Prognostic implications of CK13 and CK19 expression in the malignant transformation of oral precancerous lesions

Donghyun Yang

The Graduate School
Yonsei University
Department of Dentistry

Prognostic implications of CK13 and CK19 expression in the malignant transformation of oral precancerous lesions

Directed by Professor Jong In Yook

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Donghyun Yang

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This certifies that the doctoral dissertation
of Donghyun Yang is approved.

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Thesis Supervisor: Jong In Yook

_____

Hyun Sil Kim

_____

Eunae Sandra Cho

_____

Nam Hee Kim

_____

Xianglan Zhang

The Graduate School
Yonsei University
December 2020

DEDICATIONS

지도 교수님 이시며 큰 스승이신 육종인 교수님을 비롯하여 논문 심사를 맡아 주신 김현실, 조은애, 김남희, 장향란 교수님께 감사의 말씀을 드립니다. 구강병리학을 전공할 수 있도록 많은 가르침을 주시고 이끌어 주신 김진 교수님께도 감사의 말씀을 드립니다. 연구를 함께한 연세대학교 치과대학 구강병리학교실의 여러 선생님들께도 감사의 말씀을 드립니다.

묵묵히 지켜보고 격려해 주신 가족들에게도 깊은 감사를 드리며, 지금까지 인도하여 주신 하나님께 모든 영광을 돌립니다.

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양동현 드림

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ABSTRACT

Prognostic implications of CK13 and CK19 expression in the malignant transformation of oral precancerous lesions

Donghyun Yang

Department of Dentistry
The Graduate School, Yonsei University
(Directed by Professor Jong In Yook)

Oral cancer is one of the top 10 cancers that occur around the world. Although many treatment methods have been developed over the past decades, it has a poor prognosis and 5-year survival rate is less than 50%. In particular, compared to cancers that occur in the other organs, it reduces significantly the quality of life. Therefore, the importance of early diagnosis and prevention is being emphasized as the best method in the treatment of oral cancer. At present, it is the most effective method that surgical intervention through early detection of oral precancerous lesions with a high malignant transformation potential. Many biomarkers have been proposed to predict malignant transformation of oral precancerous lesions experimentally, but clinical significance of these markers needs to be verified.

In this study, the clinical usefulness of cytokeratin markers discovered through previous studies was verified, and the results of this study are as follows.

1. In the malignant transformation of oral precancerous lesions, age and extent of epithelial dysplasia are important clinicopathological risk factors.
2. CK13 and CK19 keratin subtypes are associated with the malignant transformation of oral precancerous lesions.
3. Two cytokeratin markers and clinicopathological factors were combined to measure the risk of the malignant transformation of oral precancerous lesions, which showed

the highest accuracy (c-index: 0.871)

4. It was revealed that CK13 and CK19 play a role in attenuating invasion and proliferation abilities of YD-10B OSCC cells and p53 mutation promotes proliferation and invasion abilities of keratinocyte.

These results support the fact that the initial atypical change from oral precancerous lesions, represented by oral leukoplakia, to oral squamous cell carcinoma begins with the changes in the skeletal differentiation patterns of oral squamous cells, such as loss of CK13 and CK19 expression. In addition, CK13 and CK19 can be used as useful markers for predicting the risk of the malignant transformation in oral precancerous lesions.

Key words: Oral precancerous lesions, keratinocyte, cytokeratin, malignant transformation, nomogram, P53

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Ⅰ. INTRODUCTION

Most carcinomas including oral squamous cell carcinoma (OSCC) occur through a multi-step carcinogenesis process. The multi-step carcinogenesis process is the concept that

genetic damage continuously accumulates in the field exposed to carcinogen and the altered epithelia change from normal state to potentially malignant lesions and invasive carcinoma, progressively (Farber 1984; Volgelstein et al., 1988). According to the World Health Organization (WHO) definition, oral precancerous lesions are denominated as morphologically altered tissues that are clearly more likely to develop oral cancer than normal oral tissues (Pindborg et al., 1997). OSCC, representative oral cancer is good example that explains the multi-step carcinogenesis process and the importance of oral precancerous lesions have been emphasized for several reasons. First, the lesions are easy to find and manage due to the anatomical location of the oral cavity. Second, it is possible to prevent progression from these lesions to oral cancer in advance (Boone, Kelloff and Steele, 1992).

Oral leukoplakia is a whitish patch of the mucosal lining of the oral cavity and the most common form of the precancerous lesion of squamous cell carcinoma (Warnakulasuriya, Johnson and Van der, 2007). In 2018, the WHO defined oral leukoplakia as "A white plaque of questionable risk having excluded other known disease or disorders that carry no increased risk for cancer" (Reibel et al., 2017). The prevalence of oral leukoplakia is approximately 2-3% and it has been reported 4-15% annual malignant transformation rate (Reibel, 2003; Petti et al., 2003; Van der et al., 2009; Warnakulasuriya et al., 2016). It is very important to detect, treat and manage oral leukoplakia appropriately because this kind

of interventions can block the progression of oral leukoplakia to oral cancer, and increases the possibility of good prognosis even if the lesion progresses to oral cancer (Cha, 2011). The current standard for the care of oral leukoplakia is to perform surgical resections and histopathological assessments. Until now, the degree of epithelial dysplasia has been regarded as the most reliable factor predicting the risk of malignant transformation (Leon et al., 2020). But even if epithelial dysplasia is present, the degree of dysplasia is subjective depending on pathologists and is not significantly related to predict the prognosis after surgical resections of the oral leukoplakia. Therefore, many studies have been conducted to predict cancer progression using specific predictive markers rather than using only histopathological findings by pathologists (Kim, 2002). However, predictive markers currently fall short in properly estimating the potential risk for developing oral squamous cell carcinoma in terms of high reproducibility and reliability (Zhang et al., 2017).

Cytokeratins (CKs) are a family of cytoskeletal intermediate filament proteins which are commonly found in oral epithelial tissue (Romano et al., 1988). CKs are classified into two sub-groups according to their molecular weight and isoelectric point: Type I and Type II. Type I cytokeratins (CK9 to CK20) are more acidic and have a smaller molecular weight (40-64 kDa); whereas Type II cytokeratins (CK1 to CK8) are considered as neutral or basic and have a relatively large molecular weight(52-68kDa) (Moll et al., 1982). They exhibit distinct expression patterns depending on the degree of epithelial cells differentiation and

maturation (Moll., 1998). For instance, cornified cells express CK1,2,10 and 11; stratified cells express CK4 and 13; basal cells express CK5 and 14; hyperproliferative cells express CK6 and 16 and simple cells express CK7, 8, 18 and 19 (Sun et al., 1983; Quinlan et al., 1985; Vaidya and Kanojua, 2007). CKs have a number of specific benefits as marker proteins. They are numerous, highly stable and extremely antigenic. Antibodies to groups of CKs are, therefore, extensively used as cellular markers of several epithelial and their corresponding neoplasms (Trask et al., 1990). Previous studies demonstrated that the expression of certain kind of CKs was associated with dysplasia grades and an unfavorable prognosis for OSCC patients. (Sakamoto et al., 2011; Gesche et al., 2016) These observations suggest that the patterns of CKs expression in normal epithelia and the change in oral leukoplakia may help predict cancer progression.

The purpose of this study was to investigate specific cytokeratins as predictive roles in oral leukoplakia. Immunohistochemical staining for CK13, CK16, and CK19 was performed using oral leukoplakia tissue samples, and the histologic patterns of CKs expression were analyzed and graded. Prediction accuracy was investigated using the level of protein expression and clinicopathological parameters by constructing a nomogram which has been used to quantitatively evaluate the risk factors in several carcinomas (Klar et al., 2008; Briganti et al., 2012; Zheng et al., 2016; Zhang et al., 2017). Molecular mechanisms related to malignant transformation of oral leukoplakia was investigated.

II. MATERIALS AND METHODS

1. Patients in OL cohort

All cases of OL included in the pathological database of Department of Oral Pathology, Dental Hospital, Yonsei University Medical Center from 1995 to 2010 were reviewed. The patients with a previous or concomitant diagnosis with OSCC (n=16) or with inadequate tissue available for analysis (n=11) were excluded from this study. The following clinical data were recorded: age, gender, tumor location, and status of dysplasia. In the OL cohort, a total of 156 patients (97 male and 59 females; median age of 54; age range of 13-89 years) with OL (median follow-up period:130months) were included. Among them 21 patients progressed to invasive squamous cell carcinoma (median follow-up period: 41 months) and 135 patients did not progress to malignancy (median follow-up period: 144 months) during follow-up (Figure 1). The clinical characteristics of patients in the OL cohort are described in Table 1. Additionally, 13 normal oral mucosa tissues were obtained from 13 patients (7 males and 6 females; median age of 31; ages 16 to 53) during their third molar tooth extraction and used as normal control (Table 1). This study was approved by the Institutional Review Board for Bioethics of Yonsei University College of Dentistry (IRB 2-2013-0045).

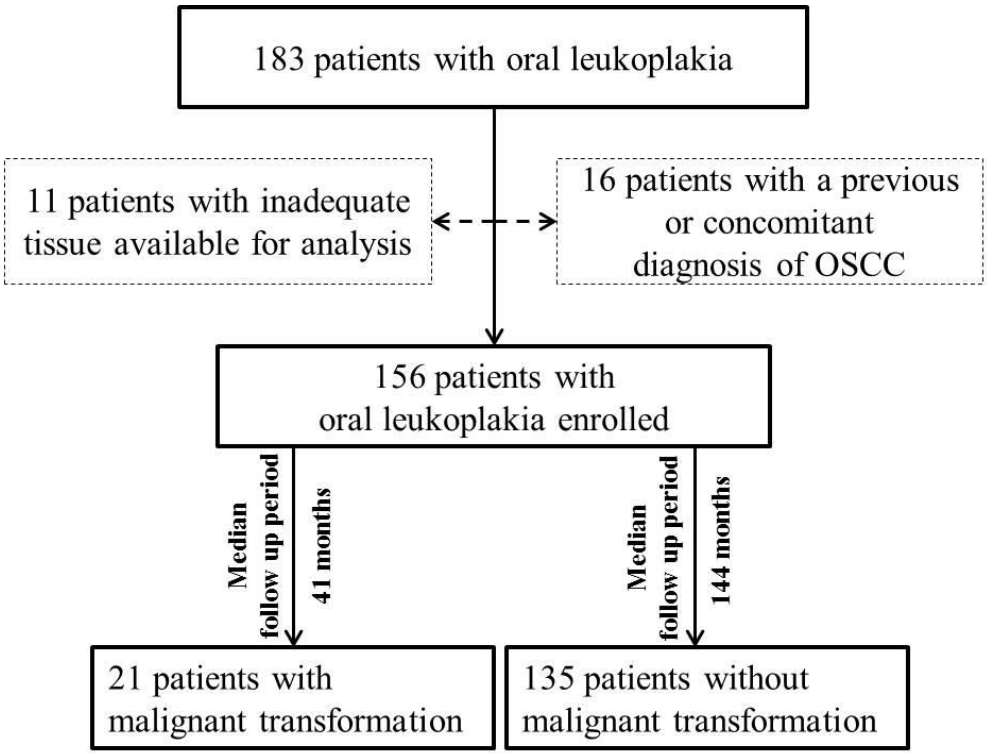

Figure 1. Flow diagram for selection and outcome of patients with OL

Table 1. Clinical characteristics of patients.

