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CD147 and Ki-67 overexpression confers poor prognosis in squamous cell carcinoma of oral tongue: A tissue microarray study

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Abstract

Squamous cell carcinoma of the oral tongue (SCCOT) exhibits high risk for recurrence and regional metastasis even after surgical resection. We assessed the clinicopathologic and prognostic significance of a group of functionally related biomarkers. We used a tissue microarray consisting SCCOT from 32 patients for this study. These patients were treated at the UT- M.D. Anderson Cancer Center from 1995 to 2008. Biomarker expression levels were examined by immunohistochemistry and graded semiquantitatively to determine their prognostic significance. CD147 and Tp63 expressions were significantly associated with a higher T-stage and Ki-67 labelling index as well as shorter overall survival (OS). Expression of Tp63 associated positively with poorly-differentiated histology. There was significant association of Tp63 with the expression levels of CD147 and Glut-1. Glut-1 overexpression was marginally associated with a higher T-stage. There was no prognostic significance of CD44v6 expression in SCCOT. SCCOT with CD147 overexpression in combination with high Ki-67 labelling index had poor OS. CD147 and Ki-67 overexpression is associated with aggressive disease with poor prognosis in SCCOT.

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CONFLICT OF INTEREST STATEMENT

The authors confirm that they have no current or potential conflicts of interest, including financial, personal, or other relationships with individuals or organizations that could inappropriately influence this study.

Keywords

Oral cancer; Squamous cell carcinoma of oral tongue; Biomarkers; CD147; Tp63; CD44v6; Glut-1; Ki-67; Overall and recurrence free survival

Introduction

Oral squamous cell carcinoma (OSCC) arises in the lip and oral cavity, representing one of the ten most frequently diagnosed cancers globally¹. Countries with the highest incidence of OSCC are India, Pakistan and Sri Lanka². In these countries, OSCC is the most common cancer in men and the third most common cancer in women². OSCC exhibits a high mortality rate of 40–50% which is due to the high incidence of disease recurrence and subsequent spread to local and distant sites³. The tongue is the most common intraoral site of OSCC in most countries and accounts for 25–40% of all OSCC³. The anterior two-thirds of the tongue, also known as the mobile tongue, is a sub-site of the oral cavity; cancers arising from this portion of the tongue are designated as squamous cell carcinomas of oral tongue (SCCOT). The posterior one third or base of the tongue is a sub-site of the oropharynx; cancers arising from this portion of the tongue are designated as squamous cell carcinomas of the base of the tongue. Tobacco and alcohol consumption are major risk factors for SCCOT which typically occurs in patients older than 50-years of age^{4, 5}. On the other hand, oncogenic human papilloma virus (HPV) infection is a major risk factor for squamous cell carcinomas of the base of the tongue⁶. The incidence of SCCOT has been steadily increasing since 1975, whereas the incidence of OSCC of other intraoral sites has been decreasing^{7–9}. SCCOT represents 75% of all tongue cancers and approximately 7,100 new SCCOT cases are diagnosed each year in the United States¹⁰. Furthermore, recent studies report an increased incidence of SCCOT in young white females who were never-smokers or never-drinkers^{7, 11}. SCCOT occurring in young patients without the traditional risk factors of tobacco and/or alcohol abuse exhibit a more aggressive clinical course characterized by higher rates of loco-regional recurrences, shorter RFS and OS^{7, 12}. Recent cancer exome sequencing studies revealed that SCCOT in young non-smokers and older smokers had similar genomic profiles and mutational spectrum except for the frequency of Tp53 mutation, which is more common in SCCOT of older smokers than young non-smokers^{13, 14}.

SCCOT is the most aggressive of all OSCC subtypes with a poor 5-year survival rate of < 50%¹⁵. Despite recent advances in cancer research and treatment modalities, prognosis of SCCOT remains poor, primarily due to high rates of distant metastasis and locoregional recurrence¹⁵. Advanced local disease and/or lymph node metastasis are associated with the poor prognosis in SCCOT patients¹⁰. SCCOT exhibits extremely high rates of occult lymph node metastases which cannot be identified by clinical and radiographic imaging studies¹⁵. The rate of occult cervical lymph node metastasis in patients diagnosed with early stage SCCOT has been reported to range from 24–42%^{16–19}. More importantly, relapse from the subclinical nodal disease is the most common cause of treatment failure in SCCOT¹⁵. Rusthoven and colleagues demonstrated that patients of early stages of SCCOT had worse survival rates, even after aggressive therapy²⁰. Most clinicians advocate elective neck

dissection, even in clinically node negative (N0) patients with early stage (T1 and T2) SCCOT, to accurately determine the stage for effective treatment^{21–23}. However, elective neck dissection represented an overtreatment in >70% of early stage SCCOT patients (T1/T2) who were found to have pathologically negative nodes^{24, 25}.

Hence, there is increased interest in identifying molecular biomarkers that are associated with disease specific survival and can be incorporated into the staging in guiding therapeutic decision making in patients with SCCOT. Tumor biomarkers are molecules produced either by tumor or the surrounding host tissue in response to tumorigenesis, which have various clinical applications that include cancer risk assessment, diagnosis and staging of cancer, prognostication and therapeutic selection^{26, 27}. Numerous studies have examined an extensive list of tumor biomarkers in SCCOT²⁸. Conflicting or unconfirmed associations for the same biomarker were found among different studies²⁸. The sample size for a single SCCOT study is usually relatively small and differences existed in the study design, patient population, and variability of the quantitative measurements of biomarkers. Moreover, a majority of these independent studies have tested only one or two biomarkers, reporting limited success in predicting the overall survival (OS) and recurrence free-survival (RFS) and have not been adapted for clinical practice. There is emerging evidence that a panel of biomarkers, rather than single markers, will be required to have sufficient sensitivity and specificity for accurate cancer staging and prediction of treatment response at the time of diagnosis²⁶.

