

Studies on the Expression of MMP-9 and Significance of a Macrophage Assay in Oral Squamous Cell Carcinoma

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OBJECTIVE To investigate the significance and relationship between matrix metalloproteinase 9 (MMP-9) and infiltration of macrophages in the process of invasion and metastasis in oral squamous cell carcinoma (OSCC).

METHODS The immunohistochemical SABC method was used to detect the expression of MMP-9 and CD68 (for labeling macrophages) in 42 cases of OSCC and in 10 normal tissues.

RESULTS The expression of MMP-9 and macrophage counts in the OSCC cases were significantly higher compared to normal tissues ($P < 0.05$). The expression of MMP-9 and macrophage counts were related to lymph-node metastasis and the TNM stage ($P < 0.05$), showing that there was a positive correlation among these parameters ($\gamma = 0.443$, $P < 0.01$).

CONCLUSION Both MMP-9 and macrophages may play an important role in the process of invasion and metastasis in OSCC, and this cellular activity may relate to the macrophages which affect the tumor cells and up-regulate the expression of MMP-9.

KEYWORDS: oral squamous cell carcinoma, matrix metalloproteinase-9, macrophage, immunohistochemistry.

INTRODUCTION

Invasion and metastasis of oral squamous cell carcinoma (OSCC) follows a complicated pathological course. The extracellular matrix (ECM) and degradation of the basal membrane (BM) are key links for invasion and metastasis of malignant tumors. The substrates of the matrix metalloproteinases (MMPs) make up the fibrous molecular network of the ECM and BM, among which the MMP-9 can specifically degrade type-IV collagen, the main constituent. This area of research has gained importance over the past few years. In addition, it has been confirmed by Feng et al.^[1] that macrophage infiltration is extensive in OSCC, and that macrophages play a very important role in growth and metastasis of the tumor. Macrophages are the major source of MMP-9. In OSCC cases, reports on the relationship of MMP-9 and macrophages, as well as related research, are rare. In our study, a concise analysis on the significance and correlation between MMP-9 and macrophages was conducted by detecting expression of MMP-9 and determination of macrophage infiltration in OSCC cases.

MATERIALS AND METHODS

Clinical materials

Forty two squamous cancer specimens from the oromaxillo-facial region were selected from surgical resections or biopsies in the Department of Oral and Maxillofacial Surgery, the Affiliated Hos-

pital of Guiyang Medical University, Guiyang, during a period from June 2004 to August 2006. There were 39 well-differentiated cancer cases, 2 moderately-differentiated cancer cases and 1 poorly-differentiated cancer case. Regional lymph node metastasis was found in 15 of these cases. The study was comprised of 24 male and 18 female cases with a mean age of 57 years. Based on the 2002 UICC TNM staging on oral carcinoma, 13 of these cases were at Stage-I and II, and 29 at Stage-III and IV. Normal oromaxillo-facial mucosas from another 10 patients (mean age of 32 years) who were nonsmokers without excessive alcohol intake, external injury or tooth extraction, were used as the controls. All specimens were verified by pathological diagnosis, without any preoperative treatment of the tumor.

Immunohistochemical staining

Routine pathological sections were prepared and microwave coctoantigen developed based on reagent specifications. Rabbit-antihuman MMP-9 monoclonal antibody (MCAB) (working concentration 1:100) was used to mark MMP-9, and mouse-antihuman CD68 MCAB (working concentration 1:100) employed to label macrophages in the tumor. The immunohistochemical SABC method was used to separately determine the expression of the MMP-9 and CD68. The rabbit-antihuman MMP-9 MCAB and compressed (mouse/rabbit) general-type SABC immunohistochemical kit was purchased from the Wuhan Boster Bioengineering Co., Ltd., and the mouse-antihuman CD68 MCAB was obtained from the Beijing Zhongshan Golden Bridge Biotechnology Co., Ltd. Available colon cancer specimens were used as a positive control, and PBS replaced the first antibody as a negative control.

Judgment of the results

The criterion for MMP-9 staining was based on a combination of two parameters, i.e. the immunohistochemical staining intensity and positive cell number^[2]. The staining-intensity scoring was conducted with an integral optical density value of positive staining: weak-staining score was 0, lower-intensity staining 1, medium-intensity staining 2 and high-intensity staining 3. At the same time, the scoring of the positive cell percentage was as follows: the positive cell number of less than 10% was 0 score, that between 10% to 30% 1; 30% to 50% 2; and over 50% 3. Summation of the two numbers equivalent to or more than 3 was deemed as an immunohistochemical positive staining.

Assessment of the macrophage number was conducted by the positive staining CD68 cell count under

a light microscope. At first, 3 regions with densest macrophage in the cancer, i.e., the hot spot region, were selected under a low power lens (10×10), then counting was conducted under a high-power lens (10×40), and a mean value was chosen for statistical analysis^[3].

Statistical analysis

The χ^2 test with the table of line×row was employed for statistics of enumeration data, and monofactorial analysis of variance for statistics of measurement data. The group t test was used for comparison between the data of the two groups. The Spearman-rank correlation analysis was employed to analyze the relationship among the indices. All statistical analyses were completed using SPSS14.0 software. A $P<0.05$ indicated statistical significance.