Clinical variables	No. of patients (%)	
	NOM	OL
Total samples (n)	13	156
Age (years)		
Median age (range)	31(16-51)	54(13-89)
< median age	6 (46.2)	76(48.7)
≥median age	7 (53.8)	80(51.3)
Gender		
Male	7(53.8)	97(62.2)
Female	6(46.2)	59(37.8)
Location		
Buccal mucosa	0(0)	44(28.2)
Tongue	0(0)	42(26.9)
Gingiva	13(100)	68(43.6)
Lip	0(0)	2(1.3)
Malignant transformation		
Yes		21(13.5)
No		135(86.5)
Duration of follow-up		
Median (range, years)		11.2(4.6-23.2)
Interval of malignant transformation		
Median (range, years)		3.4(1.1-10.3)

(NOM: normal oral mucosa; OL: oral leukoplakia)

2. Immunohistochemical staining

For immunohistochemical staining, the tissue sections were deparaffinized with xylene and hydrated using a graded ethanol. The tissue sections were incubated with a mixture of H_2O_2 and methanol at a dilution of 1:40 at room temperature for 10 min to block endogenous peroxidase activity. Antigen retrieval was performed with antigen retrieval buffer (Dako, Glostrup, Denmark) using the pressure-cooking method. After blocking with 5% bovine serum albumin at room temperature 5 min, the sections were incubated with primary antibody at room temperature for 1 h. For immunohistochemical staining, each group of cells was seeded in a chamber slide (Thermo Fisher Scientific, Waltham, MA, USA) at a density of 2×10^4. After 24 h of culture, the cells were fixed with 95% ethanol for 30 min at room temperature, and then incubated with primary antibody at RT for 1 h. Cytokeratin (CK)13 (1:100, Abcam, Cambridge, MA), CK16 (1:50, Abcam), and CK19 (1:100, Abcam) were used in this study, and REAL EnVision HRP Rabbit/Mouse Detection System (Dako, Santa Clara, CA, USA) was used as secondary antibody. The sections were visualized with 3.3'-diaminobenzidin, the sections were counterstained with hematoxylin.

Low immunoreactivity refers to no positive cells or positive cells restricted to the lower third of epithelium. By contrast, positive cells in the upper two-thirds of the epithelium

were considered as high-immunoreactivity. All slides were interpreted by two pathologists, and consistency between observers was excellent [intraclass correlation coefficient (ICCC)=0.925].

3. Cell culture and establishment for stable cell lines

One OSCC cell line (YD10B), and 2 spontaneously immortalized human keratinocyte cell lines that derived from adult skin (HaCat) and oral mucosa (IHOK) were used in this study. YD10B and HaCat cell lines were purchased from Korean Cell Line Bank (KCLB, SNU, Seoul, Korea). IHOK was provided by professor Jin Kim, which established by co-transfecting the pLXSN vector containing the E6/E7 open reading frame of HPV-16 and pLPC-puro-hcdk4-hTERT vector as previously described (Park et al., 2016). All cells were maintained in the culture medium composed of Dilbecco's Modified Eagles Medium (DMEM; Gibco BRL, USA) and F-12 Ham (Ham's-F12; Gibco BRL, USA) mixed in a 3:1 ratio, supplemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin, 0.01 ug/ml cholera toxin, 0.04 ug/ml hydrocortisone, 0.5ug/ml insulin, 0.5 ug/ml apotransferrin, and 0.2ug/ml 3'-5-triodo-1-thyroine (Sigma, MO, USA). Cultured cells were incubated at 37 °C in a 5% CO_2 humidified atmosphere.

CK13 or CK19 overexpressing YD10B cells ($YD10B^{CK13}$ and $YD10B^{CK19}$) were established using pTRIPZ lentiviral system (Open Biosystems). P53 wild type and P53 mutant (R273H) expression vectors were obtained from Addgene. An expression vector for the p53 transactivation domain deletion mutant (dTAD) was generated using polymerase chain reaction-based methods. Wild type and mutant p53 (R273 or dTAD) were subcloned into pLentilox-IRES-GFP (Addgene) transduction and transfected into both HaCat (HaCat $^{\text{wild type p53}}$, $HaCat^{R273H}$, $HaCat^{dTAD}$) and IHOK cells (IHOK $^{\text{wild type p53}}$, $IHOK^{R273H}$, $IHOK^{dTAD}$).

4. Proliferation assay

To investigate the influence of CK13, CK19 expression or p53 mutation in proliferation ability of cells, trypan blue assay was performed in each group of HaCat, IHOK, and YD10B cells. Each group of cells were seeded in a 6-well plate at a density of $1x10^4$ and counted each day for 4 days after trypan blue staining. The number of cells comparatively investigated between groups in all of the cells.

5. Matrigel invasion assay

To investigate the influence of CK13, CK19 expression or p53 mutation in invasion ability of the cells, Matrigel invasion assay was performed in each group of HaCat, IHOK, and YD10B cells. Each group of the cells were seeded in a Matrigel (BD Biosciences, San Jose, CA, USA) coated upper chamber of a transwell (BD Biosciences, Bedford, MA, USA) at a density of $5x10^4$ with culture medium containing 1% FBS. Culture medium containing 10% FBS was added in the bottom chamber. After 30 h of culture, invading cells were stained with 0.25% crystal violet and counted under the microscope.

6. Statistical analysis

The associations between protein expression and clinicopathological characteristics of tissue samples were analyzed by Chi-square and Fisher's exact tests in this study. Kaplan-Meier survival curves were plotted based on CK subtype protein expression and various clinicopathological factors, and the significance was analyzed by the log-rank test for determine the progression free survival of patients with OL. Furthermore, the strength of associations among various clinical factors and progression free survival was analyzed

using the Cox proportional hazards model. The nomogram for assessment of the risk for malignant transformation was constructed using CK13 and CK19 expression, various clinicopathological factors, evaluated the concordance index (c-index) and a calibration plot (Chen et al., 2018). R package version 3.1.1 (The R Foundation for Statistical Computing; http://www.r-project.org) with the rms (3.5.0) and eha (2.7.6) packages was used for statistical analysis. $P<0.05$ was considered to indicate a statistically significant difference.

Ⅲ. RESULTS

1. Clinicopathological risk factors for malignant transformation of OL

The clinicopathological risk factors for malignant transformation of OL were analyzed in this cohort study. Malignant progression occurred more often in patients with higher age (16, 20.0%) than patients with lower age (5, 6.6%) (P=0.018). Moreover, malignant progression was most frequently detected in patients with high grade dysplasia (10, 43.5%) than patients without dysplasia (4, 4.8%) or low grade dysplasia (7, 14.3%) (P<0.001). There is no significant association between malignant transformation and gender / location in this cohort (Table 2).

Parameters	Total, n (%)	Unprogressed	Progressed	*P*
Age (years)				
<54	76(48.7)	71(93.4)	5(6.6)	0.018
≥54	80(51.3)	64(80.0)	16(20.0)	
Gender				
Male	97(62.2)	88(90.7)	9(9.3)	0.057
Female	59(37.8)	47(79.7)	12(20.3)	
Location				
Buccal mucosa	44(28.2)	38(86.4)	6(13.6)	
Tongue	42(26.9)	33(78.6)	9(21.4)	0.259
Gingiva	68(43.6)	62(91.2)	6(8.8)	
Lip	2(1.3)	2(100)	0(0)	
Dysplasia				
Without dysplasia	84(53.8)	80(95.2)	4(4.8)	
Low grade	49(31.4)	42(85.7)	7(14.3)	<0.001
High grade	23(14.7)	13(56.5)	10(43.5)	

Table 2. Clinicopathological risk factors for malignant transformation of OL

2. CK13 protein expression in normal oral mucosa and OL tissues

CK13 expression was detected in the cytoplasm and membrane of epithelial cells of normal oral mucosa and OL tissues (Figure 2). CK13 expression was detected in all normal oral mucosa tissues and has a tendency to decrease in OL tissue samples (127, 81.4%) (Figure 3**)** (*P*=0.088). Moreover, high CK13 expression was more frequently detected in normal oral mucosa tissues (100%) than in OL (101, 64.7%), unprogressed OL (98, 72.6%), and progressed OL samples (3, 14.3%) in the present study (Figure 4, Figure 5) (*P*=0.009, 0.029, and <0.001).

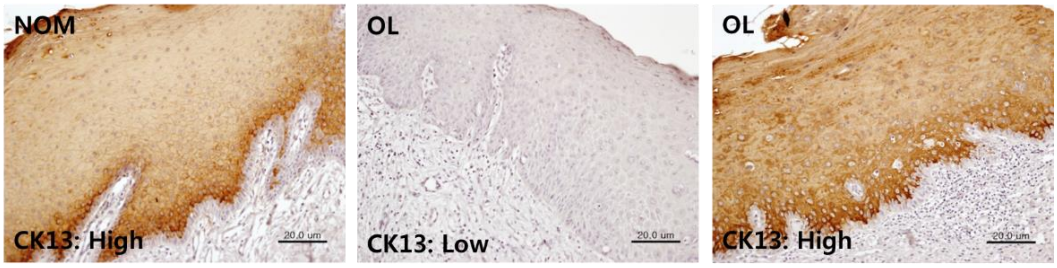

Figure 2. Cytoplasmic and membrane expression of CK13 in normal oral mucosa (NOM) and OL tissue samples: Representative expression pattern for low or high level of CK 13 in NOM and OL tissue samples (original magnification, x400, scale bar, 20 μm).

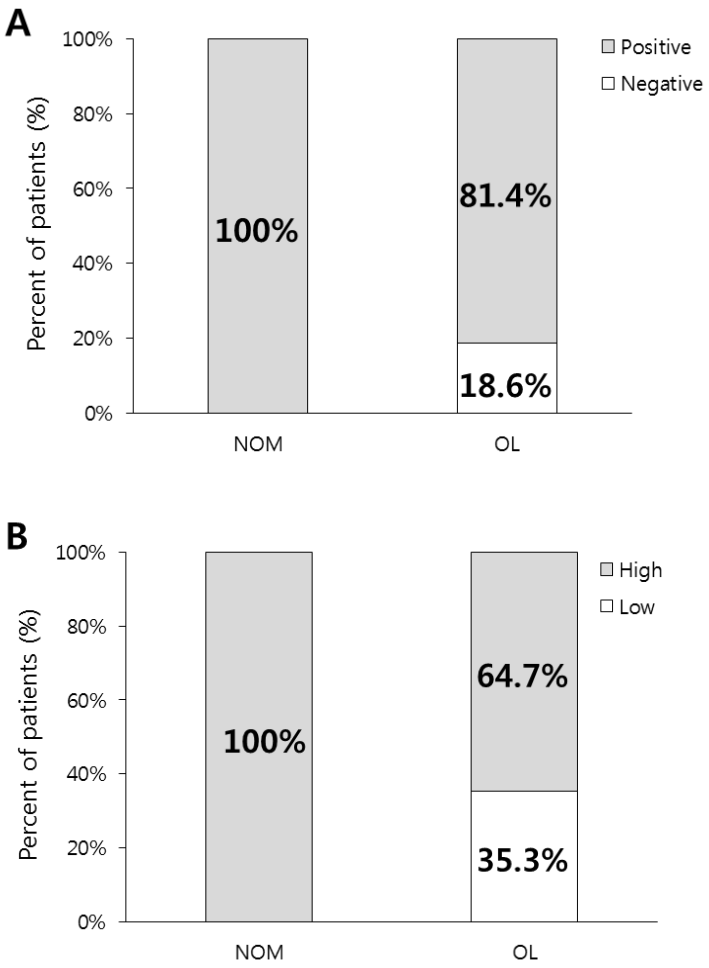

Figure 3. Comparative investigation for CK13 expression in NOM and OL tissue samples

A. CK13 expression was detected more often in NOM than in OL tissues (*P*=0.088).

B. High CK13 expression was significantly increased in NOM than OL (*P*=0.009).

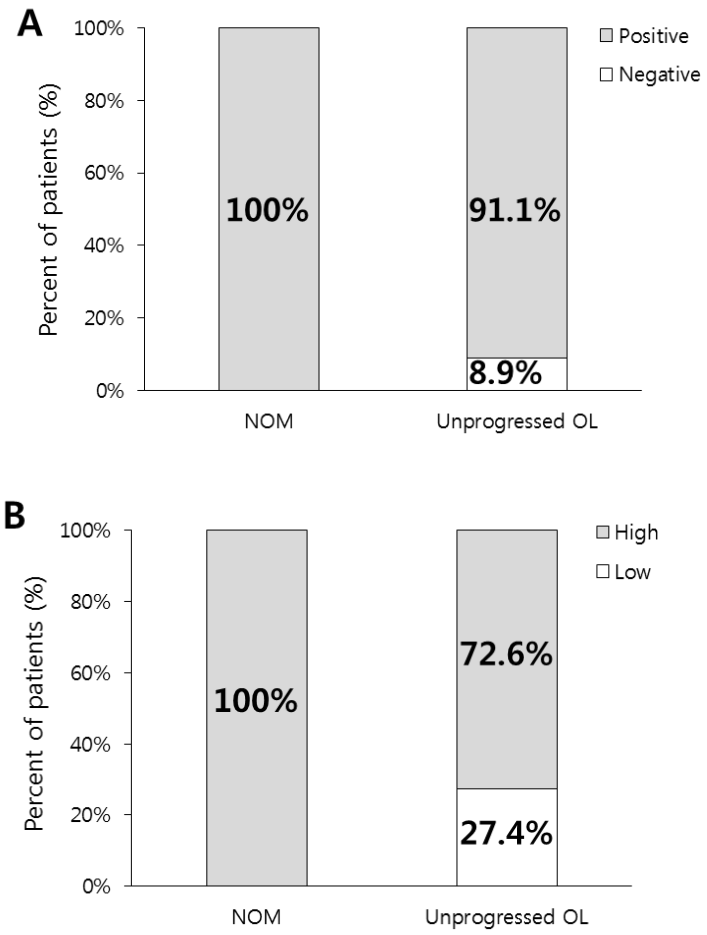

Figure 4. Comparative investigation for CK13 expression in NOM and unprogressed OL tissue samples