In this study, we tested a group of functionally related biomarkers namely, CD147, CD44v6, Glut-1, Tp63 and Ki-67, which are reported to have prognostic implications in head and neck squamous cell carcinomas^{29–34}. CD147 (also known as EMMPRIN or BSG) is a multifaceted molecule that facilitates tumor progression by several mechanisms that include tumor invasion, angiogenesis, metastasis, suppression of tumor immunity and resistance to therapy. CD44 is a cell surface glycoprotein involved in regulating cell-cell and cell-ECM (extracellular matrix) interactions. Overexpression of CD44, a putative cancer stem cell maker, confer adverse prognosis and poor response to radiation therapy in laryngeal dysplasias and carcinomas, respectively^{35, 36}. Tp63 belongs to the p53 transcription factor family (p53, Tp63 and p73) and contains a middle region of DNA binding domain that is constant among these members to regulate cell cycle arrest and apoptosis^{37, 38}. Tp63 is one of the most frequently mutated gene in head and neck SCC³⁹. The predominant Tp63 isoform expressed in head and neck SCC lacks the full N-terminal domain and is referred to as Np63⁴⁰. The transcriptionally inactive isoform Np63 promotes oral tumorigenesis by functioning as a trans-dominant negative mutant and inactivating tumor suppressor gene p53³⁷. Glucose transporter Glut-1 is frequently overexpressed in many solid malignancies to increase glucose uptake in response to aerobic and anaerobic glycolysis⁴¹. Overexpression of Glut-1 in oral squamous cell carcinomas is associated with poor prognosis^{42, 43}. The Ki67 is a nuclear protein expressed during the G2/M phase of the cell cycle and is widely used to determine the proliferative fraction in solid malignancies⁴⁴. We assessed the expression of these biomarkers in tissue microarrays of SCCOT and correlated their expression levels with clinicopathologic factors, OS and RFS.

Material and methods

Patient population and specimens

Archival SCCOT specimens with adequate clinical follow up and tissue samples were selected for the fabrication of these tissue microarrays (TMAs). Patients with SCCOT treated at the M.D. Anderson Cancer Center with archived tissue and adequate clinical data were included in this study. Clinical data obtained from chart review include tumor site, size, regional and distant metastasis status, extracapsular spread, treatment type and the disease status at the time of last follow-up. Patients' samples were not included if there were missing data. Histopathological grading of hematoxylin stained sections of primary SCCOT was made using the standard criteria into well- (Grade 1), moderately- (Grade 2) and poorly- (Grade 3) differentiated tumors⁴⁵.

This SCCOT patient population is composed of thirty-two patients with operable tongue cancers who were treated and followed up at the M.D. Anderson Cancer Center (MDACC), Houston, TX between 1995 and 2008. This research follows the tenets of the Declaration of Helsinki and all samples were obtained after informed consent from the patients. All protocols using tissue specimens were approved by the Institutional Review Boards of MDACC. Patient tissue samples were spotted on glass slides for tissue microarray immunohistochemistry (TMA-IHC) staining. Among the 32 patients, there were sixteen patients who had one specimen, fifteen patients who had two specimens, and one patient who had three specimens for the TMA-IHC analysis.

Tissue microarray (TMA)

Tumor tissues were formalin fixed, embedded in paraffin and incised into sections for the histological diagnosis and microscopic characterization. The staging was evaluated according to the American Joint Committee on Cancer (AJCC) system for the tumor (T), nodal invasion (N), and distant metastasis (M). Representative tumors were identified and a 1 mm-in-diameter tissue core was punched out and transferred to a recipient block with 1.5 mm between each core. Each tissue microarray also contained sections of normal oral mucosa, dysplastic oral mucosal, spleen and lymph nodes as internal controls. Two sets of slides were used labeled TMA-1, TMA-2, with a positive and negative control for each biomarker.

Immunohistochemical staining for biomarkers

Consecutive TMA sections were deparaffinized, rehydrated and subjected to antigen retrieval by boiling in ANTIGEN DECLOAKER (Biocare Medical, Concord, CA) solution for ten minutes. Endogenous peroxidase activity was blocked by incubating the tissue section with 3% H₂O₂ in methanol for ten minutes. Non-specific binding sites were blocked by incubating the tissue sections in BACKGROUND TERMINATOR (Biocare Medical, Concord, CA) solution for ten minutes. Tissue sections were treated with normal horse serum for ten minutes to block non-specific binding sites and then incubated diluted primary antibodies overnight at room temperature. Source and working dilutions of the primary antibodies specific for each biomarkers are provided in Table 1. Primary antibody binding sites were detected using the Vectastain Elite mouse/rabbit/goat ABC kits (Vector Labs,

Burlingame, CA) according to the manufacturer's protocol. Peroxidase reactivity was visualized using 3-3'-diaminobenzidine tetrachloride as chromogenic substrate and counterstained with hematoxylin.

Scoring of immunohistochemical staining

Expression levels of CD147, CD44v6, and Glut-1 were evaluated semiquantitatively based on the staining intensity and the percentage of tumor cells with membranous and cytoplasmic staining as previously reported⁴⁶. Immunoreactive scores (range 0 – 4) were derived by multiplying the percentage of positive cells times staining intensity score. Immunoreactive scores for Tp63 were determined based on the percentage of nuclei positive cells as follows: grade 0, <5%; grade 1, 5%–25%; grade 2, 26%–50%; grade 3; 51%–75%; and grade 4; 76%–100%. The growth fraction of SCCOT samples was determined using Ki-67 immunolabeling and the Ki-67 Immuno scoring was done as follows: grade 1, < 25% of tumor cells are positive; grade 2, > 25% of tumor cells are positive.

Statistical analysis

Frequencies and percentages are reported for categorical variables (such as gender, smoking status, and stage). To evaluate the association between histology grade and marker expression and the association between two markers, regression models using generalized estimating equations (GEE) were used to take the intra-patient correlation into account. Kaplan-Meier method was used to analyze the time-to-event outcomes, including OS and RFS. The log-rank test was used to evaluate the difference in time-to-event free rate between patient groups. Multi-covariate Cox proportional hazards model was fitted to evaluate the effect of a marker on time-to-event outcome with adjustment of other prognostic variables in the model. All tests were two-sided. P-values less than 0.05 were considered statistically significant. Statistical software SAS 9.1.3 (SAS, Cary, NC) and S-Plus 8.0 (TIBCO Software Inc., Palo Alto, CA) were used for all the analyses.

RESULTS

Clinicopathological characteristics of the TMA samples

We analyzed the expression levels of CD147, CD44v6, Glut-1, Tp63 and Ki-67 in TMA composed primary tumor sections from 32 patients with SCCOT (Table 2). There were 27 males and 5 females, with a median age of 60-years (range: 22–77 years). Twenty-eight patients were either current or former smokers and the remaining four patients had no history of tobacco use. Distribution of these patients according to their tumor size was as follows: T1=6%; T2=41%; T3=34%; T4=19%. Twenty patients (62.5%) had pathologically confirmed regional lymph node metastatic spread and all of these lymph node metastases revealed extracapsular spread (ECS) at the time of diagnosis. Four of these patients also developed distant metastasis. The remaining 12 patients were staged as N0 and none of the 20 ECS (–) patients presented with distant metastasis. These tumors were graded histologically as well differentiated or Grade 1 (n= 8; 25%), moderately differentiated or Grade 2 (n= 19; 59%) and poorly differentiated Grade 3 (n=5; 16%) (Figure 1). These patients were treated with surgery alone (n=11; 34%), surgery with radiation therapy (n=20; 63%) and surgery with radiation and chemotherapies (n=1; 3%). In all, 9 (28%) patients had

tumor recurrences which included both local and nodal recurrence and the remaining patients were free of tumor recurrence during the follow-up period. At the time of last follow-up, 12 patients were alive and 20 patients had died. Among the patients who died during the follow-up period, 13 of them died of SCCOT, one patient died due to treatment related complications and six of them died of unknown disease status. The median OS time was 18.3 months, with the lower bound of the 95% confidence interval (CI) at 7.56 months and upper bound was not reached.

