免疫組織化学的應用與評估

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博士論文

Immunohistochemical assessment of Eph/ephrin expression in oral squamous cell carcinoma and its precursor lesions
（口腔癌と前癌病変における Eph/ephrin 系の免疫組織学的検討）

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ABSTRACT

Objective: To evaluate erythropoietin-producing hepatocellular carcinoma (Eph) / Eph receptor-interaction protein (ephrin) expression in oral squamous cell carcinoma (OSCC) and oral epithelial precursor lesions (OEPLs), EphA2, EphB4, and ephrinB2 were examined and compared with microvessel density (MVD) and lymphatic vessel density (LVD).

Methods: Samples from 73 OSCC and 43 OEPLs patients were immunohistochemically analyzed with antibodies against EphA2, EphB4, ephrinB2, CD34, and D2-40. Results were compared with clinicopathological findings.

Results: Immunohistochemical reactivity for EphA2, EphB4, and ephrinB2 was detected in epithelial cells and some stromal vascular cells in OEPLs and OSCC, proportionately with the level of malignancy. Blood vessel endothelial cells stained with CD34 and lymphatic vessel endothelial cells stained with D2-40 were up-regulated in OEPLs and OSCC. In OSCC, ephrinB2 and EphB4 exhibited significant correlation with recurrence and invasion depth, respectively. MVD was significantly lower in slight lymphocytic reaction than in prominent stromal reaction. Correlation was found between LVD and T classification, postoperative metastasis, survival, mode of invasion, and invasion depth.
Conclusion: Expression of EphA2, EphB4, ephrinB2, MVD, and LVD might be associated with malignant potential of the oral epithelium. Angiogenesis and lymphangiogenesis appear to be related to progression of potentially malignant oral lesions.

Keywords

Eph/ephrin, oral squamous cell carcinomas, oral epithelial precursor lesions, angiogenesis, lymphangiogenesis
INTRODUCTION

Oral squamous cell carcinoma (OSCC) is the most common malignancy of the oral cavity, it occurs predominantly in the fifth and sixth decades, and is typically associated with risk factors, such as smoking, alcohol, and human papilloma virus (HPV) infection. Worldwide, OSCC incidence is higher among males than females. OSCC may arise in any part of the oral cavity, including the tongue, gingiva, palate, buccal mucosa, floor of the mouth, and lip, thus indicating differences in disease occurrence (Warnakulasuriya, 2009; Kouketsu et al., 2016; Takata et al., 2017). Other features include rapid growth, diffuse invasion, cervical lymph node metastasis, and other distant organ metastases, most commonly into the lungs. Radical surgery of the primary tumor with or without chemoradiotherapy is the standard treatment for OSCC. In most countries, five-year survival rates for OSCC are around 50% (Warnakulasuriya, 2009). Oral epithelial precursor lesions (OEPLs) represent morphologically altered tissues that possess a greater risk of malignant transformation than normal tissues; leukoplakia, in particular, is a well-known precursor lesion. Leukoplakia is considered a potentially malignant disorder, and use of the clinical term cannot rule out the presence or absence of histopathological features of epithelial dysplasia. The World Health Organization (WHO) defines epithelial dysplasia at this body site as comprising of a spectrum of
architectural and cytological epithelial changes caused by an accumulation of genetic changes that can be associated with an increased likelihood of progression to squamous cell carcinoma. The 2017 WHO classification divides precursor lesions into low-grade and high-grade dysplasia (Gale et al., 2017; Takata et al., 2017).

Tumor angiogenesis plays a critical role in both local growth and systemic dissemination of malignancies (O’Reilly et al., 1997). Angiogenesis is characterized by complex mechanisms involving vascular endothelial growth factor (VEGF), angiopoietin, fibroblast growth factor (FGF), and platelet-derived growth factor (PDGF) (Kumamoto et al., 2002; Bergers et al., 2003). In addition, the lymphatic system also contributes to tumor cell dissemination. Indeed, regional lymph nodes metastasis is an early event in systemic extension of the disease. Lymphangiogenesis is also driven by complex mechanisms associated with VEGF-C, D, PDGF-BB, FGF-2, angiopoietin-1, and hepatocyte growth factor (HGF) (Kubo et al., 2002; Cao et al., 2004; Morisada et al., 2005; Saito et al., 2006). Cervical lymph node metastasis is considered an important prognostic factor for patients with OSCC (Warnakulasuriya, 2009; Kreppel et al., 2010).

