Expression of CD4 and CD8 in head and neck mucosal melanoma

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Abstract

Objectives: The expression of CD3, CD4, CD8, PD-1 and PD-L1 in mucosal malignant melanoma tumors was analyzed by immunohistochemical staining. The aim was to explore the characteristics of malignant melanoma in head and neck and its correlation with the expression of these factors, to further our understanding of the disease. Design:Retrospective clinical study. Setting: Otorhinolaryngology unit of a tertiary hospital. Participants: 36 patients underwent immunohistochemical staining. Main outcome measures: In total, 24 patients underwent immunohistochemical staining of CD4, CD8, PD-1, and PD-L1, and 12 patients underwent CD3 immunohistochemical staining. Results: There was no significant difference between the expression of CD3, CD4, or CD8 and the general clinical features of mucosal melanoma. However, (1) the high rate of expression of CD8 was significantly different in different lesion locations: 85.71% in the nasal cavity/sinus/nasal cavity and sinus, and 66.67% in the nasopharynx; (2) the expression of PD-L1 was statistically significantly correlated with the combined expression of CD4, CD8 and CD4/CD8 ($r = 0.451$, $P < 0.05$); (3) the median progression-free survival times of patients with high and low expression CD4 were 24 months and 4 months, respectively, and those of patients with high and low expression of CD8 were 24 months and 6 months, respectively. Conclusions: The correlation between the expression of CD8 and PD-L1 suggests the role of tumor infiltrating lymphocytes in anti-tumor immunity and their possible use as immunotherapy markers. In addition, the expression of CD4 and CD8 in tumor tissue may play a guiding role in the prognosis of patients.

Key points:

- Expression of CD3, CD4, CD8, PD-1 and PD-L1 in mucosal malignant melanoma tumors.
- Expression of CD8 may different in different lesion locations.
- PD-L1 was correlated with the combined expression of CD4, CD8 and CD4/CD8.
- Expression of CD8 and CD4 may related with the median progression-free survival times.
- The expression of CD4 and CD8 in tumor tissue may play a role in the prognosis of patients.

INTRODUCTION

Mucosal melanoma is a tumor type with a low incidence rate but it is highly malignant. Although it is one of the most common subtypes of melanoma in China [1], only a limited number of cases have been reported with no support from large datasets. The related literature is mostly on clinical analysis of head and neck mucosal melanoma [2], and this has an impact on our understanding and clinical treatment of this disease. With the development of immunotherapy, researchers are increasingly studying the immune microenvironment of this tumor type. Current research is focused on providing theoretical support and laboratory research on the clinical application of immunotherapy in this setting. Unlike other types of melanoma, mucosal melanoma does not benefit significantly from immunotherapy as it has the characteristics of low tumor mutation load and low lymphocyte infiltration. T cells are important functional cells in tumor immunity and play an important role in tumor-related immune processes. CD3 is a specific marker on the surface of T cells, and CD3 immunohistochemical staining can effectively evaluate the infiltration level of T cells in tumor tissue [3]. CD8 + T cells are the main lymphocyte subsets infiltrating the tumor microenvironment [4]. Previous

studies have also confirmed that CD4 + and CD8 + T lymphocyte infiltration can reflect anti-tumor immune function to a certain extent [5].

Through analysis of the pathological results from patients with a previous diagnosis of mucosal melanoma and subsequent treatment, we aimed to explore the local infiltration of CD3, CD4, and CD8 lymphocytes in this tumor type. We also examined the relationship of these lymphocytes to the general characteristics of the disease and the expression of PD-1 and PD-L1 in the tumor. From this, we hope to further our understanding of the characteristics of mucinous melanoma in head and neck, and thus aid clinical practice.

METHODS

General data

This was a retrospective clinical study. In total, 36 patients with head and neck mucosal malignant melanoma were treated in [removed for blind peer review] between January 2015 and October 2018. They were all treated surgically and with the diagnosis confirmed pathologically as mucosal melanoma. Immunohistochemical staining was performed on the tumor tissue after surgery (routine staining process in the pathology department, and the results were summarized and analyzed). All 36 patients were included; samples from 24 patients underwent immunohistochemical staining of CD4, CD8, PD-1, and PD-L1; staining of CD3 was performed in samples from 12 patients. All of the related data were obtained from our otolaryngology head and neck surgery department. In total, 16 patients were male and 20 were female with age ranging from 5 to 78 years. The follow-up time ranged from 22 to 67 months. The primary sites of the tumor were the nasal cavity (17), paranasal sinus (3), the nasal cavity and paranasal sinus (12), and the nasopharynx (4). According to the eighth edition of TNM staging, there were 18 cases of T3N0M0, 16 of T4aN0M0, and 1 each of T4aN1M0 and T4bN0M0.

