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ORIGINAL ARTICLE

The presence of Merkel cells and CD10- and CD34-positive stromal cells compared in benign and malignant oral tumors

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BACKGROUND: To describe sequential changes in Merkel cells (MC), and CD10$^+$ and CD34$^+$ stromal cells (SC) during the transition from benign to malignant oral lesions and correlate with clinicopathologic parameters.
MATERIALS AND METHODS: Changes in cytokeratin 20-positive (CK20$^+$) Merkel cells, CD10$^+$ and CD34$^+$ SC were immunohistochemically examined in specimens of 28 oral verrucous carcinomas (VC), 32 squamous cell carcinomas (SCC) and 36 benign squamous lesions (BSL). Immunoreactivity and localized inflammation were measured quantitatively and/or semiquantitatively, and between-group results were statistically compared.
RESULTS: The mean number of CD34$^+$ SC was significantly lower in VC (57.36) and SCC (33.81) than BSL (351.56, $P < 0.001$). However, the three tumor types had similar staining level and number of CD10$^+$ SC. We found a significant difference in the density of MC between BSL and VC ($P < 0.001$) or SCC ($P < 0.001$). The number of CK20$^+$ MC was significantly lower in highly inflamed specimens than mildly inflamed specimens ($P = 0.001$).
CONCLUSION: CD34$^+$ SC and to a lesser extent MC, but not CD10$^+$ SC, reveal statistically different density during the transition from benign to malignant oral lesions. The correlations between the CD34$^+$ SC expression and squamous lesions may be associated with epithelial dysplasia and/or tumor invasion.
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Keywords: CD10; CD34; CK20; Merkel cell; oral cancer; stromal cell

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Introduction

Factors dictating the progression and behavior among oral benign squamous lesions (BSL) and malignancy lesions are still poorly understood. Although differences between benign, dysplastic and malignant squamous cells of BSL, verrucous carcinomas (VC) and squamous cell carcinomas (SCC) have been detected immunohistochemically (Chang *et al*, 2000; Wu *et al*, 2002; Choi *et al*, 2004; Oliveira *et al*, 2005), the roles and the changes of non-neoplastic cells among benign and malignant lesions of human oral mucosa are paid relatively less attention.

Merkel cells (MC) are neuroendocrine cells that are found in clusters in the outer root sheath of developing hair follicles or as widely scattered single cells in the basal layer of the epidermis and squamous mucosa (Kim and Holbrook, 1995; Righi *et al*, 2006). Previous studies have demonstrated that the identification of MC can be useful in distinguishing between benign and malignant adnexal neoplasms of the skin (Schulz and Hartschuh, 1997; Abesamis-Cubillan *et al*, 2000). Whether MC are present in different oral squamous lesions is unclear. Cytokeratin 20 (CK20), a low-molecular-weight major cytoskeletal polypeptide of human intestinal epithelium, was found to be a more sensitive and specific biomarker of MC than chromogranin A, a key protein of neuroendocrine cells (Chan *et al*, 1997).

CD10 (a cell surface metalloendopeptidase) and CD34 (a human progenitor cell antigen) are expressed in a wide variety of normal and neoplastic cells (Bilalovic *et al*, 2004). Although several studies revealed that patterns of CD10 or CD34 expression in the peritumoral spindle-shaped cells are different in different neoplasms (Iwaya *et al*, 2002; Chauhan *et al*, 2003; Barth *et al*, 2004; Pham *et al*, 2006), these patterns remain uninvestigated in stromal cells (SC) of benign and invasive oral squamous neoplasms.

The objectives of this study were: (i) to compare the number and distribution of MC, and CD10$^+$ and CD34$^+$ SC in oral BSL, VC, SCC and boundary

dysplastic mucosa adjacent to carcinomas and (ii) to correlate the relationships among above non-neoplastic cells, inflammation and other clinicopathologic factors.