Expression patterns of CD147, CD44v6, Tp63 and Glut-1 in SCCOT

All of the tumor samples in the TMA revealed positivity for CD147, CD44v6, and Glut-1, albeit at different expression levels. Immunohistochemical stainings for CD147, CD44v6, and Glut-1 revealed prominent cell surfaces, diffuse and weak cytoplasmic patterns in individual tumor cells (Figures 2–4). We found high levels (Immunoscore >3) of CD147, CD44v6 and Glut-1 expression in 32%, 18%, and 22% of SCCOT samples, respectively (Figures 2–4). SCCOT tumor cells revealed nuclear positivity for Tp63 and Ki-67 at varying proportions (Figures 5 & 6). Twenty-six percent of SCCOT expressed high-levels of Tp63 (Immunoscore >3) (Figure 5). Twelve of 49 SCCOT (24%) cases in the TMA revealed a higher proliferation rate (Immunoscore 2 = > 25% Ki-67 positive tumor cells) as measured by Ki-67 immunolabeling.

Association between biomarkers expression and clinicopathologic variables

Increased expression of CD147 correlated significantly with advanced tumor stage ($p = 0.004$) (Table 3). Interestingly, high levels of CD147 expression ($p = 0.058$) is observed more often in SCCOT of non-smokers (71.4%) than SCCOT of current (36.4%) and former smokers (15%). SCCOT with high Glut-1 expression levels (Immunoscore ≥ 3) presented with higher T-stages, but this association is only marginally significant ($p=0.058$). Overexpression of Tp63 in SCCOT was positively associated with large tumor size (T3/T4; $p=0.04$) and poor histologic differentiation ($p = 0.001$) (Table 3). Tumor size and histologic grading of SCCOT were also significantly ($p < 0.5$) associated with the percentage of tumor cells positive for Ki-67 antigen (growth fraction) (Table 3). Expression of CD44v6 was not significantly associated with any of the examined clinicopathologic features (Table 3). Expression levels of neither of these biomarkers revealed positive association with either pathological N stage or extracapsular spread (ECS) (Table 3).

Associations among biomarkers in SCCOT

Using the total 49 TMA-IHC records, we evaluated the association of IHC scoring between pairs of biomarkers (Table 4). CD147 expression levels in SCCOT associated positively with the expression of Tp63 and Ki-67 ($p < 0.05$). Similarly, there were significant positive associations between the expression levels of Tp63 and Ki-67 as well as Tp63 and Glut-1 ($p < 0.05$). We did not find any significant association between the expression of CD44v6 and other biomarkers.

Association between biomarker expression levels and RFS and OS

The follow-up period ranged from 0.33 to 93 months. A total of 5 patients had nodal recurrence and 20 patients died. The associations of pathological N- stage and extracapsular spread (ECS) on OS or RFS were significant ($p < 0.05$). With regard to the TMA-IHC grading of biomarkers, SCCOT with high CD147 expression levels had marginally significant association with poor OS ($p = 0.094$) and poor RFS ($p=0.092$) (Figure 6); SCCOT with high Ki-67 expression levels had marginally significant association with poor OS ($p = 0.06$) and significant association with poor RFS ($p=0.041$) (Figure 6). Moreover, CD147 expression levels, when combined with Ki-67 labelling indices, were significantly associated with OS ($p = 0.045$) and RFS ($p = 0.047$). We also fitted multi-covariate Cox proportional hazards model to evaluate the effect of CD147 or CD147 and Ki-67 combining score on OS and RFS. With the adjustment of T stage (1/2 vs. 3/4) in the model, the effect of the marker on OS or RFS was no longer significant. However, the trend persisted.

DISCUSSION

Squamous cell carcinoma of the head and neck is the sixth most common cancer worldwide and the tongue remains the most common site in the United States. The tongue has rich lymphatic drainage resulting in a high incidence of occult nodal metastases with SCCOT at the time of diagnosis than the SCCs of any other site in the oral cavity^{47, 48}. Nodal metastasis remains the most important independent prognostic factor for tongue cancer patients¹⁵. Therapeutic decisions for SCCOT are currently made based on the standard clinicopathologic variables such as the tumor size, depth of invasion, cervical lymph nodes status, extracapsular spread (ECS) and histologic grade^{28, 49}. However, the accuracy of these clinicopathologic parameters in predicting the SCCOT response to therapy remains uncertain^{18, 50}. There is a critical need to avoid overtreatment in SCCOT patients which may provide only modest benefit while causing significant morbidity. On the other hand, under-treatment or incorrect treatment that increases the risk of local recurrence and metastasis should be also avoided. There is a need to incorporate predictive biomarkers into the current tumor staging to identify SCCOT patients who are at risk for recurrence and poor survival. Such identification could aid clinicians in identifying patients who need more aggressive treatment and close monitoring. In this study, we tested the technical validity and clinical utility of five biomarkers in predicting the prognosis of SCCOT. By testing five biomarkers in the same set of tumor samples, we were able to explore the correlations and interplays among these functionally related biomarkers that are reported to be prognostic predictors in head and neck squamous cell carcinomas in previous studies^{29, 31-34}.

Our results demonstrated that CD147 and Tp63 expression levels in SCCOT were associated with a large tumor size, a high proliferation rate (Ki-67 labeling index) and shorter OS and RFS. Increased expression of CD147 in combination with high Ki-67 labeling index associated with poor OS. Moreover, poorly differentiated SCCOTs (Grade 3) revealed significantly higher nuclear labeling of Tp63 compared with well to moderately (Grade 1 and 2) differentiated tumors. Most importantly, SCCOT with elevated CD147 expression possessed significantly higher levels of Tp63. Previous studies have reported that high expression of CD147 and Tp63 is associated with poor clinical outcomes in head and neck

SCC^{51, 52}. Our study confirms the previous reports and suggests that overexpression of CD147 significantly correlated with aggressive tumor phenotype and poor clinical outcome in SCCOTs.

