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Cytoplasmic-only Expression of Maspin Predicts Poor Prognosis in Patients With Oral Squamous Cell Carcinoma

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Abstract. *Background/Aim: Maspin has tumor-suppressor functions; however, its prognostic value in patients with oral squamous cell carcinoma (OSCC) remains unknown. We aimed to assess the prognostic importance of the subcellular localization of maspin in patients with OSCC. Patients and Methods: Eighty resected specimens were analyzed by immunohistochemistry. Cytoplasmic-only expression observed in >10% of the tumor was defined as maspin-positive. Results: The maspin-positive status (25%) was significantly associated with a higher recurrence rate and shorter disease-free survival (DFS). Cox's multivariate analysis showed that maspin-positive status was an independent factor for shorter DFS. All OSCC cell lines (HSC2, HSC3, HSC4, Ca9-22 and SAS) showed maspin protein localization to both the cytoplasm and nucleus using western blot analysis. In HSC4 cells, cell invasion was significantly increased in response to maspin knockdown. Conclusion: Cytoplasmic-only expression of maspin could be an independent poor prognostic factor for patients with OSCC.*

Oral squamous cell carcinoma (OSCC) is a major health problem worldwide, with approximately 378,000 new cases and 178,000 deaths each year (1). The overall survival rate of patients with OSCC has not improved significantly over the past few decades, with a 5-year survival rate of 45-50% in most countries (2). The prognosis of patients with OSCC is greatly influenced by many factors, such as pathological TNM stage, nodal involvement, primary tumor site, tumor thickness, and surgical margins (2, 3). Many molecular prognostic markers for patients with OSCC have also been reported; however, the practical application of these markers is controversial (3). Therefore, it is necessary to identify new reliable prognostic markers to make proper treatment choices.

Mammary serpin protease inhibitor (maspin) was originally identified as a tumor-suppressor protein expressed in normal human breast epithelial cells but not in breast carcinoma (4). Maspin inhibits tumor growth, cell motility, invasiveness, angiogenesis, and metastasis in multiple cancer cell lines and animal models (4, 5). Although many studies have reported an association between maspin expression and clinicopathological factors in several cancer types, including OSCC, there are conflicting results regarding whether maspin expression is a favorable or unfavorable indicator for patients (6). The most influential factors contributing to this complexity may be the difference of "positive" criteria including cut-off point, subcellular localization, and staining intensity. We previously reported that cytoplasmic-only expression of maspin is a poor prognostic indicator for patients with breast (7), lung (8-10), and pancreatic cancer (11). Several studies have investigated the relationship between maspin expression and prognosis in patients with OSCC (12-17); however, only one study focused on the correlation between subcellular localization of maspin and patient' prognosis in 56 cases (12). Taken together, we explored the correlation between the subcellular localization of maspin and prognosis in patients with OSCC. The aim of the present study was to clarify whether cytoplasmic-only expression of maspin is an unfavorable prognostic indicator in patients with OSCC. We also investigated the subcellular localization of maspin and its invasive abilities in several human OSCC cell lines.

Patients and Methods

Patients and tumor specimens. From January 2005 to December 2015 at Tottori University Hospital (Yonago, Japan), 127 consecutive patients underwent curative surgical resection of OSCC. A total of 47 patients were excluded for the following reasons: in nine cases, induction chemotherapy was performed before surgery

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and the effect was more than grade 2 according to the criteria of general rules for clinical and pathological studies on oral cancer (18); in 29 cases, radiation therapy and/or superselective intra-arterial chemotherapy were performed before surgery; and in nine cases, invasive lesion was not found in immunostained slides. Thus, 80 OSCC cases were included in the present study. The clinicopathological data of the patients were obtained from their hospital medical records. Tumor stage was determined based on the 7th edition of the American Joint Committee on Cancer staging system (19). Informed consent was obtained using the opt-out method by publishing at the Tottori University Hospital (Yonago, Japan) website, and the present study was approved by the Ethics Committee of the Faculty of Medicine, Tottori University (approval no.:18A046; May 11, 2018).

