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Original

Investigation of Cell Migration and Invasion Using Real-time Cell Analysis, as well as the Association with Matrix Metalloproteinase-9 in Oral Squamous Cell Carcinomas

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Abstract: The recently developed technology of real-time cell analysis (RTCA) was designed to analyze cell migration and invasion in vitro. In this study, we investigated these cellular factors in oral squamous cell carcinomas (OSCCs) of the tongue and floor of the mouth with RTCA. We also examined the associated matrix metalloproteinases (MMPs) and integrins. We used the cell lines SCC-4 and SAS, which are human poorly differentiated OSCCs from the tongue, and HO-1-u-1, which are human poorly differentiated OSCCs from the floor of the mouth. Using RTCA, cell migration was assessed on fibronectin–coated CIM-Plates, and invasion was assessed on fibronectin- and matrigel-coated CIM-Plates. SCC-4 cells demonstrated a high ability for cell migration and invasion compared with SAS and HO-1-u-1 cells. The SCC-4 cells also expressed high levels of MMP-9 and integrin $\alpha 1$ mRNA compared with SAS and HO-1-u-1 cells. The MMP inhibitor Marimastat blocked migration and invasion of all OSCCs. The findings suggest that MMP-9 is associated with cell migration and invasion in OSCCs, and indicate that RTCA will be useful for analyzing the metastatic capability of OSCCs and developing more effective new drugs for this disease.

Key words: oral squamous cell carcinoma, migration, invasion, matrix metalloproteinase, real-time cell analyzer

Introduction

Oral squamous cell carcinoma (OSCC) is a well-known malignancy that accounts for more than 90% of all oral cancers and has a high incidence of cervical micrometastases[1, 2)]. The presence of lymph node metastases is one of the most important and widely accepted prognostic factors related to the survival of patients with OSCC[2)].

The metastatic cascade is a complex, multistage process involving modulation of cell phenotype, cell migration, and dynamic homeotypic and heterotypic cell–cell interactions[3)]. Moreover,

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increased tumor size and microvascular invasion are the most important, independent prognostic factors for predicting survival in patients with OSCC[4].

Matrix metalloproteinases (MMPs) are a family of zinc-dependent proteases involved in cancer proliferation and metastasis due to their roles in extracellular matrix (ECM) degradation, cellular migration, tissue remodeling, and angiogenesis[5]. Activated MMPs degrade many ECM and basement membrane components[6, 7], and as tumor cells secrete MMPs to degrade the interstitial matrix, microtracks of weakened and digested matrix result, enabling tumor cell migration[8]. In particular, MMP-2 and MMP-9 are highly expressed in OSCC compared with normal oral mucosa tissues, with tumor progression producing further increases at the mRNA and protein levels[9, 10]. In addition, MMP-2 and MMP-9 are closely associated with tumor invasion and metastasis in a variety of human tumors[11–13].

Integrins are transmembrane heterodimeric receptors that interact with the ECM. By associating laterally on the cell membrane with other proteins and by signaling to modify the expression of molecules such as matrix-modifying proteolytic enzymes, integrins coordinate the interaction of a cell with the ECM[14]. Expression of integrins and proteases of the MMP and plasminogen activator families is altered in both healing and malignant epithelium, implicating these molecules as key factors in the process of re-epithelialization, cell migration, and tumor invasion[14, 15].

Many different experimental methods have been developed to assay tumor cell migration and invasion in vitro[16], with real-time monitoring a major technical challenge. The recently developed xCELLigence real-time cell analysis (RTCA; Roche Applied Science, Mannheim, Germany) system has emerged as an alternative non-invasive and label-free approach that uses impedance detection for the continuous monitoring of cellular proliferation, migration, and invasion in real time on a cell culture level[17, 18]. In this study, we applied RTCA to OSCCs of the tongue and floor of the mouth, and examined the associated MMPs and integrins.

Materials and Methods

Cell lines and reagents

SCC-4 (JCRB9118)[19] and SAS (JCRB0260)[20] cells were established from a poorly differentiated OSCC in the tongue. HO-1-u-1 (JCRB0828) cells were established from a poorly differentiated OSCC in the floor of the mouth[21]. All cell lines were provided by the Health Science Research Resources Bank (Osaka, Japan). Marimastat, a broad-spectrum MMP inhibitor, was obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

Cell culture

SAS and HO-1-u-1 cells were cultured at 37°C in humidified 5% CO_2 and 95% air in 45% Dulbecco's modified Eagle's medium, 45% Ham's F12 medium (DMEM/F12), 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin. The medium was changed every 3days. SCC-4 cells are considered undifferentiated cancer cells and were cultured in the same conditions in DMEM/F12 with 0.4 μg/ml hydrocortisone, 10% FBS, 100 U/ml penicillin, and

100 μg/ml streptomycin. The medium was changed every 3 days.

