Prognostic role of CD11b+ myeloid-derived suppressor cells in oral squamous cell carcinoma

Type
Research paper

Keywords
CD11b, prognostic biomarker, oral squamous cell carcinoma, myeloid derived suppressor cells

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High density of CD11b+ MDSCs at CT or IM significantly associated with inferior overall and disease-free survival (Kaplan-Meir, P < 0.05, Log-rank test). CD11b CT and CD11b IM were identified as independent prognostic predictors for patient survival. The prediction accuracy and specificity of CD11b CT and CD11b IM were superior to other prognostic parameters.

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Our data indicated that increased densities of CD11b+ MDSCs at CT and IM regions significantly associated with poor prognoses which might be novel prognostic factors for OSCC.
Prognostic role of CD11b+ myeloid-derived suppressor cells in oral squamous cell carcinoma

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a. These two authors contributed equally to this work.

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Running title: Prognostic value of CD11b+ MDSCs in OSCC

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Introduction

Until recently, the 5-year survival for patients with primary oral squamous cell carcinoma (OSCC) remains approximately 60%, which has not been markedly improved in the past decades.¹ Such dismal prognosis is in part due to high proportion of patients presented with advanced disease at initial diagnosis. Insufficient and inaccurate evaluation of this malignancy hamper effective treatment planning and proper prognostic prediction. Nowadays, patients with OSCC are commonly staged according to the TNM staging system based on the tumor dimension, lymph node and metastasis.² However, owing to the heterogeneity of OSCC, patients with the same TNM stage usually present a significant variety of clinical outcome.³ Thus, identification of novel prognostic factors is urgently needed to better estimate survival and guide treatment planning.

Tumor microenvironment of solid cancer including OSCC is usually characterized by significant infiltrating of both innate and adaptive immune cells including but not restricted to T cells, B cells and macrophages.⁴ Mounting evidence has demonstrated that various types of tumor-infiltrating immune cells promote cancer initiation, overgrowth and metastatic dissemination.⁵ Moreover, quantifications of these cancer-associated
immune cells have robust prognostic significance in a broad spectrum of human cancer. In particular, myeloid-derived suppressor cells (MDSCs), one of the major components of tumor microenvironment, have emerged as a key regulatory cell population that critically participates in tumorigenesis. Intensive studies have indicated MDSCs as a negative predictor of clinical outcomes in various cancers and a potential predictive biomarker in malignancies including melanoma, gastrointestinal and bladder cancer. 

Currently, human MDSCs are usually labelled as CD11b+CD33+HLA-DR-Lin and consist of two groups of cells termed polymorphonuclear (PMN-MDSCs, CD11b+CD33+CD14-CD15+HLA-DR-Lin) and monocytic (M-MDSCs, CD11b+CD33+CD14+CD15+HLA-DR-Lin). Several previous studies have used the CD11b to label tumor-infiltrating MDSCs by immunohistochemistry in various cancer contexts. Evaluation of CD11b+ MDSCs in tumor center and microenvironment have been utilized to predict patients’ clinical outcome. For example, high densities of CD11b+ MDSCs significantly associated with unfavorable survival of patients diagnosed with hepatocellular carcinoma. However, to the best of our knowledge, it remains largely unknown regarding the prognostic value of CD11b+ MDSCs infiltration in OSCC.
The present study was designed to evaluate the densities of CD11b+ MDSCs at both center of tumor (CT) and invasive margin (IM) regions by immunohistochemistry and assess their prognostic significance in a retrospective cohort of OSCC from a tertiary referral oral cancer center.
Material and methods