CD147 is a cell surface transmembrane protein that regulates cancer development and progression by mediating tumor-microenvironmental cross-talk. CD147 induces peritumoral fibroblasts to produce matrix metalloproteinases (MMPs) which initiate tumor invasion⁵³. Tumor cell surface CD147 functions as a receptor for $\alpha 3\beta 1$ and $\alpha 6\beta 1$ integrins that facilitate cancer metastasis⁵⁴⁻⁵⁶. Tumor cell surface CD147 stimulates the production of vascular endothelial growth factor (VEGF) by both tumor and stromal cells and promotes tumor angiogenesis^{57, 58}. Moreover, CD147 acts as chaperone for the cell surface expression of monocarboxylate transporters-1&4, which in turn promotes MMP induction, lactate-induced angiogenesis and hyaluronan production⁵⁹. In a previous study, we reported that CD147 is a promising molecular biomarker of oral mucosal carcinogenesis⁶⁰. CD147 is the most constantly upregulated mRNA in metastatic cells⁶¹ and plays critical roles in tumor metastasis and resistance to chemoradiation therapy^{53, 62-65}. CD147 is overexpressed in various solid malignancies and its overexpression is reported to correlate with tumor progression and poor prognosis^{51, 66-69}. Tumor cells engineered to overexpress CD147 demonstrate increased invasiveness *in vitro* and accelerated growth rate and metastatic progression in animal models⁶⁵. RNAi mediated silencing of CD147 inhibits proliferation, invasion, angiogenesis and metastatic propensity of malignant melanoma cells⁷⁰. In concordance with these published reports, our study reveals CD147 overexpression is a reliable predictor of loco-regional failure and poor OS in SCCOT, lending credence to the proposed link between CD147 and maintenance of cancer stem cells niche and poor response to therapy^{71, 72}. CD147 stimulates the production of hyaluronan by tumors cells, which not only promotes tumor cell survival during anchorage-independent growth,⁷³ but also aids in the maintenance of cancer stem cell niche by being the primary ligand for cancer stem cell marker CD44^{72, 74-77}.

The expression levels of CD44, which is a putative marker of cancer stem cells in head and neck carcinomas, failed to show prognostic significance in SCCOT patients⁷⁸. CD44 is encoded by at least 20 exons and 10 variant exons (v1-10) which can be alternatively spliced in various combinations, thereby producing numerous spliced variants (VD44v1-10) from a single gene^{79, 80}. The standard CD44 (CD44s), which is not encoded by variant exons, is exclusively expressed by hematopoietic and mesenchymal cells^{79, 80}. On the other hand, CD44v6, which is a spliced variant of CD44 containing variant exon 6, is frequently overexpressed in carcinomas and is reported to be associated with tumor progression and metastasis⁸¹. However, there are contradictory data in validating the association between the expression levels of CD44v6 and poor prognosis in several types of malignancies including head and neck SCC^{32, 75, 82, 83}. Although CD44v6 was expressed in about > 80% (Immunoscore >2) of our tumor samples, it surprisingly did not show any significant correlations with the main clinical prognostic factors for SCCOT nor with the survival functions.

Expression of another stem cell related molecular marker, Tp63, is associated with aggressive tumor phenotype in SCCOT. Tp63 gene is expressed as multiple isoforms due to

alternative splicing and differential promoter usage and these isoforms have variable N- and C-terminal regions^{37, 38}. Tp63 is crucial for the proliferation of stem cells in stratified squamous epithelium⁸⁴ and is one of the most frequently mutated gene in head and neck SCC³⁹. The predominant Tp63 isoform expressed in head and neck SCC lacks the full N-terminal domain and is referred to as Np63⁴⁰. This N-terminal region of Tp63 harbors the transactivation domain (TA) which is present in transcriptionally active isoforms but is absent in the Np63 isoform³⁷. Deregulated expression of Tp63 leading to an increased Np63/Tp63 ratio promotes tumor development by inhibiting cellular senescence⁸⁵. Inactivation of p53 function, combined with the activation of Wnt/ β -catenin pathway, results in the overexpression of Np63 that promotes oral mucosal tumorigenesis and early metastatic progression^{30, 86}. Furthermore, in squamous cell carcinomas, the transcription factor SOX2, which is critical for the maintenance of embryonic and adult stem cells, preferentially interacts with Np63 and regulates gene expression that governs undifferentiated cancer stem cells-like properties⁸⁷.

Interestingly, there is a coordinated expression of CD147 and Tp63 limited to the basal cells of normal oral mucosa; the expression of both of these markers is downregulated during maturation of normal oral squamous epithelium. In contrast, both CD147 and Tp63 are aberrantly overexpressed spreading to the upper layers of the epithelium during the early stages of oral carcinogenesis suggesting that they may act to maintain an immature cell state in SCCOT and its precursors^{30, 60}. In this study, we document a close correlation between the expression levels of CD147 and Tp63 in SCCOT which has significant prognostic implication.

Moreover, Tp63 expression was significantly correlated with the expression levels of Glut-1 which is functionally related to CD147. Most solid cancers, including SCCOT, switch from oxidative to glycolytic metabolism for energy production resulting in increased glucose uptake and enhanced production of intracellular lactic acid. Glut-1 is the main transporter involved in glucose influx, whereas monocarboxylate transporters (MCT) mediate the efflux of lactic acid from the tumor cells. Maturation and tumor cell surface expression of MCT are dependent on CD147 expression⁵⁹. Moreover, increased expression of Glut-1 in association with CD147 renders poor prognosis and resistance to radiation therapy in cervical squamous cell carcinomas⁸⁸. Our data shows that Glut-1 expression associates marginally with tumor size (T-stages) but did not show any significant association with RFS or OS. We observed that the majority of the T1 and T2 SCCOT (>50%) had low levels of Glut-1 (immunoscore =1) expression, while most (> 70%) of T3 and T4 cases were intensely positive (immunoscore 2 & 3) for Glut-1. The association between Glut-1 expression levels and tumor size in SCCOT is consistent with previous studies in oral squamous cell carcinomas³³.

CONCLUSION

Our data show that SCCOT with high expressions of CD147 and Ki-67 have increased risk for loco-regional recurrence resulting poor OS and aggressive tumor phenotypes, respectively. Sample size is the major limitation of this study and further studies on larger cohorts of SCCOT are needed to determine the prognostic value of CD147 and Tp63. In

addition to their prognostic relevance, correlation between the expression levels of CD147 and Ki-67 within the same dataset suggests a potential functional cross-talk between these two molecules. Further research is needed to elucidate the underlying biologic mechanisms.

