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ORIGINAL ARTICLE

Immunological and Clinicopathological Significance of MFG-E8 Expression in Patients with Oral Squamous Cell Carcinoma

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Abstract

Milk fat globule-epidermal growth factor 8 (MFG-E8) is a glycoprotein secreted by the activated macrophages and acts as a bridge between apoptotic cells and phagocytes. Aside from macrophages, a variety of malignant cells also express MFG-E8. The objective of this study is to elucidate the clinical relevance and significance of MFG-E8 in the tumor microenvironment (TME) of patients with oral squamous cell carcinoma (OSCC). We investigated MFG-E8 expression in 74 patients with OSCC by immunohistochemistry and evaluated the relationship between MFG-E8 expression and various clinicopathological factors including immune cell infiltration. MFG-E8 expression was detected in 34 of 74 (45.9%) patients with OSCC and a significant correlation was observed with levels of infiltrating T cells, macrophages, and immunosuppressive M2 macrophages. Furthermore, MFG-E8 expression was also associated with clinical stage, lymphatic/vascular invasion, and Ki-67+ tumor cells but not with survival. Our results suggest that MFG-E8 may play an important role in shaping the immune suppressive network in TME as well as tumor progression.

Keywords Milk fat globule-epidermal growth factor 8 (MFG-E8) · Oral squamous cell carcinoma (OSCC) · Tumor microenvironment (TME) · Immune suppression

Introduction

The interaction between the tumor cells and stromal cells in the tumor microenvironment (TME) directly and indirectly affects the tumor growth, metastasis, and treatment resistance [1–3]. The role of immune cells in TME has attracted much attention following the emergence of immune checkpoint inhibitors for the treatment of cancers. Immune cells such as lymphocytes, macrophages, natural killer (NK) cells, and dendritic cells (DCs) form a complicated network known as the tumor immune microenvironment, wherein these cells exert tumor-suppressive or -promoting properties [1, 4, 5]. In cancer immunity cycle, the capture of tumor cells and tumor antigen presentation by macrophages and DCs are some of the important processes for the effective induction of antitumor immune responses [6]. In these processes, the uptake of apoptotic tumor cells by macrophages and DCs may evoke tolerance, while that of tumor cells undergoing immunogenic cell death may enhance the antitumor immune responses.

Milk fat globule-epidermal growth factor 8 (MFG-E8) is a glycoprotein secreted by the activated macrophages and acts as a bridge between apoptotic cells and phagocytes and promotes the engulfment of apoptotic cells by phagocytes [7, 8]. MFG-E8-mediated phagocytosis has been shown to facilitate tolerogenic immune responses by the induction of regulatory T cell-inducing cytokines [9]. Aside from macrophages, various types of malignant cells also express MFG-E8 [10–13]. In several carcinomas, MFG-E8 expression is not only upregulated but also correlates with tumor development, invasion,

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and metastasis and poor prognosis. Moreover, MFG-E8 has been known to promote angiogenesis through the activation of vascular endothelial growth factor (VEGF) signaling [13, 14]. Thus, MGF-E8 possesses multifunctional properties and may play pivotal roles in the immune status of TME.

In this study, we investigated MFG-E8 expression in oral squamous cell carcinoma (OSCC) and evaluated the relationship between MFG-E8 expression and clinicopathological factors including immune status in TME.

Materials and Methods

Patients

Seventy-four OSCC samples surgically resected at the Gunma University Hospital between November 2000 and January 2012 were analyzed. All samples were primary tongue cancer. Patients that received preoperative neoadjuvant chemotherapy or radiation were excluded. All surgical specimens were classified according to the World Health Organization (WHO) classification by a pathologist that was blinded to the clinical findings and were diagnosed as squamous cell carcinoma. Pathological tumor-node-metastasis (TNM) stages were established using the International System for Staging adopted by the American Joint Committee on Cancer and the Union Internationale Centre le Cancer (UICC). Clinicopathological variables, including age, sex, primary tumor (pT), nodal metastasis (pN), TNM stage, histological grade, lymphatic/vascular invasion, and Ki-67 staining as well as progression-free survival (PFS) and overall survival (OS), were evaluated. This study was approved by the Institutional Review Board of Gunma University (No. 12–12) and performed in line with the Declaration of Helsinki of 1996.