According to previous studies, angiogenesis and lymphangiogenesis are essential processes within the tumor growth processes (Bunget et al., 2013). In OSCC, VEGF is related to angiogenesis (Shao et al., 2008), and the VEGF-C/VEGF receptor (VEGFR)
-3 axis is associated with lymph node metastasis through lymphangiogenesis (Naruse et al., 2015).

Erythropoietin-producing hepatocellular carcinoma (Eph) receptors, a class of endothelial receptor tyrosine kinases, have been implicated in the control of blood vessel formation. The ligands of Eph receptors, called Eph receptor-interaction proteins (ephrins) are bind to the receptors on the cell membrane. Each Eph receptor and its ephrin ligand is divided into A and B subclasses based on sequence homology and binding affinities (Gale et al., 1999; Holder et al., 1999). Eph receptor-ephrin signaling has been implicated in many processes, including embryonic development and human diseases (Pasquale et al., 2005). EphrinA1 and Eph receptor A2 (EphA2) are associated with tumor vasculature in several cancers (Ogawa et al., 2000; Kataoka et al., 2004). Overexpression of EphA2 is related to malignancy in tongue squamous cell carcinoma (Shao et al., 2008). In contrast, endothelial cell expression of Eph receptor B4 (EphB4) indicates that vascular development is mediated by ephrinB2 signaling (Wang et al., 1998). Overexpression of EphB4 and ephrinB2 has been described in papillary thyroid cancer, uterine cervical cancer, and urogenital cancer (Alam et al., 2009; Ozgür et al., 2011; Sharma et al., 2015). In addition, EphB4 and ephrinB2 are involved in the formation of both blood and lymphatic vessels under normal and pathologic conditions.
Systemic administration of ephrinB2-blocking antibodies caused a drastic reduction in the number of blood and lymphatic vessels in xenografted mice and a concomitant reduction in tumor growth (Abéngozar et al., 2012). However, to our knowledge, the expression of EphA2, EphB4, and ephrinB2, as well as their relationship, during angiogenesis and lymphangiogenesis in OSCC remain poorly understood.

The aim of this study was to use immunohistochemistry to investigate the expression of EphA2, EphB4, and ephrinB2 in OSCC and OEPLs, and determine if there was a correlation with angiogenesis and lymphangiogenesis. Moreover, the finding were compared with clinicopathological features of patients with OSCC.
MATERIALS AND METHODS

The study protocol was reviewed and approved by the Research Ethics Committee of Tohoku University Graduate School of Dentistry (26-52).

Tissue preparation

Specimens were surgically removed from 73 patients with primary OSCC and 43 patients with OEPLs at the Department of Oral and Maxillofacial Surgery, Tohoku University Hospital, between 2010 and 2013. None of the OSCC patients had received any chemotherapy or radiotherapy before excision. Ages ranged from 33 to 93 years (mean, 68 years). Thirty three patients were men and 40 were women. Forty-two carcinomas were located in the tongue, 7 in the upper gingiva, 9 in the lower gingiva, 3 in the lower lip, 1 in the palate, 8 in the buccal mucosa, and 3 in the floor of the mouth. The TNM disease stage was classified according to the Union for International Cancer Control (UICC) system (Brierley et al., 2017). Patients were classified as: T1 (27 cases), T2 (24), T3 (6), and T4 (16); N0 (54 cases), N1 (8), N2 (10), and N3 (1); and M0 (70 cases), and M1 (3). During follow-up, 17 patients had local recurrence, and 9 had postoperative metastasis (regional lymph node metastasis in 5 patients and pulmonary metastasis in 4). Five patients died of OSCC, and 6 patients died of another disease.
None of the OEPLs patients was diagnosed as OSCC at any site before surgery. OEPLs patients (21 men and 22 women) were aged between 30 and 89 years (mean, 68 years); 16 OEPLs were located in the tongue, 3 in the upper gingiva, 15 in the lower gingiva, 2 in the hard palate, 6 in the buccal mucosa, and 1 in the oral floor. Two patients had local recurrence, and 8 had cancerization from OEPLs to OSCC. Ten samples of normal mucosa at distant points from the excised neoplastic tissues were used as controls.

