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Expression of basal cell keratin 15 and keratin 19 in oral squamous neoplasms represents diverse pathophysiologies

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Summary. Human epithelium contains keratin, which is expressed during differentiation. Depending on the target cell type, different types of keratin are expressed, and their alterations seem to represent changes in cell properties. The basal cells of oral epithelium express keratin 5 (K5), K14, K15 and K19, but their alterations in tumors are unclear. To address this issue and to seek possible diagnostic application, we examined the expression of these keratins in oral squamous cell carcinoma (OSCC) and squamous intraepithelial neoplasm (SIN). cDNA microarray analysis of 43 OSCC revealed slight upregulation of *KRT14*, downregulation of *KRT15* and *KRT19*, and unaltered *KRT5* expression. There were great variations in *KRT15* and *KRT19* expression across each cancer. Well-differentiated OSCC tended to express more *KRT15* and less *KRT19* compared to moderately- or poorly-differentiated OSCC. *KRT15* was positively correlated with differentiation-related keratin, *KRT13*. These observations were further investigated by immunohistochemical examination. K5 and K14 were ubiquitously expressed in all 50 OSCC and 50 SIN examined. K15 and K19 were generally downregulated, but were considerably retained in about half of the cases and showed diverse expression patterns. K15-positive cancers tended to show a well-differentiated phenotype, and K19-positive cancers tended to show more invasive tumor fronts. Most K19-positive cancers appeared to develop with little associating SIN. K19 was consistently downregulated in SIN, while K15 was downregulated mainly in high grade SIN. In summary, K15 and K19, unlike K5 or K14, are expressed variably in both SIN and OSCC, which reflects the differences in their pathogenesis and biological behaviors, suggesting their prospective applications as markers for subclassifying OSCC and SIN.

Key words: Keratin 15, Keratin 19, Oral squamous cell carcinoma, Squamous intraepithelial neoplasm, Epithelial dysplasia

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Introduction

Intermediate filaments are one of three types of cytoskeletal elements that are essential for cell shape, motility and structural integrity (Moll et al., 2008). Intermediate filaments belong to a large and diverse gene family, and they are expressed selectively in specific cell types. For example, vimentin, desmin and glial acidic protein are expressed mainly in mesenchymal, muscle and glial cells, respectively (Coulombe et al., 2001). Keratins are epithelial-specific intermediate filament proteins that comprise the largest numbers of subtypes (Moll et al., 2008). Classified as acidic or basic, they are arranged in heterotypic pairs and integrated into multimeric filaments (Moll et al., 2008). These subtypes seemingly have evolved from the simple epithelial keratin pairs, keratin 8 (K8) and K18, through a series of tandem gene duplications (Blumenberg,

1988). These keratins are expressed depending on cell type and differentiation (Moll et al., 1982, 2008; Fuchs, 1995; Moll, 1998). Although the mechanism of selective expression is unclear, it facilitates cell typing, and its alteration represents certain changes in cell property. Furthermore, accumulating evidence suggests that keratin expression itself elicits alterations in cell property (Hutton et al., 1998; Wawersik and Coulombe, 2000; Kim et al., 2006; Depianto et al., 2010). In a stratified epithelium, basal and suprabasal layers express different sets of keratins. In the non-cornified squamous epithelium of oral mucosa, the basal cells express one basic keratin, K5, and three acidic keratins, K14, K15 and K19 (Sakamoto et al., 2011). In the suprabasal layer, these basal cell keratins disappear, and K4 and K13 become a dominant pair. Our previous study showed that this strict control of differentiation and stratification is invariably affected in oral epithelial neoplasms, and the expression of K4 and K13 is consistently downregulated in oral squamous cell carcinoma (OSCC) and squamous intraepithelial neoplasm (SIN, oral epithelial dysplasia and carcinoma in situ). This indicates that aberrant terminal differentiation is a common feature in these lesions, which is represented by loss of K4 and K13 expression (Sakamoto et al., 2011). OSCC and SIN show great variations in their histologies and biological behavior. We asked whether the keratin patterns reflect those differences across tumors. For example, whether ectopic induction of K1 and K10 in the suprabasal layer correlates with orthokeratotic lesion (Sakamoto et al., 2011). Here, we focused on the keratins expressed in the basal cells. We thought that these keratins are more essential than the keratins expressed in the suprabasal layer because the basal cell properties determine the epithelial differentiation fate and the interaction with stroma, which influence tumor behavior. The expression of these keratins in OSCC has not been systematically investigated, except that changes of K14 and K19 expression in OSCC have been documented in several studies, in which K14 was either downregulated (Vaidya et al., 1996; Farrar et al., 2004) or upregulated (Su et al., 1996; Ohkura et al., 2005), and K19 was also either downregulated (Su et al., 1996; Crowe et al., 1999; Kobayashi et al., 2009) or upregulated (Lindberg and Rheinwald, 1989; Xu et al., 1995; Fillies et al., 2007; Zhong et al., 2007; Safadi et al., 2010). The results reported to date are confusing, and the roles of the altered keratin expression in the pathogenesis are still unknown.

In this study, we examine the expression of K5, K14, K15 and K19 in OSCC and SIN. We show that their expression is altered in different manners. K5 is largely unchanged, whereas K14 is upregulated. K15 and K19 are downregulated, but unlike K14, they show great variations depending on the case, which correlate to histopathological features of each OSCC. The significance of these keratin expressions in oral epithelial neoplasms is discussed, and possible applications to pathology practice are shown.

Materials and methods

cDNA microarray analysis

Cancer cells were collected from surgically-excised specimens from 43 patients by laser capture microdissection of frozen sections and were subjected to cDNA microarray analysis, as previously described (Tomioka et al., 2006). Histological grade of differentiation was evaluated by oral pathologists, and the 43 cancers consisted of 17 WHO grade I, 20 grade II and 6 grade III OSCC. Epithelial portions free of cancer, dysplasia or any histopathological alterations were collected from 7 specimens and microdissected, and they were used as normal controls.

