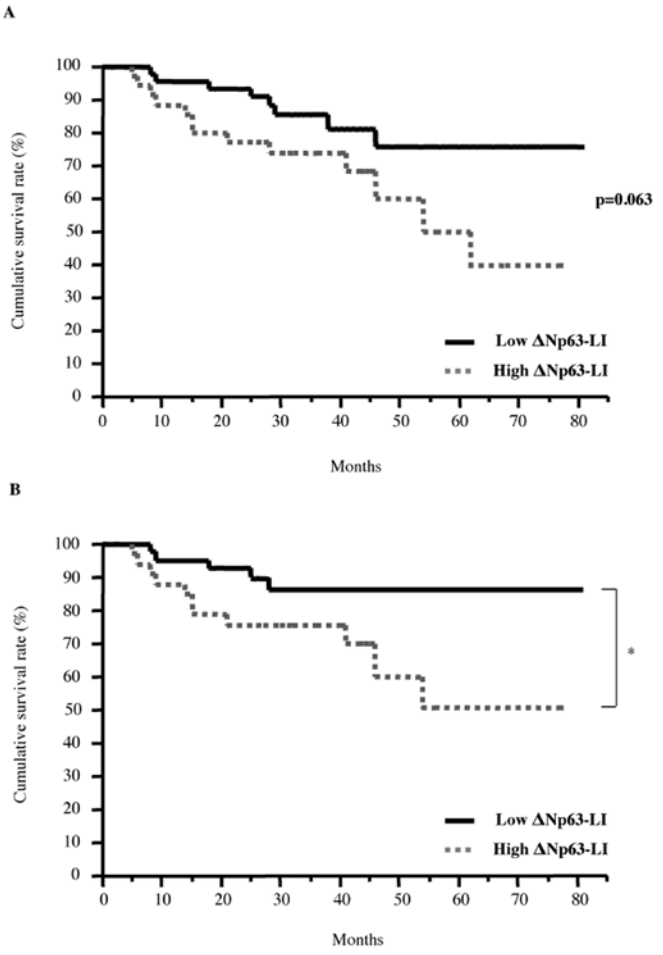

Figure 5. Survival curves according to the ΔNp63-LI in the OSCC. (A) No significant difference between these groups is found in the overall cumulative survival curves (p=0.063). The overall cumulative survival rates for 5 years in the high and low ΔNp63-LI groups were 40.0 and 76.1%. In the cause-specific survival curves, the prognosis in the high ΔNp63-LI group is poorer than that in the low ΔNp63-LI group as shown in (B) (*p<0.05). The cumulative survival rates for 5 years in the high and low ΔNp63-LI groups are 51.9 and 86.0%, respectively. Statistical analyses were performed by log-rank test.

malignant transformation was significantly higher than that of the OL without malignant transformation (49.3 vs. 34.2%; Mann-Whitney *U* test, p<0.01; Table III).

Expression of ΔNp63 proteins in OSCC. In the OSCC patients, ΔNp63, Ki-67, and CK14 were over-expressed, compared with the NOE (ΔNp63-LI, 73.8 vs. 30.4%; Ki-67-LI, 31.4 vs. 10.3%; CK14-LI, 71.5 vs. 36.8%; Mann-Whitney *U* test, p<0.0001, respectively). The expression patterns of these markers in OSCC patients varied according to the histologic grade of the carcinoma. Indeed, in the well differentiated OSCC, ΔNp63 was detected only in the outer layers of the cancer nest, whereas the carcinoma cells with keratinization in the center lacked immunoreactivity for ΔNp63 (Fig. 3b). Ki-67 and CK14 was also localized in the outer layers of the cancer nest as well as ΔNp63 (Fig. 3c and d). In the moderately and poorly differentiated OSCCs, ΔNp63, Ki-67, and CK14 were expressed in almost all of the cancer cells (Fig. 3f-h and j-l). In the association of these LIs with the histologic grade, each LI of ΔNp63, Ki-67, and CK14 increased significantly in association with the histologic grade (p<0.0001, Fig. 4A). Meanwhile, each of these LI was not significantly associated with the mode of tumor invasion (Fig. 4B).

Association of ΔNp63-LI with clinical characteristics of patients with OSCC. The associations of the ΔNp63-LI with the clinical characteristics of the patients with OSCC were further examined. The prevalence of cervical lymph node or distant metastasis in the high ΔNp63-LI (mean value >73.8%) group was significantly higher than that in the low ΔNp63-LI (mean value ≤73.8%) group (Table IV). On the contrary, other clinical factors including gender, primary site, clinical growth pattern, clinical T classification, clinical stage, and local recurrence, did not show significant differences between these groups.