Patients and specimens

This study was in accordance with Declaration of Helsinki and approved by the Research Ethic Committees of Affiliated Stomatological Hospital, Nanjing Medical University. Informed consent was obtained from each patient or their guardians. Records of patients with primary OSCC who underwent ablative surgery at Affiliated Stomatological Hospital of Nanjing Medical University between April 2011 to December 2013 were reviewed. Patients enrolled here suffered from primary OSCC and treatment-naive. Formalin-fixed paraffin-embedded (FFPE) specimens from ablative resection of OSCC and detailed clinical, pathological as well as follow-up data were available for all eligible patients. Histopathological grading and clinical staging of each case were evaluated according to the WHO classification and American Joint Committee on Cancer Staging System 7th edition, respectively.¹⁴

Immunohistochemical staining of CD11b in OSCC

The FFPE specimens of all patients enrolled were collected for slide preparation. Immunohistochemical staining of CD11b was performed on 4-μm thickness sections as
described previously. Briefly, FFPE specimens were consecutively sliced into sections followed by deparaffinized in xylene and standard gradient ethanol. Subsequently, the slides were immersed in Tris-EDTA buffer (pH 8.0) for 15 minutes for antigen retrieval and incubated in 3% H₂O₂ for the blockage of endogenous peroxidase activity. The sections were further incubated with primary antibody (anti-CD11b, 1:200 dilution, CST, 49420) at 4°C overnight followed by phosphate-buffered saline washing and biotinylated secondary antibody incubation (Maxim, China). Finally, the antigen detection was conducted by a color reaction with 3.3-diaminobenzidine (DAB) under the microscopic monitoring and counterstained with haematoxylin. Negative controls without primary antibody incubation were included.

**Evaluation of immunohistochemical staining**

Images of selected areas were acquired using an upright microscope (Leica DM4000B, Germany), and the immunohistochemical staining results of CD11b+ MDSCs in OSCC samples were independently assessed by two senior oral pathologists without knowledge of patients’ clinicopathological data. When an agreement was difficult to reach, final judgment was made by reevaluating the slides. For each tumor specimen,
slides containing invasive margin (IM) and tumor center (CT) regions were selected. Five representative fields per slide including both of the regions were selected at 200× magnification after initial screen under a low power filed (100×). As illustrated in Supplementary Figure 1, similar with previous reports, the invasive margin (IM) was defined as a region of 500 μm width on each side of the border between malignant cells and tumor stroma, while the tumor center (CT) was defined as a region in the center of tumor which was full of malignant cells and excluded the first 250μm adjacent to the tumor border.16,17 The density of CD11b+ MDSCs in OSCC specimens was recorded independently at CT and IM and presented as the mean of positively stained cells per mm², similar to our previous report.18-20 As shown in Figure 1, cell counts of CD11b+ MDSCs were automatically quantified using ImageJ (version 2.0). The optimal cut-off values for the densities of CD11b+ MDSCs at CT and IM were determined after analyzing the association between cell amount and overall survival (OS) by X-tile software with a minimum $P$ value approach (version 3.6.1, https://medicine.yale.edu/lab/rimm/research/software/). X-tile program is a commonly-
used statistical tool for the assessment of biological relationships between a biomarker and outcome and the discovery of optimal cut-point based on marker expression.\textsuperscript{21}

**Statistical analysis**

The X-tile program was applied to determine the optimal cut-off value of the CD11b\textsuperscript{+} MDSCs density with the minimum \(P\) value. Analysis of the association between classified densities of CD11b\textsuperscript{+} MDSCs at CT or IM and multiple clinicopathological parameters was conducted using Chi-square test or Fisher’s exact test. Overall survival (OS) and disease-free survival (DFS) were calculated with the Kaplan-Meier method and comparison between groups was performed with the Log-rank test. Univariate and multivariate Cox proportional hazards regression model was used to determine the hazard ratio of different prognostic factors and the association between these factors with OS and DFS for OSCC. Receiver operating characteristics (ROC) curve of indicated prognostic factors were plotted and the area under the curve (AUC) was calculated to identify the predictive performance of each individual marker. All tests were two-sided, and \(P\) values less than 0.05 were considered statistically significant. All statistical analysis was performed with GraphPad Prism 8.0, IBM SPSS 22.0 software and R 3.5.3.
Results

Epidemiological and clinicopathological characteristics of patients

One hundred and forty-four patients (76 males and 68 females) who were diagnosed with primary OSCC and underwent ablative surgery were included. They were aged from 30 to 82 years. The median follow-up duration was 45 months (ranging from 6 to 138 month). Sixty-nine patients died during the follow-up and 8 patients with local recurrence or cervical node metastasis remained alive until the last follow-up. Detailed epidemiological and clinicopathological characteristics of these patients enrolled were summarized in Table I.

