Abstract: Background. TP73, a p53 homologue gene, shares similar structural sequences with p53. The aim of this study was to investigate the p73 expression for human buccal epithelial dysplasia (ED) and squamous cell carcinoma (SCC).

Methods. Seventy-five samples of human buccal ED, including mild, moderate, and severe ED (25 samples in each category), were analyzed for p73 protein expression by means of immunohistochemistry. Twenty-five samples of human buccal SCCs were analyzed for p73 mRNA expression with reverse transcription-polymerase chain reaction (RT-PCR) and were also analyzed for protein expression with immunohistochemical analysis.

Results. By use of immunohistochemical analysis, nuclear staining of p73 protein was detected in a subset of normal mucosa, buccal ED, and SCC specimens. p73 nuclear staining was noted for the basal cells of normal buccal mucosa. For buccal lesions deriving from mild, moderate, and severe ED, p73 protein was observed in basal and parabasal layers and in more superficial cell layers corresponding to the spinous layer. For well-differentiated carcinomas, p73 immunoreactivity was chiefly observed among the less-differentiated cells in the periphery of carcinomatous clusters, whereas moderately differentiated carcinomas revealed homogeneous staining, involving nearly all of the tumor cells. On RT-PCR, the expression of p73 mRNA from buccal SCC was noted to be compatible with the findings of immunohistochemical analysis. An electrophoretic band with a 180-bp PCR product corresponding to p73 mRNA has been observed. The expression of p73 seemed to be significantly elevated for specimens of buccal ED (protein level) and SCC (protein and mRNA levels) compared with the analogous expression for normal control tissue (Fisher’s exact test, \( p < .001 \)).

Conclusions. The data suggest that p73 expression may be (1) associated with the differentiation of oral stratified squamous epithelium, (2) an early event in human oral carcinogenesis, and (3) associated with the nodal status of patients with oral carcinoma and a possible indicator for malignant change of oral ED.
TP73 has been cloned and mapped at chromosome 1p36.2-3, a region that reveals highly frequent loss of heterozygosity for various human cancers.1 This gene encodes a protein the structure of which is highly homogeneous to p53 in the three domains: transcriptional activation domain, DNA-binding domain, and oligomerization domain.2 p73 also shares some common functions with the p53 protein, including inhibiting cell growth and inducing apoptosis.2 Mutations at the TP53 are detected for more than 50% of many different types of cancers,3 including human head and neck squamous cell carcinoma (SCC).4 By contrast to p53, a mutant TP73 has rarely been found among human tumors.5,6 In addition, unlike p53, p73 has multiple splice variants (α, β, γ, and δ), which may have different biologic characteristics and cellular specificities.7

In this study, we examined the p73 expression for human buccal epithelial dysplasia (ED) and SCC to obtain more data for elaboration of its role in human oral squamous cell carcinogenesis.

source of tissue. The tissue specimens used in this study were obtained from patients who had visited our institution. Patients were followed up for a maximum of 2 years. All of the patients had been exposed to risk factors such as betel-quid chewing, cigarette smoking, or alcohol consumption. All tissues (including those normal samples) were obtained with the patients’ informed consent and with the approval of the Ethics Committee for Scientific Research on Human Beings at this institution. For all cases, both test and control, no treatment had been undertaken before the removal of any of the oral tissue.

Specimens of the diseased buccal mucosa were obtained from samples from 75 male patients aged 36 to 65 years (mean, 44 years). The diseased buccal mucosa involved in this study was composed of mild, moderate, and severe ED (25 samples in each category). A representative part of each specimen was fixed in 10% neutral buffered formalin solution for approximately 24 hours, dehydrated in various graded alcohol solutions, cleared in xylene, and embedded in paraﬁn for histologic diagnosis of various degrees of severity of ED and subsequent immunohistochemical analysis for p73 protein. The histopathologic characteristics of ED include: (1) basal layer hyperplasia, (2) nuclear enlargement and hyperchromatism, (3) loss of intercellular adhesion and normal polarization, (4) abnormal mitoses above the basal cell layer, (5) individual cell keratinization within the spinous layer, (6) cellular pleomorphism, (7) drop-shaped epithelial ridges, (8) irregular stratification, and (9) altered nuclear–cytoplasmic ratio.8

Among these histologic changes, the presence of basal cell hyperplasia, nuclear enlargement and hyperchromatism, and drop-shaped rete ridges are regarded as the minimal criteria for the histologic diagnosis of ED.9 Diagnosis was successfully achieved, and the degrees of dysplasia were graded with reference to the following criteria10: (1) mild ED, dysplastic alterations conﬁned to the lower third of the buccal epithelium; (2) moderate ED, dysplastic changes observed for up to two thirds of the thickness of the buccal epithelium; and (3) severe dysplasia, more than two thirds but less than the whole thickness of the buccal epithelium contains the dysplastic cells.