Immunohistochemistry. All specimens were fixed in 10% neutrally buffered formalin and embedded in paraffin. Sections (4 μm-thick) were deparaffinized, treated with hydrogen peroxide to inhibit endogenous peroxidase activity, and then microwaved in citrate buffer (0.01 M, pH 6.0) for 15 min. Next, we performed immunohistochemical staining using a monoclonal anti-human maspin antibody (clone EAW24, diluted 1: 150; Leica Biosystems, Newcastle upon Tyne, UK), as described previously (7).

Evaluation of immunohistochemical findings. Cells were considered positive if strong staining, defined as a staining intensity equal to that in mammary myoepithelial cells, was observed only in the cytoplasm. Maspin expression in the normal oral mucosa varies from sample to sample; therefore, myoepithelial cells of normal breast tissue were used as an internal positive control. If positive cells accounted for more than 10% of the tumor, the case was considered maspin-positive, as previously described (20). The subcellular localization of maspin was classified into four categories: cytoplasmic-only, pancellular (combined nuclear and cytoplasmic), nuclear-only, and no staining. All slides were independently evaluated by M.K. and Y.U., without knowledge of the clinicopathological data of the patients.

Cell culture. HSC2, HSC3, HSC4, Ca9-22, and SAS cell lines were purchased from the Riken Bio Resource Center Cell Bank (Riken RBC Cell Bank, Ibaraki, Japan). HSC2, HSC3, and Ca9-22 cells were maintained in Gibco Minimum Essential Media (Thermo Fisher Scientific, Waltham, MA, USA), whereas HSC4 and SAS cells were maintained in RPMI 1640 medium (Thermo Fisher Scientific). All cell lines were maintained at 37˚ in a humidified incubator containing 5% CO_2. Each medium was adjusted by adding 10% fetal bovine serum (FBS; Biological Industries Ltd. Kibbutz Beit Haemek, Israel), 5% glutamine, and 1% penicillin-streptomycin.

RNA extraction and quantitative polymerase chain reaction. Total RNA from HSC2, HSC3, HSC4, Ca9-22, and SAS cells was extracted using TRIzol reagent (Thermo Fisher Scientific) and reverse-transcribed into complementary DNA (cDNA) using a high-capacity RNA-to-cDNA kit (Thermo Fisher Scientific) according to the manufacturer's protocol. Gene expression levels were measured using the TaqMan Gene Expression Assays (Thermo Fisher Scientific) with the following gene-specific primers: *β-actin (ACTB)* (Hs01060665_g1, Thermo Fisher Scientific) and *maspin* (Hs00985285_m1, Thermo Fisher Scientific).

Western blot analysis. Whole-cell lysates were prepared using RIPA lysis buffer with a protease inhibitor cocktail (Roche Applied Science, Indianapolis, IN, USA) and phosphatase inhibitor cocktail (Roche Applied Science). Subcellular protein fractions were obtained using a subcellular protein fractionation kit (Thermo Fisher Scientific) according to the manufacturer's instructions. Proteins were separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to a 0.45 μm pore size polyvinylidene difluoride membrane (Merck Millipore, Burlington, MA, USA). Blocking was performed with 5% ECL prime blocking agent (Cytiva, Tokyo, Japan) for 90 min. The primary antibodies used were mouse monoclonal anti-human maspin antibody (clone EAW24; 1:1000 dilution, Leica Biosystems), mouse monoclonal anti-β-actin (ACTB) antibody (8H10D10; 1:1000 dilution, Cell Signaling Technology, Danvers, MA, USA), mouse monoclonal anti-HSP90 antibody (C45G5; 1:1,000 dilution, Cell Signaling Technology), and rabbit polyclonal anti-HDAC1 antibody (10E2; 1:1,000 dilution, Cell Signaling Technology). Horseradish peroxidase (HRP)-conjugated anti-mouse IgG (cat. No.NA934; 1:3,000 dilution, GE Healthcare, Boston, MA, USA) or HRP-conjugated anti-rabbit IgG (cat. No. NA931; 1:3,000 dilution, GE Healthcare) was used as the secondary antibody. The signals were visualized using the Immobilon Western chemiluminescent substrate (Millipore, Billerica, MA, USA) and quantified using the Image Quant LAS 4000 mini (GE Healthcare).

