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Running title: Loss of maspin is a negative factor

Key words: oral squamous cell; carcinoma; maspin; serine protease inhibitor; tumor suppressor

KUNIO YOSHIZAWA*, SHINICHI NOZAKI[b], AYAKO OKAMUNE, HIROKO KITAHARA, TERUHISA OHARA, KOROKU KATO, SHUICHI KAWASHIRI, ETSUHIDE YAMAMOTO

Department of Oral & Maxillofacial Surgery, Kanazawa University Graduate School of Medical Science, 13-1 Takaramachi, Kanazawa, Ishikawa 920-8641, Japan

[b]Oral & Maxillofacial Surgery, National Hospital Organization Kanazawa Medical Center, Kanazawa, Ishikawa 920-8650, Japan

*Correspondence to:

Department of Oral & Maxillofacial Surgery, Kanazawa University Graduate School of Medical Science, 13-1 Takaramachi, Kanazawa, Ishikawa 920-8641, Japan

Tel.: +81-76-265-2444; Fax: +81-76-234-4268

E-mail: yoshizawa@oral.m.kanazawa-u.ac.jp (K. Yoshizawa)

Abstract:

BACKGROUND: Maspin, a 42-kDa protein, belongs to the serpin family of protease inhibitors and is known to have tumor-suppressor function. In the current study, we investigated the interrelationship between clinicopathological findings and maspin expression in oral squamous cell carcinoma (OSCC).

METHODS: Using immunohistochemical techniques to examine the expression levels of maspin in OSCC, maspin expression in OSCC was detected in 46 (64.8%) of 71 cases. We also compared the clinicopathological features of OSCC cases with maspin expression levels. Moreover, we examined expression of maspin in eight cell lines derived from OSCC using Reverse-Transcription PCR (RT-PCR) and western blotting.

RESULTS: There was a significant correlation between decreased maspin expression and T-category ($p < 0.01$), lymph metastasis ($p < 0.0001$), and mode of invasion ($p < 0.0001$). Patients with positive maspin expression had a significantly better prognosis ($p < 0.001$). Lower expression of maspin was also seen in cell lines derived from grade 4D, which shows stronger invasive potential than other grades of OSCC.

CONCLUSION: Maspin may be a useful marker to identify the potential for progression in OSCC.

Introduction

Maspin, which was originally identified in normal human breast epithelial and myoepithelial cells, is a 42-kDa cytoplasmic protein that belongs to the serpin family of protease inhibitors (1, 2). The gene has subsequently been localized to epithelial cells in a variety of tissues, including oral squamous epithelium, and its protein specifically functions as a protease inhibitor by blocking tissue plasminogen activator (PA) in vitro (3-5). It has been proposed that tumor cells produce PA in order to facilitate cellular invasion and migration (6, 7). These reports suggest that maspin, which inhibits tissue PA, is a tumor-suppressive factor. A correlation between the absence of maspin expression and poor prognosis has been reported for several tissues, including colon, uterus, breast, prostate, skin and thyroid (2-6, 8, 9).

The TNM classification is a good system for describing the condition of cancer patients. However this system cannot predict the prognosis of individual patients and cannot describe the biological characteristics of tumor cells. It is therefore important to look for new objective prognosis factors that add information about the biological characteristics of tumors. It is believed that invasion and metastasis are the most crucial characteristics of malignant tumors. Thus, mode of invasion is used as a histopathological classification category in oral SSC (OSCC), as described by

Yamamoto et al., and this classification is frequently used to predict progression, metastasis and prognosis (10-15). To provide proper treatment, it is also important to examine the characteristics of cancer cells at the invasive front of OSCC. We thus immunohistochemically examined the expression of maspin in vivo and compared maspin expression in cell lines derived from invasive OSCC in vitro with regard to mode of invasion of OSCC.

Materials and methods

Patients

Seventy-one biopsy specimens of primary OSCC were obtained from patients undergoing surgical resection at the Department of Oral and Maxillofacial Surgery of Kanazawa University Hospital between 1989 and 2006. The patients (38 males, 33 females) ranged in age from 27 to 92 years (mean age, 64 years).

Staining methods

Biopsy specimens were fixed in periodate-lysine-paraformaldehyde solution or 10% formalin solution, and were then embedded in paraffin to prepare serial sections (4 μm). Hematoxylin and eosin (H-E) staining was used for histological examination.

