Original

Comparison of MMP2 and MMP9 expression levels between primary and metastatic regions of oral squamous cell carcinoma

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(Received October 9, 2015; Accepted December 1, 2015)

Abstract: Matrix metalloproteinases (MMPs) and tumor-associated macrophages (TAMs) play important roles in tumor growth. The present study investigated the expression levels of MMP2 and MMP9 in relation to the distribution of TAMs in the primary and metastatic regions of oral squamous cell carcinoma. Twenty-nine cases of oral squamous cell carcinoma (OSCC) with regional lymph node metastasis were selected from available documents in the archives of the Department of Pathology, Nihon University School of Dentistry. Four-micrometer-thick sections were prepared from the primary and metastatic regions. Each section was subjected to immunohistochemical staining using anti-MMP2, anti-MMP9, and anti-CD68 antibodies. The distribution and localization of MMPs and TAMs were compared between primary and metastatic regions. The expression levels of both MMPs were higher in the metastatic regions of lingual and gingival cancers. Statistically significant differences were observed in both T1 and T2 cases. In contrast to the higher expression of MMPs in metastatic regions, a higher number of TAMs were distributed in the primary regions. From these results, MMP expression levels

and the numbers of TAMs were expected to have an inverse relationship between the primary and metastatic regions of OSCC. (J Oral Sci 58, 59-65, 2016)

Keywords: oral squamous cell carcinoma (OSCC); matrix metalloproteinases (MMPs); tumor-associated macrophages (TAMs).

Introduction

The complex interplay between tumor cells and their microenvironment plays a pivotal role in carcinogenesis and cancer progression (1). Matrix metalloproteinases (MMPs) are the most important participants of extracellular matrix remodeling (1,2). They belong to the zinc-dependent family of endopeptidases which are implicated in a variety of physiological processes (2,3). One of the most relevant functions of MMPs is the degradation of physical barriers such as the basement membrane (4-7). As the metastatic potential of various cancers depends on the ability of tumor cells to degrade the basement membrane, MMPs are the main targets of therapeutic agents (3). Among them, MMP2 and MMP9 have the ability to cleave type IV collagen, the main component of the basement membrane and to contribute to the metastasis of tumor cells (4-7).

Tumor-associated macrophages (TAMs) have various effects on tumor growth (5-8). Although the existence of TAMs in the metastatic region has been recognized, the role of these cells has only recently been appreciated.

Oral squamous cell carcinoma (OSCC) has the highest

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doi.org/10.2334/josnusd.58.59

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incidence rates among malignant tumors of the head and neck (9) and is one of the six most common cancers. Although the expression of MMPs and distribution of TAMs in OSCC have been intensely studied (8,10,11), a comparison of the expression levels and distribution patterns in the primary and metastatic regions has not been well defined. Research in these fields will help provide insight into the clinical behavior of OSCC. Thus, the aim of this study was to compare the expression levels of MMP2 and MMP9 in relation to TAM distribution in the primary and metastatic regions of OSCC.

Materials and Methods

Case selection

Twenty-nine OSCC cases with regional lymph node metastasis were chosen for this study. The surgical specimens were obtained from the Dental Hospital of Nihon University School of Dentistry. The histopathological diagnoses of these specimens were independently confirmed by two oral pathologists. Additionally, the differentiation degree (grades I-III) of the tumors was also assessed. The locations of the primary tumors were lingual ($n = 13$), gingival ($n = 14$), and buccal mucosa ($n = 2$). This study was conducted in accordance with the Declaration of Helsinki with respect to medical protocol and ethics, and the regional Ethical Review Board of Nihon University School of Dentistry approved this study (EP2015-11).

Antibodies

Anti-MMP2, anti-MMP9, and anti-CD68 antibodies (Abs) were purchased from Abcam (Cambridge, UK), Santa Cruz (CA, USA), and Dako (Tokyo, Japan), respectively. Anti-MMP2 and anti-CD68 Abs were diluted to 1/50 and anti-MMP9 Ab was diluted to 1/100 using 10 mM Tris-HCl (pH 7.5)-buffered saline supplemented with 1% bovine serum albumin (1% BSA-TBS).