Tissue samples were fixed in 10% buffered formalin for several days, and embedded in paraffin. Tissue blocks were sliced into 4μm-thick sections for routine histological examination and subsequent immunohistochemical analyses. OSCC samples were classified into 54 well differentiated types and 19 moderately differentiated types, according to the WHO classification of tumors of the oral cavity and mobile tongue (El-Naggar et al., 2017). The degree of stromal lymphocytic reaction was classified as slight for 4 specimens, moderate for 64, and severe for 5. The mode of invasion was classified according to Yamamoto et al. (Yamamoto et al., 1984), and the carcinoma grade was 2 for 18 specimens, 3 for 43, 4C for 9, and 4D for 3. Invasion depth was defined as microinvasion (6 specimens), invasion within mucosal tissue (30), and invasion into submucosal tissue (37). The OEPLs pathological diagnosis was made according to the WHO classification of tumors of the oral cavity and mobile tongue.
(Gale et al., 2017; Takata et al., 2017). Fourteen patients had leukoplakia without epithelial dysplasia (hyperplasia; LP), 15 had low-grade epithelial dysplasia (mild to moderate dysplasia; LD), and 14 had high-grade epithelial dysplasia (severe dysplasia or carcinoma in situ; HD).

**Immunohistochemistry**

Immunohistochemical studies were done using the following primary antibodies: rabbit anti-EphA2 polyclonal antibody (1:50; Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit anti-EphB4 polyclonal antibody (1:100; Proteintech, Rosemont, IL, USA), rabbit anti-EphrinB2 polyclonal antibody (1:50; Santa Cruz Biotechnology), mouse anti-CD34 monoclonal antibody (prediluted; Nichirei, Tokyo, Japan; subclass IgG1) and mouse anti-D2-40 monoclonal antibody (prediluted; Biolegend, San Diego, CA, USA; subclass IgG1). Tissue sections were deparaffinized and immersed in methanol with 3% hydrogen peroxide. Specimens for EphA2, EphB4, and ephrinB2 analyses were heated in 0.01 M citrate buffer (pH 6.0) for 10 min by autoclave (121°C, 202 kPa). Then, sections were incubated with primary antibodies at 4°C overnight. These sections were allowed to react with peroxidase-conjugated anti-rabbit or mouse IgG polyclonal antibody (Histofine SimpleStain MAX-PO, Nichirei) for 45 min, and the
reaction products were visualized by immersing the sections in 0.03% diaminobenzidine (DAB) solution containing 2mM hydrogen peroxide for 3 min. Nuclei were lightly stained with Mayer’s hematoxylin. For control studies of the antibodies, serial tissue sections were treated with phosphate-buffered saline (PBS), normal rabbit IgG, and mouse anti-desmin monoclonal antibody (Nichirei; subclass IgG1) instead of primary antibodies was confirmed to be unstained.

**Evaluation of staining for EphA2, EphB4, and ephrinB2**

Immunohistochemical reactivity for EphA2, EphB4, and ephrinB2 was evaluated and classified into three groups: (-) negative, (+) positive (reactive at the same level as adjacent normal part), and (++) strongly positive (strongly reactive as compared with adjacent normal part).

**Microvessel density (MVD) and lymphatic vessel density (LVD) determination**

MVD and LVD were estimated using CD34-positive blood vessel endothelial cells and D2-40-positive lymphatic endothelial cells, respectively. After scanning 5 areas displaying the highest neovascularization (vascular hotspots) in subepithelial mesenchymal tissue or tumor stromal tissue at 20-fold magnification, the number of
CD34-positive blood vessels and D2-40-positive lymphatic vessels in the hotspots were counted at 200-fold magnification, and the average count was recorded as MVD and LVD for each case. Based on the criteria of Weidner et al. (Weidner et al., 1991), highlighted endothelial cells or a cell cluster clearly separated from adjacent microvessels, epithelial cells, and other connective tissue elements were regarded as distinct countable microvessels. The presence of a lumen or red blood cells was not required, and single cell sprouts were included in the counts.