Plasmid and siRNA transfection. HSC3 and HSC4 cells were seeded in 6-well plates and incubated for 24 h at 60-80% confluence at transfection. Cells in each well were transfected with 10 nM siRNA targeting maspin (Silencer Select siRNA, s10468, Thermo Fisher Scientific), or control siRNA (Silencer Negative Control #1; Thermo Fisher Scientific) using 9 μl Lipofectamine RNAiMAX (cat. No. 13778030, Thermo Fisher Scientific) according to the manufacturer's instructions.

Cell invasion assays. Cell invasion assays were performed using the CytoSelect 24-well cell invasion assay (Cell Biolabs, Inc., San Diego, CA, USA) according to manufacturer's instructions. HSC3 and HSC4 cells (1.8×10^5 cells/well) were cultured in a medium containing 10% FBS for 24 h, transfected with siRNA, and further cultured for 24 h. The medium was replaced with a serum-free medium, and the cells were further cultured for 24 h. Cells transfected with siRNA were seeded in serum-free medium in the upper chamber containing polycarbonate membrane inserts (8 μm pore size) in a 24-well transwell plate. The lower chamber was filled with medium containing 10% FBS. After incubation for 48 h, the infiltrating cells at the bottom of the chamber were removed with a stripping solution. Invasive cells in the striping solution were read with a fluorescence plate reader using CyQuant GR Dye (Cell Biolabs). Three independent experiments were performed, and the mean values were statistically analyzed.

Statistical analysis. All statistical analyses were performed using the SPSS ver.25 software program (IBM SPSS, IBM, Armonk, NY, USA). The association between maspin status and clinicopathological factors were analyzed using non-parametric tests. The chi-square test or Fisher's exact test were used when there were two categorical variables of interest, whereas the Kruskal-Wallis test was applied when there were three variables.

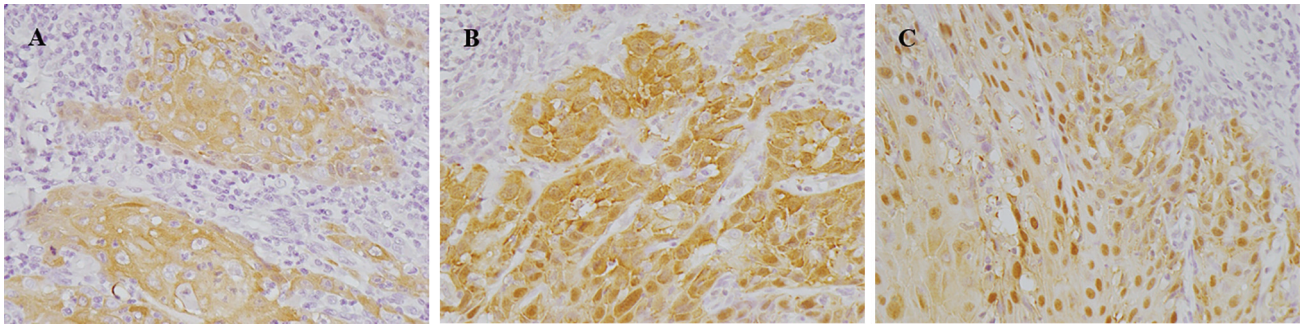

Figure 1. *Immunohistochemical staining pattern of maspin in oral squamous cell carcinoma. A) Cytoplasmic-only expression of maspin. B) Pancellular (both nuclear and cytoplasmic) expression of maspin. C) Nuclear-only expression of maspin.*