Moreover, to examine the correlation between the ΔNp63-LI and the prognosis of the patients with OSCC, survival rates were calculated by the Kaplan-Meier method. Cause-specific cumulative survival curves indicated that patients in the high ΔNp63-LI group had a significantly more unfavorable outcome than patients in the low ΔNp63-LI group (log-rank test, p<0.05; Fig. 5A), and the cumulative survival rates for 5 years in the high and low ΔNp63-LI groups were 51.9 and 86.0%, respectively.

Table III. Comparison of the ΔNp63-LIs between the OL with and without malignant transformation into OSCC.

	Cases (%)	HYP	MLD	MOD	SED	ΔNp63-LI
Malignant transformation						
Yes	6 (5.4)	0	0	5	1	49.3%[a]
No	106 (94.6)	76	22	3	5	34.0% [a]
Total	112	76	22	8	6	

[a]Statistical analyses were performed by Mann-Whitney *U* test; p=0.0035.

Table IV. Relationship between the ΔNp63-LIs and the clinical characteristics of the patients with OSCC.

	Cases	Low ΔNp63-LI	High ΔNp63-LI	p-value[a]
Cases	81	41	40	
Gender				
Male	58	29	29	N.S.
Female	23	12	11	
Primary site				
Tongue	42	29	13	N.S.
Gingiva	27	9	18	
Floor of mouth	9	2	7	
Buccal mucosa	3	1	2	
Clinical growth pattern				
Superfacial type	8	3	5	N.S.
Exophytic type	15	6	9	
Endophytic type	58	32	26	
Clinical T stage				
T1	16	9	7	N.S.
T2	35	21	14	
T3	8	4	4	
T4	22	7	15	
Cervical lymph node metastasis				
No	45	27	18	p<0.05
Yes	36	14	22	
Distant metastasis				
No	74	40	34	p<0.05
Yes	7	1	6	
Clinical stage				
I	15	9	6	N.S.
II	28	18	10	
III	12	5	7	
IV	26	9	17	
Local recurrence				
No	69	37	32	N.S.
Yes	12	4	8	

[a]Statistical analyses were performed by χ^2 test.

Furthermore, the overall cumulative survival rates in the high and low ΔNp63-LI groups were 40.0 and 76.1%, respectively, though no significant differences between these groups was found (log-rank test, p=0.063; Fig. 5B).

Discussion

In this study, we first demonstrated the different expression patterns of ΔNp63, Ki-67 and, CK14 in NOE and OL. In the NOE and HYP, proliferating cells positive for Ki-67 were localized mainly in the parabasal layer rather than basal layer, which was different from the expression pattern of ΔNp63 as a maker of keratinocyte stem cells. These different expression patterns might be caused by asymmetric cell division in keratinocyte stem cells located in the basal layer. In previous studies, keratinocyte stem cells have been shown to be slow-cycling *in vivo* and possess the abilities to self-renew and generate a rapidly dividing progenitor cells, so-called transit-amplifying (TA) cells, through asymmetric cell division (33-39). After several rounds of cell division, all TA cells withdraw permanently from the cell cycle and undergo terminal differentiation. Thus, Ki-67$^+$ cells identified in parabasal layer of the NOE might be TA cells, whereas ΔNp63$^+$Ki-67$^-$ cells in the basal layer might be stem cell-rich subpopulation.

In the different degrees of epithelial dysplasia, it was observed that the number of ΔNp63$^+$ cells increased in association with the severity of epithelial dysplasia. These results are in accordance with those of previous studies (40-42). Koster *et al* have reported that ΔNp63 maintains the phenotype of the basal cells by preventing their differentiation (43,44). Thus, in the normal oral stratified squamous epithelium, the expression of ΔNp63 proteins should be down-regulated and rarely detected in upper layers of epithelium. However, in the dysplastic epithelium, it is considered that dysplastic keratinocytes above the basal layers remain expressing ΔNp63 providing an anti-differentiation effect and a proliferative capacity, and thus architectural disturbance occurred in the epithelium. Recent studies have also indicated that ΔNp63 directly binds to a specific DNA sequence in the *CK14* enhancer and thereby induces the transcription of *CK14* (45,46). In this study, the expression pattern of ΔNp63 was almost consistent with that of CK14 in NOE and OL, suggesting that ΔNp63 directly governs the expression of CK14 and regulates differentiation of the basal cells in NOE and dysplastic keratinocytes in OL. Furthermore, the ΔNp63-LI in the OL with the malignant transformation into the OSCC was higher than that in the OL without. Chen *et al* have reported that subsets of moderate and severe dysplasia showing ΔNp63-positive staining have undergone malignant transformation into OSCC within 5 years of follow-up (41). Takeda *et al* have demonstrated that distributional disturbance of ΔNp63-positive cells in epithelial dysplasia might play a role in oral tumorigenesis (40). Based on these results, it is suggested that ΔNp63 is possibly involved in the tumorigenesis of OSCC. Moreover, these results suggest that ΔNp63 is a useful marker that is indicative of the degree of epithelial dysplasia and predictive of malignant transformation.