Statistical analysis

Statistically significant differences between the percentages of cases with EphA2, EphB4, and ephrinB2 reactivity, as well as differences between mean values of MVD and LVD were analyzed by the Mann-Whitney $U$-test for differences between two groups or the Kruskal-Wallis $H$-test for differences among three or more groups. $P$-values of less than 0.05 were considered to indicate statistical significance.
RESULTS

_Immunohistochemical reactivity for EphA2, EphB4, ephrinB2, CD34, and D2-40 in normal mucosa, LP, LD, HD, and OSCC_

Expression of EphA2, EphB4, and ephrinB2 was detected in the cell membrane and cytoplasm of epithelial cells and some stromal vascular endothelial cells (Fig1: A1-6, B1-6, C1-6). In normal mucosa, LP, and LD, EphA2 was detected in epithelial cells except for the superficial layer (Fig1: A1 -3); whereas EphB4 was detected in epithelial cells near the basement membrane (Fig1: B1 -3, C1 -3). HD exhibited stronger reactivity for EphA2, EphB4, and ephrinB2 in dysplastic cells than in normal cells (Fig1: A4, B4, C4). In OSCC, EphA2, EphB4, and ephrinB2 were expressed in carcinoma cells except for keratinized areas (Fig1: A5 -6, B5 -6, C5 -6).

The results of immunohistochemical analyses for EphA2, EphB4, and ephrinB2 in normal mucosa, LP, LD, HD, and OSCC are summarized in Table1. EphA2 immunoreactivity was significantly lower in normal mucosa than in HD ($P < 0.01$) and OSCC ($P < 0.001$). EphA2 reactivity was significantly lower in LP than in LD ($P < 0.05$), HD ($P < 0.01$), or OSCC ($P < 0.001$). EphB4 immunoreactivity was significantly lower in normal mucosa than in HD ($P < 0.05$) or OSCC ($P < 0.001$), in LP compared to
HD ($P < 0.01$) or OSCC ($P < 0.001$), and in LD compared to HD ($P < 0.05$) or OSCC ($P < 0.001$). EphrinB2 immunoreactivity was significantly higher in OSCC than in normal mucosa, LP, or LD ($P < 0.001$, respectively).

Positive staining for CD34 was observed in blood vessel endothelial cells of normal mucosa, LP, LD, HD, or SCC (Fig 1: D1 -6). MVD was significantly higher in OSCC than in normal mucosa ($P < 0.05$), LP ($P < 0.01$), LD ($P < 0.01$), or HD ($P < 0.05$) (Table 1). Positive staining for D2-40 was observed in lymphatic endothelial cells and epithelial cells neighboring the basement membrane of normal mucosa, LP, LD, HD, and SCC (Fig 1: E1 -6). LVD was significantly lower in normal mucosa than in HD ($P < 0.05$) or OSCC ($P < 0.001$) (Table 1), but significantly higher in OSCC than in LP ($P < 0.001$) or LD ($P < 0.05$) (Table 1).

**Correlation between clinicopathological variables and the results of immunohistochemical analysis in OSCC**

EphrinB2 immunoreactivity was significantly higher in patients without recurrence than in those with recurrence ($P < 0.05$). LVD was significantly higher in T1 than in T3 ($P < 0.05$) or T4 cases ($P < 0.01$), whereas T2 cases showed significantly higher LVD than T4 cases ($P < 0.01$). LVD was significantly higher in patients without
postoperative metastasis and surviving cases than in patients with postoperative metastasis and dead cases \((P < 0.01,\text{ respectively})\) (Table 2).