Materials and methods

Tumor material

Specimens of 138 oral tumors identified as BSL, VC or SCC were retrieved from the files of the Department of Pathology at Tri-Service General Hospital, Taipei, Taiwan. After approval of the study protocol by the Hospital Review Board and excluding patients without proper clinical information [e.g. age, gender, lesion location or tumor node metastasis (TNM) stage] or specimens that lacked adjacent non-neoplastic mucosa or underlying stroma, or loss of morphologic orientation, 96 patients were included: 28 VC patients (27 men and 1 woman, mean age 57 years, range 40–74 years), 32 SCC patients (30 men and 2 women, mean age 50 years, range 29–72 years; 18 well-differentiated, 10 moderately differentiated and four poorly differentiated specimens) and 36 BSL patients (28 men and 8 women, mean age 39 years, range 16–64 years; 18 squamous papilloma, 12 irritated fibroma and six condyloma acuminatum specimens).

Immunohistochemistry

For immunohistochemical staining, tissue blocks were sectioned into 5-μm slices, which were placed on 3-(triethoxysilyl)-propylamin-coated slides. The slides were placed in preheated 10 mM citrate buffer (pH 6.0) and heated under pressure for 5 min for antigen retrieval. All primary antibodies used in this study were monoclonal: CK20 (Neomarkers Ab-1, clone K20.4, 1:50 dilution, Fremont, CA, USA), CD10 (Neomarkers Ab-2, clone 56C6, 1:50 dilution) and CD34 (Dako, clone QBEnd 10, 1:100 dilution, Glostrup, Denmark). After incubation with primary antibody for 60 min at room temperature, the staining was visualized using 3-amino-9-ethylcarbazole chromogen + substrate chromogen for 8 min (Dako). Red-brown precipitates within the cytoplasm for CK20 and on the cell surface for CD10 and CD34 were seen. The slides were counterstained with 4% Mayer hematoxylin. Paraffin sections of colon adenocarcinoma for CK20 (tumor cells) and CD34 (vascular endothelium) and of normal breast tissue for CD10 (myoepithelial cells) served as positive controls. Omitting the primary antibody served as negative control. One section of each specimen placed on a regular slide was stained with hematoxylin and eosin (H&E) alone.

Evaluation and statistics

Routine H&E sections were evaluated by two pathologists (HW Gao and CP Yu) and used for diagnosis of VC, BSL or SCC. The density of immunostained $CK20^+$ MC and $CD10^+$ and $CD34^+$ SC were compared between the tumor and adjacent normal/dysplastic mucosa.

Immunoreactivities of CK20, CD10 and CD34 were measured quantitatively and/or semiquantitatively. Immunoreactivity was defined semiquantitatively as follows: (i) for CK20: negative (−) indicated absence of stained cells in the basal layer or periphery of the tumor nests; positive (+) indicated one stained cell per 301 or more basal or peripheral cells on average, (++) indicated one stained cell per 101–300 basal or peripheral cells on average and (+++) indicated one or more positive cells per 100 or less basal or peripheral cells on average; (ii) for CD10 and CD34, ratings were based on the number of stained spindle SC per 10 high power fields (HPF) (field diameter 0.59 mm diameter/0.274 mm^2 area; one end of the field moving along the basal layer or the tumor base while measuring): negative (−) indicated 20 or less cells, (+) indicated 21–100, (++) indicated 101–400, (+++) indicated 401 or more. To prevent overestimation, endothelial cells of vessels and intermuscular SC were not counted. Additionally, inflammation both in the lesion and adjacent mucosa were rated 0 (no inflammation), 1 (mild), 2 (moderate) and 3 (severe).