In the OSCC, the number of ΔNp63$^+$ cells was remarkably increased in comparison with NOE and OL. Furthermore, the expression of ΔNp63 showed the same distribution as Ki-67 and CK14, being observed at the periphery of the cancer nest of well differentiated OSCC. These immunohistochemical results are consistent with those of previous studies on OSCC and lung squamous cell caricinoma (47,48). However, to our knowledge, few studies have examined the association of ΔNp63 immunoreactivity with the clinicopathogic behaviors of the OSCC. Thus, the present study was undertaken to elucidate whether the ΔNp63 positive rate could be related to the clinicopathologic findings of OSCC. Our analyses indicate that the higher the histologic grade, the higher the ΔNp63 positive rates were. Lo Muzio *et al* also demonstrated positive correlations between the histologic grade and ΔNp63 positive rate in head and neck carcinoma (49). Moreover, other studies found significant association between the ΔNp63 positive rate and the degree of differentiation in nasopharyngeal carcinoma, lung carcinoma, and epidermal carcinoma (23,24,50,51). These results suggested that ΔNp63 was closely associated with the cell maturation of cancer cells as well as normal stratified squamous epithelium and the positive staining might reflect the immaturity of the cancer cell lineage.

In the association of the ΔNp63-LI with the clinical characteristics in the OSCC, significant differences between the high and low ΔNp63-LI groups also showed in the frequencies of cervical lymph node and distant metastasis. In OSCC, it has been widely accepted that the incidence of cervical lymph node metastasis is closely associated with mode of tumor invasion in the tumor-host borderline based on Yamamoto-Kohama's criterion (32). Our results also indicated significant association of the frequency of cervical lymph node metastasis with mode of tumor invasion (data not shown). However, no significant association of the ΔNp63-LI with mode of tumor invasion was shown in this study. These results suggested that ΔNp63-LI, independently of mode of tumor invasion, was useful to predict cervical lymph node metastasis.

In the cause-specific survival analysis by the Kaplan-Meier method, the patients in the high ΔNp63-LI group had unfavorable outcomes. Several studies on the assessment of correlation between the positive rate of p63 and patient prognosis in head and neck carcinoma have been reported (25,49,52-55). Lo Muzio *et al* and Moergel *et al* also have found that the high expression of p63 is significantly associated with the poor prognosis (49,52). In contrast, some reports have shown that reduced p63 expression associates with poor prognosis in the head and neck carcinoma (53-55). Such discrepancies might be due to the monoclonal A4A antibody used in immunohistochemical analyses. Foschini *et al* have been suggested that A4A antibody recognizes all of p63 isoforms (TAp63, ΔNp63) that may have different functions (56). Therefore, this antibody was not able to discriminate between the different isoforms. In the present study, the monoclonal A4A antibody made by Dako Cytomation, which is considered to recognize ΔNp63 isoforms, was used. According to the manufacturer's specification, this antibody was raised against amino acids 1-205 mapping at the N-terminal portion of ΔNp63. Several studies using the monoclonal A4A antibody made by this company showed similar findings to our results, meanwhile previous studies using other antibodies had opposite findings and outcomes (25,49,52). Foschini *et al* also analyzed the expression of TAp63 in the excised OSCC specimens by reverse transcription-polymerase chain reaction and nested polymerase chain reaction, and found that the patients with TAp63 expression have favorable outcomes (56). Therefore, as indicated in this study, it is suggested that high expression

of ΔNp63 which had dominant negative activity for TAp63 associates with poor prognosis in the OSCC.