Immunohistochemical (IHC) Staining

The list of antibodies used in this study are shown in Supplementary Table 1. Surgical specimens were fixed in 10% formaldehyde and routinely processed for paraffin embedding. Serial histological sections (5-μm thick) were deparaffinized in xylene and hydrated in descending dilutions of ethanol. Antigen retrieval was achieved by autoclaving the samples at 121 °C for 20 min in citrate buffer (pH 6.0), followed by incubation at room temperature for 30 min, or by the application of Proteinase K (DAKO, Glostrup, Denmark) at room temperature for 5 min for MFG-E8 and CD68 staining. Endogenous peroxidase was blocked with peroxidase blocker (DAKO, Glostrup, Denmark) at room temperature for 5 min, and sections were covered with 1% bovine serum albumin (Sigma-Aldrich, St. Louis, MO, USA)/5% normal horse serum (Immuno-Biological Laboratories, Fujioka, Japan) at room temperature for 10 min. Slides were overnight incubated at 4 °C with primary antibodies shown in Table 1 or at room temperature for 4 h with monoclonal mouse anti-human MFG-E8 antibody. Labeled polymer-horseradish peroxidase (HRP) anti-mouse/rabbit (DAKO) was applied to the slides at room temperature for 45 min. The reaction products were detected with 3,3′-diaminobenzidine (DAB, DAKO) and counterstained with Mayer's hematoxylin (Wako Pure Chemical Industries, Ltd., Osaka, Japan). After being dehydrated by ascending dilutions of ethanol, the slides were immersed in xylene and mounted with DPX (Merck, Darmstadt, Germany).

Evaluation of IHC Samples

Slides were evaluated by two independent investigators (K.S. and H.T.) in a blinded manner using a Zeiss Axioscope light microscope (Carl Zeiss Microscopy GmbH, Jena, Germany). Staining for MFG-E8 was assessed on the basis of the proportion of the stained cells and immunostaining intensity. The proportion of cells was graded as 0 (negative), 1 (< 1% positive), 2 (1–5% positive), and 3 (> 5% positive), and staining intensity was graded as 0 (negative), 1 (weaker staining than vascular endothelial cells), and 2 (stronger than them), as per a previous report [11] with some modifications. The scores for the proportion of stained cells and staining intensity were multiplied to provide total score. Microvessel density

Table 1 Clinicopathological features of patients

Parameters	*n* = 74 (%)
Age	33–92, median: 69
Sex	
Male	47 (63.5)
Female	27 (36.5)
TNM	
T1/T2	63 (85.1)
T3/T4	11 (14.9)
N0	53 (71.6)
N+	21 (28.4)
M0	73 (98.6)
M1	1 (1.4)
Stage	
I/II	47 (63.5)
III/IV	27 (36.5)
Differentiation	
Well/moderate	66 (89.2)
Poorly	8 (10.8)
Invasion	
Lymphatic	35 (47.3)
Vascular	25 (33.8)
Ki-67	5–72, median: 22
MVD	2–29, median: 15

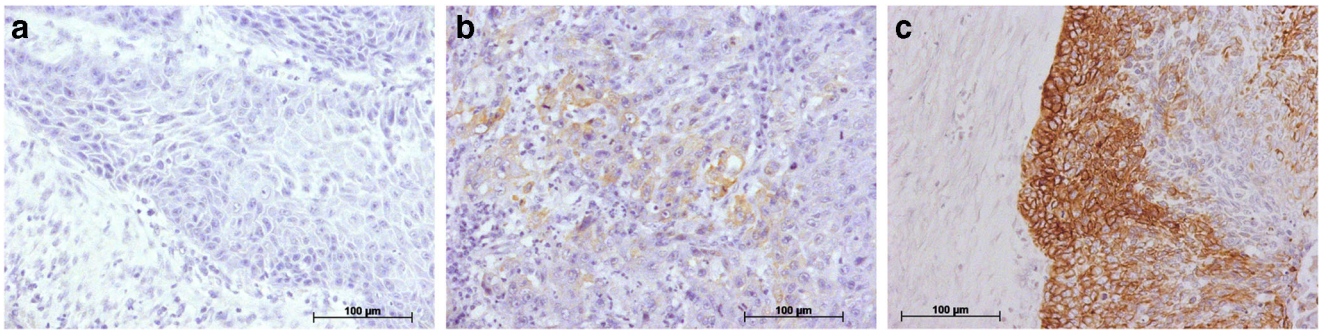

Fig. 1 Demonstrative immunohistochemical images of MFG-E8. MFG-E8 was stained in the membrane and cytoplasm of tumor cells (×200 magnification). MFG-E8 staining intensity was graded as 0 (negative) (**a**), 1 (weak) (**b**), and 2 (strong) (**c**). Total score was calculated by multiplying the proportion of stained cells and staining intensity as described in materials and methods

(MVD) was defined as the mean CD34+ vessel count in four selected high power fields (HPF; ×400, 0.26 mm^2 filed area).