Specimens of buccal SCC were obtained from fresh tissue samples of surgically resected tumors from 25 male patients aged 32 and 70 years (mean, 55 years). Cases were selected from patients (with buccal SCC) who had undergone elective neck dissection; 17 of them demonstrated histologically conﬁrmed cervical lymph node involvement. Tumors were graded according to the World Health Organization classiﬁcation11; 15 samples were well differentiated, and 10 were moderately differentiated. A portion of the surgically resected buccal tissue was immediately frozen in liquid nitrogen for subsequent RNA extraction and use in reverse transcription-polymerase chain reaction (RT-PCR) studies for p73 mRNA. Another portion was ﬁxed in 10% neutral buffered formalin solution for histologic diagnosis and subsequent immunohistochemical analysis for p73 protein.

Ten samples of normal buccal mucosa tissue taken from sex-matched and age-matched healthy individuals aged 36 to 62 years (mean, 47 years) were used as controls; none of these control subjects had ever chewed betel-quid or smoked cigarettes.

immunohistochemical analysis. Immunohistochemical analysis was performed by a standard avidin-biotin peroxidase complex (ABC) method.12 The primary antibody used was polyclonal antibody raised against p73 (Santa Cruz Biotechnology, Santa Cruz, CA; 1:100 dilution). Subsequent to deparaffinization in xylene and ethanol, tissue sections were treated three times with microwaves in 10 mM citrate buffer (pH = 6.0) for
5 minutes each to retrieve the antigenicity. The tissue sections were then treated in 0.3% H2O2-methanol and 10% normal goat serum (Dako, Santa Barbara, CA). All sections were subsequently incubated with the primary antibody at room temperature for 60 minutes. These sections were then incubated for a further 30 minutes at room temperature in the presence of biotinylated goat anti-rabbit immunoglobulin (Ig) G for p73 and biotinylated goat anti-mouse IgG for p53 (both at a dilution of 1:100; Vector, Burlingame, CA). The sites of peroxidase binding were visualized as brown reaction products as a result of a benzidine reaction. The sections were subsequently counterstained with hematoxylin. Positive and negative controls were used for each experiment. An esophageal SCC known to express high levels of p73 was used as positive controls. Negative controls were obtained by omitting the primary antibodies. Because p73 is a nuclear protein, only nuclear positivity was assessed. Samples with 10% or more positive nuclear-stained keratinocytes were classified as positive staining.13,14

Reverse Transcription-Polymerase Chain Reaction.
Total RNA was extracted by homogenizing the buccal tissue specimens in guanidium isothiocyanate followed by ultracentrifugation in cesium chloride, as described previously.15 The RNA concentration was determined by way of the sample’s optical density at a wavelength of 260 nm (by use of an OD260 unit equivalent to 40 μg/mL RNA).

Isolated total RNA (1 μg) was reverse-transcribed to cDNA in a reaction mixture (with a final volume of 20 μL) containing 4 μL of MgCl2 (5 mM), 2 μL of 10 × reverse transcription buffer (10 mM Tris-HCl, [pH = 9.0], 50 mM KCl, 0.1% Triton X-100 [Promega Corp., Madison, WI]), 2 μL of dNTP mixture (1 mM each), 0.5 μL of recombinant RNasin ribonuclease inhibitor (Promega Corp., Madison, WI; 1 μ/μL), 15 units of avian-myeloblastosis-virus (AMV) reverse transcriptase (high conc.) (15 μ/μg), 0.5 μg of oligo(dT)15 primer (Promega, catalogue no. A3500, Madison, WI). The reaction mixture was incubated for 15 minutes at 42°C. The AMV reverse transcriptase was inactivated by heating for 5 minutes at 99°C and then incubated at 0°C to 5°C for a further 5 minutes.