Immunohistochemical staining was performed by the labeled streptavidin-biotin (LSAB) method after deparaffinization and rehydration. Endogenous peroxidase was blocked using 0.3% hydrogen peroxide for 30 min. Sections were washed with PBS. Excess blocking solution was drained and the sections were incubated with primary antibody overnight at 4°C. Sections were reacted with primary antibody; anti-maspin monoclonal antibody (LAB VISION, Fremont CA, USA) 300-fold diluted with PBS at 4°C for 24 h (9). Sections were then reacted with secondary antibody; biotin-labeled goat anti-mouse immunoglobulin polyclonal antibody (Dako Japan, Kyoto, Japan) at RT for 60 min. After reacting with peroxidase-conjugated streptavidin (Dako) for 60 min, sections were washed with PBS. Immunohistochemical reactions were developed in 0.01% 3,3'-diaminobenzidine tetrahydrochloride. For nuclear staining, hematoxylin was used. A section of normal oral epithelium previously identified as having strong staining was used as a positive control with each batch. As a negative control, PBS treatment was used instead of maspin antibody.

Cell culture and cell lines

All cell lines were maintained at 37°C in a humidified incubator containing 5% CO_2. OSCC cell lines HSC-2, HSC-4, OSC-20, OBC-01, OSC-19, OTC-04, HOC313 and

TSU were maintained in minimal essential medium (MEM; Sigma-Aldrich, Ayrshire, UK) supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin. Cell lines were derived from OSCC with the following grades of invasiveness, according to Yamamoto-Kohama criteria (11) (Table 1): HSC-2, HSC-4 and OSC-20 cells from grade 3, described as weakly invasive; OBC-01, OSC-19 and OTC-04 from grade 4C, described as mildly invasive; HOC313 and TSU from 4D, described as highly invasive.

RNA extraction and reverse transcriptase-polymerase chain reaction (RT-PCR)

RNA was extracted from cultured cells using an RNeasy kit (Qiagen, Hilden, Germany). A 1-μg sample in 10 μl RNase-free water was incubated for 5 min at 60°C and was then quickly chilled on ice for 5 min. RNA samples were reversed-transcribed into first-strand cDNA at 40°C for 40 min in RT solution from the RNeasy kit. The cDNA samples were amplified following addition of the PCR mixture solution from the RNeasy kit and the following primers (16-18): for maspin, 5'-CAG GCA CAA CAA AAC TCG AA-3' (forward) and 5'-AAT CGG CAT CCA CAG AAA AG-3' (reverse); for E-cadherin, 5'-AGC CAT GGG CCC TTG GAG-3' (forward) and 5'-CCA GAG GCT CTG TCA CCT TC-3'; and for β-actin, 5'-GAA AAT CTG GCA CCA CAC CTT-3' (forward) and 5'-TTG AAG GTA GTT TCG TGG AT-3' (reverse). PCR was

carried out under the following conditions: 3 min at 94°C, followed by cycles (32 for maspin, 30 for E-cadherin and 20 for β-actin) of 1 min at 94°C, 1 min at 58°C, and 1 min at 72°C. All reactions were completed with a final incubation at 72°C for 10 min. The lengths of the amplified fragments for maspin, E-cadherin and β-actin were 104, 653 and 592 bp, respectively. PCR products were detected by 3.0% agarose gel electrophoresis and staining with ethidium bromide.

Western blot analysis

Parent- and resistant-cells on 80% confluent plates were used as protein samples. Samples (25 μg) that were extracted from the whole cellular structure using M-PER Mammalian protein extraction reagent (Pierce, Rockford, IL, USA) were heated at 95°C for 5 min before 10% SDS-PAGE. After electrophoresis, samples ware transferred onto PVDF membranes (ATTO Co., Tokyo, Japan) and incubated for 1 h with 500-fold diluted polyclonal anti-rabbit antibody against maspin (BD Biosciences, San Jose, CA, USA) and 5000-fold diluted polyclonal anti-mouse antibody β-actin (Sigma, St. Louis, MO, USA), respectively. The membrane was washed three times with PBS and was then incubated for 1 h with 2000-fold diluted horseradish peroxidase-conjugated anti-mouse IgG (Amersham, Buckinghamshire, UK) to detect maspin and β-actin,

respectively. Blots were revealed by enhanced chemiluminescent detection carried out according to the manufacturer's instructions.