Tissue specimens

Fifty formalin-fixed paraffin-embedded specimens of OSCC (32 OSCC with SIN and 18 OSCC without SIN) plus 18 SIN specimens and 5 epulis specimens were collected from the archives of the dental hospital at Tokyo Medical and Dental University. As a normal control, paraffin-embedded archival specimens of tongue, pharynx, skin and submandibular gland taken by autopsy were used. The experimental procedures were approved by the Tokyo Medical and Dental University Ethics Committee. The OSCC patients were 29 males and 21 females, aged 32-84 years (mean 61.7), and the primary sites of cancer were the tongue (34), gingiva (5), buccal mucosa (6), oral floor (4) and palate (1). The SIN patients were 11 males and 7 females, aged 40-76 (mean 64.7), and the sites of SIN were the tongue (12), gingiva (4) and buccal mucosa (2). Histological grading of OSCC and SIN was done according to WHO criteria (Barnes et al., 2005). The pattern of invasion was evaluated according to the criteria used in the malignancy grading system developed by Anneroth (Anneroth et al., 1987).

Immunohistochemical staining

Immunohistochemical staining was conducted as previously described (Sakamoto et al., 2011). In brief, sections were heat-treated in alkaline buffer (10 mM Tris (pH 9.0) and 1mM EDTA) at 120°C for 20 minutes. The primary antibodies used in this study were anti-K5 (EPR1600Y, Epitomics); K14 (LL002, Abcam); K15 (EPR1614Y, Epitomics); K19 (EP1580Y, Epitomics); Ki-67 (MIB-1, Dako) and CD44 (EPR1013Y, Epitomics) antibodies. They were diluted 1/500 in 50 mM Tris (pH 7.4) and 0.1% Tween 20 and used for overnight incubation at 4°C. As a negative control, the primary antibodies were replaced by normal rabbit or mouse IgG. After washing with TBST (10 mM Tris (pH 7.4), 50 mM NaCl, 0.1% Tween 20), the sections were incubated with EnVision Dual Link (Dako) at room temperature for 1 hr. Coloration was done in DAB substrate. For immunofluorescent double staining, two antibodies (K15

and Ki-67 or K19 and Ki-67) were mixed for primary antibody reaction, and Alexa Fluor 488 anti-rabbit IgG (Invitrogen) and Alexa Fluor 594 anti-mouse IgG (Invitrogen) were mixed for secondary antibody reaction. Fluorescence images were digitally obtained from three representative areas of each specimen by Axiocam (Zeiss). For evaluation of the Ki-67 labeling indices, numbers of K15+ cells and K15+Ki-67+ cells, or numbers of K19+ cells and K19+Ki-67+ cells were counted on the computer screen, and the ratios of K15+Ki-67+ / K15+ and K19+Ki-67+ / K19+ were calculated.

Statistical analyses

Microarray data was analyzed by Pearson's product moment correlational analysis or Student's t-test. G factor, D factor and S factor were analyzed by Pearson's chi-square test. I factor was analyzed using the Cochran-Armitage test. A p-value less than 0.05 was considered statistically significant. A p-value less than 0.1 was considered to indicate a tendency.