Immunohistochemistry

All the samples were fixed in 10% formalin and embedded in paraffin. Four-micrometer-thick sections were prepared, de-paraffinized in xylene, and rehydrated with 100% ethanol. Endogenous peroxidase activity was inactivated with 0.3% hydrogen peroxide in methanol for 20 min at room temperature (RT). The sections were then boiled in 10 mM citrate buffer (pH 6.0) for 20 min and cooled. To block non-specific binding, the sections were incubated with 1% BSA-TBS for 1 h at RT. Thereafter, the blocking solution was removed, and the first antibodies were applied and incubated for 1 h at RT or overnight at 4°C (Table 1). Negative control studies were performed

with 1% BSA-TBS instead of the first antibody. The sections were then incubated with horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG or anti-rabbit IgG (diluted to 1/500) for 1 h at RT. After the sections were washed, they were developed with freshly prepared diaminobenzidine chromogen solution (Sigma, Tokyo, Japan) for 7 min, counterstained with hematoxylin for 10 s, dehydrated in a series of ethanol dilutions, cleared in xylene, and mounted on glass coverslips.

Immunohistochemical evaluation

The sections were evaluated in three different randomly selected fields, each of which contained tumor nests and stroma in approximately equal proportions. For the evaluation of MMP expression levels, the sections were scored by determining the highest score number. The staining intensities were classified into four different levels: 0, negative; 1, low; 2, moderate; and 3, high. For the CD68-positive TAMs, the sections were scored by determining the average number of stained cells observed under a light microscope using a micrometer (Olympus, Tokyo, Japan) at 200× magnification. The results have been expressed as mean ± standard deviation values.

Statistical analysis

Statistical analysis was performed using a two-tailed Student's *t*-test, and *P* values less than 0.05 were considered to be significant.

Results

Table 2 shows the clinical data of the OSCC cases examined in this study. The age of the patients ranged from 18 to 88 years, with a mean age of 62 years. The patients included 17 men and 12 women, at a ratio of 1.4:1. Clinicopathological data were determined according to the guidelines of the Union Internationale Contre le Cancer (UICC) (12). Immunohistochemical staining showed that both MMP2 and MMP9 were present in the cytoplasm of the tumor cells (Fig. 1). The expression levels of the MMPs were compared between the primary and metastatic regions. In lingual cancer, equivalent levels of MMP9 expression were detected in both regions (Fig. 1A, B), and there were no significant differences between the regions (Fig. 1C: $P = 0.482$). In contrast, 1.7-fold higher MMP9 expression was observed in the metastatic region of gingival cancer (Fig. 1D, E) than in the primary region, but this difference was not statistically significant (Fig. 1F: $P = 0.082$). In spite of the clear expression of MMP9, MMP2 expression was barely detectable in the primary regions of both lingual and gingival cancer (Fig. 1G, J). Slight MMP2 expres-

Table 1 Primary antibodies used in this study

Antibody	Host	Clone	Dilution	Antigen retrieval	Incubation	Supplier
MMP2	mouse	CA-400 /CA719E3C	50×	CB, 98°C; 20 min	4°C, 18 h	Abcam
MMP9	rabbit	C-20	100×	CB, 98°C; 20 min	RT, 1 h	Santa Cruz
CD68	mouse	PG-M1	50×	CB, 98°C; 20 min	RT, 1 h	Dako