Assessment of immunohistochemical staining of maspin proteins

Expression of maspin in tumor cells was evaluated as present or absent. Only cases in which at least 50% of the tumor cells were immunoreactive were scored as positive. Evaluation was performed by an observer completely blinded to patient characteristics. Mann-Whitney's U test was used to analyze the association between maspin expression and clinico-pathological factors, such as tumor site, differential type, T-category, lymph node metastasis and mode of invasion. Survival rates of maspin-positive and -negative patients were calculated by the Kaplan-Mayer method, and were examined for statistical significance using the log-rank test. Differences were considered significant at p values of <0.05.

Results

Immunohistochemistry and evaluation

The relationship between the clinico-pathological parameters and expression of maspin are summarized in Table 2. Maspin immunostaining was observed in cytoplasm,

membrane and nuclear of tumor cells (Fig. 1). Immunohistochemical staining showed that 46 of the 71 specimens (64.8%) examined were positive for maspin. There was a significant negative correlation between maspin and mode of invasion ($p < 0.0001$); the number of maspin-positive cases was 1 (100%) for grade 1, 11 (100%) for grade 2, 16 (80%) for grade 3, 8 (66.7%) for grade 4C, and 1 (10%) for grade 4D.

Moreover, the number of maspin-positive cases was 18 (43.9%) with lymph node metastasis and 28 (93.3%) without lymph node metastasis. Therefore, maspin expression showed a negative correlation with lymph node metastasis ($p < 0.0001$).

As shown in Figure 2, patients with high maspin expression had better survival rates than those with low levels of maspin expression ($p < 0.001$).

Analysis of maspin mRNA and E-cadherin levels in OSCC cell lines by RT-PCR

We further examined the levels of maspin and E-cadherin in these eight cell lines by RT-PCR. Expression of maspin was significantly lower in HOC313 and TSU cell lines (grade 4D) than in other cell lines (Fig. 3).

Analysis of maspin protein levels in OSCC cell lines by western blotting

Levels of maspin protein expression were examined by western blotting (Fig. 4).

Expression of maspin protein was significantly lower in HOC313 and TSU cell lines (grade 4D) than in other cell lines.

Discussion

OSCC is characterized by a high degree of invasion into local tissues, as well as a high incidence of lymph node metastasis. However, there have been few reports regarding the possible association between expression of maspin and invasive potential in OSCC. In this study, maspin expression also showed a negative correlation with T-category, lymph node metastasis and mode of invasion. Xie et al. (19) and Yasumatsu et al. (20) reported that decreased maspin expression in OSCC is associated with an unfavorable clinical outcome due to invasion and lymph metastasis, which is supported by the results of this study. Moreover, Xu et al. (3) reported that maspin plays a role in disease progression from in situ to invasive carcinoma of the uterine cervix. On the other hand, Cho et al. (21) reported that maspin expression in early oral tongue cancer was not correlated with survival. Thus, we believe that expression of maspin weakens as the tumor enlarges and becomes more invasive.

Ets-1 proteins constitute a family of transcription factors implicated in the regulation of several matrix-degrading proteinases, including maspin. Ets-1 has been reported to activate the expression of MMP-1,-3 and -9, as well as urokinase-type plasminogen activator (uPA) and maspin, via the Ets-1-binding sites in the promoters of these genes (22-24). Moreover, Ets-1 functions as an effector for EMT, and it enhances the

malignancy of SCC cells through the regulation of proteolytic enzymes, including maspin in the course of EMT (23-25). In this study, cases and cell lines were derived from grade 4D, which shows EMT characteristics such as spindly shape and decreased expression of E-cadherin, and they expressed lower levels maspin than other invasion grades. Thus, EMT may be related to proteolytic enzymes, including maspin, and is correlated with invasion by regulating expression of Ets-1.

Moreover, the adhesion protein E-cadherin plays an important role in homophilic cell-cell adhesion and is known as an invasion suppressor gene. It has been reported that expression of E-cadherin is down-regulated during the acquisition of metastatic potential during the late stages of epithelial tumor progression (26-28). In our study, maspin and E-cadherin were expressed at significantly lower levels in two cell lines established from grade 4D OSCC, compared with cell lines derived from other invasive grades.

In conclusion, maspin may be a useful marker to identify the potential for progression of OSCC, as lower immunoreactivity is associated with larger tumor and greater invasive potential, particularly for grade 4D. It is necessary to clarify the mechanisms between maspin expression and progression of OSCC to apply to clinical applications.