RESULTS

Expression and characteristics of the MMP-9 in OSCC

Table 1. shows that the expression of MMP-9 was significantly higher in OSCC than in the normal controls ($P<0.05$). In OSCC tissues, the main expression of MMP-9 was found in the cytoplasm of the tumor cells. MMP-9 expression could also be seen in a few mesenchymal cells or fibroblasts, most being at the verge of a cancer nest or in the parenchymal cells of the tumor margin, sometimes with a diffused distribution. The expression of MMP-9 was negative or weakly positive in normal oral mucosa, markedly less than in the OSCC tissues (Figs.1 and 2). In comparing the expression by the TNM staging, there was a significant difference in the expression of MMP-9 between the two groups ($P<0.05$). The expression of MMP-9 was significantly higher in the group with lymphatic metastasis compared to the group without metastasis ($P<0.05$). The MMP-9 expression was significantly higher in the male OSCC patients than in the females ($P<0.05$), however, no significant difference could be seen in the comparison between various age groups (Table 2).

Counting and character of the macrophages in OSCC

Table 3. shows that the macrophage count was significantly higher in OSCC samples compared to the normal mucosa ($P<0.01$). In the tumor tissue, the macrophages concentrated mainly in the zone of tumor necrosis and the matrix of tumor margin, but few were located inside the tumor tissue. Macrophage infiltration was rarely found in normal tissue (Fig.3

Table 1. The expression of MMP-9 in different oral tissues.

Tissue types	Cases	Expression of MMP-9		Positive rate %	χ^2	P
		Positive	Negative			
Normal mucosa	10	3	7	30.00	5.203	<0.05
OSCC	42	29	13	69.05		

Table 2. Correlation between the expression of MMP-9 and clinical indices in OSCC.

Item	Cases	Positive expression of MMP-9 (%)	χ^2	P
Sex			5.347	<0.05
Male	24	20 (83.33)		
Female	18	9 (50.00)		
Age			0.001	<0.05
<60 years	26	18 (69.23)		
≥60 years	16	11 (68.75)		
Lymphatic metastasis			6.439	<0.05
No	27	15 (55.56)		
Yes	15	14 (93.33)		
Clinical stages			4.617	<0.05
Stage I and II	13	6 (46.15)		
Stage III and IV	29	23 (79.31)		

Table 3. The mean macrophage count in different tissues.

Classification	Cases	Average macrophage count ($\bar{x}\pm s$)	t	P
Normal mucosa	10	7.10±1.197	28.536	<0.01
OSCC	42	42.05±7.548		
SqCa metastasis	15	48.67±8.415	4.872	<0.01
No SqCa metastasis	27	38.41±3.544		
Stage I and II SqCa	13	37.46±5.190	-2.857	<0.01
Stage III and IV SqCa	29	44.10±7.599		

Note: SqCa means squamous carcinoma

Table 4. The relationship between the expression of MMP-9 and macrophage count in OSCC.

Expression of MMP-9	Cases	Average macrophage count ($\bar{x}\pm s$)	t	P
Positive	29	43.86±8.184	3.325	<0.01
Negative	13	38.00±3.559		

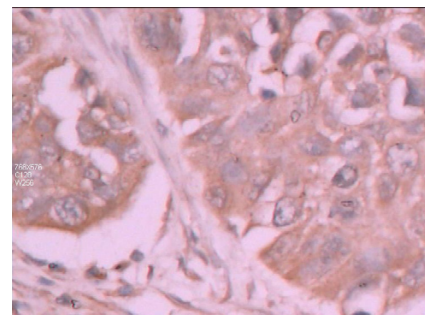


Fig.1. Expression of MMP-9 in OSCC (SABC×400).

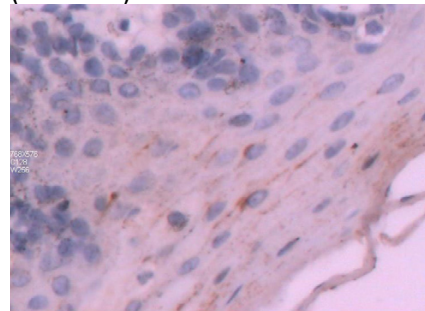


Fig.2. Expression of MMP-9 in normal oral tissue (SABC×400).

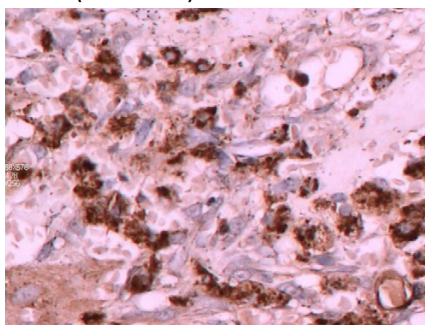


Fig.3. Expression of CD68 in OSCC (SABC×400).