EphB4 immunoreactivity was significantly higher in cases invaded into submucosal tissue than in those showing intramucosal invasion \((P < 0.05)\). MVD was significantly lower in slight stromal lymphocytic reaction than in moderate and severe stromal lymphocytic reaction \((P < 0.05,\text{ respectively})\). LVD was significantly higher in mode of invasion grade 2 cases than in mode of invasion grade 3 or 4C cases \((P < 0.05,\text{ respectively})\). Finally, LVD was significantly higher in cases with microinvasion than in those with submucosal invasion \((P < 0.01)\) (Table 3).
DISCUSSION

OEPLs and OSCC develop as a result of increased genetic instability that manifests through stimulation of oncogenes and switching off of tumor suppressor genes.

Increased malignant potential in the oral epithelium is characterized by loss of cellular control systems that regulate cell cycle progression, pitting cell death against growth imbalance, as well as inadequate parenchymal-stromal interaction, abnormal angiogenesis and/or lymphangiogenesis. These aberrations cause a variety of poor prognosis (Khan et al., 2009). In the present study, we immunohistochemically examined angiogenesis- and/or lymphangiogenesis-related factors, EphA2, EphB4, and ephrinB2 in 43 cases of OEPLs and 73 cases of OSCC. Their expression was compared with tumor angiogenesis and lymphangiogenesis. In previous studies, EphA2 was found to be overexpressed in tongue and colorectal cancer (Kataoka et al., 2004; Shao et al., 2008), whereas overexpression of EphB4 and ephrinB2 was reported in papillary thyroid, uterine cervical, and urogenital cancer (Alam et al., 2009; Ozgür et al., 2011; Sharma et al., 2015). In our study, EphA2, EphB4, and ephrinB2 were detected chiefly in epithelial cells in OEPLs and OSCC, and their expression increased according to the level of malignancy, especially in HD and OSCC. These results suggest that angiogenetic and/or lymphangiogenetic factors EphA2, EphB4, and ephrinB2 might be
associated with the malignant potential of the oral epithelium. Moreover, our study reports increased angiogenesis in OSCC, and lymphangiogenesis in OSCC and epithelial dysplasia. Previous studies have reported higher numbers of blood vessel endothelial cells stained with CD34 and lymphatic vessel endothelial cells stained with D2-40 in stromal tissue of colon and breast cancer (Lai et al., 2014; Planeix et al., 2015). Angiogenesis and lymphangiogenesis in oral potentially malignant lesions are regarded as key components in tumor multistep progression. In addition, our present study suggests that EphA2, EphB4, and ephrinB2 expression is implicated in not only angiogenesis but also lymphangiogenesis.

Correlation analyses with clinical variables, such as age, sex, and site, in OSCC patients indicated no apparent association with EphA2, EphB4, and ephrinB2 immunoreactivity, MVD, and LVD. However, EphB4 and ephrinB2 tended to increase in grade 3 and 4 T-classified tumors; whereas EphA2, EphB4 and ephrinB2 expression tended to increase in metastatic cases in N and M tumors, suggesting that a higher TNM staging coincides with increased expression of these factors in OSCC. Our date show that ephrinB2 immunoreactivity was significantly higher in patients without recurrence than in those with recurrence; whereas the opposite was true for EphA2 and EphB4. Also, expression of EphA2, EphB4, and ephrinB2 was slightly greater in postoperative
metastasis-positive cases and patients who died of OSCC. These findings suggest that EphA2, EphB4, and ephrinB2 expression in OSCC is a poor prognostic marker. Even though MVD was slightly higher in T3 cases, no other association with clinical variables was detected in our study. In contrast, LVD inversely correlated with progression of TNM classification, postoperative metastasis, or patient survival. A previous study has demonstrated higher LVD in T3/T4 than in T1/T2 cases (Miyahara et al., 2007), whereas another report has showed decrease in LVD according to T and N classification (Watanabe et al., 2013). The exact reasons for these discrepancies remain to be determined; however, at present, advanced OSCC cases might suppress lymphangiogenesis rather than activate it.