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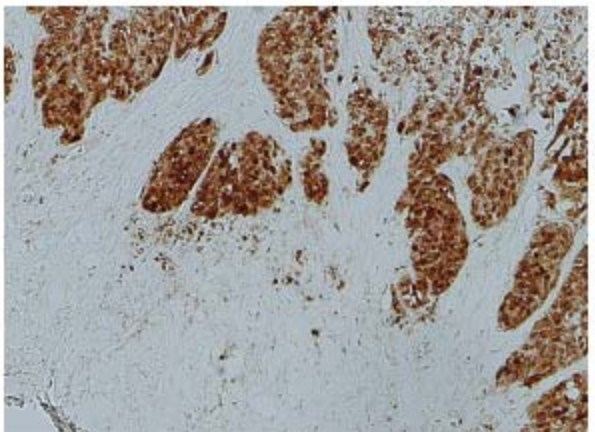
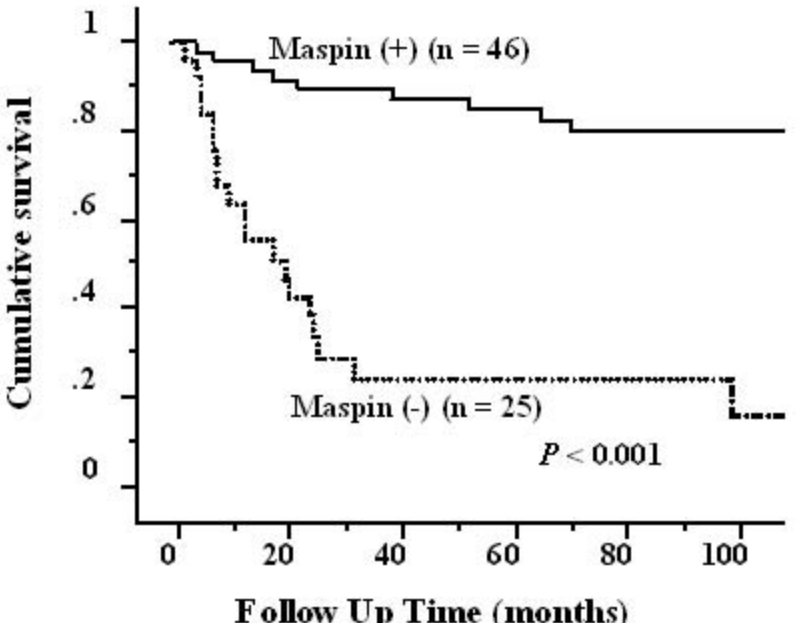
Table 1. Histological grading of mode of cancer invasion.

Grade[a]
1. Well-defined borderline
2. Cords, less marked borderline
3. Groups of cells, no distinct borderline
4. Diffuse invasion
4C : Cord-like type
4D : Widespread type

[a]Yamamoto-Kohama classification

Parameter	Positive no. (%)	Negative no.(%)	Total
Tumor site			
Tongue	23 (67.6)	11 (32.3)	34
Gingiva	9 (45.0)	11 (55.0)	20
Oral floor	7 (100)	0 (0)	7
Buccal	6 (75.0)	2 (25.0)	8
Others	1 (50.0)	1 (50.0)	2
Differential type			
Well	31 (70.5)	13 (29.5)	44
Moderately	10 (62.5)	6 (37.5)	16
Poorly	5 (45.5)	6 (54.5)	11
T-category			
T1	17 (89.5)	2 (10.5)	19
T2	19 (61.3)	12 (38.7)	31
T3	4 (75.0)	2 (25.0)	6
T4	6 (40.0)	9 (60.0)	15
Lymph node metastasis			
Positive	18 (43.9)	23 (56.1)	41*
Negative	28 (93.3)	2 (6.7)	30*
Mode of invasion			
1	10 (100)	0 (0)	10*
2	11 (100)	0 (0)	11*
3	16 (80.0)	4 (20.0)	20*
4C	8 (66.7)	12 (33.3)	20*
4D	1 (10.0)	9 (90.0)	10*
Total	46 (64.8)	25 (35.2)	71




Cumulative survival
1
.8
.6
.4
.2
0
Maspin (+) (n = 46)
Maspin (-) (n = 25)
P < 0.001
0
20
40
60
80
100
Follow Up Time (months)