Statistical significance of differences in CK20, CD10 and CD34 expression were tested using the chi-squared

Table 1 Immunohistochemical expression of CK20, CD10, and CD34 in BSL, VC and SCC

		Semiquantitative estimate of expression levels			
Diagnoses	*Ab*	−	+	++	+++
BSL (n = 36)	CK20	20 (55.5%)	8 (22.2%)	6 (16.7%)	2 (5.6%)
	CD10	8 (22.2%)	8 (22.2%)	12 (33.3%)	8 (22.2%)
	CD34	4 (11.1%)	0	16 (44.4%)	16 (44.4%)
VC (n = 28)	CK20	28 (100%)	0	0	0
	CD10	16 (57.1%)	0	12 (42.9%)	0
	CD34	24 (85.7%)	2 (7.1%)	0	2 (7.1%)
SCC (n = 32)	CK20	29 (90.6%)	3 (9.4%)	0	0
	CD10	4 (12.5%)	12 (37.5%)	14 (43.8%)	2 (6.2%)
	CD34	18 (56.3%)	12 (37.5%)	2 (6.2%)	0

BSL, benign squamous lesions;VC, verrucous carcinomas; SCC, squamous cell carcinomas; Ab, antibodies; HPF, high power fields.
CK20 for detection of MC of basal/peripheral layer: −, absence of stained cells; +, one stained cell detected per 301 or more basal or peripheral cells; ++, one stained cell found in 101–300 basal or peripheral cells; +++ one or more positive cells per 100 or less basal or peripheral cells. CD10 and CD34 for detection of stained spindle-shaped stromal cells per 10 HPF beneath the basal layer or lesional base: −, 20 or less; +, 21–100; ++, 101–400; +++, 401 or more.