For CD1a + DCs and CD56+ NK cells, more than five areas of a representative field were counted at ×200 magnification. For CD3+ T cells, infiltration in more than five ×400 HPF was graded as grade 1 (< 10 positive cells/HPF), grade 2 (10–30/HPF), grade 3 (31–100/HPF), or grade 4 (> 101/HPF), as previously reported [15]. More than four areas of a representative field adjacent to cancer cells were counted at ×400 magnification for CD68+ and CD163+ macrophages and the average was calculated. Final scores of the immune infiltrates were defined as the average of the number of stained cells in each field and CD3 positivity was defined as grade 2 and over.

The highly cellular area of the stained sections was evaluated for Ki-67. Approximately 1000 nuclei were counted on each slide and the proliferative activity was assessed as the percentage of Ki-67-stained nuclei (Ki-67 labeling index) in the sample [16].

Statistical Analysis

Data were analyzed using Statcel 3 (OMS Publishing, Tokorozawa, Japan). Mann-Whitney's U test was used to examine the statistical significance of MFG-E8 score in categorical variables. Spearman's rank correlation test was used to examine the correlation between MFG-E8 score and continuous variables. Significance was defined at $p < 0.05$. Kaplan-Meier survival curves and log-rank statistics were used to evaluate PFS and OS.

Results

Patient Characteristics

The clinicopathological characteristics of all 74 patients are summarized in Table 1. Postoperative adjuvant chemotherapy with the oral administration of S-1 (Taiho Pharmaceutical, Tokyo, Japan), tegafur, or docetaxel was provided to 9, 10, and 3 patients, respectively. The median follow-up duration was 915 days (range, 85–3452 days).

Expression of MFG-E8 and Correlation with Immune Cell Infiltration in OSCC

Figure 1 shows the membranous staining image of MFG-E8 protein in OSCC tissues. MFG-E8 was stained in the membrane and cytoplasm of tumor cells. As shown in Table 2, 34 of 74 (45.9%) patients with OSCC were positive for MFG-E8 protein expression. Moreover, weak and strong MFG-E8 staining were observed in 16 (21.6%) and 18 (24.3%), respectively (Table 3). MFG-E8 score was calculated by multiplying the proportion of stained cells and staining intensity (Table 4). Demonstrative IHC staining of immune cell infiltrates and CD34 endothelial cells is shown in Fig. 2. The correlation between MFG-E8 expression in tumor tissues and immune cell infiltration is presented in Table 5. A significant positive correlation was observed between the expression of MFG-E8 in OSCC and CD3+ T cells, CD68+ pan-macrophages, and CD163+ immunomodulatory/immunosuppressive M2 macrophage subsets but not CD1a + DCs and CD56+ NK cells.

Table 2 Proportion of MFG-E8 positive cells in 74 OSCC cases

Score	% in cancer cells	n	(%)
0	0%	40	(54.1)
1	0.1% - 1%	16	(21.6)
2	1% - 5%	13	(17.6)
3	5% -	5	(6.7)

Table 3 Intensity of MFG-E8 positive cells in 74 OSCC cases

Score	Intensity	n	(%)
0	No staining	40	(54.1)
1	Weak	16	(21.6)
2	Strong	18	(24.3)

Table 4 MFG-E8 expression score in 74 OSCC cases

Total score	n	(%)
0	40	(54.1)
1	11	(14.9)
2	10	(13.5)
4	8	(10.8)
6	5	(6.7)

Table 5 Association between MFG-E8 and immunological parameters

	the number of positive cells (median)	rs	*p* value
MFG-E8 vs			
CD1a	0–127.2 (20.5)	−0.00387	0.974
CD3	1–4 (3)[#]	**0.261**	**0.0260**
CD56	0–104.8 (6.6)	0.0611	0.602
CD68	1–542 (203.5)	**0.250**	**0.0350**
CD163	0–195 (64)	**0.261**	**0.0260**

[#], CD3+ cells was evaluated by a grading system as described in Materials and Methods; Bold entries show statistically significance ($p<0.05$)

Correlation between MFG-E8 Expression and Clinicopathological Parameters in OSCC

The expression of MFG-E8 was significantly and positively correlated with T-factor, nodal status, clinical stage, lymphatic invasion, vascular invasion, and Ki-67 labeled index representative of the proliferative potential of cancer cells. On the other hand, MFG-E8 expression in OSCC showed no correlation with age, sex, differentiation, and MVD defined by CD34+ cells (Table 6). Furthermore, survival analyses by Kaplan-Meier method and log-rank test revealed that MFG-E8 positive patients showed a tendency towards worse PFS ($p = 0.07$, Fig. 3a), but there was no significant difference in OS between patients with MFG-E8 positive and negative ($p = 0.32$, Fig. 3b).