All oligonucleotide primers were purchased from Genset Corp. (La Jolla, CA). Primers for p7316 were 5′-CTCCCCGCTCTTGAAGAAAC-3′ and 5′-GTTGAAGTCCCTCCCGAGC-3′. Primers for β-actin17 were 5′-AAC CGC GAG AAG ATG ACC CAG ATC ATG TTT-3′ and 5′-AGC AGC CGT GGC CAT CTC TTG CTC GAA GTC-3′. The 20-μL first-strand cDNA synthesis reaction product obtained from the reverse tran-

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<th>p73 protein</th>
<th>p73 mRNA</th>
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<td>(+)</td>
<td>(-)</td>
</tr>
<tr>
<td>Mild ED</td>
<td>17*</td>
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<tr>
<td>Moderate ED</td>
<td>18*</td>
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<tr>
<td>Severe ED</td>
<td>18*</td>
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<tr>
<td>Squamous cell carcinoma</td>
<td>17*</td>
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<tr>
<td>Normal mucosa</td>
<td>2</td>
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Abbreviation: ED, epithelial dysplasia.
*Statistical significance compared with normal mucosa (Fisher’s exact test, p < .001).
scriptase reaction was diluted to 100 μL with nuclease-free water. The PCR amplification reaction mixture (with a final volume of 100 μL) contained 20 μL of diluted, first-strand cDNA reaction product (<10 ng/μL), 2 μL of cDNA reaction dNTPs (200 μM each), 8 μL of 10× reverse transcription buffer (10 mM Tris-HCl, pH = 9.0, 50 mM KCl, 0.1% Triton X-100), 50 pmol of upstream primer, 50 pmol of downstream primer, and 2.5 units of Taq DNA polymerase (Promega, catalogue no. M7660).

The PCR steps were carried out on a DNA thermal cycler (TaKaRa MP, Tokyo, Japan). Thermocycling conditions included denaturing at 94°C for 1 minute (one cycle), then denaturing at 94°C (60 seconds), annealing at 55°C (60 seconds) for p73 or at 60°C (60 seconds) for β-actin, and extending at 72°C (60 seconds) for 30 cycles, and a final extension at 72°C for 7 minutes. The β-actin primers were used as positive controls. Negative controls (ie, those conducted in the absence of RNA and reverse transcriptase) were also performed. Amplification products were analyzed by electrophoresis in a 2% agarose gel along with the relevant DNA molecular-weight marker (Boehringer, Mannheim, Germany) and stained with ethidium bromide. The PCR products were visualized as bands with an ultraviolet transilluminator. Photographs were taken with a Polaroid DS-300 camera (Applied Biosystems Taiwan, Taipei, Taiwan). The PCR products were then sequenced to confirm their identities by use of a T7 Sequenase version 2.0 kit (Amersham International, Little Chalfont, UK).

### Statistical Analyses.
Fisher’s exact test was used to analyze the association between categorical variables. All tests were two sided. A p value <.05 was selected as that confidence level considered to represent statistically significant difference between data sets.

### RESULTS

#### Immunohistochemical Analysis.
With the use of immunohistochemical analysis, nuclear staining of p73 protein was detected in a subset of buccal ED, SCC, and normal buccal mucosa (Table 1). p73 nuclear staining was located in basal cells of normal stratified squamous epithelium (Figure 1A). For lesions of mild, moderate, and severe ED, p73 protein was observed in basal

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**Table 2.** Correlation of p73 protein and mRNA expression for the primary buccal squamous cell carcinoma and the status of cervical lymph node metastasis (Fisher’s exact test, p < .001).

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<th>p73 protein (+)</th>
<th>p73 protein (−)</th>
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<tr>
<td>mRNA (+)</td>
<td>mRNA (−)</td>
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<tr>
<td>Buccal SCC with cervical node metastasis</td>
<td>15/14</td>
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Abbreviation: SCC, squamous cell carcinoma.
and parabasal layers and in more superficial cell layers corresponding to the spinous layer (Figures 1B–1D). For well-differentiated carcinomas, p73 immunoreactivity was chiefly observed for the less-differentiated cells located at the periphery of carcinomatous clusters and negative for areas of keratin pearl (Figure 2). Moderately differentiated carcinomas revealed homogeneous staining involving almost all of the tumor cells (Figure 3).

Reverse Transcription-Polymerase Chain Reaction. On RT-PCR, the proportional (percentage) expression of p73 mRNA for buccal SCC was noted to be compatible with the findings with immunohistochemical analysis (Tables 1 and 2). An electrophoretic band with a 180-bp PCR product corresponding to p73 mRNA was observed for 17 specimens of buccal SCC (17 of 25; 68%) (Figures 4A and 4B). On direct sequencing, this 180-bp band was confirmed to be part of the TP73. Two samples of normal buccal mucosa revealed bands of β-actin (350-bp). Lanes M (A and B) are the DNA molecular-weight markers.