However, recent *in vitro* studies have shown that the reduced expression of ΔNp63 upregulates several genes including *N-cadherin* and *Wnt-5A* characterized as promoting tumor invasion and metastasis (57,58). Barbieri *et al* also have been found that loss of ΔNp63 induces to decrease the expression of *Wnt-4*, and Taki *et al* reported that down-regulation of *Wnt-4* and up-regulation of *Wnt-5A* expression are associated with epithelial-mesenchymal transition (EMT) involved in invasion and metastasis of cancer cells (57,59). Therefore, the reduced ΔNp63 expression in the cancer cells may lead to acquirement of EMT and promote tumor invasion and metastasis. However, we know no apparent reason for the discrepancy between our results and those of *in vitro* studies. Therefore, in order to clarify a role of ΔNp63 in the OSCC, further studies *in vivo* and *in vitro* might be expected.

Acknowledgments

This work was supported by a Grant-in-Aid (no. 20791531) from the Japanese Ministry of Education, Culture, Science, Sports and, Technology of Japan.

References

1. Yang A, Kaghad M, Wang Y, *et al*: p63, a p53 homolog at 3q-27-29, encodes multiple products with transactivating, death-inducing, and dominant-negative activities. Mol Cell 2: 305-316, 1998.
2. Augustin M, Bamberger C, Paul D and Schmale H: Cloning and chromosomal mapping of the human p53-related KET gene to chromosome 3q27 and its murine homolog Ket to mouse chromosome 16. Mamm Genome 9: 899-902, 1998.
3. Trink B, Okami, Wu L, Sriuranpong V, Jen J and Sidransky D: A new human p53 homologue. Nat Med 4: 747-748, 1998.
4. Osada M, Ohba M, Kawahara C, *et al*: Cloning and functional analysis of human p51, which structurally and functionally resembles p53. Nat Med 4: 839-843, 1998.
5. Jost CA, Marin MC and Kaelin WG: p73 is a human p53-related protein that can induce apoptosis. Nature 389: 191-194, 1997.
6. Hagiwara K, McMenamin MG, Miura K and Harris CC: Mutational analysis of the p63/p73L/p51/p40/CUSP/KET gene in human cancer cell lines using intronic primers. Cancer Res 59: 4165-4169, 1999.
7. Bourdon JC, Fernandes K, Murray-Zmijewski F, *et al*: p53 isoforms can regulate p53 transcriptional activity. Genes Dev 19: 2122-2137, 2005.
8. Hibi K, Trink B, Patturajan M, *et al*: AIS is an oncogene amplified in squamous cell carcinoma. Proc Natl Acad Sci USA 97: 5462-5467, 2000.
9. Ratovitski EA, Patturajan M, Hibi K, Trink B, Yamaguchi K and Sidransky D: p53 associates with and targets Delta Np63 into a protein degradation pathway. Proc Natl Acad Sci USA 98: 1817-1822, 2001.
10. Patturajan M, Nomoto S, Sommer M, *et al*: DeltaNp63 induces beta-catenin nuclear accumulation and signaling. Cancer Cell 1: 369-379, 2002.
11. Stiewe T, Zimmermann S, Frilling A, Esche H and Putzer BM: Transactivation-deficient DeltaTA-p73 acts as anoncogene. Cancer Res 62: 3598-3602, 2002.
12. Wu G, Nomoto S, Hoque MO, *et al*: DeltaNp63alpha and TAp63alpha regulate transcription of genes with distinct biological functions in cancer and development. Cancer Res 63: 2351-2357, 2003.
13. Yang A and McKeon F: P63 and P73: P53 mimics, menaces and more. Nat Rev Mol Cell Biol 1: 199-207, 2000.
14. Mills AA: p63: oncogene or tumor suppressor? Curr Opin Genet Dev 16: 38-44, 2006.
15. Yang A, Schweitzer R, Sun D, *et al*: p63 is essential for regenerative proliferation in limb, craniofacial and epithelial development. Nature 398: 714-718, 1999.
16. Pellegrini G, Dellambra E, Golisano O, *et al*: p63 identifies keratinocyte stem cells. Proc Natl Acad Sci USA 98: 3156-3161, 2001.
17. Barker N, Ridgway RA, van Es JH, *et al*: Crypt stem cells as the cells-of-origin of intestinal cancer. Nature 457: 608-611, 2009.
18. Zhu L, Gibson P, Currle DS, *et al*: Prominin 1 marks intestinal stem cells that are susceptible to neoplastic transformation. Nature 457: 603-607, 2009.