RTCA

We used RTCA to study migration and invasion of OSCCs[22]. Migration was analyzed in a CIM-Plate 16 (Roche) coated with fibronectin (20 mg/ml), while invasion was assessed using a CIM-plate 16 coated with Matrigel (1:80) (BD Biosciences, Erembodegem, Belgium) and fibronectin (20 mg/ml). For each analysis, SCC-4 cells (1.6×10^5), SAS cells (3.2×10^5), or HO-1-u-1 cells (3.2×10^5) were seeded in the upper chamber of a CIM-Plate 16 in 100 μl medium for each cell line without FBS and with or without Marimastat (10 or 100 μM). The upper chamber was then placed on the lower chamber of the CIM-Plate 16, which contained growth medium supplemented with 10% FBS as an attractant.

RNA isolation and quantitative real-time RT-PCR (Q-PCR)

Q-PCR was used to quantify the mRNA levels of MMP-2 and 9 and integrin α1 and β1 in OSCCs. Total RNAs were extracted with NucleoSpin RNAII (MACHEREY-NAGEL GmbH & Co. KG, Duren, Germany), and reverse transcription of total RNA was performed using PrimeScript RT Master Mix from TaKaRa Bio Inc. (Shiga, Japan). The cDNAs were amplified using primers designed by ProbeFiber software (Roche). Table 1 details the primers used, the Roche Universal ProbeLibrary Probe numbers, and accession numbers. Amplification was performed with a LightCycler (Roche) using LightCycler TaqMan Master mix (Roche). The PCR reaction parameters were as follows: 95℃ for 10 min, 45 cycles (except 18s, which was performed for 25 cycles) of 95℃ for 10s, 60℃ for 30s, and 72℃ for 1s. Fluorescence data were analyzed with LightCycler software (Roche). The mRNA levels were compared to 18s rRNA as a standard, and relative expression ratios were calculated[23].

Table 1. Nucleotide sequences of the primers used for PCR

Gene		Sequence	Accession number	Probe number
MMP-2	Sense primer	5'-gaggggcaggtcatgtag-3'	AY738117.1	#58
	Antisense primer	5'-ggctggatgagatcttgctg-3'		
MMP-9	Sense primer	5'-gaaccaatctcaccgacagg-3'	NM_004994.2	#6
	Antisense primer	5'-gccacccgagtgtaaccata-3'		
Integrin α1	Sense primer	5'-aattggctctagtcaccattgtt-3'	NM_181501.1	#14
	Antisense primer	5'-caaatgaagctgctgactggt-3'		
Integrin β1	Sense primer	5'-cgatgccatcatgcaagt-3'	NM_002211.3	#65
	Antisense primer	5'-acaccagcagccgtgtaac-3'		
18s	Sense primer	5'-gcaattattccccatgaacg-3'	X03205.1	#48
	Antisense primer	5'-gggacttaatcaacgcaagc-3'		