Associations between infiltrating CD11b+ MDSCs and clinicopathological features

Here, we employed immunochemical staining of CD11b, a common marker to label MDSCs in cancer, to characterize and quantify MDSCs in primary OSCC.\textsuperscript{7,11,12} As shown in Figure 1, CD11b positive staining MDSCs was readily detected at CT and IM regions in OSCC samples. Following image capture and automatic cell counting by ImageJ, the densities of CD11b+ MDSCs in each tumor region (CT, IM) was calculated and recorded (Figure 1). Our results revealed that the densities of infiltrating MDSCs varied in CT
(130.9±5.84 per mm$^2$) and IM (466.2±15.28 per mm$^2$), thus suggesting the significant enrichment of MDSCs at IM compartments. Additionally, no significant association between intensities of CD11b$^+$ MDSCs in CT and IM was identified (data not shown).

Then, to achieve outcome-based cut-off optimization, the "minimum $P$ value" approach was employed using X-tile software. The optimal cutoff value for CD11b$^+$ MDSC at the IM was 389.0 per mm$^2$ ($P=0.0012$) and 143.0 per mm$^2$ at the CT ($P=0.00189$) (Figure 2). Consequently, patients were classified into low or high CD11b$^+$ MDSCs subgroups.

As summarized in Table I, we analyzed the associations between CD11b$^+$ MDSCs densities and several clinicopathological parameters, but failed to identify any significant associations.

**Association between infiltrating CD11b$^+$ MDSCs and survival in OSCC patients**

To identify the possible associations between CD11b$^+$ MDSCs and patient survival, we performed Kaplan-Meier analyses and found that increased densities of CD11b$^+$ MDSCs at both CT and IM regions were significantly associated with shorter OS and DFS (Figure 3A-D). Furthermore, we verified the prognostic values of these immunological features for OSCC by Cox regression analysis. As listed in Table II, the
univariate analysis indicated that elevated densities of CD11b⁺ MDSCs at either IM or CT significantly associated with reduced OS ($P=0.001$ and $<0.001$, respectively) and DFS ($P=0.001$ and $<0.001$, respectively). Moreover, the multivariate Cox proportional regression analyses revealed that, besides pathological grade, densities of CD11b⁺ MDSCs in IM or CT were independent prognostic factors for OS (HR 3.183, 95% CI:1.780-5.689, $P<0.001$ at IM; HR 2.012, 95% CI:1.213-3.337, $P=0.007$ at CT). Similar results were found in the DFS analysis, where estimated HR for CD11b⁺MDSCs in IM was 2.762 (95% CI: 1.618-4.715) and at CT was 1.919 (95% CI: 1.181-3.119).

**Predictive performance of CD11b⁺ MDSCs for OSCC prognosis**

Next, we proceeded to determine the predictive performance of CD11b⁺ MDSCs for patients with OSCC by time-dependent ROC curve analysis. Tumor size, cervical nodal metastasis, pathological grade and clinical stage, widely adopted as prognostic factors for OSCC, were included for assessing the predictive value of CD11b⁺ MDSCs. With respect to the OS, the AUC of CD11b IM at 1 year, 3 years and 5 years were 0.69, 0.58, 0.78 and the AUC of CD11b CT were 0.72, 0.68, 0.75, respectively (Figure 4A, B).
However, the AUC of the aforementioned four clinicopathological parameters were less than 0.54 (Supplementary Figure 2).
Discussion