Immunohistochemistry

Tumor tissues were fixed with 4% paraformaldehyde, embedded in paraffin and sectioned with a thickness of 5 μm. Paraffin sections were dewaxed and hydrated with xylene and an ethanol gradient. After antigen repair with 0.01 mol/L citric acid buffer, the sections were tested with an immunohistochemistry kit (Abcam, Shanghai, China) using the following antibodies: anti-CD3 (Abcam, ab16669), anti-CD4 (Abcam, ab133616), anti-CD8 (Abcam, ab237709), anti-PD-1 (Abcam, ab52587) and anti-PD-L1 (Abcam, ab205921). They were diluted at 1:50 and incubated overnight at 4°C. After phosphate buffered saline (PBS) cleaning, biotin-labeled secondary antibody (concentration: 1:100) was added. After incubation at 37°C for 30 minutes, 3,3'-diaminobenzidine (DAB) color was developed for 15 minutes, then washed with tap water, stained with hematoxylin, sealed with xylene transparent resin, observed and photographed under a light microscope. According to the research methods in various studies, the scoring criteria for CD3-, CD4- and CD8-positive cells were from 0 to 3, of which 0 = no immune cell infiltration, 1 = only perivascular immune infiltration, 2 = perivascular + peritumoral and/or interstitial infiltration, and 3 = diffuse immune cell infiltration. Images showing immunohistochemical staining with score 1 to 3 are shown in Fig. 1A–C. The negative expression rate of PD-1/PD-L1 cell membrane or cytoplasm was <5%, and the positive expression rate was >5%. Five high power fields (HPF) were randomly selected from each section. [Figure 1 near here]

Statistical methods

Microsoft Excel 2017 (Microsoft, Redmond, WA, USA) was used to establish the database, and SPSS version 24.0 statistical software (IBM Corp., Armonk, NY, USA) was used to analyze the differences among the groups. The chi-squared test was used to compare frequency data between groups, and the differences between groups was considered to be statistically significant when $P < 0.05$. The immunohistochemical staining scores for CD3, CD4, CD8, PD-1, and PD-L1 in the 36 patients were analyzed.

RESULTS

Correlation between the expression of CD4 and CD8 and general clinical features (Table 1)

Among the 24 patients with staining of CD4 and CD8, 11 were male and 13 were female; 15 were <60 years old and 9 were [?]60 years old. The lesions were located in the nasal cavity (10), sinus (1), nasal cavity and sinus (10), and nasopharynx (3). According to TNM staging, 12 patients were T3N0M0, 10 were T4aN0M0, and there was 1 each of T4aN1M0 and T4bN0M0. In total, 19 patients had pigmented-type melanocytic lesions and 5 had non-pigmented type. By August 2020, 21 patients were still alive and 3 had died.

According to the scoring criteria for immunohistochemical staining of CD4- and CD8-positive cells discussed in the Methods section, the scores for CD4 in the 24 patients were from 1 to 3, of which 2 cases had a score of 1 point, 21 cases had a score of 2 points, and 1 case had a score of 3 points. For CD8, again the scores were from 1 to 3 with 7 patients scoring 1, 16 scoring 2, and 1 scoring 3. According to the immunohistochemical scores for CD4 and CD8, the patients were divided into two groups: a group with a low expression of CD4 and CD8 (immunohistochemistry score for CD4 and CD8 [?] 1) and a group with a high expression of CD4 and CD8 (immunohistochemistry score for CD4 and CD8 > 1). In addition, the group with a low expression of CD4/CD8 had a score with both CD4 and CD8 [?] 1 or one of them [?]1. The group with a high expression of CD4/CD8 had a score with both CD4 and CD8 > 1. The clinical characteristics of the disease (including age, sex, location, pigmentation classification and disease stage) that may be related to each group (low and high expression of CD4, CD8, CD4/CD8) were analyzed and the results are shown in Table 1. It can be seen that there were no statistically significant differences among the groups.