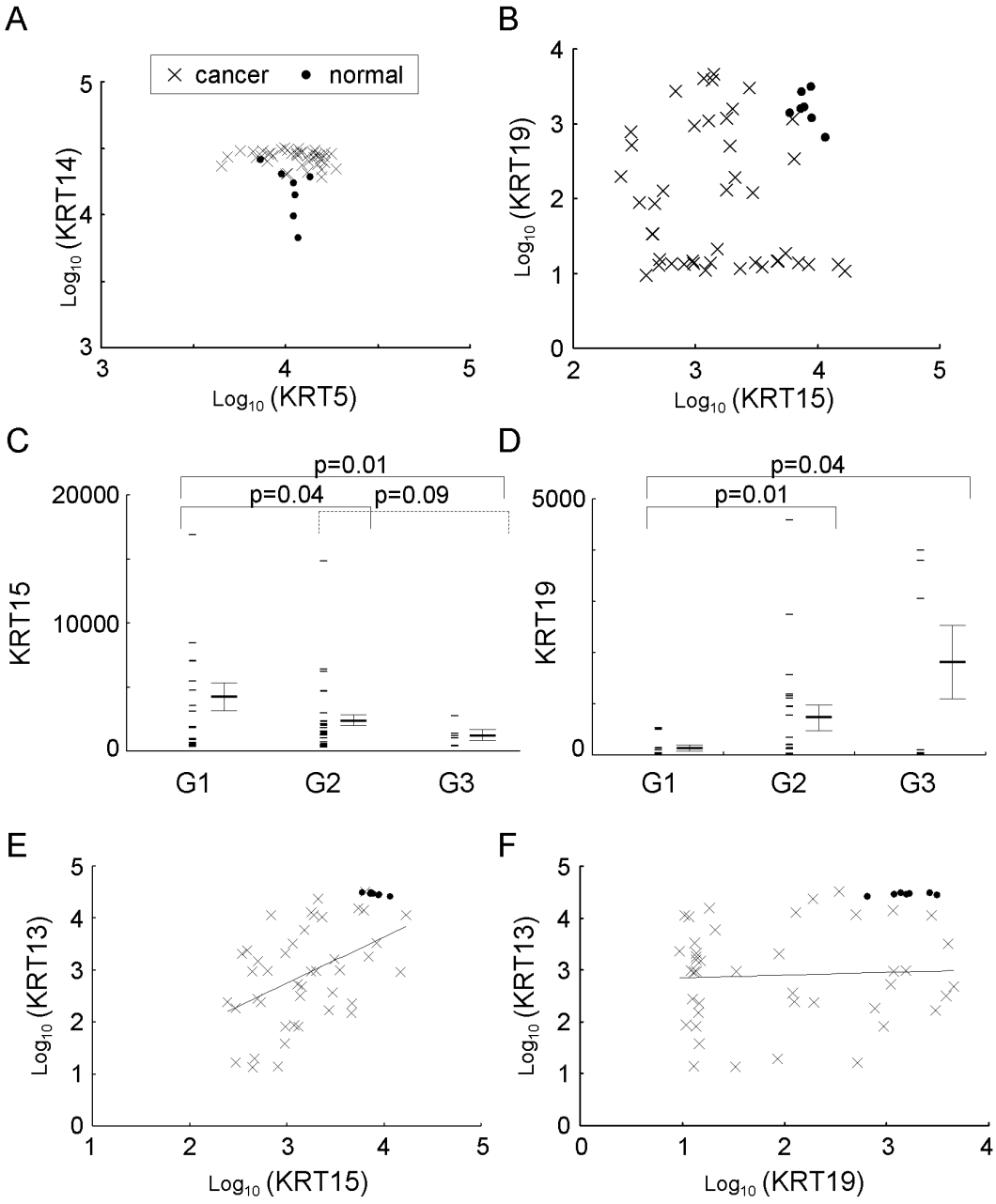

Fig. 1. cDNA microarray analysis of 43 OSCC specimens plus 7 normal controls. **A.** The expression of *KRT5* and *KRT14* in each OSCC, plotted on dual logarithmic axis charts. Crosses denote each OSCC and filled circles denote normal epithelium. **B.** The expression of KRT15 and *KRT19* in each OSCC, plotted on dual logarithmic axis charts. Crosses denote each OSCC and filled circles denote normal epithelium. **C, D**) Correlation between histological differentiation grades and *KRT15* expression (**C**) and KRT19 expression (**D**). Each OSCC specimen and averages with standard error bars are shown. G1; well-differentiated, G2; moderately-differentiated, G3; poorly-differentiated. **E.** Correlation between *KRT15* and *KRT13* in each OSCC and normal control. A regression line of cancer data calculated by linear least squares fitting is overlaid. *KRT15* and *KRT13* are positively correlated. **F)** Correlation between *KRT19* and *KRT13* in each OSCC and normal control. A regression line of cancer data calculated by linear least squares fitting is overlaid. *KRT19* and *KRT13* are not correlated.

Results

cDNA microarray analysis of basal cell keratins in OSCC

cDNA microarray analysis of OSCC collected by laser-capture microdissection revealed complex alterations in the expression of keratin subtypes. In our previous study, we evaluated the average values of the expression, and performed further studies on *keratin 4 (KRT4)* and *KRT13* that showed the most significant changes (Sakamoto et al., 2011). In this study, we focused on the keratins expressed in the basal layer; *KRT5, KRT14, KRT15 and KRT19*. To clarify the expression in each case, we plotted the globally-normalized microarray signals of each sample on dual logarithmic axis charts (Fig. 1A,B). *KRT5* expression in OSCC (11500±558; mean±s.e.) showed little diversity, as revealed by a small dispersion along the horizontal axis, and the expression did not differ (p=0.40) from that in normal epithelium (11233±777) (Fig. 1A). *KRT14* expression in OSCC (25783±992) showed a 1.6-fold upregulation over normal epithelium (16107±2165) (p=0.003). Normal epithelia co-expressed *KRT15* and *KRT19*, as revealed by the localization at the right upper corner in Fig. 1B, wherein the expression of *KRT15* (8295±605) was 5-fold higher than that of *KRT19* (1752±285). In OSCC, *KRT15* (2735±545) was reduced to 0.31-fold (p<0.001), and *KRT19* (639±180) reduced to 0.44-fold (p=0.007) compared to normal epithelium. When the expression in each OSCC was individually evaluated, the reduction rates of *KRT15* and *KRT19* were diverse, as revealed by a great dispersion of the scatter plot (Fig. 1B). Thirty-three percent of OSCC showed more than 10-fold reduction of *KRT15* (represented by a left shift of one gauge in Fig. 1B). Sixty-three percent showed more than 10-fold reduction of KRT19 (represented by a downshift of one gauge in Fig. 1B), and 42% showed more than 100-fold reduction of *KRT19* (represented by a down shift of two gauges in Fig. 1B). In contrast, there were OSCCs that considerably retained these keratin expressions: 21% of OSCC showed more than half (a left shift of approximately 0.3 gauge) of the normal *KRT15* ; and 23% showed more than half (a downshift of approximately 0.3 gauge) of the normal *KRT19*. Great variations in *KRT15* and *KRT19* expression across different cancers contrasted well with unvarying changes of *KRT5* and *KRT14*. We hypothesized that the expression levels of these keratins may reflect diverse biological properties of each cancer. With this in mind, we compared the expression of *KRT15* and *KRT19* with clinicopathological parameters, including age, sex, site, size of tumor and histological grade. Only the histological grade showed correlation with *KRT15* and *KRT19*. Well-differentiated OSCC showed higher expression of *KRT15* compared to moderately- or poorly-differentiated OSCC, and moderately-differentiated OSCC tended to express more *KRT15* than did poorly-differentiated OSCC (Fig. 1C). *KRT19* expression was significantly higher in moderately- or poorly-differentiated OSCC compared to well-differentiated OSCC (Fig. 1D). To further evaluate the correlation between keratin expression and differentiation, correlation coefficients between *KRT15* or *KRT19* and the other major basic keratins were calculated in 43 OSCC data (Table 1). Positive correlation was indicated between *KRT15* and *KRT13* (Table 1, Fig. 1E). Since K13 is a differentiation-related keratin, this suggests that cancer with high *KRT15* expression tends to achieve normal terminal differentiation. No significant correlation was observed between *KRT19* and *KRT13* (Table 1, Fig. 1F). In contrast, *KRT19* was negatively correlated with *KRT16* and *KRT17* (Table 1), which are keratins in activated keratinocytes (Wawersik and Coulombe, 2000; Kim et al., 2006). This suggests that cancer with low *KRT19* expression tends to mimic hyperplastic epithelium in terms of keratin expression.