19. Kramer IR, Lucas RB, Pindborg JJ and Sobin LH: Definition of leukoplakia and related lesions: an aid to studies on oral precancer. Oral Surg Oral Med Oral Pathol 46: 518-539, 1978.
20. Burkhardt A: Premalignant changes in the mouth mucosa. Proposals for nomenclature by an international expert commission. Pathologe 6: 126-132, 1985.
21. Schepman KP, Van Der Meij EH, Smeele LE and van Der Waal I: Malignant transformation of oral leukoplakia: a follow-up study of a hospital-based population of 166 patients with oral leukoplakia from The Netherlands. Oral Oncol 34: 270-275, 1998.
22. Nylander K, Coates PJ and Hall PA: Characterization of the expression pattern of p63α and ΔN-p63 in benign and malignant oral epithelial lesions. Int J Cancer 87: 368-372, 2000.
23. Chen YK, Huse SS and Lin LM: Differential expression of p53, p63, and p73 proteins in human buccal squamous cell carcinomas. Clin Otolaryngol 28: 451-455, 2003.
24. Chen YK, Huse SS and Lin LM: Immunohistochemical demonstration of p63 in DMBA-induced hamster buccal pouch squamous cell carcinogenesis. Oral Diseases 9: 235-240, 2003.
25. Pruneri G, Pignataro L, Manzotti M, *et al*: p63 in laryngeal squamous cell carcinoma: evidence for a role of TA-p63 down-regulation in tumorigenesis and lack of prognostic implications of p63 immunoreactivity. Lab Invest 82: 1327-1334, 2002.
26. Glickman JN, Yang A, Shahsafaei A, Mckeon F and Odze RD: Expression of p53-related protein p63 in the gastrointestinal tract and in esophageal metaplastic and neoplastic disorders. Hum Pathol 32: 1157-1165, 2001.
27. Geddert H, Kiel S, Heep HJ, Gabbert HE and Sarbia M: The role of p63 and deltaNp63 (p40) protein expression and gene amplification in esophageal carcinogenesis. Hum Pathol 34: 850-856, 2003.
28. Massion PP, Taflan PM, Jamshedur SM, *et al*: Significance of p63 amplification and overexpression in lung cancer development and prognosis. Cancer Res 63: 7113-7121, 2003.
29. Gale N, Pilch BZ, Sindramsky D, *et al*: Epithelial precursor lesions. In: World Health Organization Classification of Tumors. Pathology and Genetics of Head and Neck Tumors. Barnes L, Eveson J, Reichart P and Sidransky D (eds). Iarc Press, Lyon, pp177-179, 2005.
30. Wahi PN, Cohen B, Luthra UK, *et al*: Histological consideration. In: Histological Typing of Oral and Oropharyngeal tumors. Wahi PN, Cohen B, Luthra UK, *et al* (eds). World Health Organization, Geneva, pp15-19, 1977.
31. Sobin LH, Witte Sobin LH and Wittekind Ch (eds). TNM Classification of Malignant Tumors. Wiley-Liss, Inc., New York, NY, 2002.
32. Yamamoto E, Miyakawa A and Kohama G: Mode of invasion and lymph node metastasis in squamous cell carcinoma of the oral cavity. Head Neck Surg 6: 938-947, 1984.
33. Fuchs E and Segre JA: Stem cells: a new lease on life. Cell 100: 143-155, 2000.
34. Watt FM and Hogan BL: Out of Eden: stem cells and their niches. Science 287: 1427-1430, 2000.
35. Lehrer MS, Sun TT and Lavker RM: Strategies of epithelial repair: modulation of stem cell and transit amplifying cell proliferation. J Cell Sci 111: 2867-2875, 1998.
36. Morris RJ and Potten CS: Slowly cycling (label-retaining) epidermal cells behave like clonogenic stem cells *in vitro*. Cell Prolif 27: 279-289, 1994.
37. Morris RJ and Potten CS: Highly persistent label-retaining cells in the hair follicles of mice and their fate following induction of anagen. J Invest Dermatol 112: 470-475, 1999.
38. Taylor G, Lehrer MS, Jensen PJ, Sun TT and Lavker RM: Involvement of follicular stem cells in forming not only the follicle but also the epidermis. Cell 102: 451-461, 2000.
39. Cotsarelis G, Cheng SZ, Dong G, Sun TT and Lavker RM: Existence of slow-cycling limbal epithelial basal cells that can be preferentially stimulated to proliferate: implications on epithelial stem cells. Cell 57: 201-209, 1989.
40. Takeda T, Sugihara K, Hirayama Y, Hirano M, Tanuma JI and Semba I: Immunohistological evaluation of Ki-67, p63, CK19 and p53 expression in oral epithelial dysplasias. J Oral Pathol Med 35: 369-375, 2006.