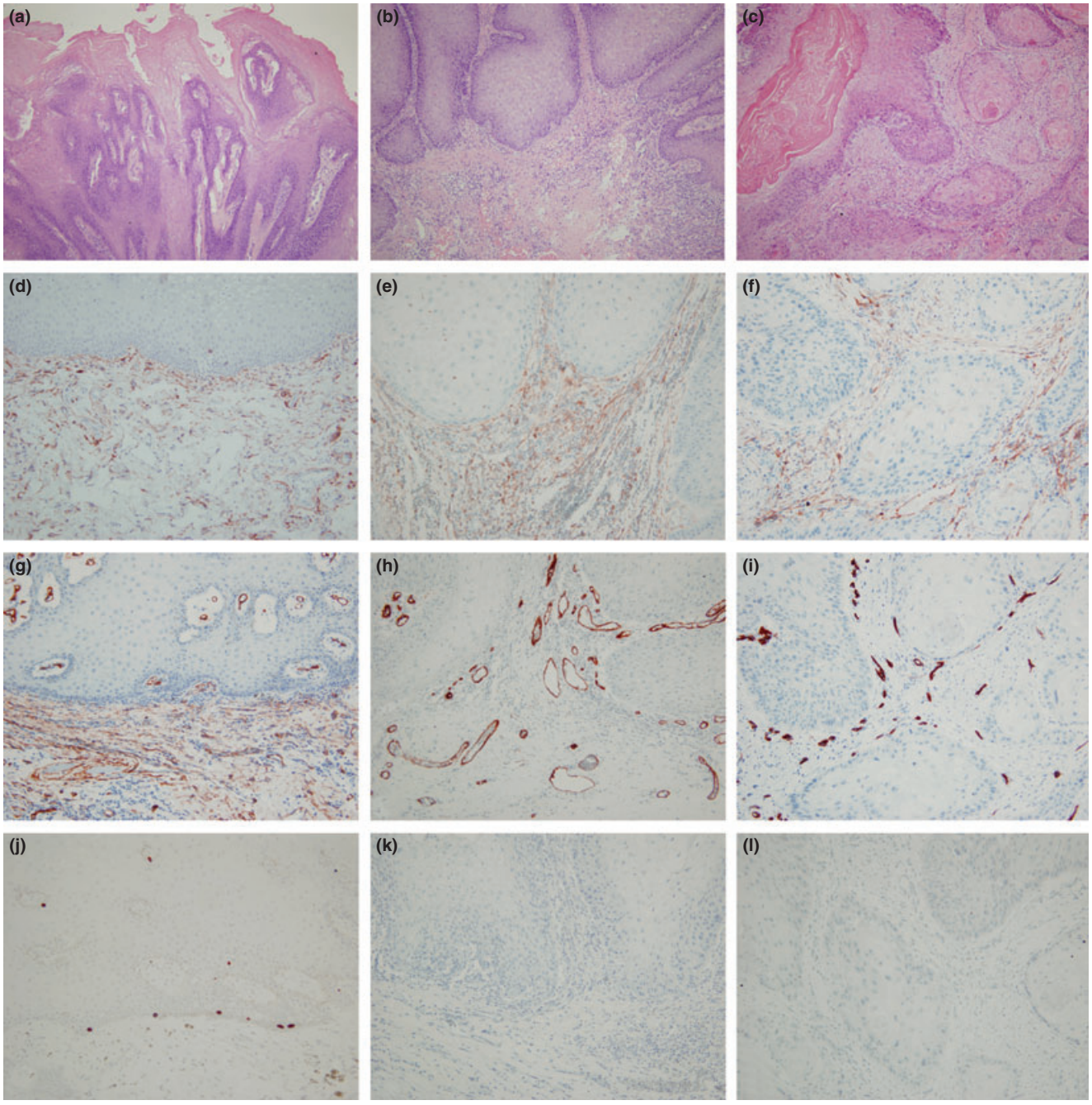

Figure 1 Immunohistochemical staining for CD10, CD34 and CK20. H&E of **(a)** BSL, **(b)** VC and **(c)** SCC. **(d, e, f)** CD10 staining in BSL, VC and SCC, respectively. CD10 expressed in some spindle-shaped stromal cells and perivascular cells. No statistical difference in the expression level and number of CD10$^+$ cells in BSL, VC and SCC. **(g, h, i)** CD34 staining in BSL, VC and SCC, respectively. CD34 expressed in vascular endothelium and some spindle-shaped stromal cells. A significant difference ($P < 0.001$) in the mean number of CD34$^+$ cells was found between BSL and VC, BSL and SCC, and VC and SCC. **(j, k, l)** CK20 staining for MC in the basal layer of BSL, VC and SCC, respectively (original magnification; **a**, **b** and **c**: 100×; **d**–**l**: 200×)

test, Student's *t*-test and Mann–Whitney *U*-test. A *P*-value of <0.05 was considered statistically significant. SigmaPlot software (Jandel Scientific, San Rafael, CA, USA) was used to analyze the relationship between CK20, CD10 and CD34 expression and clinicopathologic parameters.

Results

The mean age of patients was statistically different between the BSL (41.44 years) and VC (56.64, $P = 0.002$) or carcinoma (VC and SCC) groups

Table 2 Expression of CD10 and CD34 in non-neoplastic stromal cells in benign and malignant squamous lesions (BSL, VC and SCC)

	CD10		*CD34*	
Diagnosis	*Mean ± 2 × s.e.m.*	P*	*Mean ± 2 × s.e.m.*	P*
BSL	185.83 ± 106.82		351.56 ± 105.12	
VC	109.79 ± 50.18	0.25	57.36 ± 68.70	<0.001
SCC	163.00 ± 44.86	0.875	33.81 ± 70.89	<0.001
VC & SCC	138.17 ± 33.90	0.26	44.80 ± 29.68	<0.001

BSL, benign squamous lesions;VC, verrucous carcinomas; SCC, squamous cell carcinomas; s.e.m., standard error of the mean.
*P-values were determined using the Student's t-test for comparison between BSL and VC, BSL and SCC, BSL and VC & SCC, respectively.

(52.18, $P = 0.015$) but not between the BSL (41.44) and SCC (48.28, $P = 0.133$) groups.

Table 1 summarizes the levels of cells positively stained for CD10, CD34 and CK20 in all specimens, and Figure 1 shows representative microphotographs of BSL, VC and SCC stained for CD10, CD34 and CK20.

Staining for CD10 and CD34 revealed a band-like pattern of stained spindle-shaped SC over peritumorous stroma in BSL, VC and SCC.