Additionally, in correlation analyses with pathological variables, expression of EphA2, EphB4, and ephrinB2 tended to increase in moderately differentiated OSCC compared with well-differentiated cases, suggesting that differentiation of OSCC cells might be affected by these factors. Also, our findings indicate that MVD and LVD tended to be low in cases with slight stromal lymphocytic reaction compared with those with marked stromal reaction. High MVD and LVD values have been suggested to reflect interaction with their stromal tissues in OSCC (Miyahara et al., 2007). Moreover, EphA2, EphB4, and ephrinB2 expression was found to increase slightly in 4C and 4D
cases in the OSCC mode of invasion. EphB4 immunoreactivity was significantly higher in patients with submucosal invasion than in those with mucosal invasion. Moreover, both EphA2 and ephrinB2 tended to increase in submucosal invasive cases compared with mucosal invasive one. The above features suggest that these vascular related factors affect invasion mode and/or growth in OSCC. At the same time, LVD was significantly lower in grade 3 and 4C patients than in grade 2 patients, as well as in patients with submucosal invasion than in those with intramucosal invasion. These findings suggest that the number of lymph vessels in the tumor microenvironment is affected by repeated destruction and regeneration associated with the advanced invasion of cancer cells (Nakaya et al., 2005; Goes et al., 2012; Agarwal et al., 2014).
CONFLICT OF INTEREST

We declare that there are no financial and personal relationships with other people or organizations.

ACKNOWLEDGEMENTS

This study was approved by the appropriate ethical review boards, and participants provided written informed consent prior to participating in the study.
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FIGURE CAPTIONS

Fig. 1.2. Immunohistochemical reactivity for EphA2 (A1 -6), EphB4 (B1 -6), ephrinB2 (C1 -6), CD34 (D1 -6), and D2-40 (E1 -6) in normal mucosa, leukoplakia (LP), low-grade dysplasia (LD), high-grade dysplasia (HD), and oral squamous cell carcinoma (well-differentiated squamous cell carcinoma: WSCC; moderate differentiated squamous cell carcinoma: MSCC).

EphA2, EphB4, and ephrinB2 staining showing membranous and cytoplasmic reactivity in epithelial cells and some stromal vascular endothelial cells (A1 -6, B1 -6, C1 -6).

Normal mucosa, LP, and LD showing EphA2 reactivity in epithelial cells except for the superficial layer (A1 -3).

Normal mucosa, LP, and LD showing EphB4 and ephrinB2 reactivity in epithelial cells near the basement membrane (B1 -3, C1 -3).

HD showing stronger reactivity for EphA2, EphB4, and ephrinB2 in dysplastic cells than in normal cells (A4, B4, C4).

Oral SCC showing reactivity for EphA2, EphB4, and ephrinB2 in most carcinoma cells except for keratinized areas (A5 -6, B5 -6, C5 -6).

Positive staining for CD34 found in blood vessel endothelial cells of normal mucosa, LP, LD, HD, and oral SCC (D1 -6).
Positive staining for D2-40 found in lymphatic endothelial cells and epithelial cells neighboring the basement membrane of normal mucosa, LP, LD, HD, and oral SCC (E1-E6).

A1~E1, A2~E2, C3~E3, D4, E4, D5, E5, B6, D6, E6: × 200

A3, B3, A4~C4, B5, A6, C6: × 100

A5, C5: × 40
Fig. 1

A1
Normal

B1
Normal

C1
Normal

A2
LP

B2
LP

C2
LP

A3
LD

B3
LD

C3
LD

A4
HD

B4
HD

C4
HD

A5
WSCC

B5
WSCC

C5
WSCC

A6
MSCC

B6
MSCC

C6
MSCC
Fig. 2

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**Immunoreactivity**
- (-): negative
- (+): positive (reactive at the same level as adjacent normal part)
- (++): strongly positive (strongly reactive as compared with adjacent normal part)