High mortality in OSCC necessitates effective biomarkers development for treatment planning and prognostic prediction, ultimately improving patient management and long-term survival. Current commonly used TNM staging system fails to meet the clinical demand in accurate prognostic prediction.\(^1\) Although various genetic or epigenetic biomarkers in OSCC have been proposed for prognostic assessment including microRNA, IncRNA and alternative mRNA splicing signature, they are still far from optimal.\(^2,20,22,23\) In addition to these intrinsic features of cancer itself, tumor-infiltrating immunocytes have been established as key drivers underlying tumorigenesis as well as novel biomarkers with tremendous diagnostic and prognostic significance.\(^4,6\) Here, we determined the location and amount of MDSCs in OSCC and revealed that high amount of CD11b\(^+\) MDSCs associated with inferior survival and quantification of CD11b\(^+\) MDSCs might be a novel independent prognostic factor for OSCC.

Accumulating evidence has been demonstrated that multiple types of immune cells infiltrate in tumor itself or its microenvironment has facilitated tumor overgrowth, invasion and metastasis.\(^5\) In particular, MDSCs are a heterogeneous population of immature
myeloid cells and utilize multiple mechanisms to establish a tumor-promoting environment. They can suppress T cells by depleting amino acids necessary for T cells activation, inhibiting its migration and preventing its entry into lymph nodes or homing to tumor sites. Moreover, with respect to its indirect immunosuppressive mechanism, MDSCs altered the ability of antigen-presenting cells (APCs) to activate T cells and T regulatory cells (Tregs). Besides immune regulatory mechanisms, MDSCs also influenced tumor progression by modulating tumor microenvironment and promoting angiogenesis via VEGF, bFGF and MMP9. Consistent with these previous findings, our data revealed that MDSCs were highly enriched in invasive margins of tumor, thus suggesting that their tumor-promoting roles occurred primarily in tumor microenvironment. However, much work is needed to accurately dissect the roles of MDSCs and relevant mechanisms of action during OSCC tumorigenesis.

Until now, the prognostic impacts of several subsets of tumor-infiltrating immune cells in a broad spectrum of human cancers including OSCC have been documented such as CD3+ TIL, CD8+ TIL, CD45RO+ TIL and CD11b+ MDSCs. For example, we have reported that high densities of CD3+/CD8+ TIL, CD68+ macrophage significantly
associated with increased overall and disease-specific survival in OSCC. In the present study, we assessed the prognostic value of the density of CD11b+ MDSCs either in CT or IM regions. Our results revealed that high densities of CD11b+ MDSCs significantly associated with reduced patient survival. In addition, multivariate Cox regression assay also identified CD11b+ MDSCs as a novel independent prognostic factor affecting patient survival. These findings are well in line with previous reports wherein MDSCs accumulation correlated with poor outcome in melanoma, gastrointestinal cancers and bladder cancer. Moreover, MDSCs in non-small lung and cervical cancer patients associated with advanced tumor stage and unfavorable prognosis and served as an independent prognostic factor predicting patients' outcome. Here, we investigated the prognostic value of CD11b+ MDSCs in distinct locations (CT or IM) in OSCC with relatively long-term follow-up. Our findings revealed that high densities of CD11b+ MDSCs either at IM or CT compartment significantly associated with unfavorable prognosis in OSCC and also served as independent prognostic factors in predicting patients' survival. Previous studies mainly focused on MDSCs in whole tumor sections without discriminating different locations of infiltrating
immunosuppressive cells in the tumor sample, which may partially explain the inconsistent results among various studies.\textsuperscript{10, 31-33} Indeed, spatial distribution of immunoregulatory cells in different compartments like the CT and IM might have diverse biological functions and their predictive value of clinical outcome can be region-specific.\textsuperscript{34} Thus, we believe that our immunological evaluation of CD11b\(^+\) MDSCs by taking into its distribution account might be more accurate and powerful. In support of this, our time-dependent ROC curve analysis also revealed that the predictive performance of CD11b\(^+\) MDSCs in IM or CT was superior to those prognostic factors like tumor size, cervical nodal metastasis and pathological grade.

