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Prognostic significance of macrophage polarization in early stage oral squamous cell carcinomas ☆

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SUMMARY

Background: Polarization of tumor infiltrating macrophages is associated with the prognosis of solid malignancies and correlates with the occurrence of lymph node metastases in oral squamous cell carcinomas (oscc). Early stage (T1/T2, N0) oscc are characterized by a good prognosis and can be cured by surgery. The postoperative regime usually contains no adjuvant radio-/chemotherapy. The current pilot study was conducted to elucidate whether macrophage polarization in tumor resection specimens and diagnostic biopsies of early stage oscc is associated with tumor outcome.
Methods: Patients with T1/T2, N0, and R0 > 5 mm oscc without adjuvant therapy and 3-year follow-up after tumor resection were retrospectively selected. Tissue microarrays (TMA) containing diagnostic biopsies ($n = 17$) and tumor resection specimens ($n = 17$) were processed for immunohistochemistry in this pilot study to detect CD68-, CD11c-, CD163- and MRC1-positive macrophages. Samples were digitized, and the expression of macrophage markers was quantitatively analyzed.
Results: High infiltration of M2 polarized macrophages correlated with poor tumor outcome in early stage (T1/T2, N0) oscc. This correlation was observed in tumor resection specimens, but was also observed in diagnostic biopsies. M2 macrophage polarization in biopsies – but not in tumor resection samples – correlated with high scores in tumor grading.
Conclusion: Macrophage polarization in early stage oscc is a potential prognostic marker for tumor outcome. The correlation of M2 polarized macrophages with tumor outcome can already be detected in the initial biopsies. Furthermore, M2 polarization of macrophages in biopsies is associated with an increased dedifferentiation.

Introduction

Over the last three decades, the prognosis for oral squamous cell carcinomas (oscc) has not been substantially improved [1]. One reason for this lack of improvement might be the insufficient consideration of tumor-immunological parameters in the prognostic classification and treatment of oscc.

Despite the overall unfavorable prognosis of oscc patients, there are subgroups with good tumor outcome. Early stage (T1/T2, N0) oscc are generally characterized by a good prognosis due to a low rate of lymph node metastases and local recurrences [2,3]. The treatment regime of these tumors usually contains surgical resections and no adjuvant radio-/chemotherapy [4,5]. These cancers are generally considered to be curable by surgery. However, in clinical practice, there are some cases with early stage carcinomas that develop recurrences despite initial ideal prerequisites for curing these carcinomas. Common surgical problems, such as obtaining clear resection margins or radiation oncological questions, including different adjuvant or neoadjuvant treatment protocols, are not an issue in these cases. It seems to be plausible that immunological parameters might be involved in unfavorable clinical courses in these patients.

Macrophages are potent antigen presenting cells and are therefore essential for initiating immune responses against pathogens or tumor antigens [6]. The polarization of macrophages (M1 vs. M2) defines whether immune responses are directed toward immunity and antigen clearance (M1) or toward peripheral tolerance (M2)

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[7]. M2 polarized macrophages have immunoregulatory properties and are associated with wound healing, tissue remodeling, angiogenesis and tumor progression [8–17].

CD68 is the most commonly used generic macrophage marker [8,18–20]. M1 macrophages are frequently identified by staining the CD11c antigen [10,19,21,22]. M2 macrophages express CD163 [11,20,23,24] and MRC1 antigens [10,23,25].

In several solid malignancies, a prognostic significance for macrophage polarization is already known [26–29]. In gastric cancer, a high MRC1 density (M2 macrophages) in tumor specimens is correlated with poor tumor outcome [29]. In addition, a study in pleural mesotheliomas showed a positive correlation of the CD163/CD68 ratio with overall survival [26].

In oscc, an influence of macrophage polarization on tumor outcome is not yet proven. In head and neck squamous cell carcinomas (HNSCC) treated by definitive radiotherapy, an association of CD163 infiltration (M2 macrophages) with overall survival was shown [30]. In T1–T4 oscc stroma tissue, a semi-quantitative assessment of CD163 positive cells was correlated with tumor outcome [31]. However, an analysis of the possible prognostic influence of macrophage polarization in early stage oscc is not available.

It was shown that an increased M2 macrophage polarization in the epithelial compartment of oscc tumor specimens correlates with the occurrence of lymph node metastases [17]. An association of macrophage polarization with histomorphological parameters, such as tumor grading, was specifically observable in regional lymph nodes [32] – but not in the tumor resection specimens [17]. All of these studies analyzed macrophage polarization in oscc tumor resection specimens and not in the initial diagnostic biopsy samples.