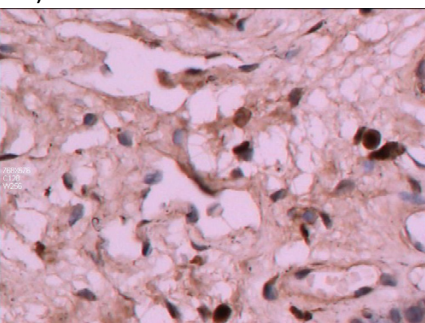


Fig.4. Expression of CD68 in normal oral tissue (SABC×400).

and 4). In OSCC tissues, the macrophage count was higher in the group with lymphatic metastasis compared to those tumors without lymphatic metastasis ($P<0.01$), and there also was a significant difference in comparison of the TNM staging ($P<0.01$).

Relationship between the expression of MMP-9 and macrophage count in OSCC

Comparison of the mean macrophage count between the cases with positive and negative MMP-9 expres-

sion showed that the macrophage count was significantly higher in the group with positive MMP-9 expression than in the group with negative MMP-9 expression ($P < 0.05$, Table 4). The correlation analysis on the expression of the MMP-9 protein and the macrophage count showed that the Spearman correlation coefficient was $\gamma = 0.443$ ($P < 0.01$), i.e., there was positive correlation between the expression of MMP-9 and macrophage count.

DISCUSSION

Our findings have shown that there was a very high expression of MMP-9 in OSCC, with the main expression in the cytoplasm of the tumor cells. The positive expression rate was much higher than in the normal controls, and it had a close correlation with TNM staging of the tumor and condition of lymphatic metastasis. Therefore, in OSCC, secretion by the tumor cells is the major source of MMP-9, and the tumor tissue may play a key role in invasion and metastasis of OSCC.

Under a normal physiological state, MMP-9 participates in the degradation and reconstruction of the basal membrane (BM) and ECM, and is regulated by multiple factors. However, MMP-9 is over-expressed in many malignant tumors^[4,5]. It was found in a study by O-charoenrat et al.^[4] on the frontline-area tumor cells of head and neck SqCa, that the expression of MMP-9 was significantly higher in OSCC than in the normal controls, and there was a positive correlation between the MMP-9 expression of the frontline tumor cells and the infiltrative tumor growth. The report of Ikebe et al.^[6] employing methods of immunohistochemical and quantitative enzymogram analysis, showed that there was a positive correlation between expression of the MMP-9 protein and the concentration of enzymogram analysis, and the content of collagenase in MMP-9 gradually stepped up with an increase of the tumor infiltration.

Results of our study and most research on MMP-9 of head and neck tumors have confirmed that MMP-9 expression does relate to tumor infiltration and lymphatic metastasis^[7,8]. So it can be presumed that in the invasion and metastasis of OSCC, with extensive presence of MMP-9 in the tumor, it may cause breakdown of the barrier shields of the tissues, i.e., the BM and ECM, resulting in a high metastatic rate of oral tumors and poor prognosis. At the same time, we also showed that there was a significant difference in comparison of MMP-9 expression between men and women, suggesting there might be a higher potential for OSCC invasion in male patients.

In addition, we have found that there were masses

of macrophage infiltration in OSCC, which is in agreement with our previous findings^[9]. A large quantity of macrophage infiltration was found in the matrix of the OSCC and the zone of necrosis, but the MMP-9 expression mainly focused in the tumor cells, indicating that macrophages are not the main source of the MMP-9 over-expression in the OSCC tissue. We found that both MMP-9 and macrophages have a relationship with the TNM tumor staging and condition of the lymphatic metastasis. Furthermore, there was a positive correlation between the macrophage count and MMP-9 expression in the tumor. These findings suggest that there may be a cooperation between macrophages and MMP-9 in OSCC, which jointly promote the invasion and metastasis of the tumor.

In vitro studies by other authors^[10] have shown that macrophages can partially or completely encase tumor cells, allowing the tumor cells to have a tissue infiltration capacity like the macrophages, and thus to produce the specific enzymes needed for passing through the BM, ECM and endothelial cells. Moreover, macrophages also provide the tumor cells with a barrier shield and monitoring for the macrophage-sourced growth factor, and for escaping immune cytotoxicity, resulting in reinforcement of the tumor's invasion and resistance. Researchers^[11] have found that, after a joint incubation of lung cancer cells with macrophages, both the potential of the tumor cell invasion and activity of matrix degradation were increased. It was discovered, through further analysis by these workers, that there were 50 cytokines in the tumor cells, including MMP-9, IL-8 and IL-6 etc., which were double the number of the cell factors before the incubation. It is thus clear that a large quantity of infiltrative macrophages in OSCC can play a role in tumor cell activity causing the tumor cells to secrete many kinds of cytokines, including MMP-9, thus providing the conditions for invasion and metastasis of the tumor.

The invasion and metastasis of tumors are regulated by quantities of factors in a cellular mixture of the OSCC. Owing to the specific relation between MMP-9 and macrophages, and their important role in the tumor tissue, it may be helpful to determine MMP-9 expression and macrophage levels for evaluating the potential invasion and metastasis of OSCC, and thus define a theoretical basis for treatment of these tumors.

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