The mean number of cells positive for CD34 per 10 HPF was significantly higher in BSL (351.56) than in VC (57.36) and SCC samples (33.81, $P < 0.001$). The difference in the mean number of $CD34^+$ cells was significant between BSL and VC ($P < 0.001$), BSL and SCC ($P < 0.001$), and BSL and VC and SCC ($P < 0.001$). By contrast, the numbers of $CD10^+$ cells were similar in all BSL, VC and SCC samples (Table 2).

Linear regression analysis was further performed to compare differences in the numbers of $CD34^+$ SC among adjacent normal oral mucosa of BSL, within BSL, surrounding dysplastic mucosa of VC and SCC, and within VC and SCC. The result revealed a correlation between number of $CD34^+$ SC and the transition from non-malignancy to malignancy in squamous mucosa ($P_{trend} < 0.001$, Figure 2).

CK20-specific monoclonal antibody stained MC along the adjacent normal mucosa and at the periphery of tumor nests in all specimens of the three tumor types.

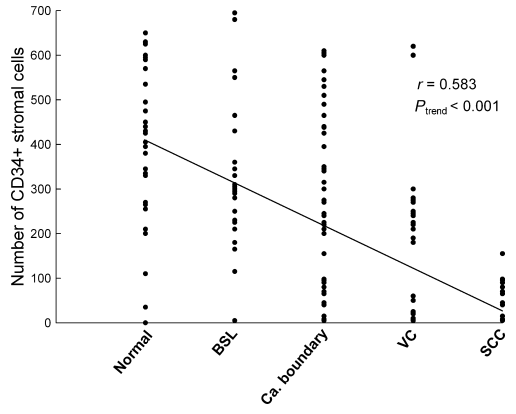

Figure 2 CD34 expression in the normal oral mucosa (normal), BSL, the boundary mucosa adjacent to carcinoma (Ca boundary), VC and SCC. Linear regression is used in this analysis

We found significant difference in the presence of MC between BSL and VC ($P < 0.001$) or SCC ($P < 0.001$). No MC could be identified in 100% of VC (28/28) and 90.6% of SCC (29/32) specimens. Staining of 1+ was found in three SCC specimens, whereas staining of up to 3+ was found in 44.4% (16/36) of BSL specimens. No statistically different expression between BSL and boundary dysplastic mucosa adjacent to carcinoma ($P > 0.1$).

The correlation between inflammation and level of CD10, CD34 and CK20 expression was analyzed by the chi-squared test. In comparison with low-score inflammation (0 or 1), high-score inflammation (2 or 3) was correlated with significantly lower numbers of $CK20^+$ MC ($P = 0.001$). There was no correlation between inflammation and expression of CD10 ($P = 0.056$) or CD34 ($P = 0.053$).

About clinicopathologic factors, immunostaining numbers of $CD34^+$ SC in SCC correlated significantly with clinical T stage ($P < 0.005$) (Figure 3). No correlation was found between expression of CD10, CD34 and CK20 and other clinicopathologic parameters, including patients' age ($P > 0.1$), tumor location ($P > 0.1$), tumor grading of SCC ($P > 0.1$), clinical TNM stages of VC ($P > 0.1$), and N and M stages of SCC ($P > 0.1$).

Discussion

For many years, researchers have concentrated almost exclusively on the malignant carcinoma cells and looked for ways to either selectively kill or restrict its growth. However, more investigations and novel therapeutic strategies that were stromal targeting (e.g. stromal tumor fibroblasts and anti-angiogenesis) were paid more attention recently (Ahmed *et al*, 2008). Recent studies have pointed out the clinicopathologic significance of the presence of non-neoplastic cells such as MC, $CD34^+$ and $CD10^+$ SC in tumors of the breast (Iwaya *et al*, 2002; Chauhan *et al*, 2003), skin (Schulz and Hartschuh, 1997; Abesamis-Cubillan *et al*, 2000; Bilalovic *et al*, 2004; Pham *et al*, 2006), and head and neck (Barth *et al*, 2004; Kojc *et al*, 2005). However, no study has focused on the relationship of non-neoplastic cells to both benign and malignant squamous neoplasms of the oral mucosa.