Oscc patients are commonly subjected to two surgical procedures (the initial diagnostic biopsy and the later tumor resection) [4]. During a time interval of days or weeks between the two procedures, alterations in tumor biology might occur. We recently found a shift toward M2-polarized macrophages in tumor resection specimens compared to diagnostic incision biopsies of the same patients [33]. Wound-healing reactions initiated by biopsy-derived tissue trauma might be responsible for the increase in M2 polarization [33]. It is not known whether macrophage polarization at an early stage of tumor progression before any diagnostic or therapeutic procedure is performed may determine tumor outcome. This initial tumor-immunological state can only be analyzed in the initial diagnostic tumor biopsy.

The current pilot study aims to answer the following questions: does macrophage polarization in initial diagnostic biopsies and tumor resection specimens of early stage oscc correlate with tumor grading and with tumor outcome 3 years after the initial treatment? Does macrophage polarization differ between diagnostic incision biopsies and tumor resections in early stage oscc?

Materials and methods

Patients and tissue harvesting

For the purposes of this study, the clinical records of oscc patients treated in our department from 2006 to 2011 were retrospectively analyzed. The inclusion criteria were: (a) primary oscc (lip carcinomas were excluded); (b) pT1 or pT2 tumors; (c) pN0 tumors; (d) grading G1 or G2; (e) R0 resection with a minimum of 5 mm of clear margins; (f) no postoperative treatment in terms of a radiotherapy, chemotherapy or radiochemotherapy; (g) follow-up for a minimum of 3 years; and (h) absence of other oral malignancies or a malignancy of the upper aerodigestive tract in the medical history of the patient. The review of our records isolated 80 patients with primary oscc and a classification of pT1/T2, pN0. Our cases were then grouped with relation to the outcome as follows: patients with a good tumor outcome (Group A, $n = 57$) and patients with a poor tumor outcome (Group B, $n = 23$ patients). A good tumor outcome was defined as absence of local recurrence, second oral tumor or lymph node and distant metastasis during the follow-up period of three years. Occurrence of local recurrence, a second oral tumor, lymph node metastasis or distant metastasis during the follow-up period was defined as a poor tumor outcome.

Twenty-four patients of the group with good outcome as well as 10 patients of the group with poor outcome had to be excluded, as they received postoperative radiotherapy (main causes: increased invasion depth, inability to reach 5 mm of clear margins, G3 tumors). Twenty-three additional patients from the group with good outcome and 5 other additional patients from the group with poor outcome could not be included in the study due to incomplete records with missing clinical data. Consequently, our collection included a total of 17 patients; 10 with good outcome (Group A) and 7 with poor outcome (Group B). Concerning group B, in 6 cases, there were local recurrences of the tumor, while in one case, a second oral tumor occurred (regional recurrence). Group A consisted of 2 females and 8 males, and group B included 3 females and 4 males. The median age of the analyzed patients was 63 years. Primary tumor localization in the floor of the mouth was the most common site (7 cases) followed by soft palate (3 cases), mandibular mucosa (2 cases), buccal mucosa (3 cases) and the tongue (2 cases).

For the purposes of the study, specimens were created from tissue samples corresponding to routine histopathological diagnostics for our patients collected from the Department of Pathology of the Friedrich Alexander University of Erlangen-Nürnberg. The ethical aspects of the study were approved by the ethical committee of the University (Ref. Nr. 45_12 Bc). Both specimens of the initial diagnostic biopsy and the definitive tumor resection were included in this study.

Immunohistochemical staining

A tissue micro-array (TMA) was performed for biopsy and tumor resection specimens. HE-stained sections of all of the samples were examined to ensure that all of the samples contained representative squamous cell carcinoma tissue.

The formalin-fixed, paraffin-embedded tissue samples were cut in consecutive, 2-μm-thick sections with a rotation microtome (Leica, Nussloch, Germany) and were then dewaxed in xylole and rehydrated in graded propanol before immunohistochemical staining. The immunohistochemical staining was performed with the LSAB (labeled streptavidin–biotin) method and an automated staining device (Autostainer Plus, Dako Cytomation, Hamburg, Germany). The staining kit (Dako Real, Cat. K5001, Dako Cytomation) was used according to the manufactureŕs instructions. Proteins were detected by incubating tissues in an autostainer (21 °C, 30 min) with specific antibodies.

The following primary antibodies were used: generic macrophage marker: anti-CD68 (11081401, clone KP1, Dako, Hamburg, Germany), M1 macrophage marker: anti-CD11c (ab52632, clone EP1347y, Abcam, Cambridge, UK), M2 macrophage markers: anti-CD163 (NCL-CD163, 6027910, Novocastra, Newcastle, USA) and anti-MRC1 (H00004360-1102, clone 5C11, Abnova).

Biotinylated immunoglobulins were used as the secondary antibody for all samples. DAB+ solution (Dako Cytomation) was used as the chromogen. Hematoxylin (Dako Cytomation) was applied to counterstain the nuclei. Two consecutive tissue samples were processed per immunohistochemical stain, with one serving as a negative control in each case (identical treatment except for the replacement of the primary antibody with an IgG-isotype of the primary antibody). An appropriate positive control was included in each series.