MMP, matrix metalloproteinase; CB, citrate buffer; RT, room temperature

Table 2 Clinical classification of 29 oral squamous cell carcinoma cases

No.	age	sex	primary site	TNM classification		M	Class of UICC	Grade	Duration for metastasis (day)
				T	N				
1	51	M	lingual	2	2b	0	IVA	II	248
2	61	M	lingual	2	2b	0	IVA	I	103
3	67	M	lingual	1	1	0	III	I	412
4	46	M	lingual	2	2b	0	IVA	II	273
5	70	M	lingual	3	1	0	III	I	109
6	81	F	lingual	2	1	0	III	I	371
7	59	M	lingual	2	2a	0	IVA	I	167
8	57	M	lingual	2	2b	0	IVA	I	231
9	51	F	lingual	1	1	0	III	I	55
10	66	M	lingual	1	1	0	III	I	148
11	52	M	lingual	4	2c	0	IVA	III	41
12	64	F	lingual	3	2c	0	IVA	I	417
13	78	F	lingual	3	2a	0	IVA	I	27
14	60	F	gingival	2	2b	0	IVA	II	32
15	18	M	gingival	2	2b	0	IVA	II	166
16	88	M	gingival	2	1	0	III	I	210
17	65	F	gingival		2b	0		II	190
18	87	F	gingival	2	2b	0	IVA	II	28
19	69	M	gingival	2	1	0	III	I	35
20	52	M	gingival	3	1	0	III	I	48
21	67	F	gingival	2	2b	0	IVA	I	19
22	74	M	gingival		1	0		I	206
23		M	gingival		2b	0		I	28
24	57	F	gingival	2	2b	0	IVA	I	9
25	60	F	gingival		2b	0		I	146
26	55	M	gingival	4	2b	0	IVA	III	59
27	65	M	gingival	2	2b	0	IVA	II	27
28	66	F	buccal	2	1	0	III	II	37
29	58	F	buccal	1	1	0	III	I	20

UICC, Union Internationale Contre le Cancer

sion was observed in the metastatic regions (Fig. 1H, K), and a statistically significant difference was observed in lingual cancer (Fig. 1I: $P = 0.039$) but not in gingival cancer (Fig. 1L: $P = 0.111$).

The expression levels of the MMPs in the metastatic regions were further compared between lingual and gingival cancers (Fig. 2A, B). MMP9 expression was found to be almost the same in both types, whereas the expression level of MMP2 was slightly higher in the metastatic region of gingival cancer (1.58-fold). However, these differences were not statistically significant for MMP2 and MMP9 ($P = 0.588$ and 0.603 , respectively).

MMP expression levels were analyzed in relation to the clinical data (Table 2). Increased expression of MMP2 (P

$= 0.046$) and MMP9 ($P = 0.016$) was evident in grade I cases. However, no other clinical data correlated with the MMP expression level.

CD68 labeling index

The distribution patterns of CD68-positive TAMs were compared between the primary and metastatic regions. TAMs were detected in the stroma of the primary and metastatic regions and were localized not only in the invasive front but also in the non-invasive area (Fig. 3A). The total number of TAMs was counted in lingual and gingival cancers and compared, the results of which are shown in Fig. 3B and C. The highest number of TAMs was detected in the primary regions of both cancers (Fig.

Fig. 1 Specimens from the primary (A, D, G, J) and metastatic regions (B, E, H, K) of OSCC samples were stained with anti-MMP9 (upper panel) and anti-MMP2 (lower panel) Abs. The staining intensities were calculated and compared (C, F, I, L). * $P < 0.05$ MMP, matrix metalloproteinase; OSCC, oral squamous cell carcinoma

Fig. 2 The MMP9 (A) and MMP2 (B) staining intensities were compared between lingual and gingival cancer. MMP, matrix metalloproteinase

3B, C). When the number in the metastatic region was set as 1, the number of TAMs in the primary regions reached 1.9 ($P = 0.006$) and 1.5 ($P = 0.013$) for lingual and gingival cancers, respectively. The number of CD68-positive cells in the primary regions was analyzed in relation to the clinical data, and a slight correlation was observed between the number of TAMs and the time intervals to neck dissection (Fig. 4).