Discussion

MFG-E8 produced from activated macrophages and immature DCs is known to express in various malignant tumors cells, including melanoma, colorectal, prostate, and oral cancer cells, and has been demonstrated to play important roles in carcinogenesis, tumor growth, and angiogenesis in TME [10–12, 17]. Using immunohistochemistry, Yamazaki et al. detected MFG-E8 expression in 83% of OSCC [11]. On the contrary, we detected MFG-E8 expression in only 45.9% OSCC samples and the difference in the observation may be attributed to the differences in IHC methods. However, it is important to note that our results showed positive correlation between MFG-E8 expression and unfavorable parameters such as T-factor, nodal status, clinical stage, lymphatic/vascular invasion, and Ki-67 proliferation index, consistent with the results of the previous report [11]. Thus, MFG-E8 expression in OSCC contributes to the acquisition and maintenance of malignant traits. MFG-E8 is also known to promote angiogenesis through VEGF signaling pathway and recognized as an essential agent for wound healing and neovascularization in ischemic disease [14, 18, 19]. Several reports have shown that MFG-E8 expression in melanoma cells is associated with tumor angiogenesis in both animal model and clinical samples [13, 20]. However, in our study, the expression of MFG-E8 in tumor cells showed no correlation with angiogenesis, as evaluated with MVD. It is likely that some other angiogenesis-related factors such as VEGF, fibroblast growth factor (FGF), or transforming growth factor

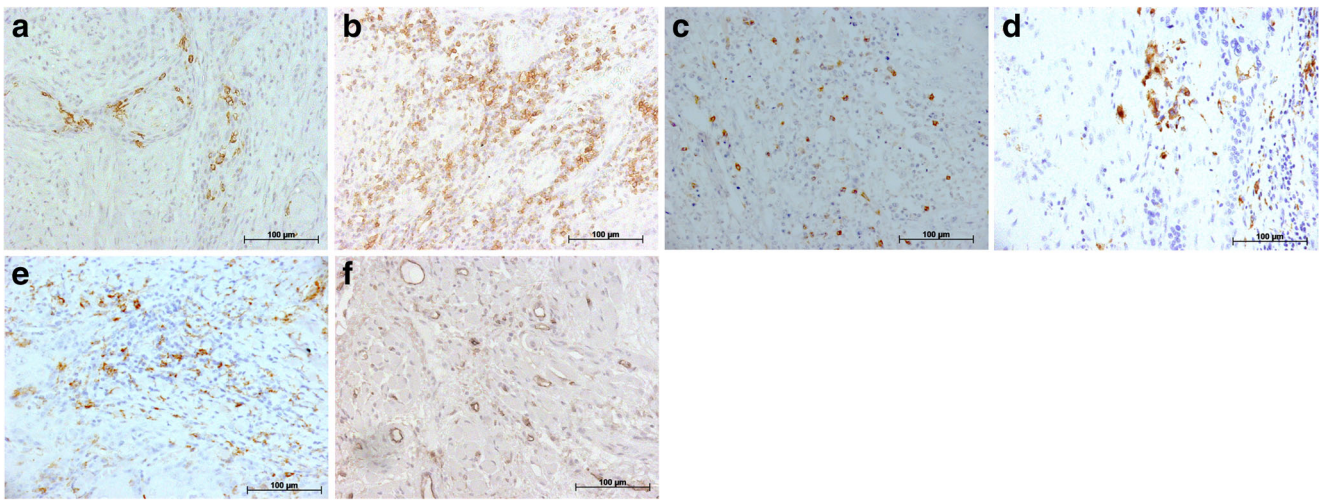

Fig. 2 Demonstrative immunohistochemical images of CD1a (**a**), CD3 (**b**), CD56 (**c**), CD68 (**d**), CD163 (**e**), and CD34 (**f**) (×200 magnification)

Table 6 Association between MFG-E8 and clinicopathological parameters

	rs	*p* value
MFG-E8 vs		
Age	0.0612	0.601
Sex	N/A	0.452
T-factor	**0.437**	**0.00019**
Nodal status	**0.370**	**0.00157**
Stage	**0.328**	**0.00502**
Differentiation	−0.201	0.0866
Lymphatic invasion	N/A	**0.0392**
Vascular invasion	N/A	**0.0321**
Ki-67	**0.350**	**0.00302**
MVD	0.131	0.268

Bold entries show statistically significance ($p<0.05$)

(TGF)-alpha/beta may be involved in tumor angiogenesis in OSCC. Alternatively, MFG-E8 expression may be increased around tumor blood vessels in TME [13]. For the comprehensive evaluation of MFG-E8 expression in TME, in addition to staining intensity and proportion of positively stained cells, localization of MFG-E8 expression should be considered.