Disease-free survival (DFS) was defined as the period from the date of initial surgery to the date of clinical or pathological cancer recurrence or last visit. DFS rates were calculated using the Kaplan-Meier method and compared using log-rank tests. Multivariate analysis of factors associated with DFS was performed using the Cox hazard test to define independent prognostic factors depending on the maspin status. Relative maspin mRNA expression was compared using one-way analysis of variance and the Tukey's honest significant difference test. Differences in cell invasion assays were evaluated using the Student's *t*-test. Differences were considered statistically significant when the *p* value was less than 0.05. All continuous values are presented as the mean±standard deviation.

Results

Immunohistochemistry. Representative immunohistochemical staining patterns for maspin expression are shown in Figure 1. The subcellular localization of maspin expression consisted of cytoplasmic-only staining (20 cases: 25%), pancellular (combined nuclear and cytoplasmic) staining (14 cases, 17.5%), nuclear-only staining (three cases: 3.7%), and no staining (43 cases: 53.8%).

Clinicopathological characteristics and association with maspin expression status. The mean age of the 80 patients at the time of surgery was 67 years (range=31-93 years); 44 patients were men and 36 patients were women (Table I). The maspin-positive status was significantly correlated with a higher population of women (p=0.038) and a higher rate of recurrence (p=0.013) than the negative group (Table II). The status of the pancellular expression of maspin was significantly correlated with a higher population of men (p=0.012), and a lower rate of recurrence rate (p=0.036) than the status of cytoplasmic-only expression of maspin (Table III).

Survival analysis. The median follow-up period was 45.5 months (range=2-133 months). A total of 17 and 21 patients experienced locoregional and distant recurrences, respectively.

Table I. *Clinicopathological characteristic of 80 patients with oral squamous cell carcinoma according to the seventh edition of the TNM classification.*

Variables	Value
Age (mean±SD, years)	67.0±13.9
Gender, n (%)	
Male	44 (55.0)
Female	36 (45.0)
Tumor site*	
Tongue	36 (45.0)
Gingiva	29 (36.2)
Oral floor	8 (10.0)
Buccal mucosa	6 (7.5)
Others	2 (2.5)
Pathological tumor status	
pT1+2	66 (82.5)
pT3+4	14 (17.5)
Lymph node metastasis, n (%)	
Present	6 (7.5)
Absent	74 (92.5)
Stage (UICC 7th), n (%)	
I+II	62 (77.5)
III+IV	18 (22.5)
Histological type, n (%)	
Well	37 (46.3)
Moderate	35 (43.7)
Poor	8 (10.0)
Recurrence, n (%)	
Present	33 (41.2)
Absent	47 (58.8)

SD: Standard deviation. *Includes 1 case of multiple cancers.

Seven patients died because of OSCC progression. The survival curves of the patients are shown in Figure 2. The five-year DFS rates of the maspin-positive and maspin-negative groups were 35.0% [95% confidence interval (CI)=14.0-56.0%)] and 56.2% (95%CI=43.1-69.3%). The five-year DFS rates were 35.0% (95%CI=14.0-56.0%) in the cytoplasm-only

Table II. *Association between maspin status and clinicopathological characteristics.*

Variables	Maspin-positive (n=20)	Maspin-negative (n=60)	*p*-Value
Age (years)			
<70	6	30	0.119
≥70	14	30	
Gender			
Male	7	37	0.038
Female	13	23	
Pathological tumor status			
pT1+2	15	51	0.308
pT3+4	5	9	
Lymph node metastasis			
Present	1	5	0.624
Absent	19	55	
Stage (UICC 7th)			
I+II	14	48	0.354
III+IV	6	12	
Histological type			
Well	10	27	0.436
Moderate	10	25	
Poor	0	8	
Recurrence			
Present	13	20	0.013
Absent	7	40	