A. CK13 expression was detected more often in NOM than in unprogressed OL tissues (*P*=0.262).

B. High CK13 expression was significantly increased in NOM than OL (*P*=0.029).

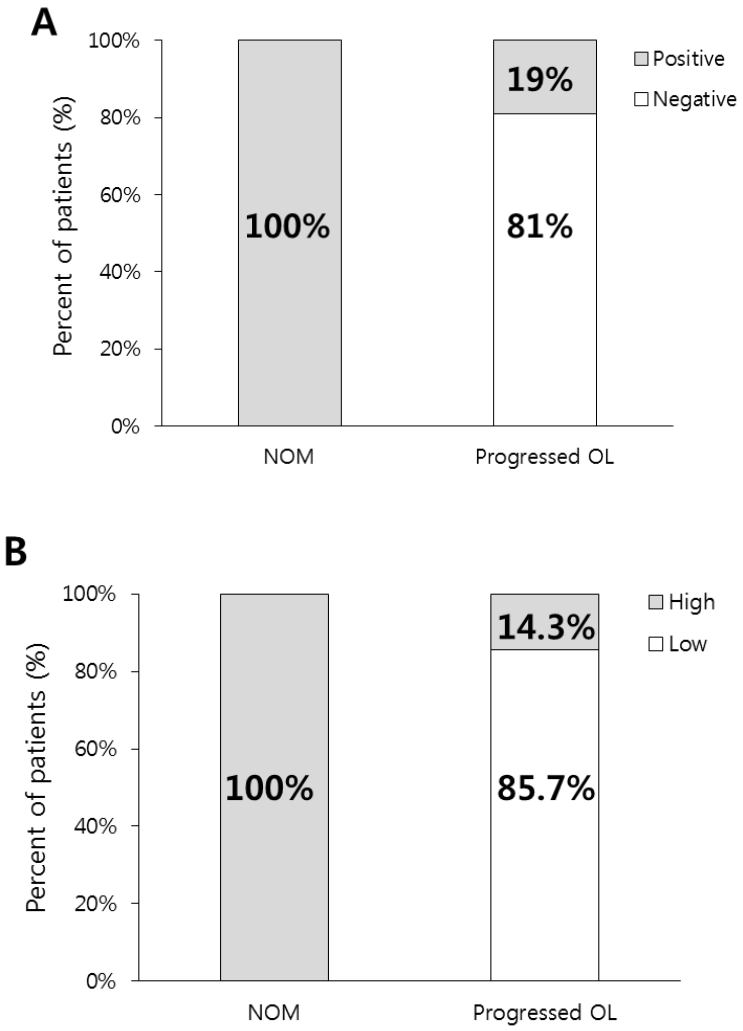

Figure 5. Comparative investigation for CK13 expression in NOM and progressed OL tissue samples

A. CK13 expression was detected more often in NOM than in progressed OL tissues (*P<0.001*).

B. High CK13 expression was significantly increased in NOM than OL (*P<0.001*).

3. Clinicopathological significance of CK13 protein expression in OL patients

High-CK13 expression was detected more often in patients with lower age (56, 73.7%) than patients with higher age (45, 56.3%) when patients were divided into two groups according to the median age (54 years) (P=0.023). Location of OL in buccal mucosa (32, 72.7%) or gingiva (50, 73.5%) showed significantly increased high-CK13 expression than OL in tongue (19, 45.2%) or lip (0, 0%) (P=0.003). Moreover, high-CK13 expression was detected more often in patients without dysplasia than patients with low- or high-grade dysplasia (P=0.005). In addition, high-CK13 expression was detected more often in patients without malignant progression than patients with malignant progression (P<0.001). Similar association tendency was also found between CK13 expression and clinical parameters when CK13 expression status was divided into two groups: negative- and positive group (Table 3). Kaplan-Meier analysis was used to estimate the probability of cancer-free survival of the patients with OL. As a result, patients with CK13 negative (median survival duration 6.1 years in CK13 negative group versus 11.8 years in CK13 positive group; P<0.001) or low CK13 expression (median survival duration 8.5 years in low CK13 expression group versus 11.6 years in high CK13 expression group; P<0.001) exhibited poor oral cancer-free survival rates (Figure 6).

Parameters	Total n (%)	CK13		*P*	CK13		*P*
		Negative	Positive		Low	High	
Age (years)							
<54	76 (48.7)	9(11.8)	67(88.2)	0.035	20(26.3)	56(73.7)	0.023
≥54	80(51.3)	20(25.0)	60(75.0)		35(43.8)	45(56.3)	
Gender							
Male	97(62.2)	14(14.4)	83(85.6)	0.087	32(33.0)	65(67.0)	0.447
Female	59(37.8)	15(25.4)	44(74.6)		23(39.0)	36(61.0)	
Location							
Buccal mucosa	44(28.2)	7(15.9)	37(84.1)	0.014	12(27.3)	32(72.7)	0.003
Tongue	42(26.9)	14(33.3)	28(66.7)		23(54.8)	19(45.2)	
Gingiva	68(43.6)	7(10.3)	61(89.7)		18(26.5)	50(73.5)	
Lip	2(1.3)	1(50.0)	1(50.0)		2(3.6)	0(0)	
Dysplasia							
Without dysplasia	84(53.8)	11(13.1)	73(86.9)	0.056	20(23.8)	64(76.2)	0.005
Low grade	49(31.4)	10(20.4)	39(79.6)		24(49.0)	25(51.0)	
High grade	23(14.7)	8(34.8)	15(65.2)		11(47.8)	12(52.2)	
Malignant transformation							
Unprogressed	135(86.5)	12(8.9)	123(91.1)	<0.001	37(27.4)	98(72.6)	<0.001
Progressed	21(13.5)	17(81.0)	4(19.0)		18(85.7)	3(14.3)	

Table 3. Clinicopathological significance of CK13 expression in 156 OL patients.

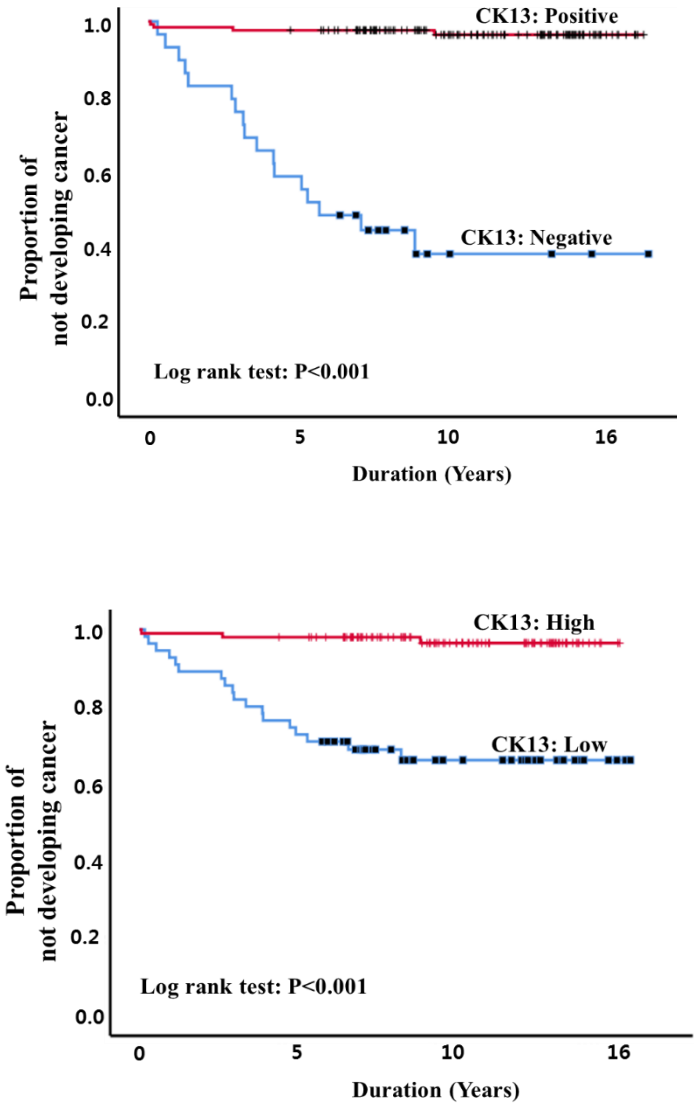

Figure 6. CK13 expression is associated with cancer-free survival of OL patients

Patients with negative for CK13 or low-CK13 expression exhibited poor oral cancer-free survival rates (Log rank test, *P<0.001* and *P<0.001*, respectively).

4. CK16 protein expression in normal oral mucosa and OL tissues

CK16 expression was detected in the cytoplasm and membrane of epithelial cells of normal oral mucosa and OL tissues (Figure 7). CK16 expression was detected in 11(84.6%) normal oral mucosa and significantly increased in OL (154, 98.7%) tissues (P=0.030). Overall, tissue immunoreactivity against CK16 was high in 112 (CK16-high, 71.8%) OL tissue samples and low in 44 (CK16-low, 28.2%). All normal oral mucosa showed CK16-low expression and there is statistical significance between normal oral mucosa and OL tissues when the CK16 expression was divided into two groups: CK16-low and CK16-high (P<0.001) (Figure 8, Figure 9, Figure 10).

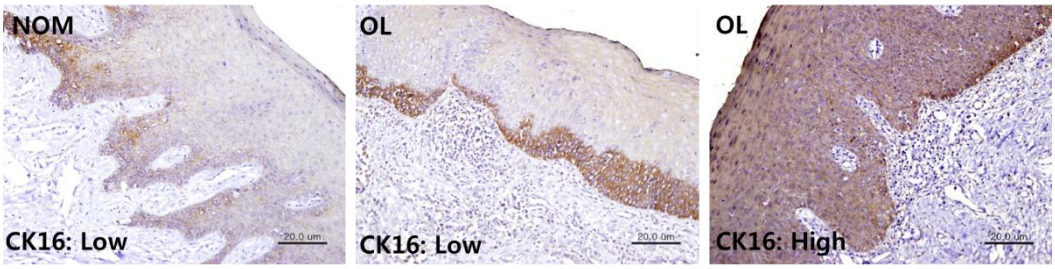

Figure 7. Cytoplasmic and membrane expression of CK16 in NOM and OL tissue samples: Representative expression pattern for low or high level of CK16 in NOM and OL tissue samples (original magnification, x400, scale bar, 20 μm).