Follow-up Data of Patients. In 2 years follow-up, neither recurrence nor death has been observed for all of the cancer patients. On the other hand, three (16.7%) of 18 cases of moderate ED and five (27.8%) of the 18 cases of severe variant showing p73 positivity have undergone malignant transformation to develop SCCs on 2 years follow-up.

Statistical Analyses. The expression of p73 seemed to be significantly elevated for specimens of buccal ED (protein level) and SCC (protein and mRNA levels) compared with the analogous expression for normal control tissue (Table 1). Also,

<table>
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<th>Table 3. Correlation of p73 protein expression for epithelial dysplasia and malignant transformation.</th>
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<tr>
<td>Moderate ED*</td>
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<tr>
<td>p73 protein (+)</td>
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<tr>
<td>With malignant transformation</td>
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<td>Without malignant transformation</td>
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Abbreviation: ED, epithelial dysplasia.

*Fisher’s exact test, $p > .05$.
†Fisher’s exact test, $p = .05$. 

FIGURE 4. Expression of p73 mRNA in buccal squamous cell carcinomas (SCCs) with reverse transcriptase-polymerase chain reaction (RT-PCR). A band of a 180-bp PCR product corresponding to p73 mRNA is observed for nine specimens of buccal SCC (lanes 1, 2, 4–7, 9, 10, and 12 in A; lanes 13, 15, 17–20, 22, and 24 in B). Lanes N (A and B) are the two normal buccal mucosa specimens showing a 180-bp PCR product corresponding to p73 mRNA. No band was observed in the negative control sample (lanes NC in A and B). All samples (lanes 1–15 and N in A; lanes 13–25 an N in B) apart from the negative control sample (lanes NC in A and B) reveal bands of β-actin (350-bp). Lanes M (A and B) are the DNA molecular-weight markers.
p73 expression (protein and mRNA levels) correlated significantly with cervical lymph node metastasis for cases of buccal SCC (Table 2). On the other hand, p73 expression for moderate ED (protein level) was statistically insignificant to SCC transformation, whereas p73 expression for severe ED was marginally insignificant to malignant change (Table 3).

DISCUSSION

Reviewing the English-language medical literature, there is only one published immunohistochemistry-based report of p73 expression in samples of ED taken from the head and neck.18 Our results are compatible with those of this small study of 16 ED lesions (five mild, six moderate, five severe). Choi et al18 demonstrated that 14 (87.5%) of the 16 samples expressed p73; however, no attempt was made to grade degree of staining with respect to the severity of the dysplastic lesions. In this study, there seemed to be no significant difference in p73 expression between the lesions of mild, moderate, and severe ED, although we found that p73 was overexpressed in buccal lesions of ED compared with normal tissue. On the other hand, our result is compatible with our earlier finding that p73 protein was overexpressed in the early stages of 7,12-dimethyl benz[a]anthracene (DMBA)-induced squamous cell carcinogenesis in hamster buccal pouch.19

To study tumor development, it is preferable to follow a tissue that has developed into a tumor. To evaluate the potential predictive value and biologic implication of p73 expression in oral carcinogenesis, it is better to use control samples from subjects with exposure to risk factors similar to the affected subjects, because this reduces the likelihood of identifying alterations indicative of only exposure but not transformation. In our experience, however, such kinds of controls (with exposure to carcinogen but without disease) are very difficult to collect, because without any oral mucosal abnormalities, these individuals are not likely to visit a dental clinic. Therefore, non–carcinogen users were selected for normal control mucosa in this study.

In 2 years follow-up, a small subset of moderate ED and severe variant showing p73 positivity have undergone malignant transformation to develop SCCs; however, no statistical significance was found. The insignificance may be the result of the follow-up period not being long enough. Nevertheless, to our knowledge, this may be the first report to provide follow-up data of p73 expression for lesions of oral ED. These preliminary data, despite statistical insignificance, suggest that p73 expression may be a potential marker to identify dysplastic lesions at risk of progression provided further studies on the characteristics of p73-positive keratinocytes and more long-term clinical follow-up data are performed.