Table III. *Comparison of clinicopathologic factors between cytoplasmic-only and pancellular (combined nuclear and cytoplasmic) expression of maspin.*

Variables	Cytoplasmic-only (n=20)	Pancellular (n=14)	*p*-Value
Age (years)			
<70	6	9	0.048
≥70	14	5	
Gender			
Male	7	11	0.012
Female	13	3	
Pathological tumor status			
pT1+2	15	12	0.378
pT3+4	5	2	
Lymph node metastasis			
Present	1	1	0.661
Absent	19	13	
Stage (UICC7th)			
I+II	14	11	0.440
III+IV	6	3	
Histological type			
Well	10	4	0.093
Moderate	10	6	
Poor	0	4	
Recurrence			
Present	13	4	0.036
Absent	7	10	

staining group, 59.9% (95%CI=31.9-87.9%) in the pancellular staining group, and 59.0% (95%CI=43.9-74.1%) in the no staining group. The log-rank test showed that a maspin-positive status was associated with significantly shorter DFS (p=0.025; Figure 2A). The cytoplasmic-only staining group was associated with a significantly shorter DFS than the no staining group (p=0.037; Figure 2B) and pancellular staining group (p=0.049). Multivariate analysis using the Cox regression hazard model showed that maspin-positive status was an independent predictor of shorter DFS (p=0.023) (Table IV).

Table IV. *Multivariate analysis of various factors of disease-free survival in 80 patients with oral squamous cell carcinoma.*

Prognostic factor	Multivariate analysis		
	HR	95%CI	*p*-Value
Age (year)			
≥70 *vs.* <70	0.684	0.353-1.326	0.260
Pathological tumor status	1.896	0.729-4.931	0.190
pT1+2 *vs.* pT3+4			
Lymph node metastasis			
Present *vs.* Absent	0.534	0.184-1.549	0.248
Maspin status			
Positive *vs.* Negative	0.462	0.237-0.901	0.023

HR: Hazard ratio; CI: confidence interval.

Maspin expression in HSC2, HSC3, HSC4, Ca9-22, and SAS cells. The mRNA and protein expression of maspin in HSC2, HSC3, HSC4, Ca9-22 and SAS cells were investigated by reverse transcription polymerase chain reaction and western blot analysis, respectively. All cells expressed both maspin mRNA and protein to various degrees (Figure 3A and 3B) and protein expression was localized to both cytoplasm and nucleus (Figure 4).

Cell invasion assays in HSC3 and HSC4. The efficacy of siRNA targeting maspin in HSC3 and HSC4 cells was determined. In both cell types, siRNA efficiently reduced the expression of maspin protein (Figure 5A). In HSC4 cells, cell invasion was significantly increased in response to maspin suppression (p=0.016), whereas no reduction was observed in HSC3 cells (Figure 5B).

Discussion

Many studies have focused on the prognostic influence and the association with clinicopathological features of maspin