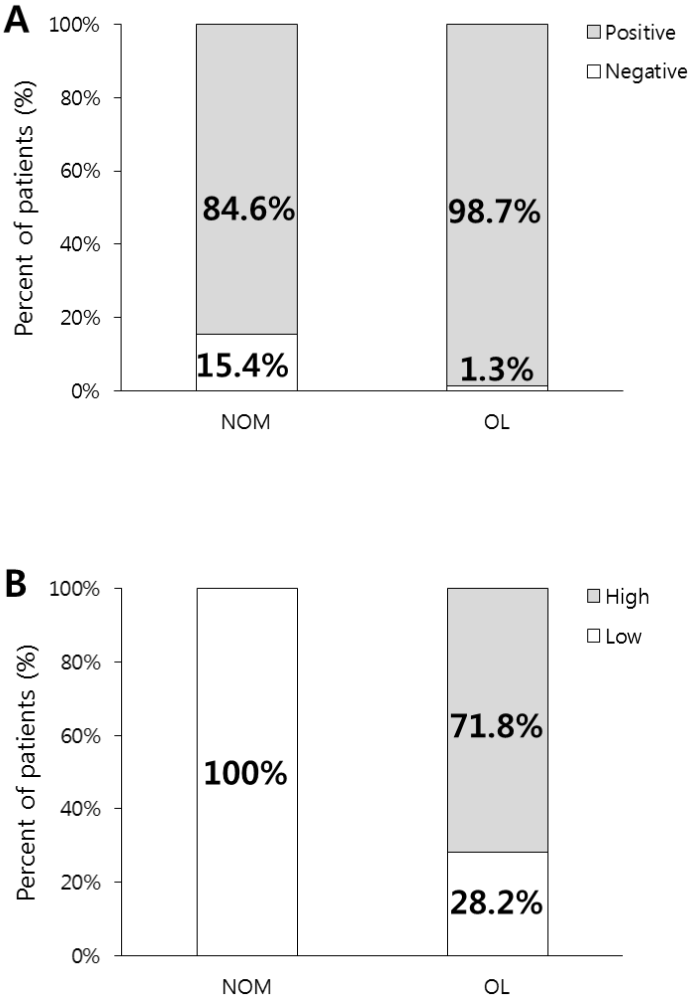

Figure 8. Comparative investigation for CK16 expression in NOM and OL tissue samples.

A. CK16 expression was detected more often in OL tissue samples than NOM tissues (P=0.03).

B. High CK16 expression was significantly increased in OL than NOM (P<0.001).

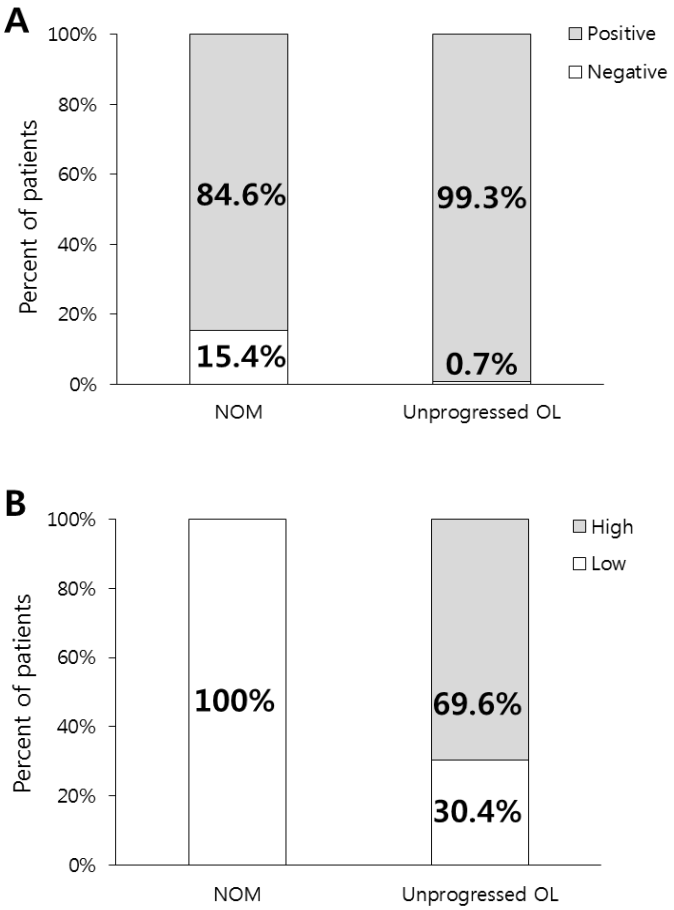

Figure 9. Comparative investigation for CK16 expression in NOM and unprogressed OL tissue samples

A. CK16 expression was detected more often in unprogressed OL tissues than in NOM. (P=0.020).

B. CK16-high expression was significantly increased in unprogressed OL tissues than NOM (P<0.001).

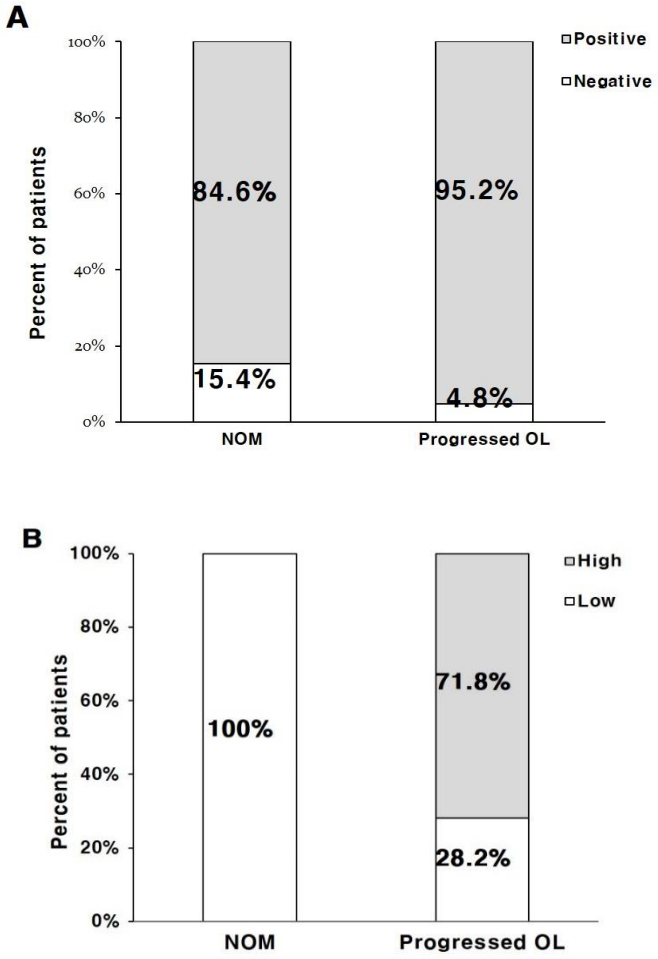

Figure 10. CK16 expression in NOM and progressed OL tissue samples

A. CK16 expression was detected more often in progressed OL tissues than in NOM, although there is no statistical significance (*P*=0.544).

B. CK16-high expression was significantly increased in progressed OL tissues than NOM (*P*<0.001).

5. Clinicopathological significance of CK16 protein expression in OL patients

The clinicopathological significance of CK16 protein expression was further evaluated in 156 OL patients. There is no significant association between CK16 expression with clinicopathological variables including age, gender, lesion site, status of dysplasia, and malignant transformation (Table 4). Kaplan-Meier analysis was used to estimate the probability of cancer-free survival of the patients with OL. As a result, there is no significant association between CK16 protein expression with cancer-free survival in OL patients (Figure 11).

Parameters	Total n (%)	CK16		*P*	CK16		*P*
		Negative	Positive		Low	High	
Age (years)							
<54	76 (48.7)	0(0)	76(100)	0.165	20(26.3)	56(73.7)	0.609
≥54	80(51.3)	2(2.5)	78(97.5)		24(30.0)	56(70.0)	
Gender							
Male	97(62.2)	1(1.0)	96(99.0)	0.721	29(29.9)	68(70.1)	0.547
Female	59(37.8)	1(1.7)	58(98.3)		15(25.4)	44(74.6)	
Location							
Buccal mucosa	44(28.2)	0(0)	44(100)	0.797	12(27.3)	32(72.7)	0.857
Tongue	42(26.9)	1(2.4)	41(97.6)		11(26.2)	31(73.8)	
Gingiva	68(43.6)	1(1.5)	67(98.5)		20(29.4)	48(70.6)	
Lip	2(1.3)	0(0)	2(100)		1(50.0)	1(50.0)	
Dysplasia							
Without dysplasia	84(53.8)	0(0)	84(100)	0.109	25(29.8)	59(70.2)	0.456
Low grade	49(31.4)	2(4.1)	47(95.9)		15(30.6)	34(69.4)	
High grade	23(14.7)	0(0)	23(100)		4(17.4)	19(82.6)	
Malignant transformation							
Unprogressed	135(86.5)	1(0.7)	134(99.3)	0.128	41(30.4)	94(69.6)	0.128
Progressed	21(13.5)	1(4.8)	20(95.2)		3(14.3)	18(85.7)	

Table 4. Clinicopathological significance of CK16 expression in 156 OL patients.

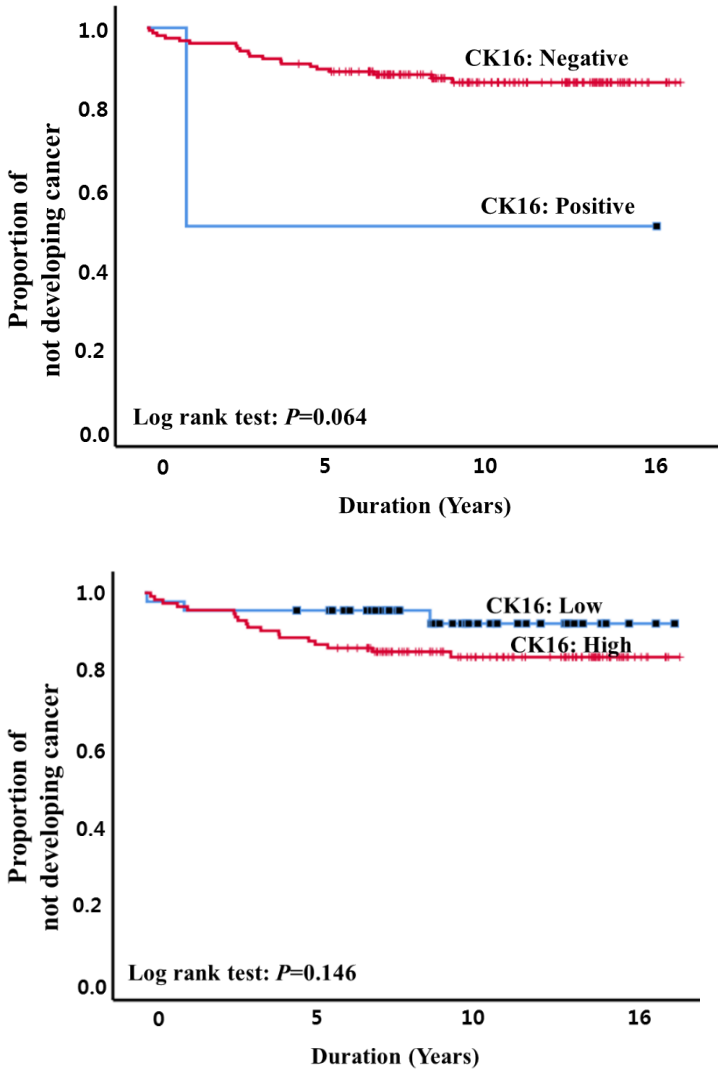

Figure 11. Probability of cancer-free survival of the patients with OL

There is no significant association between CK16 expression and cancer-free survival of OL patients.

6. CK19 protein expression in normal oral mucosa and OL tissues

CK19 expression was detected in the cytoplasm and membrane of epithelial cells of normal oral mucosa and OL tissues (Figure 12). CK19 expression was detected in all normal oral mucosa tissues and has a tendency to decrease in OL tissue samples (121, 89.6%) (Figure 13) (*P*=0.222). Moreover, high CK19 expression was more frequently detected in normal oral mucosa tissues (7, 53.8%) than in OL (60, 44.4%) and unprogressed OL (60, 44.4%), although there is no statistical significance (*P*=0.384 and *P*=0.569, respectively). Significance difference was found between normal oral mucosa tissues (7, 53.8%) and progressed OL (2, 9.5%) with high CK19 expression (Figure 14, Figure 15) (*P*=0.013).