Immunohistochemical analysis of basal cell keratins in OSCC

To validate the microarray results, we immunohistochemically examined the protein expression of K5, K14, K15 and K19 in 50 specimens of OSCC. In normal non-cornified epithelium of oral mucosa, these keratins were expressed dominantly in the basal layer (Fig. 2A). The expression patterns of K15 and K19 were different in cornified epithelium of skin. K15 was expressed in the interfollicular epithelium, but K19 was not (Fig. 2A). Both were expressed in the salivary gland, K15 was expressed only in the basal (myoepithelial) cells, and K19 was expressed both in the basal and luminal cells (Fig. 2A). K5 and K14 were expressed in the basal cells of all these tissues, accompanied with faint suprabasal expression (data not shown). In OSCC, K5 and K14 were ubiquitously expressed in all cancer cells, and there was not a single case with reduced K5 and K14 expression compared to the normal adjacent epithelium (data not shown). K15 and K19 showed diverse expression patterns. Generally speaking, both tended to be downregulated in OSCC, by which we mean that the staining intensity in individual cancer cells was less than that in normal basal cells, and the number of positively stained cells in the basal layer decreased, although there were case-dependent variations in the localization of

Table 1. Correlation coefficients between *KRT15* or *KRT19* and the other major basic keratins.

	KRT13	KRT14	KRT16	KRT17
KRT15	0.31*	-0.13	-0.09	-0.11
KRT19	-0.06	-0.37*	-0.46**	-0.31*

Correlation coefficients were calculated using cDNA microarray data of 43 OSCC specimens. *: p<0.05, **: p<0.01

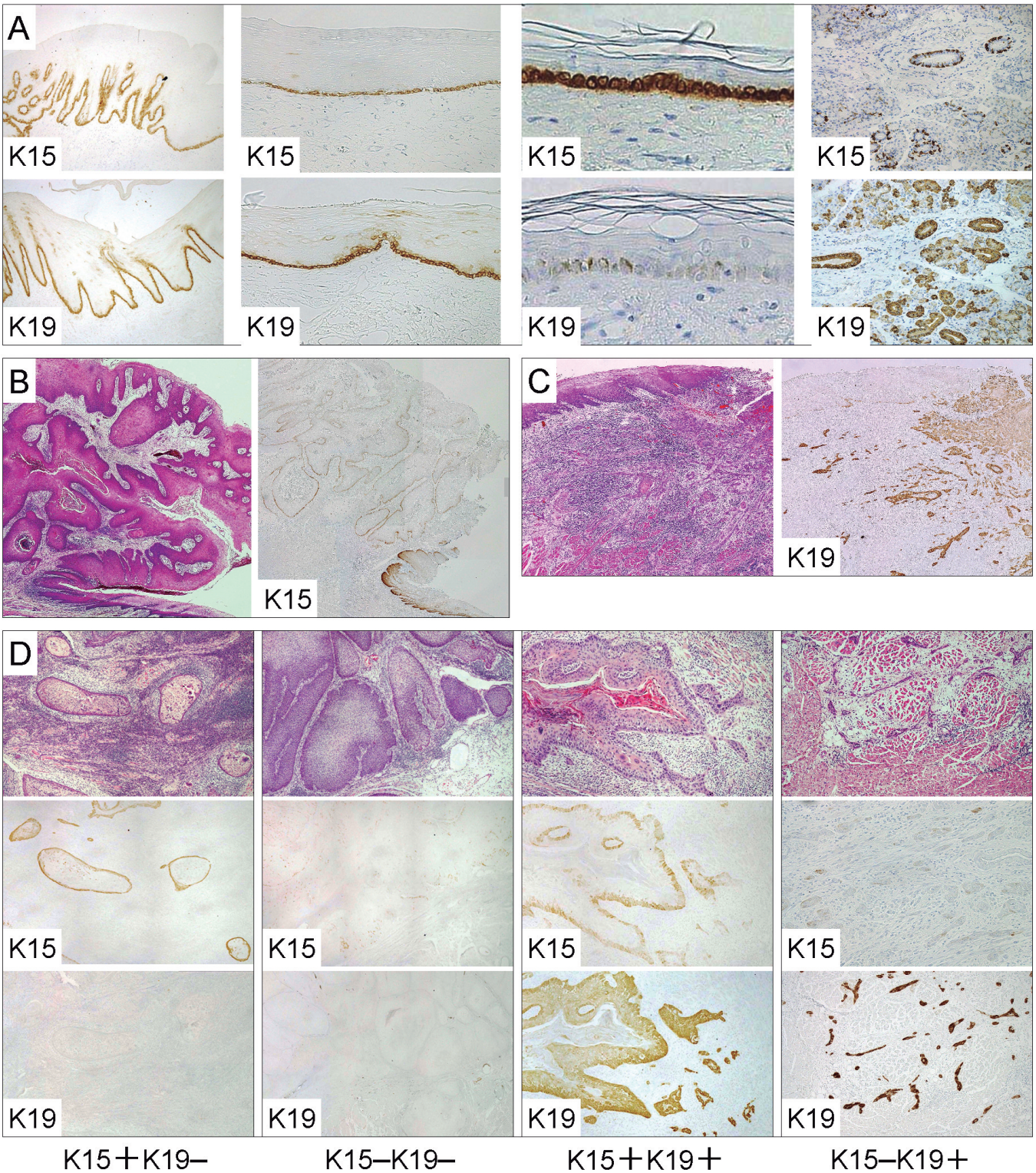

Fig. 2. Immunohistochemical expression of K15 and K19 in normal tissue and OSCC. **A.** K15 and K19 expression in normal tongue (left), buccal mucosa (middle left), skin (middle right) and salivary gland (right). K15 and K19 are expressed in the oral epithelium. In the interfollicular epithelium of skin, only K15 is expressed. In salivary glands, K15 is expressed in the myoepithelial (basal) cells while K19 is expressed in the basal and luminal cells. **B.** K15-positive OSCC, showing well-differentiated phenotype and clinically exophytic growth. This OSCC was negative for K19 (not shown). **C.** K19-positive OSCC, showing endophytic growth. This OSCC does not accompany SIN. **D.** Representative histologies of K15+K19-, K15+K19+, K15+K19+ and K15-K19+ OSCC. B, C, x 20; A, D, x 100