The CD10 antigen is a 100-kDa cell surface metalloendopeptidase that participates in inactivation of a variety of biologically active peptides. CD10 can be detected on some lymphoma/leukemia cells and in non-lymphoid cells, including fibroblasts, breast myoepithelial cells, bile canaliculi (Shousha *et al*, 2004), and the brush border of renal and intestinal epithelial cells (Loke *et al*, 1990; Moritani *et al*, 2002). Pham *et al* pointed out differences in patterns of CD10 staining in stromal and basaloid cells between basal cell carcinoma and trichoepithelioma (Loke *et al*, 1990). Some studies also showed SC expression of CD10 was associated with tumor invasion and facilitating the occurrences of metastases and local recurrences (Iwaya *et al*, 2002; Piattelli *et al*, 2006). However, this study found no

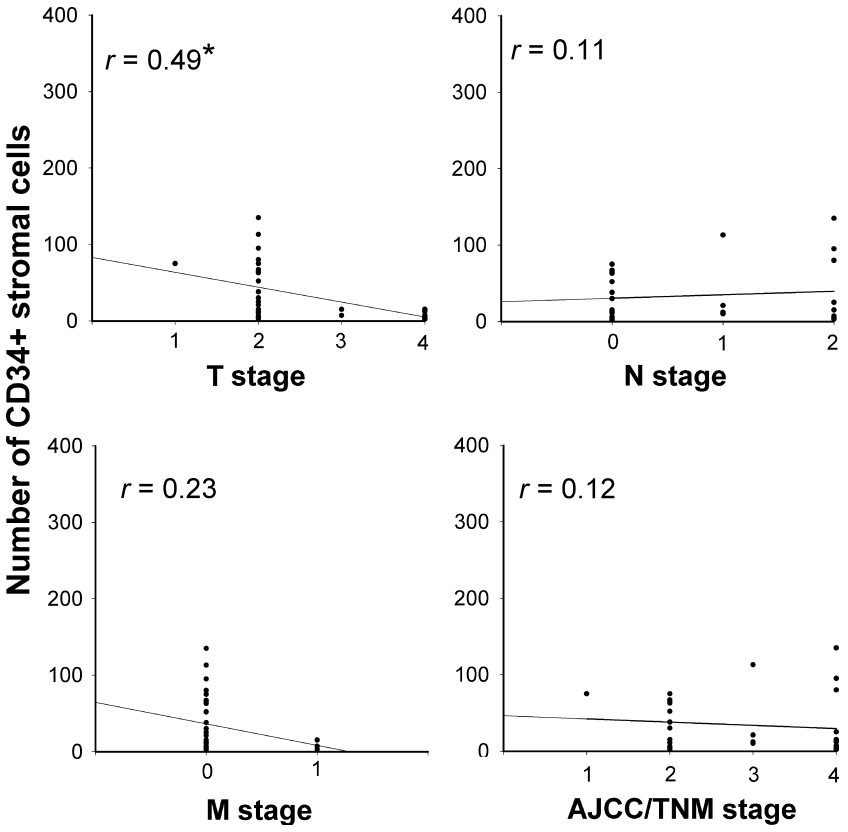

Figure 3 Clinicopathologic correlations with the number of CD34+ stromal cells in squamous cell carcinoma. *Statistical significance by linear regression testing

difference in expression of CD10 between BSL and malignant squamous lesions in the oral mucosa.