Discussion

More than 20 members of the MMP family have been characterized to date (1). Among them, MMP2 and MMP9 belong to the gelatinase family and contribute to the degradation of extracellular substrates such as collagen type IV, the main component of the basement membrane (4,13-15). Many studies have demonstrated the significance of both MMPs in the aggressive growth of OSCCs (1-3). However, there have been no reports

Fig. 3 The distribution of the TAMs in the primary and metastatic regions was compared (A). The number of TAMs was counted and compared between primary and metastatic regions (B and C). * $P < 0.05$

TAM, tumor-associated macrophages

comparing the expression levels between the primary and the metastatic regions of OSCC. In the present study, we attempted to evaluate the expression profiles of MMP2 and MMP9. Intriguingly, both MMPs were more highly expressed in the metastatic regions than in the primary regions of OSCC, and this pattern was quite prominent in gingival cancers. The increased expression of MMPs correlates with the proliferation and invasion of tumor cells (16-23). The enhancement of MMP activity in cultured cells indicates increased metastatic potential (24-26). It can be speculated that the cancer cells that metastasize to the lymph nodes still retain their metastatic potential and that this metastatic activity is much higher than that of the resident cells in the primary regions. If this is the case, then a question arises regarding factors that control the metastatic activity of cancer cells. One possibility may be the soluble factors secreted by stromal cells or other cell types (27). The susceptible sources for these factors may include TAMs. It has been known for many years that TAMs are the main cellular components of human cancers (5-8). Recent studies have described the development of two distinct subsets of macrophages in response to their tissue environment (7). Macrophages can reversibly change their functions and contribute to both cancer growth and regression. The number of TAMs was higher in the primary region of OSCC, and an inverse correlation was observed between them and MMP expression levels in the present study. The expression of the MMPs is regulated by several factors, including cytokines such as transforming growth factor- β (28,29), epidermal growth factor (30), and tumor necrosis factor- α (31,32). Transforming growth factor- β

Fig. 4 Relationship between the number of CD68-positive cells and time intervals to neck dissection.

induces the expression of MMP and also simultaneously reduces the expression of collagenase (28). Although the biological meanings of this inverse relationship and the underlying molecular mechanisms remain elusive, TAMs distributed in the primary regions of OSCCs may contribute to the reduction of MMP expression levels through some cytokines.

Most of the regulatory regions of MMP genes contain consensus binding sites for the transcription factor AP-1 (27). Analysis of this region in the MMP2 and MMP9 genes showed that the AP-1 site is only present in the latter gene. In spite of the different composition of the regulatory regions, the expression of both MMPs was low in the primary regions, and this may help explain the AP-1-independent regulatory mechanisms.

Although the MMP expression levels did not correlate with the clinical features of OSCC, a slight correlation was observed between the number of TAMs and the duration of surgery. The number of TAMs in the primary region was associated with the time to surgery. Accumulation of TAMs in the cancer stroma is correlated with angiogenesis in various cancers (33-37), including oral cancer. TAMs also increase the secretion of angiogenic factors such as vascular endothelial growth factor and contribute to the integrity of the vascular structure of OSCC (8,38). However, contradictory data has also been reported indicating a lack of correlation between TAM distribution and angiogenic activity (39). The biological functions of TAMs in OSCC require further investigation.

An inverse relationship was observed between MMP expression intensities and TAM distribution in the

primary and metastatic regions of OSCC. The results may indicate that the tumor cells in the metastatic region still maintain invasive potential, whereas the TAMs in the primary region may play other roles such as angiogenesis. Further analysis is necessary for a better understanding of OSCC behavior.

Acknowledgments

This work was supported by the following: Nihon University Joint Research Grant for 2010-2012 (to Dr. Okayama and Dr. Imamura); a grant from the Strategic Research Base Development Program for Private Universities from Ministry of Education, Culture, Sports, Science, and Technology, JAPAN (MEXT), 2010-2014 (S1001024) and 2011-2013 (23592778); Health and Labour Sciences Research Grants and Research on international cooperation in medical science; The Promotion and Mutual Aid Corporation for Private Schools of Japan (2011); a grant-in-aid for Scientific Research (C) (2011-2013).

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