positively stained cells, and positively stained cells were often observed also in the suprabasal layer. The expression of K15 tended to be restricted to the periphery of cancer nests, usually showing negative staining in the center of keratinizing cancer nests (Fig. 2B,D), but K15-positive cells often appeared also in the suprabasal layer in less differentiated cancer nests (data not shown). In contrast, when K19 is expressed in cancer, K19-positive cells were usually observed both in the basal and suprabasal layer of the cancer nests, and the basal layer was stained most strongly (Fig. 2C,D). Some OSCC specimens were almost completely negative for these keratins, while the others were composed of mixed populations of positive and negative cells. It should be noted that even in the cases with prominent overall downregulation of K15, a few K15-positive cells could be observed in the cancer nests (data not shown). We regarded a case as positive (+) when more than 20% of the basal cells of cancer nests were stained, and negative (-) when less than 20% were stained. Although this is an arbitrary threshold for convenience, it allowed us to divide the OSCC evenly into 4 groups. This immunohistochemical threshold is consistent with the microarray data. If one tenth of the normal expression level is set as the threshold, the microarray samples could also be divided evenly in half, both by *KRT15* and *KRT19* expression. Therefore, we assume this immunohistochemical threshold corresponds to approximately one-tenth the expression level of the normal epithelium, and thus is a quantitatively significant one. These groups were subsequently investigated and compared between the groups using the following histopathological parameters: 1. Whether the tumor growth was clinically exophytic or endophytic (hereafter referred to as the G factor); 2. Histological grade of differentiation (D factor); 3. Mode of invasion (I factor); 4. Whether the cancer was associated with adjacent SIN (S factor). G factor correlated with K15 (κ^2=6.2, p=0.01) (Table 2). All OSCC with exophytic growth showed positive K15 expression, and 8 out of 9 cases were K15+K19- cancers (Table 2, Fig. 2B), although endophytic tumors were composed of both K15- and K15+ cases. D factor showed a significant correlation with K15 and K19. Well-differentiated OSCC expressed K15 more often than did moderately-differentiated OSCC (κ^2=7.1, p=0.02). Notably, most of the K15+K19- cancers were well-differentiated (Table 2, Fig. 2D). Conversely, well-differentiated OSCC expressed less K19 than did moderately (κ^2=7.1, p=0.02) or poorly differentiated OSCC (κ^2=4.8, p=0.03). I factor significantly correlated with K19, and K19-positive cancers exhibited more invasive tumor fronts than did K19-negative cancers (κ^2=10.3, p=0.001) (Table 2, Fig. 2C,D). K15 did not show a significant correlation with I factor. K19 significantly correlated with S factor (κ^2=25.4, p<0.001). Most K19-positive cancers appeared to occur *de novo* with no or minimum association of SIN, whereas most K19-negative cancers accompanied a considerably large area of SIN (Table 2). A representative histology of each group is shown in Fig. 2D. K15-positive cancer tended to show well-keratinized tumor nests, while K19-positive cancer tended to show reticular or cord-like nest formation. Therefore, K15+K19- cancers showed clear keratinization with blunt tumor fronts, K15-K19- cancers showed less keratinization with blunt tumor fronts, K15+K19+ cancers showed well keratinization with cord-like tumor fronts, and K15+K19+ cancers showed scant keratinization with cord-like tumor fronts (Fig. 2D).

Expression of keratins in SIN

We next examined the expression of the keratins in SIN. For this analysis, we used the abovementioned 32 cases of OSCC with SIN, plus 18 SIN cases without cancer. In all cases, K14 positive cells expanded to the entire epithelial layer in SIN. K5 showed a similar expression pattern, although not as apparent as that of K14 (Fig. 3A). Downregulation of K19 was obvious in SIN, which was recognized as a loss or a decrease of the expression in the basal cells (Fig. 3A-C). This alteration of K19 was observed even in SIN1 (Fig. 3A,B), except in 2 cases which retained considerable expression. Both of these exceptional lesions developed at the excretory opening of a minor salivary duct with superficial spread to the duct lumen, and appeared to originate from the cells at the interface between ductal epithelium and squamous epithelium (data not shown). K15 expression seemed to correlate with the severity of SIN. Because most cases composed of SIN showed different severity

Table 2. Immunohistochemical expression of K15 and K19 in OSCC, and histopathological parameters.

	K15-K19-	K15+K19-	K15-K19+	K15+K19+	subtotal
G: exophytic/endophytic	0 / 12	8 / 9	0 / 6	1 / 14	9 / 41
D: WHO grade 1/2/3	5 / 7 / 0	14 / 1 / 2	0 / 5 / 1	5 / 7 / 3	24 / 20 / 6
I: Mode of invasion 1/2/3/4	1 / 6 / 5 / 0	1 / 10 / 4 / 2	0 / 1 / 3 / 2	0 / 4 / 5 / 6	2 / 21 / 17 / 10
S: with SIN / without SIN	11 / 1	16 / 1	1 / 5	4 / 11	32 / 18
subtotal	12	17	6	15	

The numbers of OSCC cases are shown. We regarded a case as positive (+) when more than 20% of the basal cells of cancer nests were stained, and negative (-) when less than 20% were stained. Histological grading of OSCC was done according to the WHO criteria (Barnes et al., 2005). Mode of invasion was determined according to the criteria used in the Anneroth classification system.