The most crucial role of the multifunctional protein MFG-E8 is to suppress immune responses by modulating apoptotic tumor cell uptake and processing [9]. MFG-E8 expression in tumor cells correlated with T cell (CD3+ cell), macrophage (CD68+ cell), and M2 macrophage (CD163+ cell) infiltration. Macrophages phagocytosed apoptotic cells, a process mediated by MFG-E8, and produce immunosuppressive cytokines such as granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin (IL)-10, and TGF-beta, resulting in the induction and expansion of Treg cells and inhibition of effector T cells [21]. Although we failed to analyze T cell subset or T cell function in this study, the subpopulation of Treg cells rather than effector T cells may be predominant in the infiltrated CD3+ T cells in the TME of OSCC. Furthermore, MFG-E8 expression correlated with macrophage infiltration, especially M2 macrophage infiltration. M2 macrophages are known as the key players in the establishment of the immune suppressive network in TME [22, 23]. Soki et al. have demonstrated that the co-culture of macrophages with apoptotic prostate cancer cells may result in the increase in MFG-E8 expression and induction of M2 polarization of macrophages [17]. In addition, Yamada et al. showed that the conditioned medium from the wild-type mesenchymal stromal cells that contained substantial amounts of MFG-E8 was more potent in inducing M2 macrophage polarization than the conditioned medium obtained from MFG-E8-deficient mesenchymal stromal cells [13]. Thus, our findings from tumor tissue analysis may reflect the observations of the in vitro studies. CD1a is expressed mainly in the immature DC, a type of phagocyte that has higher potential of engulfing apoptotic cells. However, MFG-E8 expression in OSCC showed no correlation with CD1a + DCs, suggesting that MFG-E8 facilitates the engulfment of apoptotic cells by immature DCs but may not recruit or induce immature DCs.

The expression of MFG-E8 in OSCC had no correlation with poor prognosis. So far, in melanoma and colorectal cancer, MFG-E8 expression has been shown to correlate with unfavorable prognosis [10, 12]. In OSCC, Yamazaki et al. have shown that MFG-E8 expression profiles correlated with clinicopathological properties, but these authors fail to indicate the prognostic significance [11]. Thus, whether MFG-E8 expression in OSCC is related to poorer prognosis is still unclear. As MFG-E8 is a multifunctional protein, the involvement of each function may be different depending on the type of cancers, tumor progression, and immune status.

Taken together, MFG-E8 expression in OSCC correlated with clinicopathological parameters such as T-factor, nodal status, clinical stage, lymphatic invasion, vascular invasion, and tumor cell proliferative potential as well as immune status in TME, indicating that MFG-E8 may play an important role in tumor-induced immune suppression and tumor progression. At present, several cancer immunotherapy-based combination studies have been ongoing and targeting MFG-E8 may serve as a strategy to overcome immune suppression in TME.

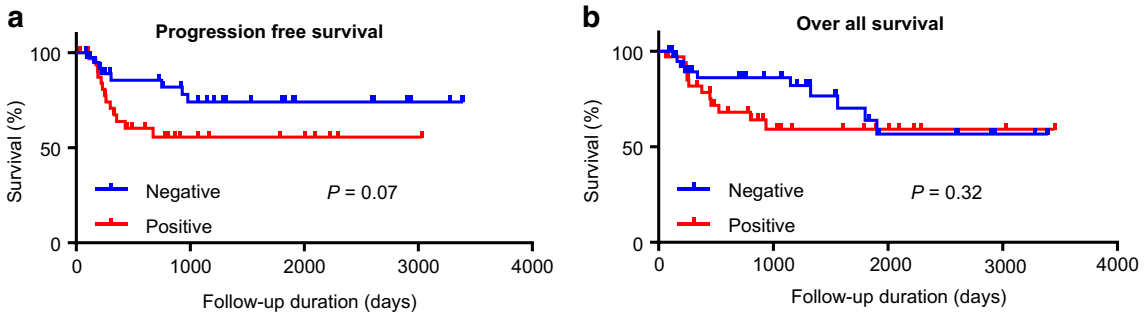

Fig. 3 Kaplan-Meier curve and Log-Rank test for PFS (**a**) and OS (**b**) for the comparison between patients with MFG-E8 positive and negative

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Compliance with Ethical Standards

Disclosure/Duality of Interest None of the authors have any financial or personal relationship with other people or organizations that inappropriately influenced this study.

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