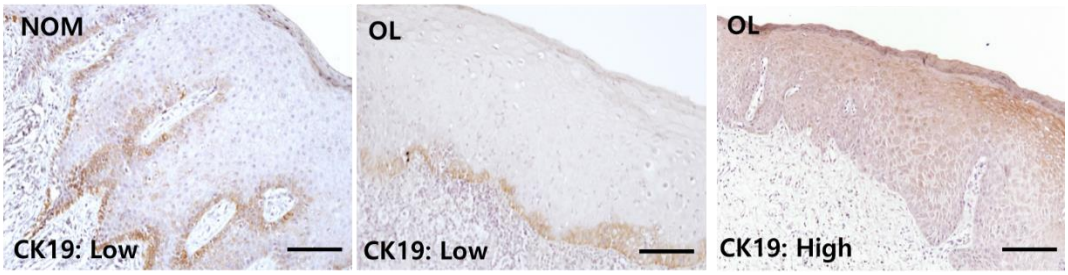

Figure 12. Cytoplasmic and membrane expression of CK19 in NOM and OL tissue samples: Representative expression pattern for low and high CK19 in NOM and OL tissue samples (original magnification, x400, scale bar, 20 μm).

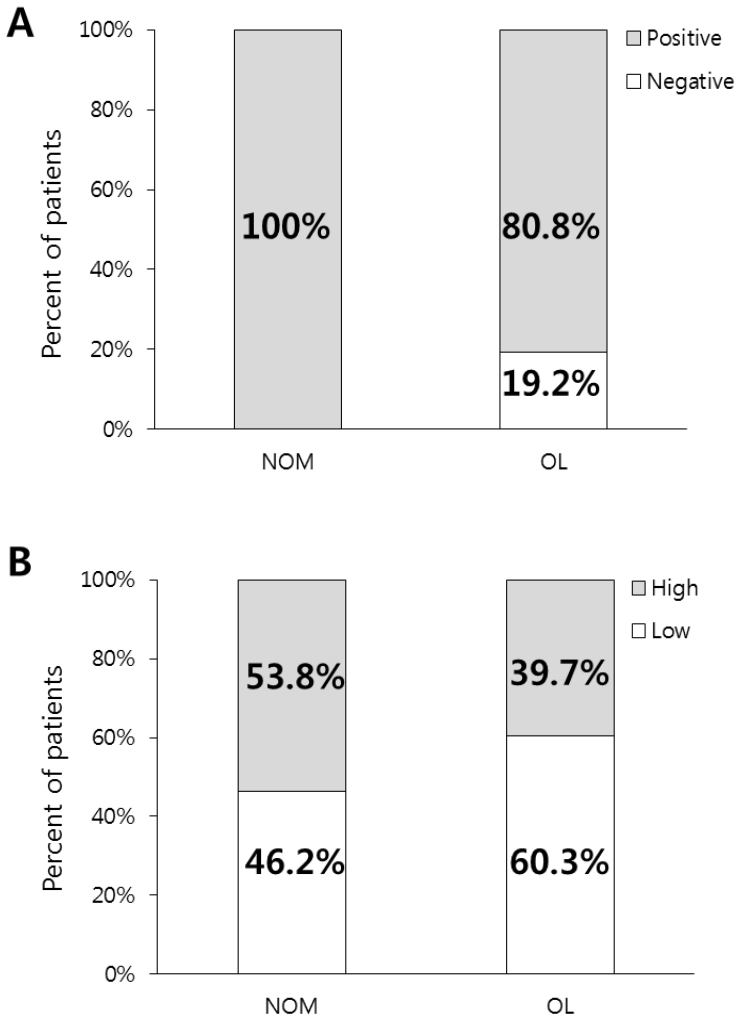

Figure 13. Comparative investigation for CK19 expression in NOM and OL tissue samples Both positive-CK19 (A) and high-CK (B) expression was detected more often in NOM than in OL tissues, although there is no statistical significance (*P*=0.081 and P=0.320, respectively).

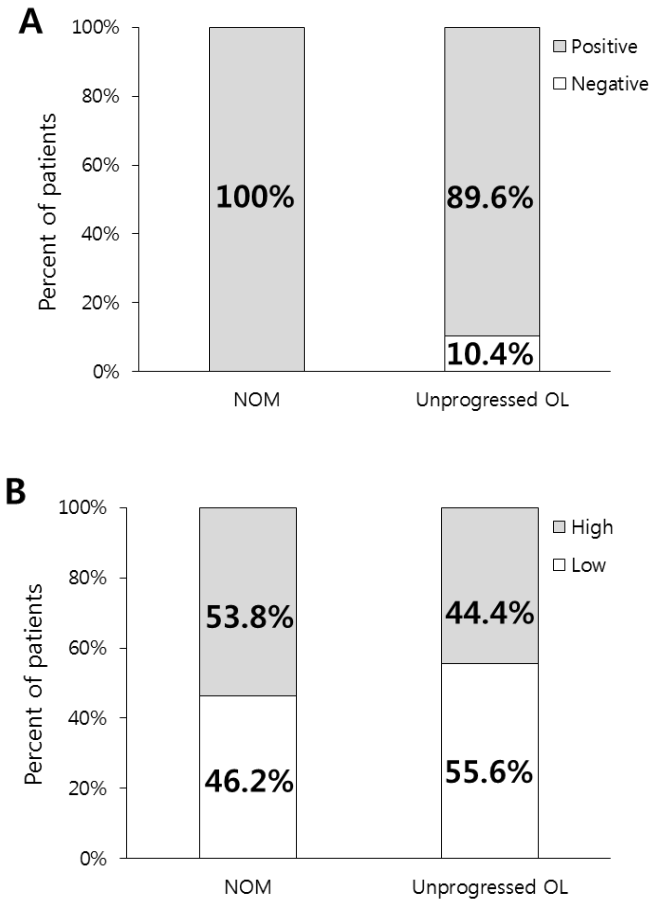

Figure 14. Comparative investigation for CK19 expression in NOM and unprogressed OL tissue samples. CK19 expression was detected more often in NOM than in unprogressed OL tissues (A & B), but there is no statistical significance between NOM and unprogressed OL tissues in CK19 expression (*P*=0.613 and *P*=569, respectively).

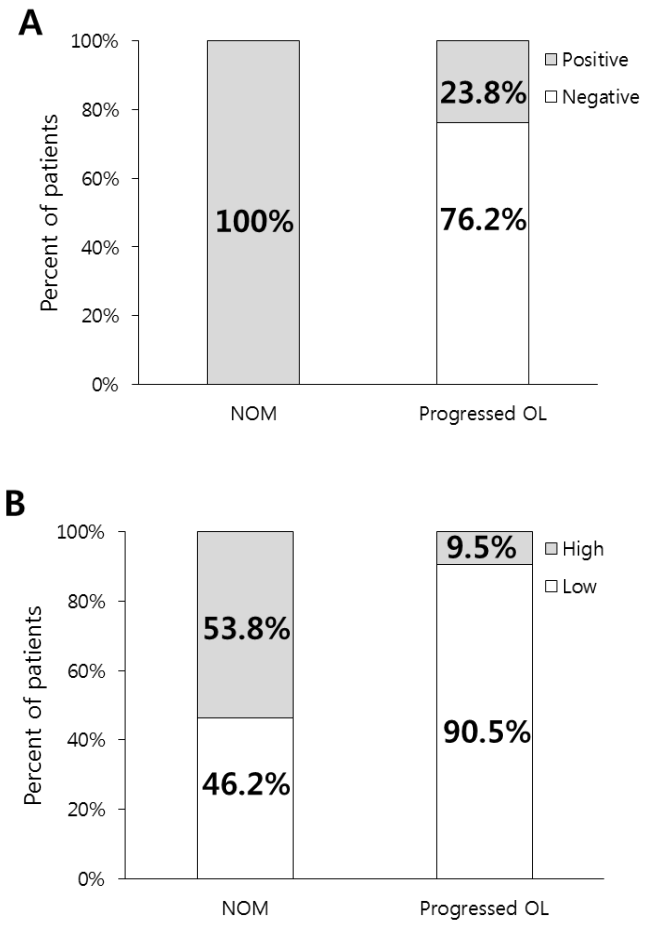

Figure 15. Comparative investigation for CK19 expression in NOM and progressed OL tissue samples. CK19 expression was detected more often in NOM than in progressed OL tissues (A) (P<0.001). Moreover, high CK19 expression was significantly increased in NOM than progressed OL (B) (P<0.013).

7. Clinicopathological significance of CK19 protein expression in OL patients

CK19 expression was detected more often in patients without dysplasia (71, 84.5%) / with low grade dysplasia (41, 83.7%) than patients with high grade dysplasia (14, 60.9%) (P=0.046). Moreover, CK19 or high-CK19 expression detected more often in patients without malignant transformation (121, 89.6% and 60, 44.4%, respectively) than patients with malignant transformation (5, 23.8% and 2, 9.5%, respectively) (P<0.001 and P<0.001, respectively). There is no significant association between CK19 expression with age, gender, and location in our cohort (Table 5). Kaplan-Meier analysis was used to estimate the probability of cancer-free survival of the patients with OL. As a result, patients with CK19 negative (median survival duration 8.6 years in CK19 negative group versus 11.0 years in CK19 positive group; P<0.001) or low CK19 expression (median survival duration 9.6 years in low CK19 expression group versus 13.5 years in high CK19 expression group; P=0.002) exhibited poor oral cancer-free survival rates (Figure 16).

Parameters	Total n (%)	CK19		*P*	CK19		*P*
		Negative	Positive		Low	High	
Age (years)							
<54	76 (48.7)	12(15.8)	64(84.2)	0.288	46(60.5)	30(39.5)	0.946
≥54	80(51.3)	18(22.5)	62(77.5)		48(60.0)	32(40.0)	
Gender							
Male	97(62.2)	16(16.5)	81(83.5)	0.266	61(62.9)	36(37.1)	0.389
Female	59(37.8)	14(23.7)	45(76.3)		33(55.9)	26(44.1)	
Location							
Buccal mucosa	44(28.2)	6(13.6)	38(86.4)	0.445	29(65.9)	15(34.1)	0.09
Tongue	42(26.9)	11(26.2)	31(73.8)		29(69.0)	13(31.0)	
Gingiva	68(43.6)	13(19.1)	55(80.9)		36(52.9)	32(47.1)	
Lip	2(1.3)	0(0)	2(100)		0(0)	2(100)	
Dysplasia							
Without dysplasia	84(53.8)	13(15.5)	71(84.5)	0.046	45(53.6)	39(46.4)	0.127
Low grade	49(31.4)	8(16.3)	41(83.7)		35(71.4)	14(28.6)	
High grade	23(14.7)	9(39.1)	14(60.9)		14(60.9)	9(39.1)	
Malignant transformation							
Unprogressed	135(86.5)	14(10.4)	121(89.6)	<0.001	75(55.6)	60(44.4)	0.002
Progressed	21(13.5)	16(76.2)	5(23.8)		19(90.5)	2(9.5)	

Table 5. Clinicopathological significance of CK19 expression in 156 OL patients

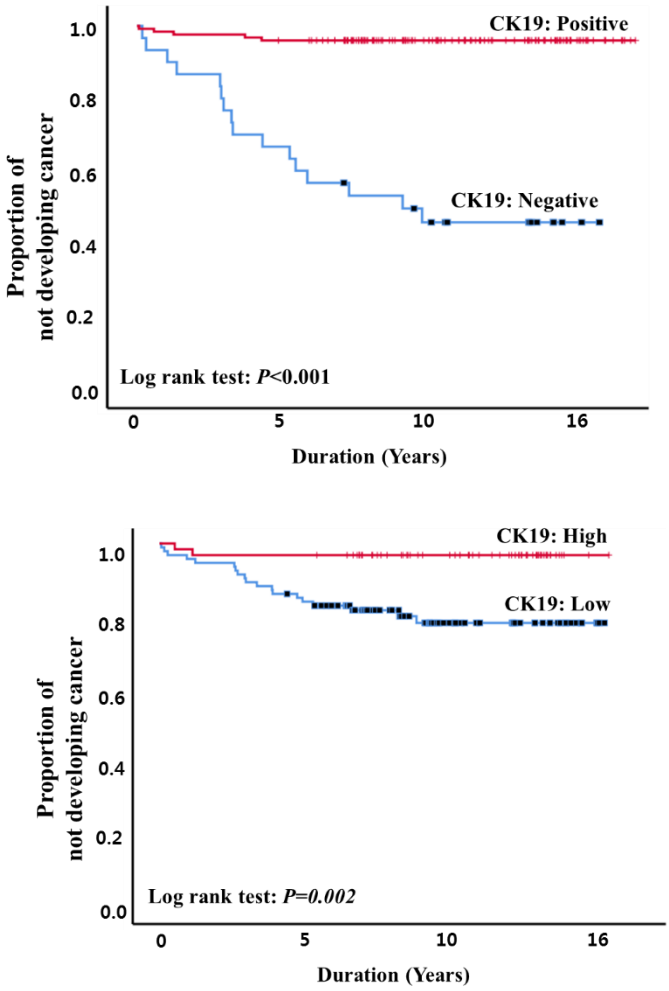

Figure 16. CK19 expression is associated with cancer-free survival of OL patients. Patients with negative for CK19 or low-CK19 expression exhibited poor oral cancer-free survival rates (Log rank test, $P<0.001$ and $P=0.002$, respectively).