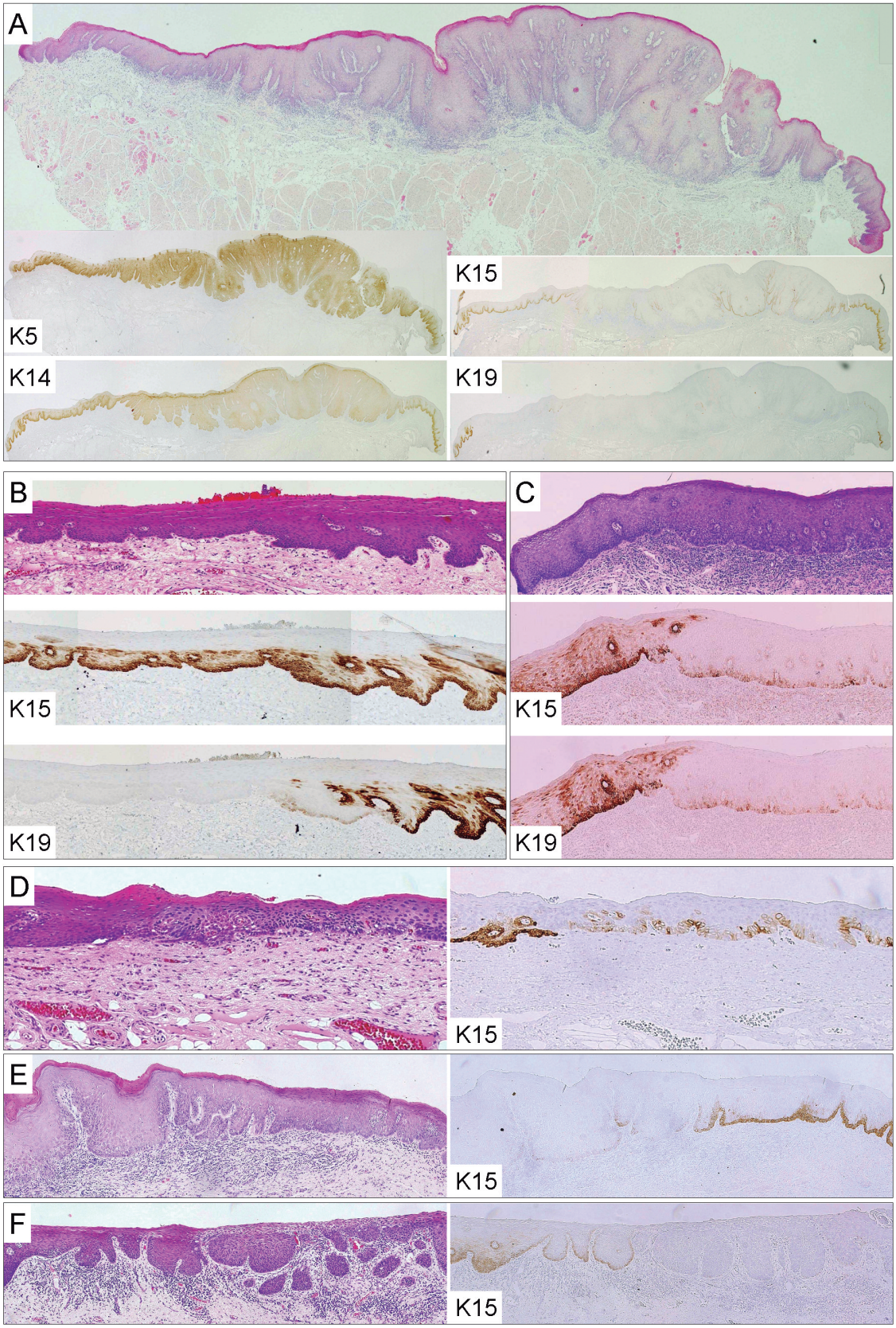

Fig. 3. Immunohistochemical expression of K15 and K19 in SIN. **A.** This neoplastic lesion of the tongue is composed of SIN1-3. K5 and K14 are detected in the whole layer of the lesion. K15 expression is missing in SIN2 and SIN3. K19 expression is missing in SIN1, SIN2 and SIN3. **B.** SIN1. The periphery of a large lesion that accompanies OSCC (not included in this photo). K19 expression is absent in the lesion, while K15 is retained. **C.** SIN2. K15 and K19 are concomitantly downregulated. **D, E, F.** SIN showing different histologies and loss of K15 expression. **D.** SIN3 adjacent to normal epithelium. A distinct reduction of K15-positive cells is observed specifically in SIN3 lesion. **E.** SIN showing hyperkeratosis. This type of lesion is frequently experienced in the oral cavity. K15 expression is unaffected in SIN1 (right) but is downregulated in SIN3 (left), suggesting the progression of the lesion. **F.** K15 is reduced in SIN2 (left) and is almost entirely absent in SIN3 or early SCC (right). A, x 20; B-F, x 100

in one lesion, often in a sequential manner, we could not quantify this observation, but a distinct tendency was observed. K15 was largely retained in SIN1 (Fig. 3A,B), and was reduced in about half of SIN2 lesions (Fig. 3A, C) and in most SIN3 (Fig. 3A,D,E,F). We did not observe differences in the expression patterns between SIN with OSCC and SIN without OSCC (data not shown). The overall pattern of the keratin expression is summarized in Table 3. To see whether these alterations are specific for neoplasms, we examined K15 and K19 expression in reactive hyperplasia using 5 specimens of epulis granulomatosa. K19 expression was disturbed in all cases; it was lost completely or intermittently, with occasional K19(+) cells in the suprabasal layer (data not shown). In contrast, K15 was largely retained in hyperplasia, and K15(+) cells in the suprabasal layer were occasionally observed in immature regenerative epithelium (data not shown). These results indicate that the expression patterns of both can be altered in non-neoplastic conditions, and that K19 expression is affected even in reactive changes, whereas K15 is more resistant to these stimuli.

K15-positive cells are slow-growing cells

To gain an insight into the cellular properties of K15- or K19-positive cells, the expression of the proliferation marker Ki-67 was assessed in 10 representative specimens. In normal oral epithelia, Ki-67-expressing cells locate mostly in the parabasal layer (Fig. 4A), and the Ki-67 labeling index of K15-positive cells was less than 10% (Fig. 4A,B). In K15-positive cancers, most Ki-67-expressing cells were negative for K15, and the Ki-67 labeling index of K15-positive cells was similar to that in normal epithelium (Fig. 4C,D), suggesting that K15(+) cancer cells have a proliferation activity similar to normal basal cells. Most K19-positive cells were negative for Ki-67 in normal epithelia (data not shown), but many cancer cells were positive both for K19 and Ki-67 in the basal and suprabasal layers (Fig. 4E). These results indicate that K15-positive cells are slow-growing basal cells in normal epithelium and that the K15-positive cancer cells retain the slow-growing feature, while K19-positive cancer cells do not. Finally, we examined the expression of a putative stem cell marker, CD44. CD44 was expressed in the basal and suprabasal layer of normal epithelium (Fig. 4A) and cancers (data not shown), indicating that it overlaps with K15 expression, although K15-positive cells are fewer in number than CD44-positive cells.