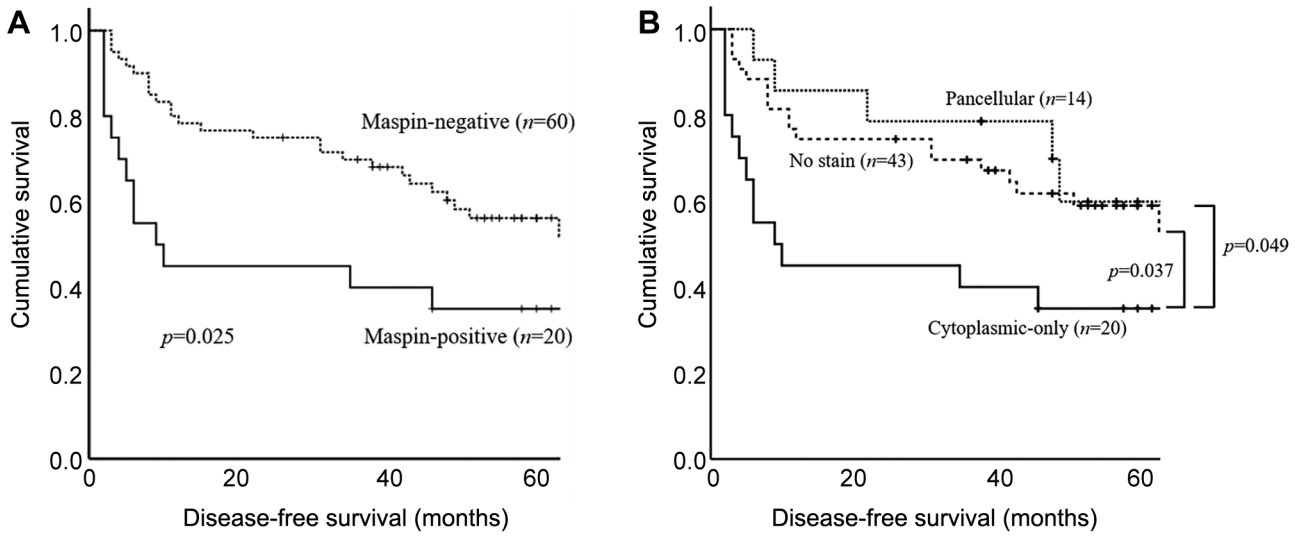

Figure 2. *Kaplan-Meier survival curves for the disease-free survival of 80 patients based on maspin-positive (cytoplasmic-only) versus maspin-negative (all other categories) status (A) and subcellular localization of maspin expression (B).*

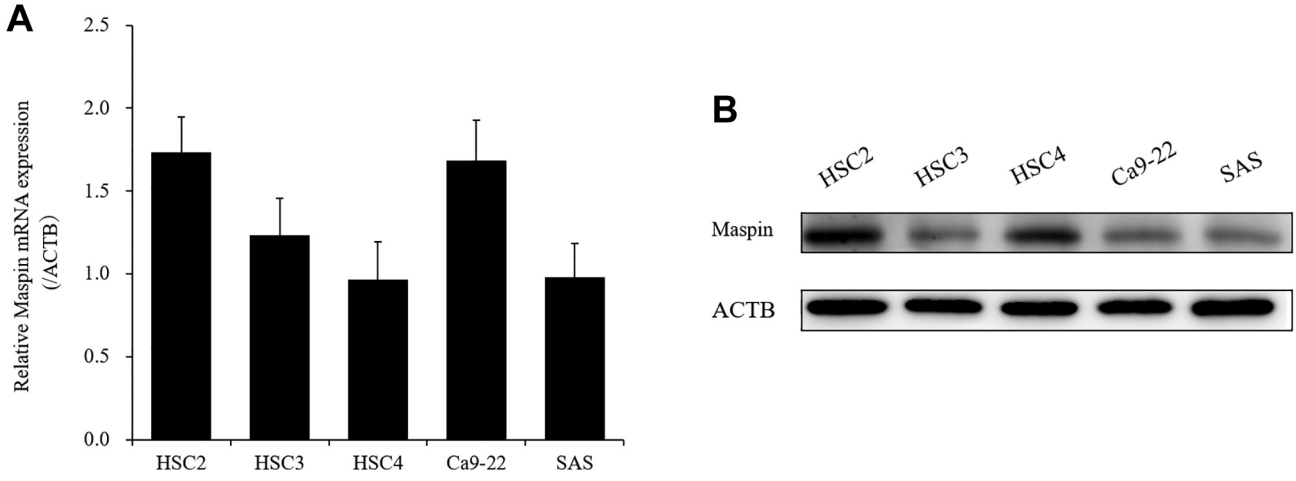

Figure 3. *Maspin expression was assessed by RT-PCR (A) and western blot analysis (B) in HSC2, HSC3, HSC4, Ca9-22 and SAS cells. Maspin mRNA expression was normalized to the expression of ACTB expression.*