8. Construction of prediction model for malignant transformation of OL

Meanwhile, among the factors including age (*P*=0.018), degree of dysplasia (*P*<0.001), CK13 (P<0.001), and CK19 (*P*=0.009) expression in univariate analysis, the multivariate analysis indicated that degree of dysplasia (OR = 12.28, 95% CI = 3.38–44.6; *P* <0.001), CK13 expression (OR = 0.1, 95% CI = 0.027–0.371; *P* = 0.001), and CK19 expression (OR = 0.14, 95% CI =0.029–0.683; *P* = 0.015) were significantly associated with the risk of malignant transformation in patients with OL (Table 6).

This study constructed a nomogram for predicting the risk of malignant transformation by combining age, degree of dysplasia, CK13, and CK19 expression. The c-index of nomogram was approximately 0.76 with only degree of dysplasia, and was increased to approximately 0.87 in the nomogram that including age, degree of dysplasia, CK13, and CK19 expression (Table 7).

A nomogram was constructed to predict 3-, 5-, 10-, and 15-year progression-free survival using clinical parameters and the expression of CK13 and CK19 expression (Figure 17). Including CK13 and CK19 expression, the c-index was 0.87 in the nomogram, the probability of progression was increasing as total points decreased. Thus, if the total points were 151 for a patient, the probabilities of 5-, 10-, and 15-year periods for progression free survival revealed 91.9%, 83.9%, and 77.9%, respectively. Meanwhile, if the total points

were 100, the probabilities of 5-, 10-, and 15-year periods for progression-free survival were found to be 99%, 64.1%, and 54.5%, respectively. For practical usage of the nomogram, I constructed a nomogram in Hypertext Markup Language (HTML) format and populated it with calculated predictions and probabilities (Figure 18). This nomogram, a useful decision-making tool for the diagnosis of patients, was calibrated to further investigate the correlation between its predictions and the actual outcomes in patients with OL. To do so, predictions calculated by the nomogram were assigned to the x-axis and the actual probability of disease progression was assigned to the y-axis. An ideal nomogram would yield a line of y = x representing a perfect correspondence between nomogram and actual clinical outcome (Figure 19).

Variable	Univariate		Multivariate	
	Hazard ratio (95% CI)	*P*	Hazard ratio (95% CI)	*P*
Age	3.382(1.237-9.242)	0.018	1.932(0.648-5.757)	0.237
Gender	2.326(0.980-5.522)	0.056	1.819(0.615-5.382)	0.280
Site				
Buccal mucosa	1	0.393	1	0.160
Tongue	1.554(0.553-4.366)	0.403	0.337(0.091-1.249)	0.104
Gingiva	0.627(0.202-1.945)	0.419	0.188(0.045-0.795)	0.023
Lip	0.000(0.000-0.000)	0.982	0.000(0.000-0.000)	0.986
Degree of dysplasia				
Without dysplasia	1	<0.001	1	<0.001
Low grade	3.430(1.000-11.768)	0.050	1.594(0.444-5.719)	0.474
High grade	13.207(4.096-42.582)	<0.001	12.281(3.381-44.605)	<0.001
Biomarkers				
CK13	0.076(0.022-0.258)	<0.001	0.100(0.027-0.371)	0.001
CK16	2.409(0.710-8.178)	0.159	2.157(0.526-8.850)	0.286
CK19	0.144(0.033-0.618)	0.009	0.140(0.029-0.683)	0.015

Table 6. Significance of clinical factors and molecular markers by Cox regression analysis

Factors	Prediction accuracy
Degree of dysplasia	0.76
Age and dysplasia	0.77
CK13 and CK19	0.81
Age, CK13 and CK19	0.83
Age, CK13, CK19 and dysplasia	0.87

Table 7. Summarization of prediction accuracies upon combining each factor

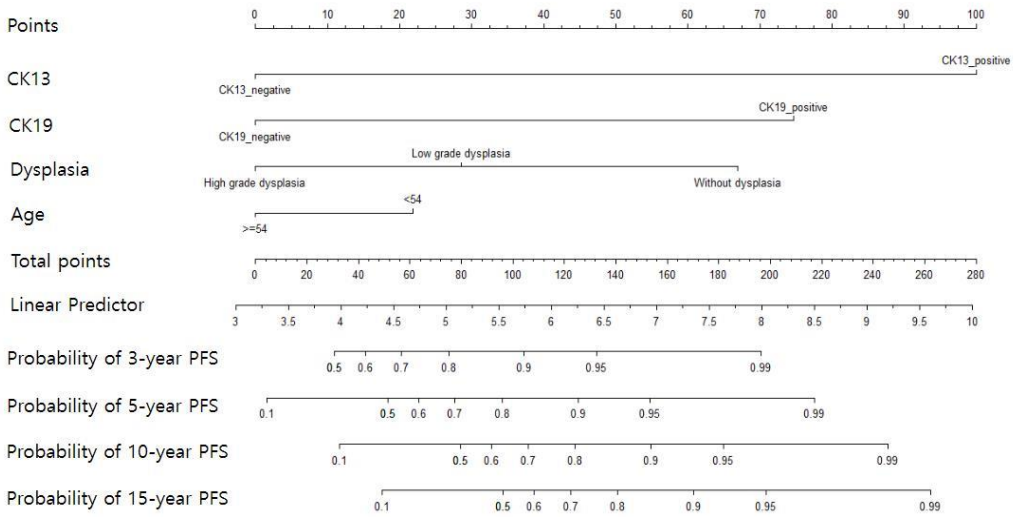

Figure 17. Nomogram for predicting the probability of progression-free survival (PFS): A nomogram was constructed to predict 3-, 5, 10, and 15-year progression-free survival using clinical parameters and the expression of CK13 and CK19.

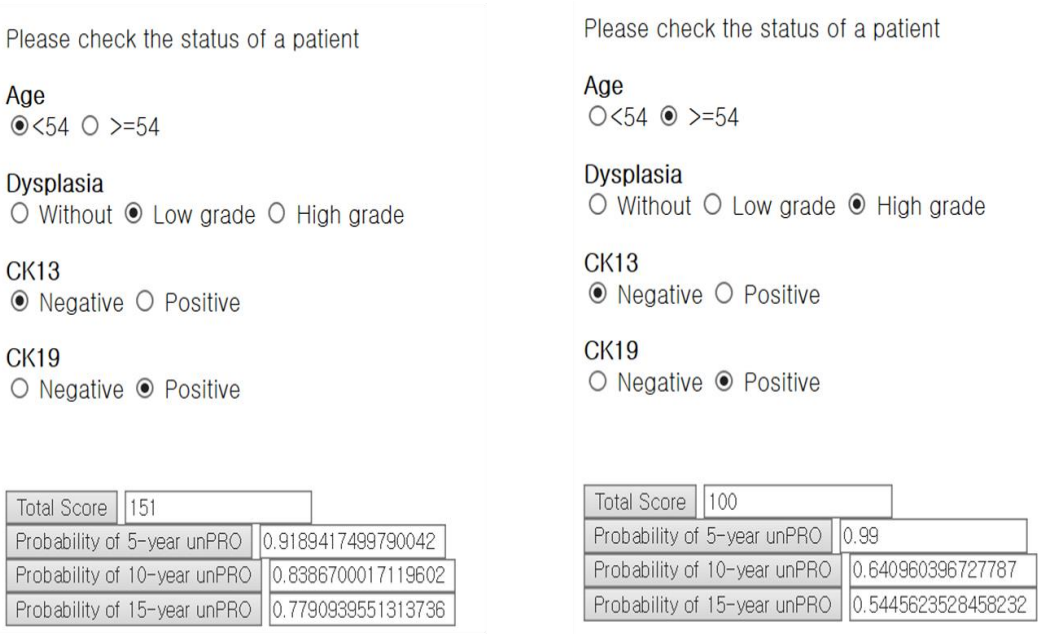

Figure 18. The practical usage of the nomogram is available in the Hypertext Markup Language (HTML) format.

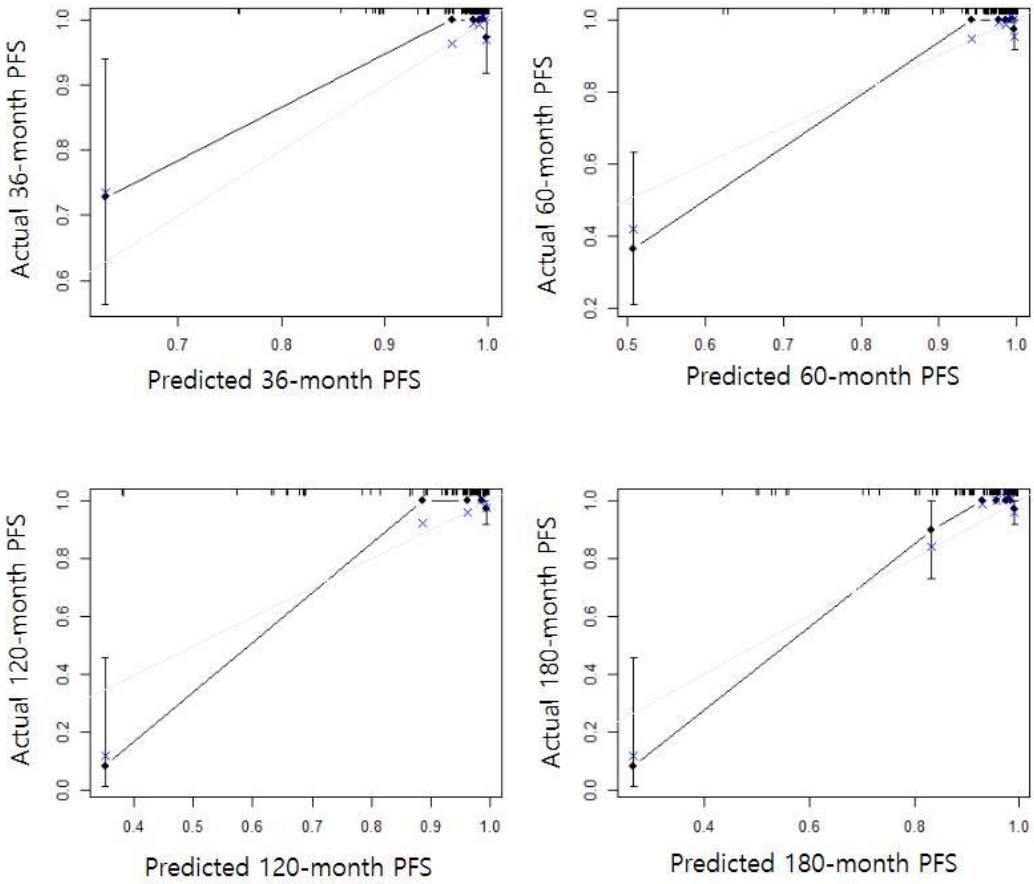

Figure 19. Calibration of the nomogram: high levels of correlations were found between nomogram predictions and the actual outcomes in patients with OL.

9. CK13 or CK19 overexpression attenuated proliferation ability of YD10B cells

Both CK13 and CK19 expression were induced by 5ug/ml doxycycline (Dox) (Sigma, USA) and the expression status of both CK13 and CK19 were confirmed by immunostaining in each group of YD10B cells (Figure 20).