Although there was no correlation between the expression levels of CD4 and CD8 and the clinical features of the various diseases, the following difference can be seen in patients with different primary tumor locations: the high expression rate of CD8 in patients with tumors in the nasal cavity/sinus/nasal cavity and sinus was 85.71%, and that of patients with tumors in the nasopharynx was 66.67% (Fig. 2).[Figure 2 near here]

In addition, the median progression-free survival was 24 months and 4 months respectively in the groups with high and low expression of CD4, and 24 months and 6 months respectively in the groups with high and low expression groups of CD8, although the difference was not statistically significant (P = 0.522). However, the expression of CD4 and CD8 in tumor tissue may be related to the prognosis of patients (Fig. 3).[Figure 3 near here]

Correlation analysis between PD-1/PD-L1 and CD4, CD8 expression (Table 2)

The correlations between the expressions of PD-1, PD-L1 and CD4, CD8 were analyzed using the Chi-squared test (Table 2). Among the 24 patients, 9 cases were PD-1 positive and 15 cases were negative; 17 cases were PD-L1 positive and 7 cases were negative. The expression of CD4 and CD8 was positively correlated with the expression of PD-L1 (Pearson correlation coefficient r = 0.451, P = 0.027).

Correlation analysis of CD3 with general clinical features (Table 3)

The immunohistochemical staining score for the 12 patients with staining of CD3 was from 0 to 3. According to the immunohistochemical score for CD3, the patients were divided into a group with low expression of CD3 (immunohistochemical staining score for CD3 [?]1) and a group with high expression of CD3 (immunohistochemical staining score for CD3 >1). Among the 12 patients, 5 were male and 7 were female; 5 were <60 years old and 7 were [?]60 years old. The lesions were located in the nasal cavity in 7 patients, the paranasal sinus in 2 patients, the nasal cavity and paranasal sinus in 2 patients, and the nasopharynx in 1 patient. According to TNM staging, there were 6 patients with T3N0M0 and 6 patients with T4aN0M0. As of August 2020, 6 patients were still alive and 6 had died. The differences between the immunohistochemical staining scores for CD3 and the clinical characteristics of the above diseases were analyzed (Chi-squared test) and the results are shown in Table 3. There was no statistically significant difference between the groups with low and high expression of CD3 for each factor.

DISCUSSION

Tumor infiltrating lymphocytes are divided into three major lymphocyte subsets, namely CD4 + T cells, CD8 + T cells and CD3 lymphocytes [6]. The CD3 molecule is an important molecular marker on the surface of mature T cells, and about 95% of mature T cells express CD3 molecules. The CD3 molecule and

T cell receptor (TCR) constitute a TCR/CD3 complex, which plays a key role in T cell stimulation signal transduction and T cell activation [7]. Therefore, CD3 is considered to be a specific marker of T cells in immunohistochemical staining. Some studies have also confirmed that the expression level of CD3 in tumor tissue may be positively correlated with the immune response level of patients. A low expression of CD3 in tumor tissue may be associated with poor T cell function, immune monitoring and immune clearance function [8]. In other tumors, the local immunohistochemical score of CD3 was confirmed to be related to the tumor stage and degree of differentiation [9]. In this study, we did not find a correlation between the expression of CD3 and the general characteristics of the disease; however, the small sample size in our study may have affected our results. Effector CD8 + T cells widely infiltrate many tumor types, such as colon cancer, ovarian cancer, cervical cancer and malignant melanoma, and directly kill tumor cells, and have been shown to be effective in the treatment of malignant melanoma [10]. The corresponding literature [11] also confirms the significance of CD8 detection in immunotherapy. However, existing studies have focused on the levels of CD4 and CD8 lymphocytes in peripheral blood to detect the immune status of patients during immunotherapy, and this effect has been confirmed [12]. The increase in CD4 + and CD8 + T cell percentage in melanoma patients 8–14 weeks after the application of ipilimumab is related to the improvement in survival rate. The number of infiltrating CD8 + T cells was positively correlated with the prognosis of tumor patients [13,14]. In terms of patient survival, some studies have found that patients with more CD8 + T cell infiltration in malignant melanoma have longer PFS, which is consistent with the conclusion reported in other studies that the effective CD8 + T cells are positively correlated with the prognosis of various tumors [15,16]. This trend was also found in this study. Although there was no statistically significant difference between study groups, we could see that the level of expression of CD8 in tumors affected the PFS of patients.