expression in various types of cancers including OSCC (12-17); however, it remains controversial whether maspin expression is a favorable or poor prognostic indicator (6). The main factors contributing to the potential complexity of the prognostic significance may be differences in the positive criteria, the antibodies used, and the study population. In particular, the lack of standardization in evaluating the positivity of maspin at the subcellular level may lead to profound discrepancies when evaluating prognostic significance. We have reported that cytoplasmic-only expression of maspin is an independent poor prognostic

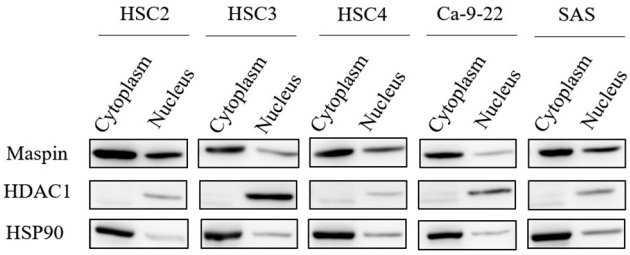

Figure 4. *Analysis of subcellular localization of maspin protein expression in HSC2, HSC3, HSC4, Ca9-22 and SAS cells by western blot analysis.*

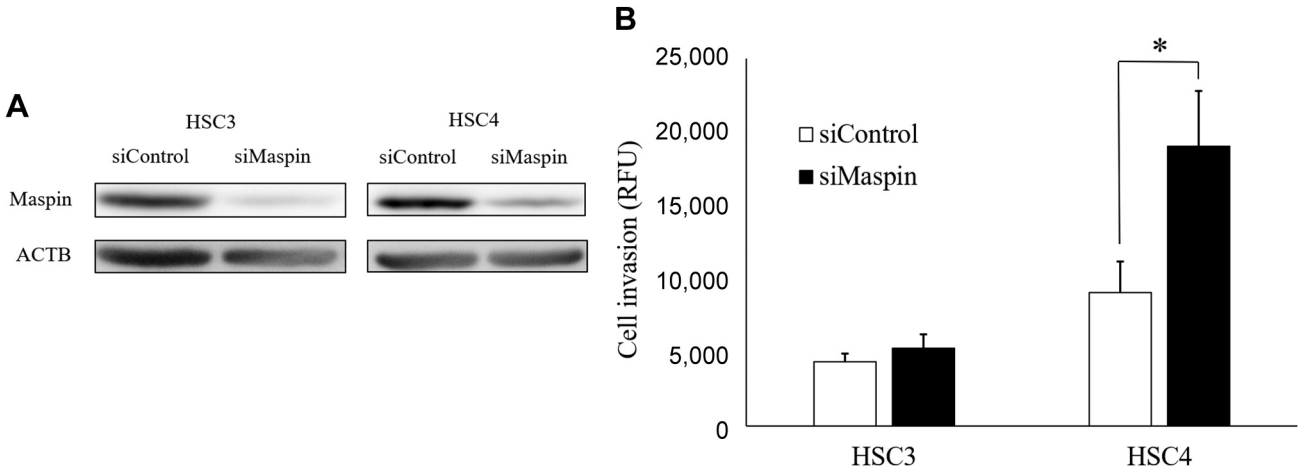

Figure 5. *The association between maspin expression and cell invasion. Knockdown of maspin by siRNA targeting maspin in HSC3 and HSC4 cells (A). Cell invasion abilities of HSC3 and HSC4 cells were analyzed using transwell assays (B). *p=0.016.*