CD34 is called the human progenitor cell antigen. In normal oral mucosa, it is present in endothelial cells, in perivascular/interstitial dendritic cells mainly in the reticular dermis, and in spindle-shaped cells around the skeletal muscle fibers. Several studies proposed that invasive carcinomas of oropharyngeal and laryngeal areas induce stromal remodeling characterized by loss of $CD34^+$ fibrocytes and subsequent gain of α-SMA- and $TGF\beta1^+$ myofibroblasts (Barth *et al*, 2004; Kojc *et al*, 2005). In this study, $CD34^+$ SC could be identified in most normal mucosa, in non-malignant squamous lesions and in boundary dysplastic mucosa of carcinoma of the oral cavity, but there was significantly decreased or complete loss of $CD34^+$ SC beneath malignant epithelial lesions. Some reports hypothesized that $CD34^+$ SC may have roles as antigen-presenting cells and multipotent mesenchymal cells (Chauhan *et al*, 2003; Barth *et al*, 2004). In this study, positive linear regression analysis (Figure 3) indicates sequential change in decreasing the number of $CD34^+$ SC from normal oral mucosa through BSL, boundary mucosa around carcinoma to malignant squamous tumor. Some researches confirmed distinctive association between increased density of stromal myofibroblasts and the development of carcinoma in human hypopharynx and in a rat 4NQO-induced tongue rat carcinogenesis model (Kojc *et al*, 2005; Vered *et al*, 2007). Contrary to this study, above findings about myofibroblast indicate that the $CD34^+$ SC in our current research may come from different cell origin other than stromal myofibroblasts. Defining the mechanism of $CD34^+$ SC suppression by oral mucosa dysplasia and carcinogenesis is expected to further broaden our knowledge on the micro-environmental events occurring during the malignant transformation.

Merkel cells are neuroendocrine cells that are a permanent constituent of the epidermis and hair follicles (Kim and Holbrook, 1995). Their preferential localization near the bulge areas of hair follicles led to the presumption of a regulative role of MC in the follicular cycle (Narisawa *et al*, 1994). We used CK20, a sensitive immunohistochemical marker for MC, to detect MC in squamous lesions of the oral mucosa. Similar to previous report (Righi *et al*, 2006), this study found no MC in 100% (28/28) VC and 90.6% (29/32) SCC (up to 1+ density for all three positive cases) specimens. There were statistically lower density levels of MC in VC and SCC than in BSL. However, MC can be detected in only 30% (32/96) of adjacent mucosa of BSL, VC and SCC with low density levels [1+ density in 87.5% (28/32) of positive cases] in our research. Besides, we also identified that the decrease of staining density levels could also be ascribed to background inflammatory response. Relatively scant number of MC in oral mucosa and inflammatory effect would practically limit its significance in distinguishing BSL from malignant lesions. The exact mechanism by which decrease or loss of MC in VC and SCC is not known but it is likely that it is closely related to inflammatory destruction by host immune reaction and/or tumor invasion.

In conclusion, by focusing on non-neoplastic cells, this study demonstrates a significant difference in the expression of $CD34^+$ SC between benign and malignant squamous lesions of the oral mucosa. As above characteristic distributing patterns and statistically transitional change from normal mucosa through BSL, boundary mucosa of carcinoma to malignant lesion, the associations between the expression of $CD34^+$ SC and squamous dysplasia and/or tumor invasion need further investigation. In spite of statistically decreasing MC in VC and SCC of oral cavity in our study, the presence or absence of MC should not be used as a major criterion in distinguishing benign from malignant lesions, as MC are not frequent in oral mucosa and can be affected by local inflammation. No significant difference in the expression of $CD10^+$ SC can be detected between BSL and VC or SCC in this study.

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Conflict of interest

We declare that we have no conflict of interest.

Author contributions

HW Gao handled the study concepts, literature search, clinical studies, data acquisition and manuscript preparation. HW Gao, CP Yu are the guarantors of the integrity of the study and they took care of definition of intellectual content and manuscript editing. HW Gao, HS Lee, CP Yu handled the study design. HW Gao, HS Lee performed the experimental studies. HW Gao, JY Ho, HS Lee, CP Yu performed data analysis. HW Gao, JY Ho performed statistical analysis. CP Yu, HS Lee reviewed the manuscript.

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