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Abbreviations

OSCC	Oral squamous cell carcinoma
SCCOT	Squamous cell carcinoma of the oral tongue
TMA	Tissue microarray
OS	Overall survival
RFS	Recurrence free survival
ECS	Extracapsular spread

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Statement of Clinical Relevance: CD147, Tp63, as well as CD147 combined with Ki-67 are potential predictive biomarkers in early stages of squamous cell carcinomas of oral tongue and can be used as adjunct for conventional tumor staging for optimal therapeutic management.

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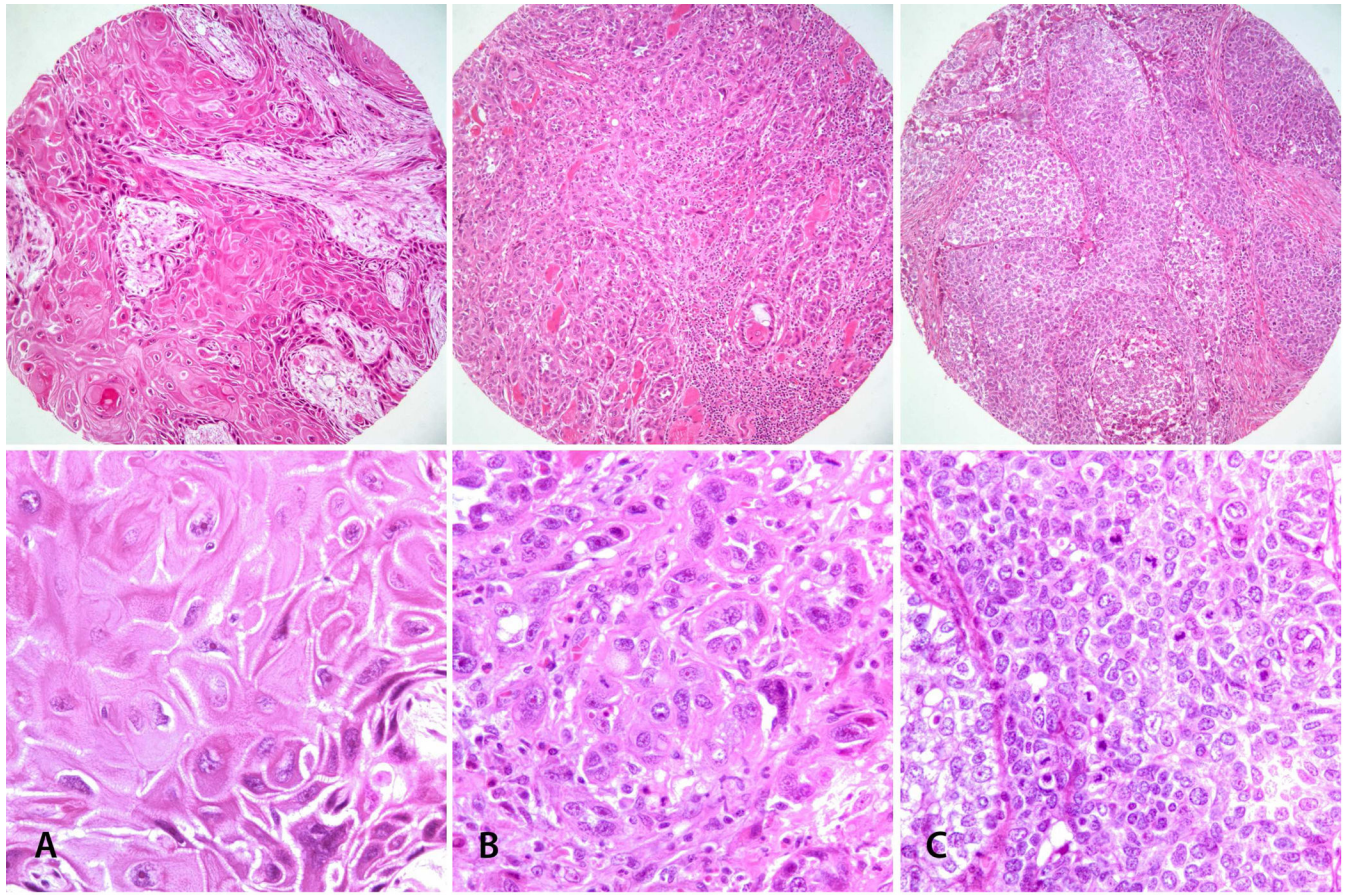


Figure 1. Hematoxylin and eosin (H&E) stained images of TMA cores. Representative images of SCCOT with well differentiated (A: Grade 1), moderately differentiated (B: Grade 2) and poorly differentiated (C: Grade 3) histologic grades. Top: Low-power view (original magnification $\times 100$); Bottom: High-power view of the same tumors (original magnification $\times 200$).