factor for patients with breast cancer (7), lung adenocarcinoma (8, 10), lung squamous cell carcinoma (9), and pancreatic ductal adenocarcinoma (11). Therefore, we hypothesized that cytoplasmic-only expression of maspin may correlate with poor prognosis in patients with OSCC. In OSCC, Yoshizawa *et al*. (14) reported that maspin-positive expression (50% cut-off) was a significantly better prognostic factor in patients with OSCC using 71 biopsy samples. Xia *et al*. (15) reported that higher expression of maspin (50% cut-off) was significantly correlated with longer overall survival (OS) in 44 resected specimens. Yasumatsu *et al*. (16) showed that patients with maspin-positive tumor had a longer DFS and OS using 37 biopsy specimens. Meanwhile, two studies failed to show significant differences in DFS (13) and OS (17) using 30 and 33 OSCC resected specimens, respectively. Considering the small cohort size or the use of biopsy samples in these studies, the clinical significance of maspin expression remains to be determined. To our knowledge, only one study has investigated the relationship between the subcellular localization of maspin and prognosis in patients with OSCC using 56 resected specimens (12). In their study, the positive ratios of maspin in cytoplasmic, nuclear-cytoplasmic, and nuclear patterns were 51.8%, 1.8%, and 5.3%, respectively, whereas our results were 25%, 17.5% and 3.7%, respectively. They failed to demonstrate a correlation between the subcellular expression of maspin and DFS or OS. These discrepancies may depend on the differences in the subcellular distribution pattern, antibody used, the rate of recurrence rate (33.9% *vs*. 41.2%), follow-up time, and cohort size. To the best of our knowledge, this is the first study to reveal that cytoplasmic-only expression of maspin is an independent predictive factor for shorter DFS of patients with OSCC. It has been reported that the nuclear localization of maspin in cancer cells is necessary for its tumour-suppressor activity, and that it does not exhibit tumour-suppressor activity when maspin is excluded from the nucleus (21). Thus, the nuclear localization of maspin, irrespective of the cytoplasmic localization, might be correlated with a favorable prognosis, whereas cytoplasmic-only expression is correlate with poor prognosis. In turn, cell invasion was significantly increased in response to maspin suppression *via* transfection with siRNA targeting maspin in HSC4 cells showing the pancellular expression pattern of maspin. Although the cancer types are different, this result contrasts with our previous study, in which cell invasion was significantly reduced in response to maspin suppression *via* transfection with siRNA targeting maspin in pancreatic ductal adenocarcinoma cell lines showing cytoplasmic-only localization of maspin (11). Although further *in vitro* studies using OSCC cell lines with cytoplasmic-only localization of maspin are required, it is hypothesized that the nuclear localization of maspin, irrespective of the cytoplasmic localization, or cytoplasmic-only localization of maspin, might be correlated with invasive abilities. Although our results are not conclusive, owing to the limitations of the study, including the small number of patients and retrospective nature of the analysis, our findings that cytoplasmic-only expression of maspin was an independent poor prognostic indicator in patients with OSCC may support these experimental findings. Further studies regarding the molecular mechanisms regulating the subcellular localization of maspin could facilitate the development of targeted therapies to suppress the progression or metastasis of OSCC.

In conclusion, this is the first study demonstrating that cytoplasmic-only expression of maspin is an independent

poor prognostic indicator in patients with OSCC. Although further studies with a larger series of cohorts are required, our findings suggest that the immunohistochemical detection of cytoplasmic-only expression of maspin could be helpful in predicting aggressive behavior in patients with OSCC.

Conflicts of Interest

The Authors declare that there are no conflicts of interest regarding this study.

Authors' Contributions

Conception and design: M.K. and Y.U.; acquisition of data: M.K.; experiments: M.K. and T.S.; analysis and interpretation of data: M.K., T.S., and Y.U.; writing, review, and/or revision of manuscript: M.K, T.S., I.K., and Y.U. All Authors read and approved the final version.

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