There are different opinions on the impact of CD4 expression in the local environment of tumors on the prognosis of patients. Some studies have shown that patients with high CD4 + T lymphocyte infiltration have a poor prognosis [17]. Other studies have also shown that the degree of CD4 + T lymphocyte infiltration is significantly positively correlated with the prognosis of patients [18]. However, many studies conclude that the prognosis of cancer patients depends on the ratio of CD4 + T cells to CD8 + T lymphocytes. Both CD4 + and CD8 + T lymphocytes maintain a dynamic balance to preserve the stability of the cellular immune function. They change the local immune microenvironment of the tissue, aggravate the immunosuppression and weaken the immune monitoring function, which is conducive to the proliferation of tumor cells, thus conducive to the progress, invasion or metastasis of the tumor [19]. It has also been independently confirmed that CD4 + and CD8 + T lymphocyte infiltration can reflect anti-tumor immune function to a certain extent, and can be used as a marker for predicting long-term survival [20]. In this paper, the expression of CD4 and its correlation with various factors was not statistically significant, but this may also be related to the sample size in this study.

For the most widely used PD-1/PD-L1 inhibitors at this stage, the evaluation indexes and predictive markers of these inhibitors have been the focus of research and exploration for a long time. Some studies have shown that the expression of PD-L1 in tumors is related to its immunohistochemical response to PD-1/PD-L1 inhibitors, and this may become a clinically relevant predictive biomarker [21]. The correlation between tumor infiltrating lymphocytes and the expression of PD-1/PD-L1 also reveals the status of local immune activity. In the microenvironment of melanoma, the upregulation of PD-L1 expression depends on CD8 + T cells. This is mainly due to the production of CCR4 binding chemokines and the additional contribution of induced proliferation. The presence of these immunosuppressive factors in melanoma metastasis is innate and driven by CD8 + T cells [22]. In the immunohistochemical study of melanoma, the expression of tumor PD-L1 was found in 21% of patients (14/66) [23]. The expression of tumor PD-L1 was correlated with tumor thickness (P = 0.0041), tumor CD8 (P = 0.0084) and interstitial CD8 (P = 0.0001) T cell counts [23]. The expression of PD-L1 was correlated with the expression of CD8. Other studies [24] have also found that the presence of CD8 (+) T cells on the edge of invasive tumors is associated with the expression of the PD-1/PD-L1 immunosuppressive axis, and may predict the response to treatment. Some scholars believe that CD8 + T cell density is the best predictor of PD-1 inhibition therapy in melanoma. The second best predictors were PD-1 + density of tumor and infiltration margin, and PD-L1 + density of tumor and

infiltration margin. The CD4 + density of tumor and infiltration margin were the worst predictors [25]. However, the direct correlation between PD-L1, PD-1, CD3 +, and CD8 + status and survival rate needs further study.

LIMITATIONS

This paper summarizes and analyzes the results of pathological immunohistochemistry in patients with previous surgery, so the sample size is small, and the detection of immunohistochemical indicators has certain limitations, and a more comprehensive comparative analysis cannot be undertaken. Postoperative adjuvant treatment was governed by the patients' consent, so there is no analysis of the combination of immunohistochemical findings and the efficacy of clinical treatment. We shall continue this line of study in subsequent more targeted research. This paper only describes and analyzes the existing data, hoping to provide ideas for the future in-depth study of head and neck mucosal melanoma.

In conclusion, the analysis of local tumor markers will contribute to a deeper understanding of the characteristics of mucosal melanoma. The correlation between CD8 expression and PD-L1 expression in tumor tissue also indicates the significance of effector T cell infiltration in the tumor immune microenvironment and its role in the prognosis of patients, but further research is required. We need to expand the sample size to verify our conclusions on the incidence rate of lower head and neck mucosa melanoma.

Conflicts of interest :There are no conflicts of interest to report

Data Availability Statement :The data that support the findings of this study are available on request from corresponding author.

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Figure legends

Table 1. Correlation between the expression of CD4 and CD8 and the general clinical features of head and neck mucosal melanoma.

Table 2. Correlation between PD-1/PD-L1 and CD4, CD8 expression.

Table 3. Correlation analysis of CD3 and general clinical features of head and neck mucosal melanoma.

Figure 1. Immunohistochemical staining of tumor samples (X100): A: score=1 (only perivascular immune infiltration); B: score=2 (perivascular + peritumoral and / or interstitial infiltration): C: score=3 (diffuse immune cell infiltration).

Figure 2. Expression of CD8 in different primary locations of head and neck mucosal melanoma.

Figure 3. Correlation between the expression of CD4 and CD8 and progression-free survival.

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