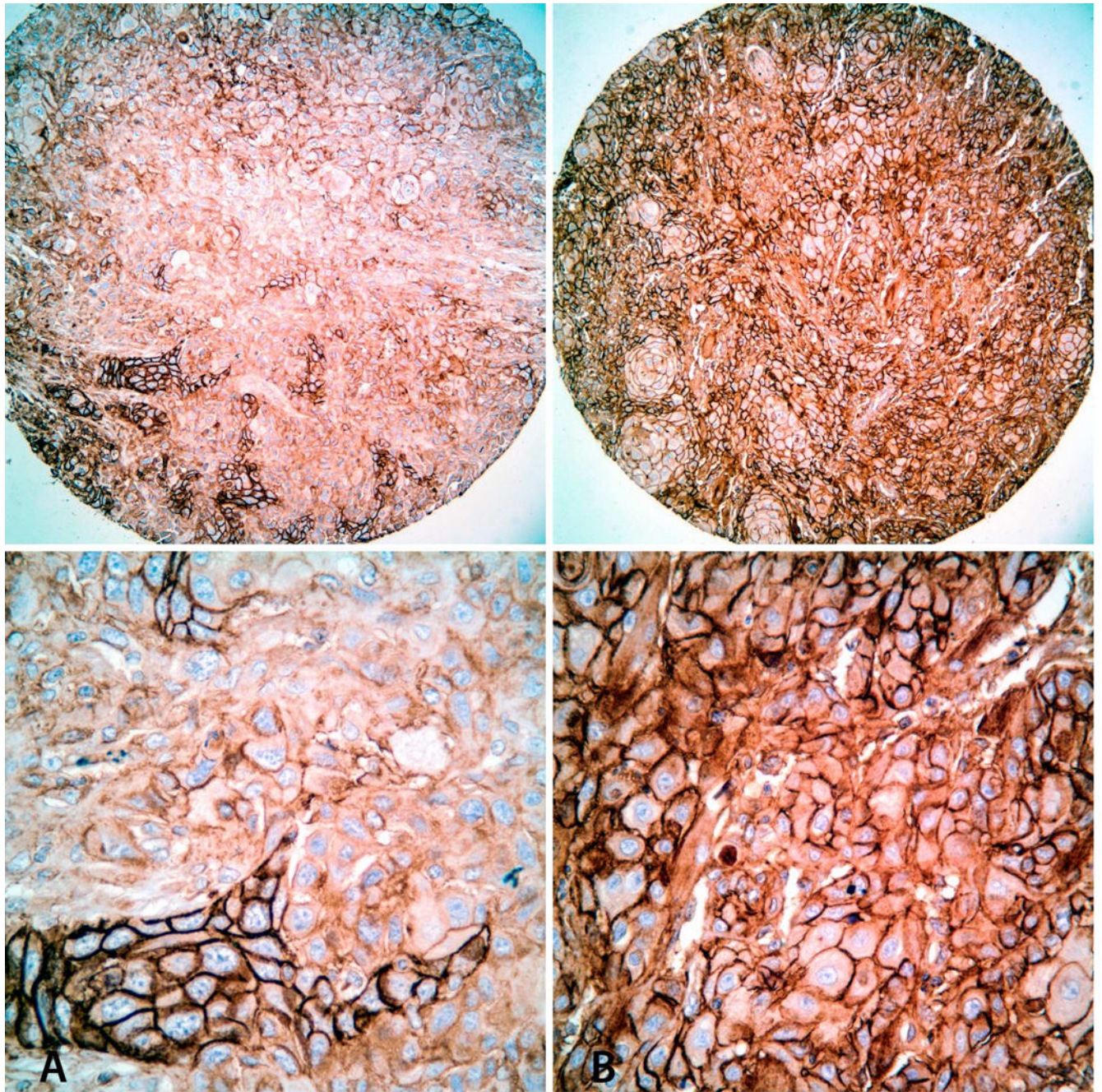


Figure 2. Immunohistochemical staining for CD147 in TMA cores. Representative images of SCCOT with low (A: Immunoscore = 1) and high (B: Immunoscore = 4) CD147 expression levels. Top: Low-power view (original magnification $\times 100$); Bottom: High-power view of the same tumors (original magnification $\times 200$).

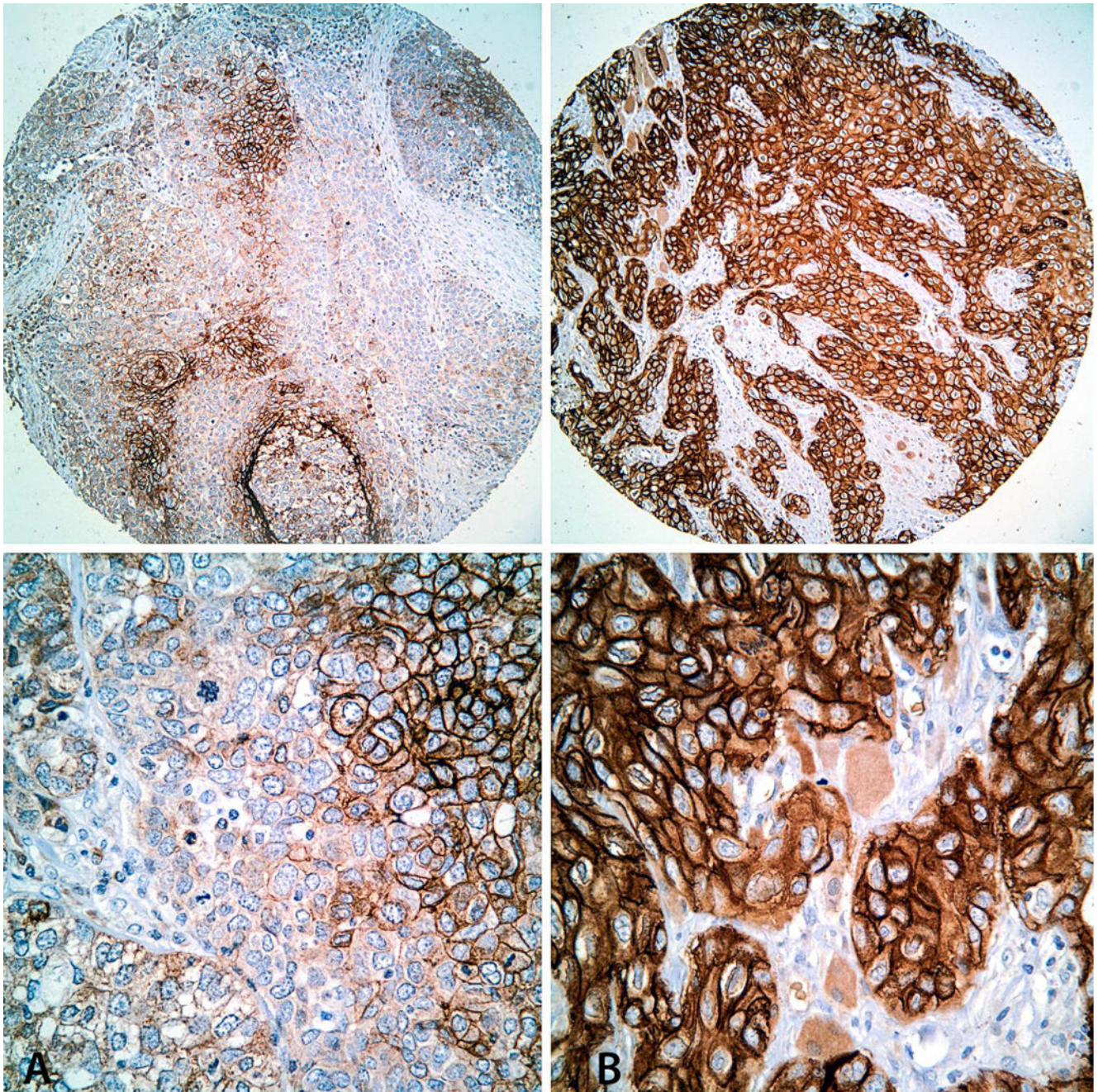


Figure 3. Immunohistochemical staining for Glut-1 in TMA cores. Representative images of SCCOT with low (A: Immunoscore = 1) and high (B: Immunoscore = 4) Glut-1 expression levels. Top: Low-power view (original magnification $\times 100$); Bottom: High-power view of the same tumors (original magnification $\times 200$).