Although a number of studies have investigated the role of p73 in human carcinomas from different organs,20–22 three studies have examined p73 in head and neck SCC, including oral variants of the disease.13,14,18 Our finding supports those previous observations in head and neck SCC.13,14,18 However, the rate of p73 expression in this study was different from that in earlier studies on head and neck SCC.13,14,18 Because such differences may be due to the heterogeneity of the head and neck tumors enrolled in the previous studies,13,14,18 a homogeneous and well-characterized series of buccal SCCs was used for our investigation. Therefore, the proportion of p73 expression of buccal SCC estimated in this study was, in fact, different from that in previous studies of head and neck SCCs,13,14,18 even though the examined specimens included oral variants of the disease.

In addition, despite an intensive search of the available literature, mutation of TP73 has rarely been found in human cancers.5,6 Judging from these results, it remains possible that wild-type p73, but not mutant p73, might have been overexpressed for oral dysplastic and cancerous keratinocytes in this study, alluding to the possibility that p73 may play an important role in oral carcinogenesis through the overexpression of wild-type p73 rather than of mutant form as a tumor suppressor. This idea is further supported by the discovery of a new isoform of human p73, ▲Np73, with possible oncogenic potential.23 Furthermore, the findings of this study corroborate earlier work pertaining to p73 for other cancers.20–22

Results of immunohistochemical analyses in this study indicate that p73 proteins are chiefly restricted to less-differentiated cells situated in the basal layers of normal stratified squamous epithelium. Furthermore, p73 proteins are found in less-differentiated cells in the periphery of carcinomatous clusters of well-differentiated carcinomas, whereas moderately differentiated carcinomas revealed more homogeneous (cell) staining, involving almost all of the tumor cells. These observations suggest that p73 protein may be related to the differentiation of oral stratified
squamous epithelium. These findings seem to be consistent with the results deriving from the works of Faridoni-Laurens et al.\textsuperscript{15} and Choi et al.\textsuperscript{18}

In this study, a significant correlation of p73 protein expression with cervical lymph node metastasis for buccal SCC has been identified. The same correlation has also been demonstrated at an mRNA level. This is in contrast to the finding of Choi et al.\textsuperscript{18} who failed to report a significant correlation of p73 expression and lymph node metastasis. As aforementioned, such disparity may be due to the heterogeneity of the head and neck tumors enrolled in the previous study of Choi et al.\textsuperscript{18}, a homogeneous and well-characterized series of buccal SCCs was used for our investigation. The positive correlation demonstrated between p73 expression and nodal status was underestimated in the previous research of Choi et al.\textsuperscript{18} even though the examined specimens included oral variants of the disease. On the other hand, Choi et al.\textsuperscript{18} reported an association of p73 expression with distant metastasis of head and neck SCCs; however, the data of distant metastasis have not been obtained in our series of buccal SCCs.

To the best of our knowledge, no studies have examined the effects, if any, of betel-quid chewing on p73 expression for various types of cancer. All patients in this study were betel-quid chewers. It may be of interest, therefore, to test the association between betel-quid chewing and p73 expression. A more corroborative conclusion could be achieved by comparing p73 activity between patients with oral cancer who are not habitual betel-quid chewers and those who do chew betel-quid but exhibit no disease in their buccal mucosa.

TP73 encodes protein isoforms caused by alternative splicing and promoter use, which results in retention of the transcription-activation domain (TA isoforms) and those that do not (\textsuperscript{▲}N isoforms).\textsuperscript{24,25} Whereas some isoforms of p73 (\textsuperscript{▲}Np73) are capable of transactivating p53 target genes and inducing apoptosis, other isoforms (\textsuperscript{▲}Np73) function as dominant negative fashion to counteract the transactivation-competent isoforms of not only p63 and p73, but p53 as well.\textsuperscript{24,25} Therefore, further studies that use different antisera that are specific for the p73 variants can identify which p73 isoform or isoforms are involved in human oral carcinogenesis.

In summary, the data suggest that p73 expression may be (1) associated with the differentiation of oral stratified squamous epithelium caused by the lack of p73 immunoreactivity for areas of keratin pearl and positive p73 expression for the less-differentiated cells of carcinomatous tissue; (2) an early event in human oral carcinogenesis and upregulated during development from normal mucosa to dysplastic or carcinomatous lesions; (3) associated with the nodal status of patients with oral carcinoma, as well as a possible indicator for malignant change of oral epithelial dysplastic lesions.

**Acknowledgment.** The authors acknowledge the technical assistance of Ms. N.Y. Dai.

**REFERENCES**