Proliferation ability was comparatively investigated between cells with or without Dox treatment. I found that the number of the cells were 2.5-, 3.8-, and 4.6-fold increased after seeding at 48 h, 72 h, and 96 h time points in Dox (-) YD10B^{CK13} cells than Dox (+) YD10B^{CK13} cells, respectively (P<0.001, P<0.001, and P<0.001, respecitively) (Figure 21A). By contrast, the number of YD10B^{CK19} cells were 1.6-fold increased after 96 h of seeding in Dox (-) YD10B^{CK19} cells than Dox (+) YD10B^{CK19} cells (P=0.035) (Figure 21B).

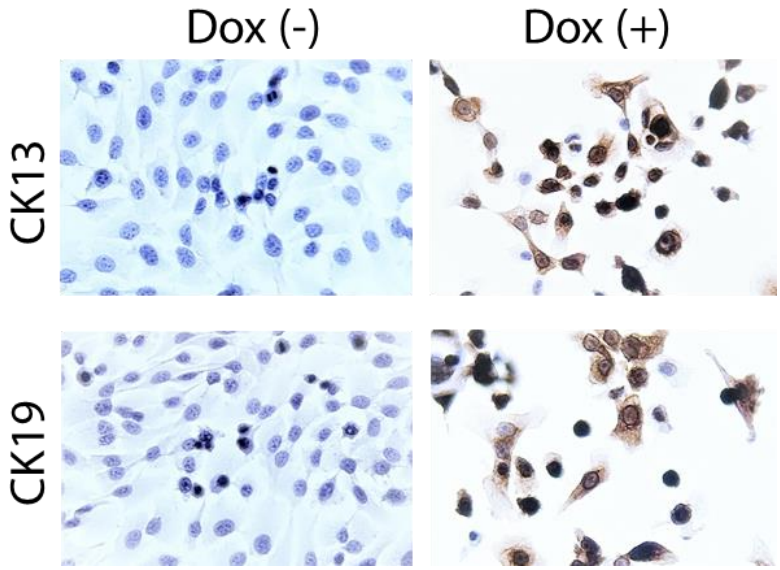

Figure 20. Expression status of both CK13 and CK 19 in each group of YD10B cells.

Both CK13 and CK19 expression were found in Dox (+) YD10B^{CK13} or YD10B^{CK19} cells, but not in Dox (-) cells.

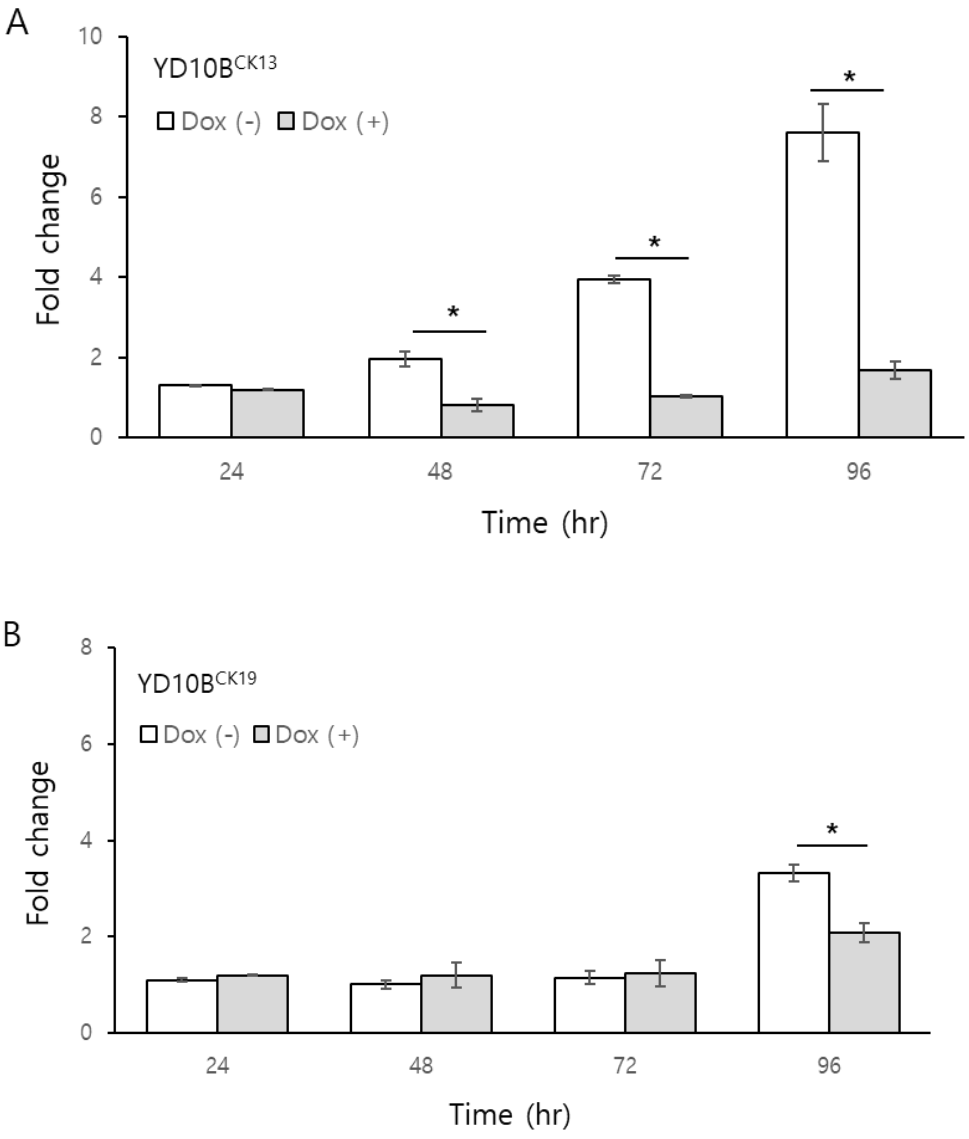

Figure 21. The influence of CK13 and CK19 expression on proliferation ability of YD10B cell (*P<0.001).

10. CK13 or CK19 overexpression attenuated invasion ability of YD10B cells

Invasion ability was comparatively investigated between cells with or without Dox treatment in both YD10B^{CK13} and YD10^{CK19} cells and the representative area was showed in Figure 22A. Invasion ability was increased 14.3- and 8.9-fold increased in Dox (-) YD10B^{CK13} and YD10^{CK19} cells than those Dox (+) (P<0.001 and P<0.001, respectively) (Figure 22B). Quantitative reults indicate average values of three independent experiments, each of which was conducted in triplicate (n=9). The results were shown as mean values±SD (n=9) and were analyzed by the Mann-Whitney U test (P<0.001 and P<0.001).

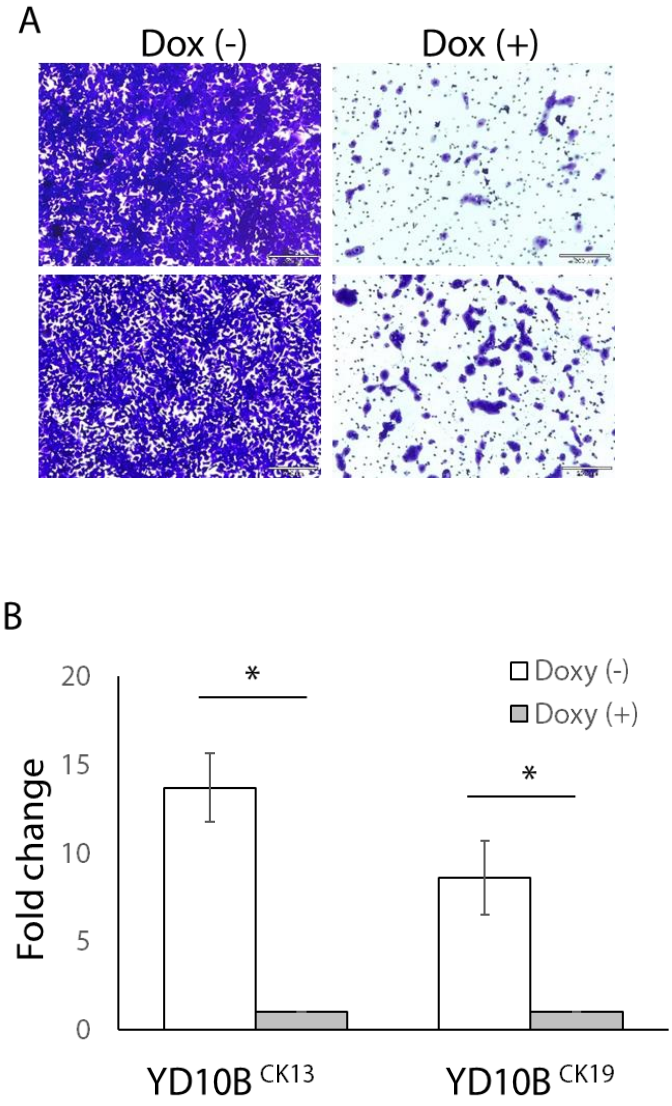

Figure 22. The influence of CK13 and CK19 expression on invasion ability of YD10B cell (**P*<0.001).

11. P53 mutation promotes proliferation ability of keratinocyte

Proliferation ability was increased 1.7- and 1.5-fold at 72 h and 96 h of time point, respectively, in HaCat^{R273H} cells and HaCat $^{\text{wild type p53}}$ (P=0.03 and P=0.04, respectively) (Figure 23A). By contrast, the number of IHOK^{dTAD} cells was increased 1.7- and 1.9-fold at 72 h and 96 h of time point, respectively (P=0.02 and P=0.03, respectively) (Figure 23 B).

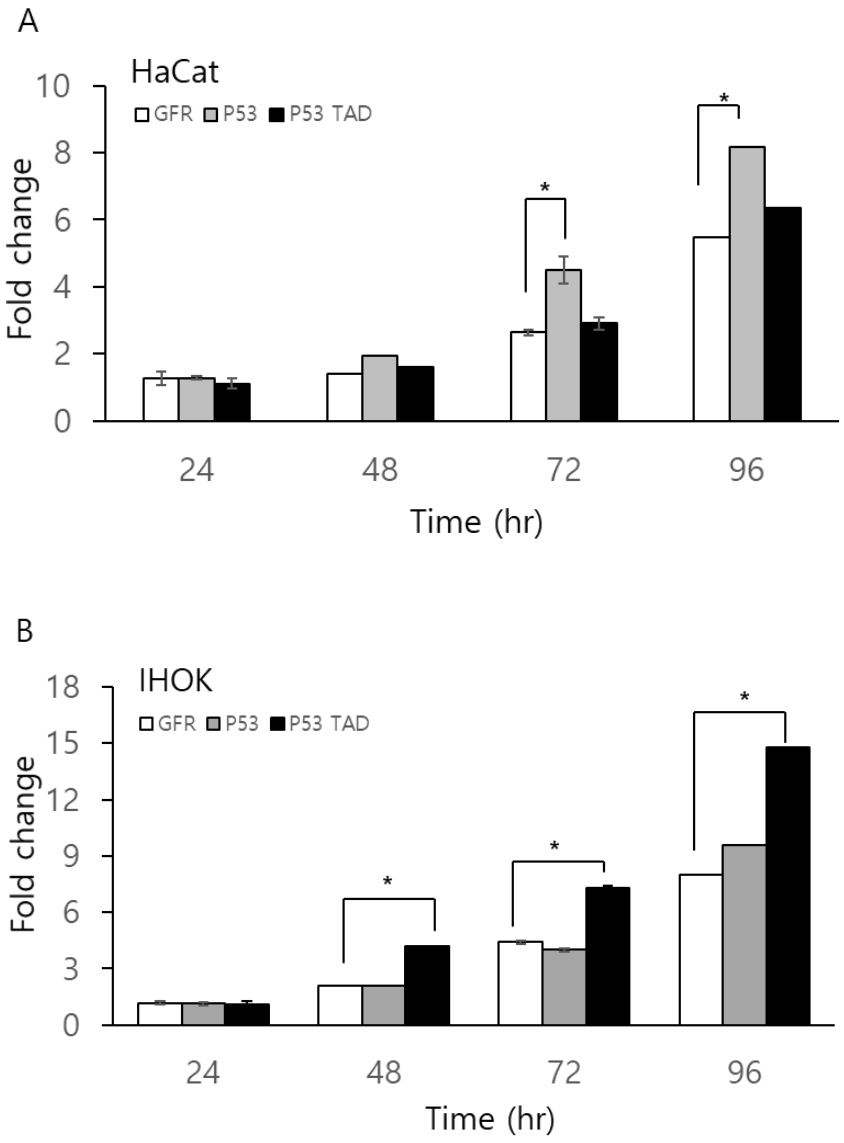

Figure 23. The influence of P53 mutation on proliferation ability of HaCat and IHOK cells (*P<0.05).