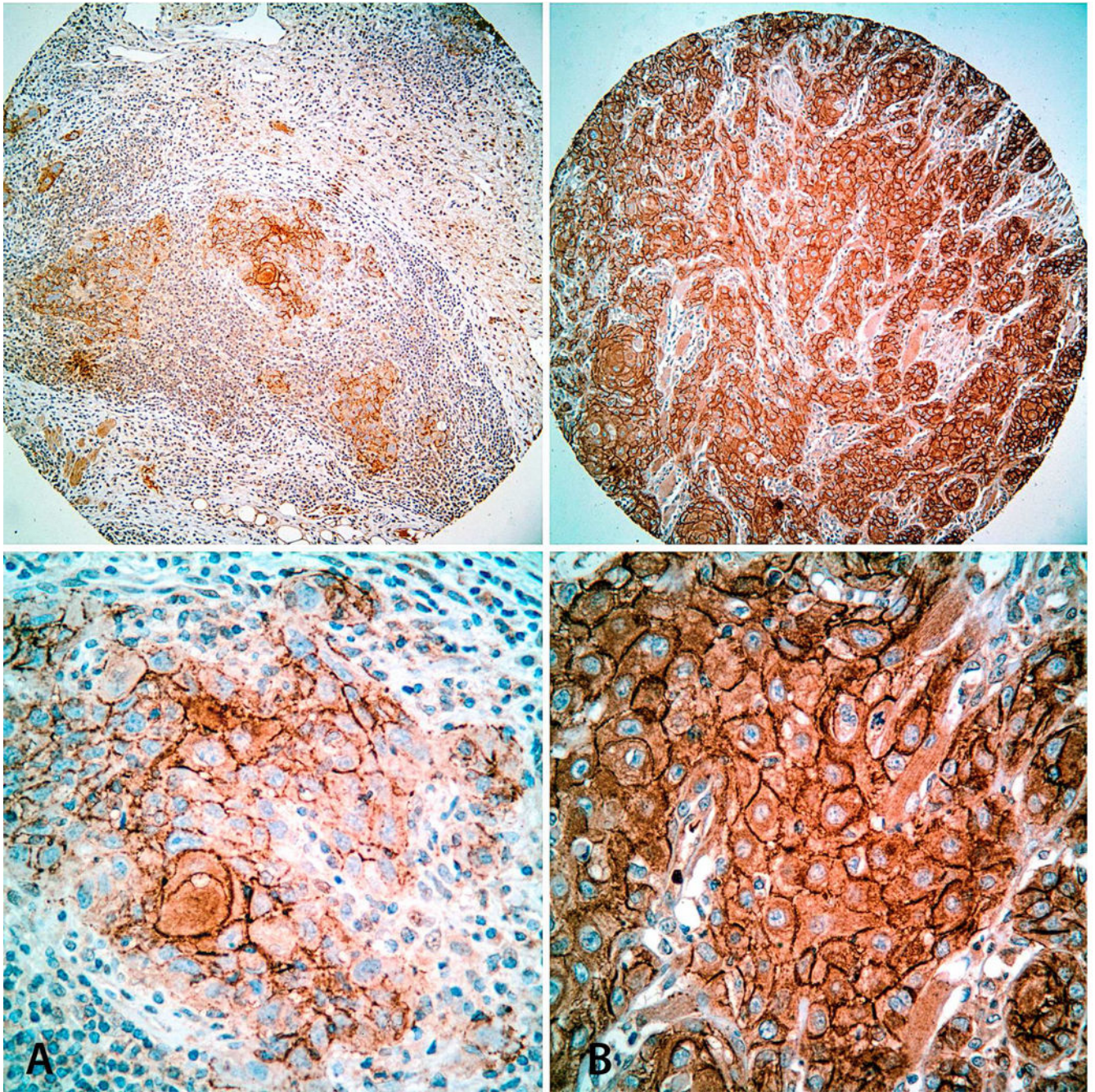


Figure 4. Immunohistochemical staining for CD44v6 in TMA cores. Representative images of SCCOT with low (A: Immunoscore = 1) and high (B: Immunoscore = 4) CD44v6 expression levels. Top: Low-power view (original magnification $\times 100$); Bottom: High-power view of the same tumors (original magnification $\times 200$).