Although our data revealed the prognostic values of CD11b\(^+\) MDSCs in IM or CT for OSCC patients, there are some limitations. Firstly, potential bias may remain due to the retrospective nature and limited sample size. Our findings are needed to be confirmed in a prospective study including a large, multicenter patient population. Single marker for MDSCs labeling might be not adequate to evaluate their diverse subtypes as well as functional status. Combinations of two or more markers might be better and more accurate to dissect the clinical and biological significance of MDSCs during OSCC.
initiation and progression. In addition, some pathological factors such as depth of invasion, extracapsular extension or perineural invasion were not included in prognostic analyses.

Besides, whether CD11b+ MDSCs in IM or CT can be predictive for response to various treatments as well as adverse events of adjuvant therapies in OSCC remains unknown.

Conclusion

Our data indicated that high densities of CD11b+ MDSCs in tumor center or invasive margins significantly correlated with unfavorable survival in patients with primary OSCC. Our finding also suggests that this infiltrating immune subsets has pro-tumorigenic effects in OSCC which might be exploited for therapeutic intervention.
Acknowledgments

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Conflict of interest

The authors declare no conflict of interest.
References


31. Singh MM, Johnson B, Venkatarayan A et al. Preclinical activity of


Figure legends:

**Fig.1** Immunohistochemical staining of CD11b+MDSCs in OSCC.

**A**, Representative staining of CD11b+ MDSCs in the tumor center (CT) of OSCC. Scale bar: 100μm. **(B, C)** The number of CD11b+ MDSCs in tumor CT is semiautomatically quantified by ImageJ software. Scale bar: 50μm. **D**, Representative staining of CD11b+ MDSCs in invasive margins (IM) of OSCC. Scale bar: 100μm. **(E, F)** The number of CD11b+ MDSCs in tumor IM is semiautomatically quantified by ImageJ software. Scale bar: 50μm.

**Fig.2** The optimal cutoff values of CD11b+ MDSCs in the CT (A) and IM (B) were determined by X-tile software using OS as the primary outcome in our patient cohort. CT, tumor center; IM, invasive margin.

**Fig.3** Prognostic significance of CD11b CT and CD11b IM in patients with OSCC.
The Kaplan-Meier analysis of overall survival (OS) and disease-free survival (DFS) in patients stratified by CD11b CT (A, C) and CD11b IM (B, D). $P$ values were calculated with the log-rank test.

**Fig.4 Predictive ability of CD11b CT and CD11b IM for the prognosis of patients with OSCC.**

ROC curves and AUC at 1 year, 3 years and 5 years were used to estimate the sensitivity and specificity of CD11b CT (A) and CD11b IM (B) in the prognostic prediction of overall survival (OS).
Supplementary Figure legends:

Supplementary Fig.1 Representative IHC image of CD11b+MDSCs in OSCC.

The dot red line marks the border between tumor and the surrounding stroma. Two red lines mark a region (IM) with 500μm wide (dot red line with arrow) on each slide of the border between malignant cells and the surrounding nontumor stroma. CT comprised the tumor section excluding the first 250μm adjacent to the tumor border.

Supplementary Fig.2 Predictive ability of tumor size, cervical node metastasis, pathological grade and clinical stage for the prognosis of patients with OSCC.

The sensitivity and specificity of four clinicopathological parameters in the prognostic prediction of overall survival (OS) were estimated by ROC curves.
Table I. Associations between density of CD11b\(^+\) MDSCs and clinicopathological parameters in OSCC

<table>
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*All P-values in Table 1 were obtained using Chi-square test or Fisher’s exact test.