**MVD**
Microvessel density

**LVD**
Lymphatic vessel density

**Statistical significance**
* P < 0.05
** P < 0.01
*** P < 0.001
| Table 2: Correlation between clinical variables (non-parametric test) for Subtyping and survival duration in oral squamous cell carcinoma |
|---|---|---|---|---|---|---|
| **Variables** | **No.** | **P** | **R** | **P** | **R** | **P** | **R** |
| Age | 0-39 | 7 | 0.21 | 0.33 | 0.21 | 0.33 | 0.21 | 0.33 |
| | 40-49 | 6 | 0.24 | 0.26 | 0.24 | 0.26 | 0.24 | 0.26 |
| | 50-59 | 8 | 0.27 | 0.28 | 0.27 | 0.28 | 0.27 | 0.28 |
| | 60-69 | 10 | 0.30 | 0.31 | 0.30 | 0.31 | 0.30 | 0.31 |
| | 70-79 | 8 | 0.33 | 0.34 | 0.33 | 0.34 | 0.33 | 0.34 |
| | 80-89 | 15 | 0.36 | 0.37 | 0.36 | 0.37 | 0.36 | 0.37 |
| | 90-99 | 1 | 0.39 | 0.40 | 0.39 | 0.40 | 0.39 | 0.40 |
| Sex | Male | 59 | 0.41 | 0.42 | 0.41 | 0.42 | 0.41 | 0.42 |
| | Female | 48 | 0.44 | 0.45 | 0.44 | 0.45 | 0.44 | 0.45 |
| Tumor stage | T1 | 62 | 0.48 | 0.49 | 0.48 | 0.49 | 0.48 | 0.49 |
| | T2 | 18 | 0.51 | 0.52 | 0.51 | 0.52 | 0.51 | 0.52 |
| | T3 | 7 | 0.54 | 0.55 | 0.54 | 0.55 | 0.54 | 0.55 |
| | T4 | 13 | 0.57 | 0.58 | 0.57 | 0.58 | 0.57 | 0.58 |
| Lymph node | N0 | 63 | 0.17 | 0.18 | 0.17 | 0.18 | 0.17 | 0.18 |
| | N1 | 20 | 0.20 | 0.21 | 0.20 | 0.21 | 0.20 | 0.21 |
| | N2 | 3 | 0.23 | 0.24 | 0.23 | 0.24 | 0.23 | 0.24 |
| | N3 | 10 | 0.26 | 0.27 | 0.26 | 0.27 | 0.26 | 0.27 |
| | N4 | 16 | 0.29 | 0.30 | 0.29 | 0.30 | 0.29 | 0.30 |
| Site | Floor of mouth | 24 | 0.32 | 0.33 | 0.32 | 0.33 | 0.32 | 0.33 |
| | Buccal mucosa | 47 | 0.35 | 0.36 | 0.35 | 0.36 | 0.35 | 0.36 |
| | Lower gingiva | 42 | 0.38 | 0.39 | 0.38 | 0.39 | 0.38 | 0.39 |
| | Lower lip | 3 | 0.41 | 0.42 | 0.41 | 0.42 | 0.41 | 0.42 |
| | Palate | 21 | 0.44 | 0.45 | 0.44 | 0.45 | 0.44 | 0.45 |
| Recurrence | Negative | 78 | 0.47 | 0.48 | 0.47 | 0.48 | 0.47 | 0.48 |
| | Positive | 4 | 0.50 | 0.51 | 0.50 | 0.51 | 0.50 | 0.51 |
| Postoperative mutations | Negative | 64 | 0.53 | 0.54 | 0.53 | 0.54 | 0.53 | 0.54 |
| | Positive | 8 | 0.56 | 0.57 | 0.56 | 0.57 | 0.56 | 0.57 |
| Survival | Alive | 62 | 0.60 | 0.61 | 0.60 | 0.61 | 0.60 | 0.61 |
| | Dead | 13 | 0.63 | 0.64 | 0.63 | 0.64 | 0.63 | 0.64 |

**MVD**: Microvessel density  
**LVD**: Lymphoid vessel density

Notes:  
(1) negative  
(+) positive (reactive at the same level as adjacent normal part)  
(++) strongly positive (strongly reactive as compared with adjacent normal part)
Table 3: Correlation between pathological variables, immunoreactivity for Eph/ephrin, and vessel densities in oral squamous cell carcinoma

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**Immunoreactivity**
- (-): negative
- (+): positive (reactive at the same level as adjacent normal part)
- (++): strongly positive (strongly reactive as compared with adjacent normal part)

**MVD**
- Microvessel density

**LVD**
- Lymphatic vessel density

**Statistical significance**
P<0.05
P<0.01