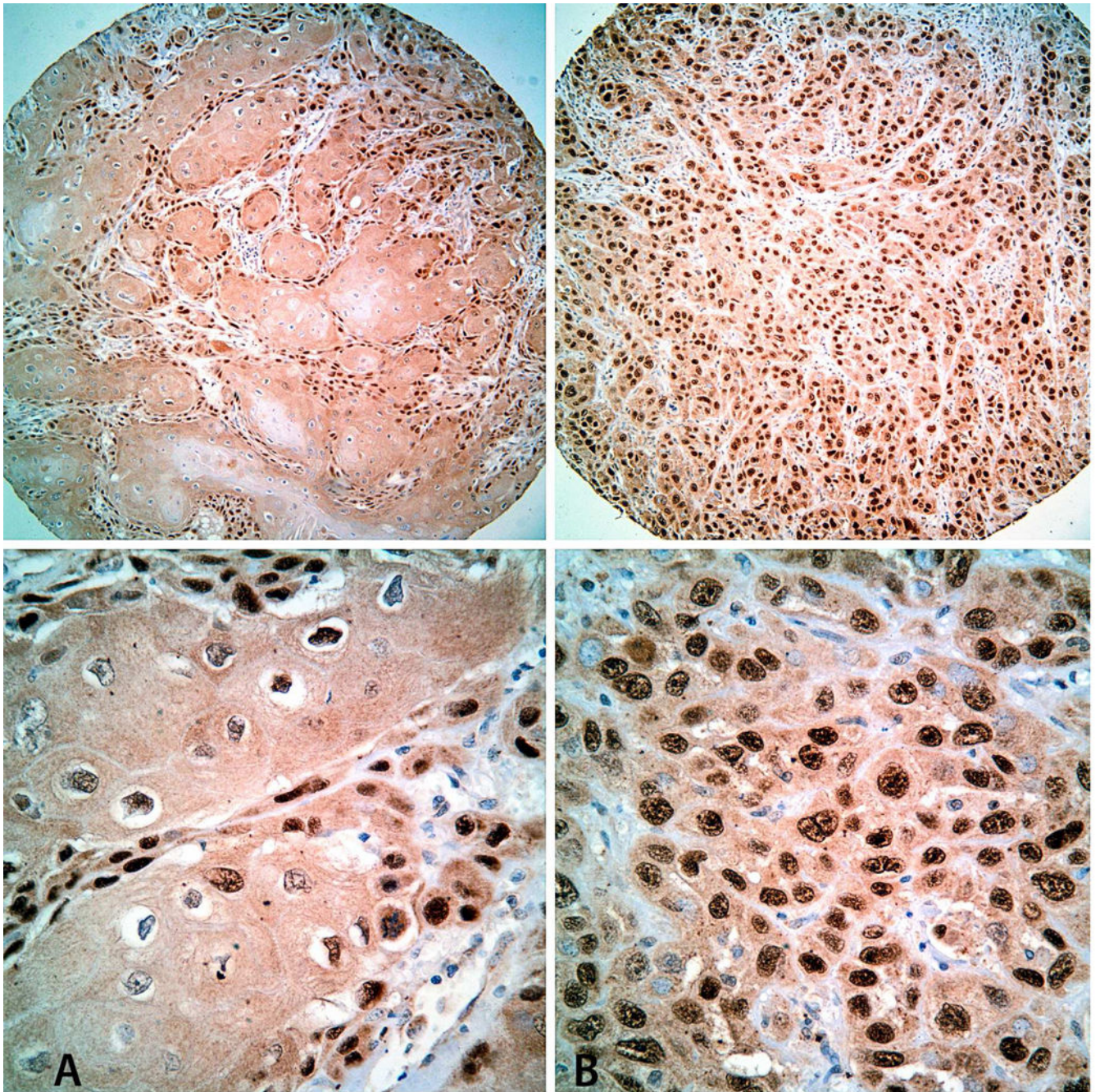


Figure 5. Immunohistochemical staining for Tp63 in TMA cores. Representative images of SCCOT with low (A: Immunoscore = 1) and high (B: Immunoscore = 4) Tp63 expression levels. Top: Low-power view (original magnification $\times 100$); Bottom: High-power view of the same tumors (original magnification $\times 200$).

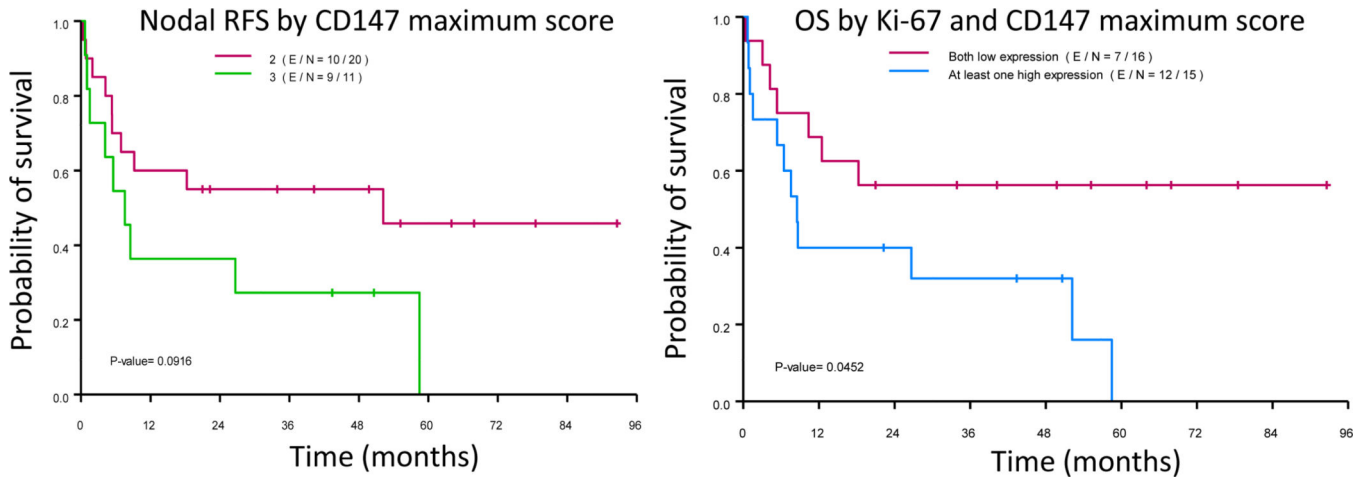


Figure 6. Kaplan-Meier analysis of recurrence free survival (RFS; right; $p = 0.0916$) and overall survival (OS; left) of patients with squamous cell carcinoma of the tongue according to CD147 and CD147 + Ki-67 expression levels, respectively ($p = 0.0452$).

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Table 1

Sources of antibodies and the working dilutions that were used for immunohistochemical analysis of tissue microarrays

Antibody	Type and Dilution	Source
Tp63	Mouse Ig 1:100	Santa Cruz, Dallas, TX, USA (4A4 clone; sc-8431)
Ki-67	Rabbit Ig 1:100	Life Technologies, Grand Island, New York, USA (18-0191Z)
CD44var (v3–v10)	Goat Ig 1:200	eBioscience Inc, San Diego, CA, USA (BMS 124)
CD147	Goat Ig 1:200	R & D Systems, Minneapolis, MN, USA AF972
Glut-1	Rabbit Ig 1:250	Thermo Scientific. Waltham, MA, USA RB-9052

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Table 2

Clinicopathologic characteristics of the SCCOT samples in the tissue microarrays

Variables	N (%)
Age	
< 40	1 (3)
40–49	5 (16)
50–59	8 (25)
60–70	12 (38)
>70	6 (18)
Gender	
Male	27 (84)
Female	5 (16)
Smoking	
Current	14 (44)
Never	4 (12)
Former	14 (44)
T Stage	
T1–2	15 (47)
T3–4	17 (53)
N Stage	
N0	12 (38)
N1–2C	20 (62)
Extracapsular Spread	
No	20 (62)
Yes	12 (38)
Treatment	
Surgery	11 (34)
Surgery+RT	20 (63)
Surgery+CRT	1 (3)
Histology Grade	
1 (Well differentiated)	8 (25)
2 (Moderately differentiated)	19 (59)
3 (Poorly differentiated)	5 (16)
Distant Metastasis	
M0	24 (75)
M1-X	4 (12.5)
Missing	4 (12.5)
Survival	
Alive	12 (37)
Dead	20 (63)

Table 3

Correlation between the traditional prognostic parameters and biomarkers

Variables	Biomarkers association (p-value from GEE model)				
	p63	Ki-67	CD147	CD44v6	Glut1
T-stage	0.042	0.048	0.004	NS	NS
N-stage	NS	NS	NS	NS	NS
ECS-Stage	NS	NS	NS	NS	NS
Histology grade	0.001	0.022	NS	NS	NS

Table 4

Positive correlation between biomarkers expression levels in SCCOT-TMA

Biomarker	(p-value)
Ki-67 & p63	0.018
Ki-67 & CD147	0.035
Ki-67& CD44v6	NS
Ki-67 & Glut1	NS
p63 & CD147	0.003
p63 & CD44v6	NS
p63 & Glut1	0.035

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Table 5

Prognostic markers associated with worse overall and recurrence free survival

Variables	Overall survival (p-value)	Recurrence-free survival (p-value)
N-stage	0.005	0.010
ECS-status	0.0005	0.001
p63	0.022	0.011
CD147	0.094	0.092
Ki-67 ± CD147	0.045	0.047

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