Discussion

K15 has been proposed to be a marker for the stem cells of skin, which reside in the hair follicle bulge (Liu et al., 2003). It is still a matter of debate where epithelial stem cells reside in oral epithelium that has no hair follicles (Webb et al., 2004). We propose that K15-positive cells may correspond to, or at least involve, the oral epithelial stem cells, since the following observations are consistent with the putative stem cell features: 1. *KRT15* is positively correlated with *KRT13*, which is the terminal differentiation marker of oral keratinocytes; 2. K15-positive cells are slow-growing basal cells; 3. High K15 expression leads to well-differentiated phenotype, while reduction of K15-positive cells is associated with aberrant epithelial differentiation; 4. The expression of a putative stem cell marker CD44 overlaps with that of K15. Another hypothesis is that remaining K15-positive cells in the cancer nests may represent the cancer stem cells. These hypotheses must be tested in future research.

K5 and K14 are universal keratins that are observed in basal cells of many stratified epithelia (Moll et al., 2008). Although we observed increased mRNA of *KRT14* in OSCC, it was not drastically upregulated compared to that of normal oral epithelium (1.6 fold). The modest increase of *KRT14* mRNA seems inconsistent with the immunohistochemical findings, in which K14 protein was detected in all the cancer cells. The discrepancy between mRNA and protein expression appears to stem from the dynamics of keratin filament turnover. Although *KRT14* mRNA synthesis is restricted to the basal layer, the protein is also weakly detected in the suprabasal layer, suggesting that K14 protein remains integrated in the keratin cytoskeleton after the cell ceases to express *KRT14* mRNA. In a normal suprabasal layer, abundant synthesis of differentiation-related K13 would eventually replace K14 (Lloyd et al., 1995), since the keratin subtypes are basically interchangeable (Sakamoto et al., 2011). In SIN and OSCC, where the cells cease to express K13, K14 would remain over a considerable period. Variable and weak suprabasal expression of K5, K15 and K19 proteins appears to be explained by the same mechanism. We assume that suprabasal staining may only represent aberrant keratin turnover and may not indicate upregulation of the protein synthesis and, therefore, it should not be overinterpreted and may rather be ignored.

K19-positive cancers tended to be graded as G2 or G3, rather than as G1. This seems due to the fact that K19-positive cancers showed more invasive tumor fronts compared to K19-negative cancers. The underlying

Table 3. Simplified summary of basal cell keratin expression in normal epithelium, SIN and OSCC.

	Normal	SIN1	SIN2	SIN3	OSCC
K5	++	++	++	++	++
K14	++	++	++	++	++
K15	++	++	+	-	variably
K19	++	-	-	-	downregulated

Note that this table describes generalized tendency and may not be applicable to all the cases. ++: strong expression; +: reduced expression; -: almost no expression.