12. P53 mutation promotes invasion ability of keratinocyte

Invasion ability was comparatively investigated between each group of keratinocyte and the representative area was showed in Figure 24A. Invasion ability was 4.2- and 2.8-fold increased in HaCat^{R273H} and HaCat^{dTAD} cells than HaCat $^{wild\ type\ p53}$ ($P<0.001$, and $P<0.001$, respectively). Similary, both IHOK^{R273H} and IHOK^{dTAD} cells showed 5.8- and 3.1-fold increased invasion ability than IHOK $^{wild\ type\ p53}$, respectively ($P<0.001$, and $P<0.001$, respectively) (Figure 24B).

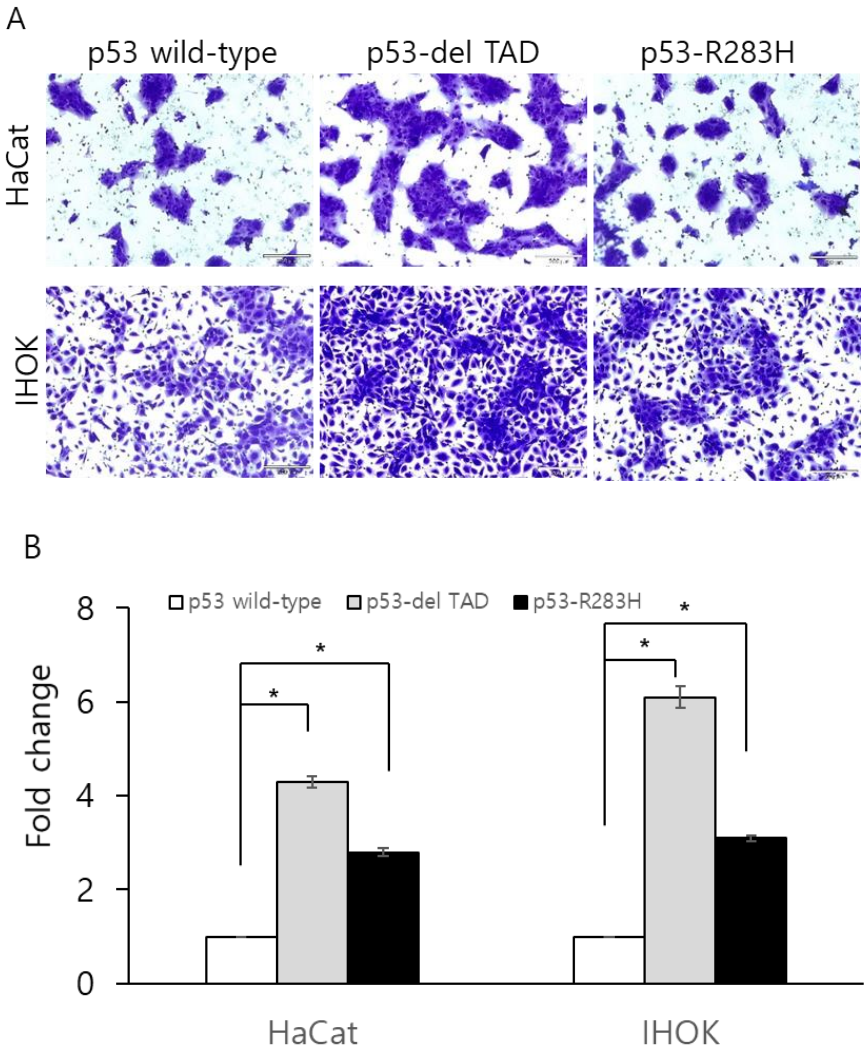

Figure 24. The influence of P53 mutation on invasion ability of HaCat and IHOK cells (*P<0.05).

DISCUSSION

Early detection of malignant lesion has a profound effect on prognosis as well as treatment due to the nature of oral cancer. For early detection of oral squamous cell carcinoma, various methods are developed using specific molecular markers (Cha, 2011). Abundant evidences have suggested that the CKs expression profile is associated with wide range of different cancers including breast, colorectal, prostate and head and neck (Chu, Wu and Weiss, 2000; Abd et al., 2004; El-Rehim et al., 2004; Knosel et al., 2006). Abd et al. (2004) showed that several cytokeratin profiles of breast carcinoma reflect alternative pathways of epithelial differentiation during carcinogenesis. Also, Knosel et al. (2006) evaluated specific cytokeratin markers which related with epithelial mesenchymal transition as powerful tools of diagnostic and prognostic signature in colorectal carcinoma.

The biomarker is based on the "multi-step carcinogenesis theory" and by estimating unique biological indicators related to each step, I can predict the possibility the progression of oral cancer. Biological markers can be classified into several categories, including genetic indicators, proliferative indicators, and differentiation indicators (Kim, 2002). In this study, I have focused on the cytokeratins as predictive biomarkers in malignant transformation of oral leukoplakia.

CK13, an acidic keratin, is assembled with CK4, its basic partner, to form intermediate cytoskeletal filaments of most internal stratified epithelia. Previous studies revealed that the loss of CK13 is associated with the malignant transformation process of the oral mucosa from epithelial dysplasia to carcinoma in situ (Sawaf et al., 1991; Hiroko et al., 2012). As CK13 has been mainly localized from the third basal layer up to the surface in normal oral mucosa, the loss of CK13 is considered to be related with the severity of dysplastic changes (Shinohara et al., 1998; Sakamoto et al., 2011). CK13 is required for healthy oral epithelium, and germline loss-of-function mutations in the KRT13 gene cause white sponge nevus, a rare genetic disorder that is mainly characterized by the presence of soft, white, spongy plaques in the oral mucosa (Richard et al., 1995). The previous study conducted by Sakamoto et al. (2011) revealed that expression of CK13 mRNA was downregulated and CK13 mRNA was downregulated in oral squamous cell carcinoma (Whipple et al., 2004). The role of CK13 in OL should be assessed in further molecular biological studies. In this study I revealed that CK13 was an independent risk factor for malignant transformation of oral leukoplakia (hazard ratios, 0.100; 95% CIs, 0.027-0.371, *P*=0.001).

CK16 has been reported to be a marker for activated keratinocytes or their hyperproliferative conditions not only in the oral cavity, but in other organs (Leigh et al., 1995). Safadi et al. (2019) insisted that degree of epithelial dysplasia was related to CK16

expression. But, in this study, there is no significant relationship between CK16 and malignant transformation (hazard ratios, 2.157; 95% CIs, 0.526-8.850, *P*=0.286).

CK19 has been utilized as an indicator gene in the diagnosis of breast, lung, stomach, and colorectal cancer. In addition, an attempt was made to evaluate squamous epithelial carcinomas based on localization of CK19 (Yoshida et al., 2015). The study conducted by Saha (2017) revealed that CK19 interacts with β-catenin/RAC1 complex to regulate the stability and translocation of β-catenin. β-catenin, in other words, binds to the NUMB promoter and initiates its expression in breast cancer cells. Modulation of NUMB expression by CK19 is therefore involved in the NOTCH pathway-mediated regulation of breast cancer and cancer stem cell properties. Also, Huang et al. (2016) insisted that CK19 expression is regulated by EMT in pleural effusion-derived lung cancer cells and related to cancer cell invasion and metastasis. In addition, Frohwitter et al. (2016) revealed that expression of CK19 protein was associated with squamous intraepithelial neoplasia and a decreased survival rate of OSCC patients. Molecular biological studies concerning CK19 role in OL should be conducted in the near future. In this study I revealed that CK19 was an independent risk factor for malignant transformation of oral leukoplakia (hazard ratios, 0.140; 95% CIs, 0.029-0.683, *P*=0.015).

Therefore, previous studies and my research results strongly suggest that the loss of CK13 and CK19 in oral leukoplakia plays an important role in oral cancer development,

and serve as biomarkers predicting malignant potential. Also, these genes may constitute therapeutic targets of oral leukoplakia having high risk of malignant transformation.

Furthermore, in this cross-sectional and retrospective cohort study, I constructed a nomogram to evaluate the quantified oral cancer risk for individual patients with oral leukoplakia. The c-index of the nomogram was approximately 0.76 using only degree of dysplasia, increasing approximately 0.87 in the nomogram that included age, dysplasia, CK13 and CK19 expression. Moreover, the calibrated nomogram showed a high level of predictive ability in this cohort of patients with oral leukoplakia.

It is well known that p53 mutation is involved in the malignant transformation of oral leukoplakia (Kim, 2002). Therefore, in this study, in vitro experiment was conducted to explorer the role of p53 as a transcription factor in the expression of specific cytokeratins such as CK13 and CK19. Trypan blue assay and Matrigel invasion assay were performed using two types of immortalized keratinocytes. I could confirm that the p53 mutation promotes the proliferation and invasion of keratinocytes, and further experiments are needed to determine the role of p53 in the regulation of biomarker cytokeratin expressions.

In conclusion, these results support that early atypical changes of oral squamous cell carcinoma may begin on the skeleton of oral squamous cells such as loss of CK13 and CK19 expressions. Also, CK 13 and CK19 expressions are useful biomarkers for predicting

the risk of malignant progression in patients with oral leukoplakia. The role of CK13 and CK19 in OL should be assessed in further molecular biological studies.

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ABSTRACT (IN KOREAN)

구강 전암병소의 악성변환에서 CK13 과 CK19 발현의 예후적 의미

<지도교수 육종인>

연세대학교 대학원 치의학과

양 동 현

구강암은 전 세계에서 발생하는 10 대 암중의 하나로 지난 수십년 동안 많은 치료방법들이 발전되어 왔지만, 5 년 생존률이 50%에 미치지 못하는 예후가 불량한 암 중에 하나이다. 특히 타 장기에 발생하는 암에 비해서, 구강암은 완치가 되더라도, 환자의 삶의 질을 현저하게 감소시키는 특징을 가지고 있다. 따라서 구강암의 치료에 있어서 가장 좋은 방법으로 암의 조기 진단 및 예방의 중요성이 강조되고 있으며, 현재로서는 암이 발생할 가능성이 높은 구강 전암병소의 조기 탐지를 통한 외과적 수술방법이 가장 효과적인 암 예방법으로 알려져 있다. 따라서, 악성변환의 가능성이 높은 병소를 초기에 발견하고 동정하는 것이 임상적으로 매우 중요하다고 할 수 있다. 현재 실험적으로 악성변환을 예측하기 위한 많은 바이오 마커가 제시되고 있으나, 이에 대한 임상적인 유의성에 대한 검증이 필요하다.

본 연구에서는 선행연구들을 통하여 발굴해 낸 사이토케라틴 마커들의 임상적 유용성에 대해서 검증하였으며, 연구결과는 다음과 같다.

1. 구강 전암병소의 악성변환에 있어서 연령과 상피이형성의 정도가 중요한 임상-병리학적 위험 요소이다.

2. 구강 전암병소의 악성변화에 있어서 CK 13 과 CK19 가 연관되어 있다.

3. 구강 전암병소의 악성 변환의 위험도를 계측화하기 위하여 두가지 사이토케라틴 마커와 임상병리학적 요소들을 결합하였고, 이것은 높은 예측 정확도를 보여주었다.

4. CK13 과 CK19 가 YD10B OSCC cell 의 침윤 및 증식능력을 억제하는 역할을 한다는 사실을 밝혔으며, P53 mutation 이 keratinocyte 의 증식 및 침윤능력을 촉진한다는 사실을 확인하였다.

이러한 연구결과들은 구강 백반증으로 대표되는 구강 전암병소에서 구강편평상피세포 암종으로의 초기 비정형적인 변화가 CK13 및 CK19 발현소실과 같은 구강 편평상피세포의 골격분화 패턴의 변화에서 시작된다는 사실을 뒷받침한다. 또한 CK13 및 CK19 는 구강 전암병소의 암 진행 위험도를 예측하는 유용한 마커로써 활용될 수 있다.

핵심어: 구강 전암병소, Keratinocyte, Cytokeratin, 악성변환, 노모그램, P53