41. Chen YK, Hsue SS and Lin LM: Expression of p63 protein and mRNA in oral epithelial dysplasia. J Oral Pathol Med 34: 232-239, 2005.
42. Vered M, Allon I and Dayan D: Maspin, p53, p63, and Ki-67 in epithelial lesions of the tongue: from hyperplasia through dysplasia to carcinoma. Oral Pathol Med 38: 314-320, 2009.
43. Koster MI and Roop DR: The role of p63 in development and differentiation of the epidermis. J Dermatol Sci 34: 3-9, 2004.
44. Koster MI, Kim S, Mills AA, De Mayo FJ and Roop DR: p63 is the molecular switch for initiation of an epithelial stratification program. Genes Dev 18: 126-131, 2004.
45. Candi E, Rufini A, Terrinoni A, *et al*: Differential roles of p63 isoforms in epidermal development: selective genetic complementation in p63 null mice. Cell Death Differ 13: 1037-1047, 2006.
46. Romano RA, Birkaya B and Sinha SJ: A functional enhancer of keratin14 is a direct transcriptional target of deltaNp63. J Invest Dermatol 127: 1175-1186, 2007.
47. Choi HR, Batsakis JG, Zhan F, Sturgis E, Luna MA and El-Naggar AK: Differential expression of p53 gene family members p63 and p73 in head and neck squamous tumorigenesis. Hum Pathol 33: 158-164, 2002.
48. Wang BY, Gil J, Kaufman D, Gan L, Kohtz DS and Burstein DE: p63 in pulmonary epithelium, pulmonary squamous neoplasms, and other pulmonary tumors. Hum Pathol 33: 921-926, 2002.
49. Lo Muzio L, Santarelli A, Caltabiano R, *et al*: p63 overexpression associates with poor prognosis in head and neck squamous cell carcinoma. Hum Pathol 36: 187-194, 2005.
50. Tsujita-Kyutoku M, Kiuchi K, Danbara N, Yuri T, Senzaki H and Tsubura A: p63 expression in normal human epidermis and epidermal appendages and their tumors. J Cutan Pathol 30: 11-17, 2003.
51. Crook T, Nicholls JM, Brooks L, O'Nions J and Allday MJ: High level expression of deltaN-p63: a mechanism for the inactivation of p53 in undifferentiated nasopharyngeal carcinoma (NPC)? Oncogene 19: 3439-3444, 2000.
52. Moergel M, Abt E, Stockinger M and Kunkel M: Overexpression of p63 is associated with radiation resistance and prognosis in oral squamous cell carcinoma. Oral Oncol 46: 667-671, 2010.
53. Oliveira LR, Ribeiro-Silva A and Zucoloto S: Prognostic impact of p53 and p63 immunoexpression in oral squamous cell carcinoma. J Oral Pathol Med 36: 191-197, 2007.
54. Oliveira LR, Ribeiro-Silva A, Costa JP, Simões AL, Matteo MA and Zucoloto S: Prognostic factors and survival analysis in a sample of oral squamous cell carcinoma patients. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 106: 685-695 2008.
55. Takahashi Y, Noguchi T, Takeno S, Kimura Y, Okubo M and Kawahara K: Reduced expression of p63 has prognostic implications for patients with esophageal squamous cell carcinoma. Oncol Rep 15: 323-328, 2006.
56. Foschini MP, Gaiba A, Cocchi R, *et al*: Pattern of p63 expression in squamous cell carcinoma of the oral cavity. Virchows Arch 444: 332-339, 2004.
57. Barbieri CE, Tang LJ, Brown KA and Pietenpol JA: Loss of p63 leads to increased cell migration and up-regulation of genes involved in invasion and metastasis. Cancer Res 66: 7589-7597, 2006.
58. Cavallaro U: N-cadherin as an invasion promoter: a novel target for antitumor therapy? Curr Opin Investig Drugs 5: 1274-1278, 2004.
59. Taki M, Kamata N, Yokoyama K, Fujimoto R, Tsutsumi S and Nagayama M: Down-regulation of Wnt-4 and up-regulation of Wnt-5a expression by epithelial-mesenchymal transition in human squamous carcinoma cells. Cancer Sci 94: 593-597, 2003.