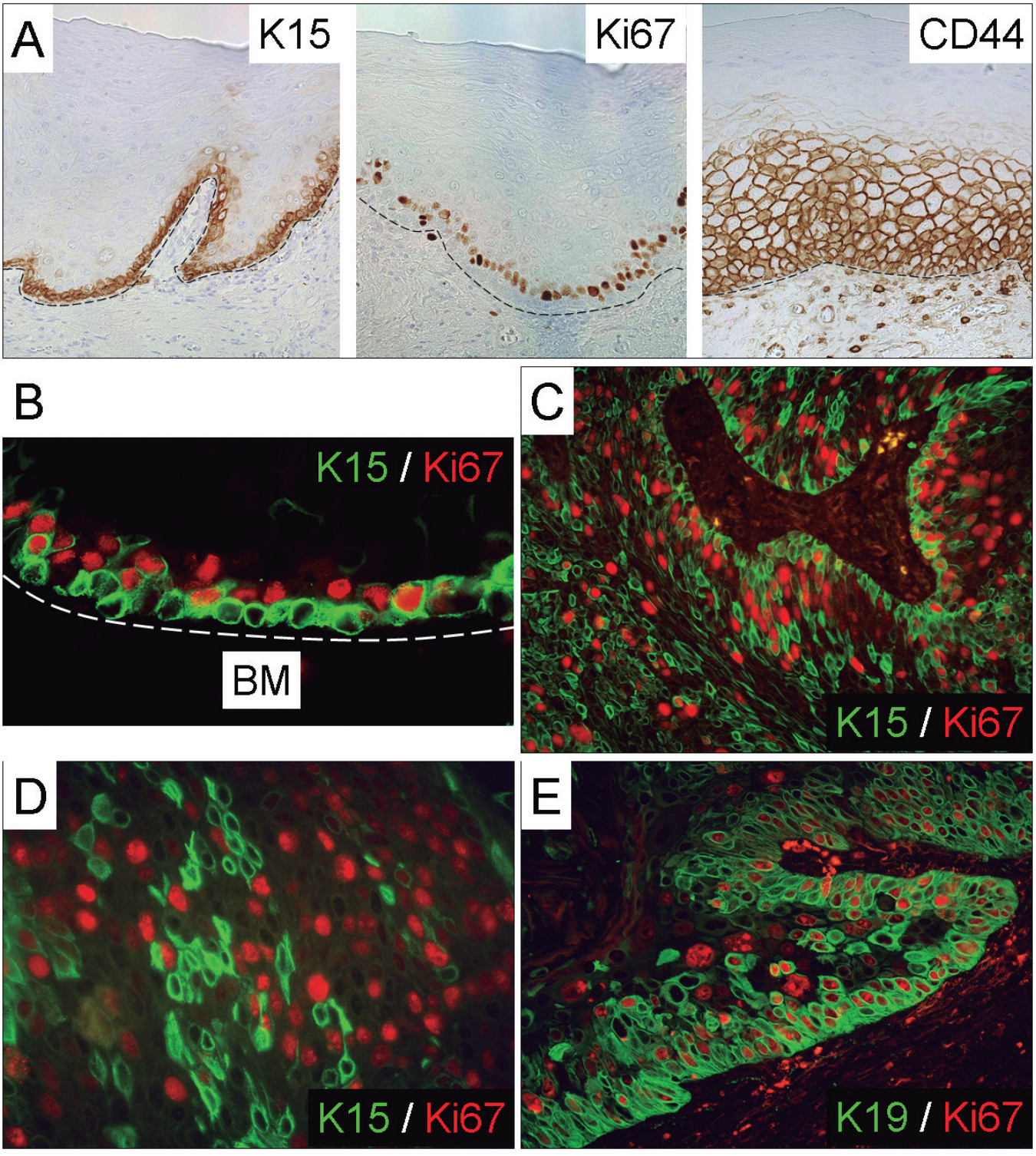

Fig. 4. A. Immunohistochemical expression of K15, Ki-67 and CD44 in normal oral epithelium. Basement membrane is indicated by a dotted line. K15-positive cells are aligned on the basement membrane. Ki-67-positive cells are in the second layer from the basement membrane. CD44 is expressed broadly in the basal and suprabasal layer. **B.** K15 (green) and Ki-67 (red) expression in normal oral epithelium, revealed by fluorescent double staining. K15 expression is restricted to the basal cells. Ki-67-expressing cells are observed mainly in the parabasal layer, although fewer Ki-67-expressing cells are observed in the basal layer. BM, basement membrane. **C.** K15 (green) and Ki-67 (red) expression in OSCC. In this K15-positive OSCC, patchy expression of K15 is observed in the basal and suprabasal layers. Cells expressing both K15 and Ki-67 are few. **D.** High magnification view of **C)**. Most of the K15-expressing cells are negative for Ki-67. **E.** K19 (green) and Ki-67 (red) expression in OSCC. In this K19-positive cancer, many basal cells, as well as parabasal cells, co-express Ki-67 and K19. A-C, E, x 100; D, x 200

mechanism is unclear, but the localization of K19-positive cells in the other sites may help us to better understand what is taking place. K19 is widely expressed in ductal and glandular epithelia. These structures are formed by epithelial cell dynamics such as invagination and branching. In the oral cavity, minor salivary glands and odontogenic epithelia, both of which originate from invaginated oral epithelia, strongly express K19 (Aragaki et al., 2010). K19-positive cells in cancer may have these potentials for invagination and branching, which are reminiscent of cancer invasion.

K19 positivity in the suprabasal layer was reportedly correlated with premalignant change in oral epithelium (Lindberg and Rheinwald, 1989), but this was later contradicted by Coltrera et al. who discussed that it may only represent metaplastic changes (Coltrera et al., 1992). We also observed variable suprabasal staining of K19 even in normal epithelia (as seen in Fig.2A). In this study, we assessed K19 expression in the basal layer and found that it was significantly downregulated in SIN, regardless of its severity. This finding gives a hint for considering the mechanism of K19-positive and K19-negative carcinogenesis. Most cases of OSCC that occurred in association with SIN were negative for K19. This could be interpreted to mean that these cancers originated from precursors that had already been transformed into K19-negative cells in SIN, whereas OSCC without SIN, displaying as if it arose *de novo*, might have originated from the precursors that skipped transformation into K19-negative cells. This suggests that a K19-positive cancer develops with fewer steps of genetic or epigenetic alterations than a K19-negative cancer, leading to the different pathophysiological features between these cancers.

K15 expression correlated with the severity of SIN. In general, SIN1 retains K15 expression, SIN2 shows its reduction and SIN3 shows its significant loss. Although the morphological criteria of SIN grading and the K15-based evaluation do not completely match, K15 could be used as a marker for objective grading of precancer and assessment of its oncogenic potential.

K19 can also be applied to pathological diagnosis of SIN. Although it cannot be used to distinguish a neoplastic lesion from a reactive lesion, its unaffected expression would ensure that it is a normal epithelium. Thus it could be used to subjectively evaluate the surgical margin, especially in lesions with minimum histological alterations.

In neoplastic lesions of oral epithelium, downregulation of K4 and K13 occurs in association with upregulation of the other keratins, such as K1, K10, K16 and K17, which can be used to aid in the diagnosis of oral malignancies (Mikami et al., 2011; Sakamoto et al., 2011). We compared these keratins in representative specimens and found that K19 was the most sensitive and easily altered, K4 was the second most sensitive, and K13 was the third, while the other keratins were reciprocally upregulated in the absence of K4 and K13 (our unpublished observation). K15 was less sensitive than these keratins and tended to be retained. We think the differentiation state in the suprabasal layer can be monitored by K4 expression, and the property of the basal cells can be monitored by K15 and K19. Upregulation of the other keratins occurs only in the absence of the inherent keratins, and thus it could be regarded as compensation for the loss of the inherent keratins. Therefore, we think that the presence of these keratins, K4, K15 and K19, is sufficient for assessing the phenotypic alterations of oral epithelium. The anti-K15 and anti-K19 antibodies used in the present study, as well as the anti-K4 antibody used in the previous study (Sakamoto et al., 2011), yield consistent and intense staining, making them appropriate for pathology practice. Furthermore, our results suggest the possibility of classifying OSCC and SIN by combination of K15 and K19 expression. For example, SIN may be classified by K15 expression, and OSCC may be classified by a combination of K15 and K19 expression. We plan to analyze the correlation between these keratin expressions and the clinical outcome, such as chemo-radio resistance, metastasis and prognosis, in a future study.

In conclusion, K15 and K19, unlike K5 or K14, are expressed differentially in each OSCC and SIN. Although K15 and K19 are co-expressed in the basal cells of normal oral epithelium, their expression patterns are different in other tissues: K15 tends to be expressed in basal cells, and K19 in glandular cells. We think that the diversity of their expression in OSCC and SIN reflects the difference in the tumor cell properties, a hypothesis which is supported by the present data showing that K15 expression correlates to differentiation and K19 expression correlates to invasion pattern. K15 and K19 can be good tools for assessing tumor cell properties and aiding diagnosis, and moreover, they may possibly be used for classifying oral epithelial neoplasms.

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