MDSCs, myeloid derived suppressor cells; OSCC, oral squamous cell carcinoma; CT, tumor center; IM, invasive margin.
Table II. Univariate and multivariate survival analyses of prognostic factors associated with OS and DFS for OSCC

<table>
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<td>Tumor size (T3-T4, T1-T2)</td>
<td>1.264</td>
<td>0.712-2.243</td>
</tr>
<tr>
<td>Pathological grade (II-III, I)</td>
<td>1.942</td>
<td>0.958-3.938</td>
</tr>
<tr>
<td>Cervical node metastasis (N+, N0)</td>
<td>0.993</td>
<td>0.583-1.692</td>
</tr>
<tr>
<td>Clinical stage (III-IV, I-II)</td>
<td>1.217</td>
<td>0.749-1.978</td>
</tr>
<tr>
<td>CD11b CT (high, low)</td>
<td>2.283</td>
<td>1.390-3.750</td>
</tr>
<tr>
<td>CD11b IM (high, low)</td>
<td>3.285</td>
<td>1.850-5.835</td>
</tr>
<tr>
<td><strong>Multivariate survival analyses</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pathological grade (II-III, I)</td>
<td>1.643</td>
<td>0.774-3.487</td>
</tr>
<tr>
<td>Cervical node metastasis (N+, N0)</td>
<td>0.599</td>
<td>0.269-1.332</td>
</tr>
<tr>
<td>Clinical stage (III-IV, I-II)</td>
<td>1.542</td>
<td>0.760-3.130</td>
</tr>
<tr>
<td>CD11b CT (high, low)</td>
<td>2.012</td>
<td>1.213-3.337</td>
</tr>
<tr>
<td>CD11b IM (high, low)</td>
<td>3.183</td>
<td>1.780-5.689</td>
</tr>
</tbody>
</table>

The numbers in bold indicate statistical significance with P-values less than 0.05.

HR, hazard ratio; CI, confidence interval; OS, overall survival; DFS, disease-free survival; OSCC, oral squamous cell carcinoma; CT, tumor center; IM, invasive margin.
Fig.1 Immunohistochemical staining of CD11b+MDSCs in OSCC.
A, Representative staining of CD11b+ MDSCs in the tumor center (CT) of OSCC. Scale bar:100μm. (B, C) The number of CD11b+ MDSCs in tumor CT is semiautomatically quantified by ImageJ software. Scale bar: 50μm. D, Representative staining of CD11b+ MDSCs in invasive margins (IM) of OSCC. Scale bar:100μm. (E, F) The number of CD11b+ MDSCs in tumor IM is semiautomatically quantified by ImageJ software. Scale bar: 50μm.
Fig. 2 The optimal cutoff values of CD11b+ MDSCs in the CT (A) and IM (B) were determined by X-tile software using OS as the primary outcome in our patient cohort. CT, tumor center; IM, invasive margin.
Fig. 3 Prognostic significance of CD11b CT and CD11b IM in patients with OSCC. The Kaplan-Meier analysis of overall survival (OS) and disease-free survival (DFS) in patients stratified by CD11b CT (A, C) and CD11b IM (B, D). P values were calculated with the log-rank test.
Fig. 4 Predictive ability of CD11b CT and CD11b IM for the prognosis of patients with OSCC. ROC curves and AUC at 1 year, 3 years and 5 years were used to estimate the sensitivity and specificity of CD11b CT (A) and CD11b IM (B) in the prognostic prediction of overall survival (OS).
Supplementary Fig. 1 Representative IHC image of CD11b+MDSCs in OSCC. The dot red line marks the border between tumor and the surrounding stroma. Two red lines mark a region (IM) with 500μm wide (dot red line with arrow) on each slide of the border between malignant cells and the surrounding nontumor stroma. CT comprised the tumor section excluding the first 250μm adjacent to the tumor border.
Supplementary Fig. 2 Predictive ability of tumor size, cervical node metastasis, pathological grade and clinical stage for the prognosis of patients with OSCC. The sensitivity and specificity of four clinicopathological parameters in the prognostic prediction of overall survival (OS) were estimated by ROC curves.