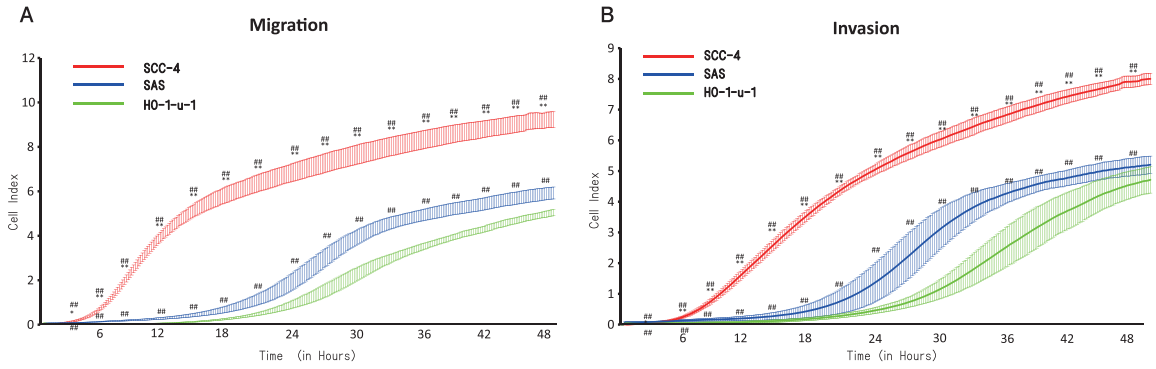

Fig. 1. This graph shows migration (A) and invasion (B) in SCC-4, SAS, and HO-1-u-1 cell lines as indicated by the color code. Cells were resuspended in their respective growth media and seeded into the wells of a CIM-Plate 16 (SCC-4 ; 1.6×10^5/well, SAS and HO-1-u-1 ; 3.2×10^5/well) of the RTCA instrument. Changes in impedance resulting from cells that had migrated to the bottom side of the membranes were recorded every 15 min and monitored for a total of 48 hours. Data are the mean values±SD of eight (SCC-4 and SAS), four (A ; HO-1-u-1), and three (B ; HO-1-u-1) wells. * ; $P<0.05$ vs. SAS cells, ** ; $P<0.01$ vs. SAS cells, ## ; $P<0.01$ vs. HO-1-u-1 cells.

Statistical analysis

Data were analyzed with the Bonferroni test following one-way analysis of variance (ANOVA). All data were expressed as the mean ± standard error (SE) or standard deviation (SD). *P* values of less than 0.05 were considered significant.

Results

The migratory response of OSCCs was observed with RTCA on a cell culture level (Fig. 1A). This response was increased early during the culture period of SCC-4 cells (approximately 12 hours), and we observed a high cell index level compared with SAS and HO-1-u-1 cells. SAS cells showed a higher migratory response than HO-1-u-1 cells. The invasion response was slightly weaker and later compared with the migratory response in OSCCs by RTCA (Fig. 1B). The order of the migratory and invasion abilities was clearly observed with RTCA as SCC-4 > SAS > HO-1-u-1.

Figures 2 and 3 show the expression profiles of integrin and MMP mRNA, respectively. The level of each mRNA in HO-1-u-1 cells was set at 100%. Integrin $\alpha1$ mRNA levels in SCC-4 cells were 15.8 times ($P<0.01$) higher than levels in HO-1-u-1 cells (Fig. 2A), whereas integrin $\beta1$ (Fig. 2B) and MMP-2 (Fig. 3A) mRNA levels were not significantly different among SCC-4, SAS, and HO-1-u-1 cells. MMP-9 mRNA levels in SCC-4 cells were 4.91 times ($P<0.01$) and 1.90 times ($P<0.05$) higher than levels in HO-1-u-1 and SAS cells, respectively (Fig. 3B).

No cell death or morphological changes were seen in OSCCs treated with 100 μM Marimastat, an MMP inhibitor. Lombard *et al.*[24] also reported that human colon adenocarcinoma treated with 100 μM Marimastat inhibited shedding of tumor necrosis factor-alpha receptors. Therefore, there is little toxicity to OSCCs at concentrations less than 100 μM Marimastat (data not shown). Figures 4 and 5 show that Marimastat added to the upper chamber at a concentration

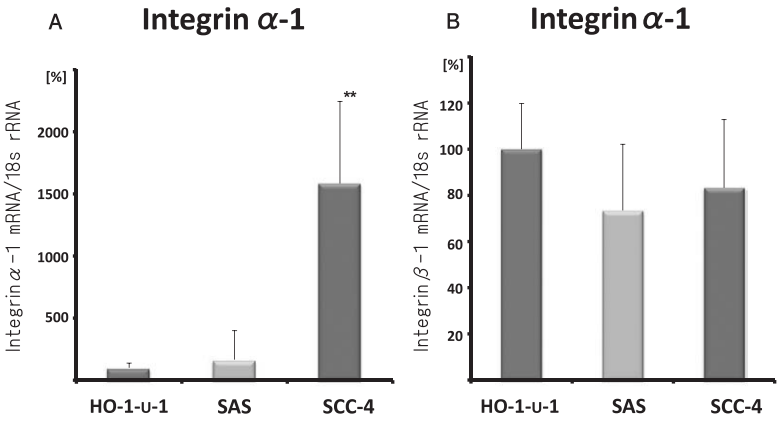

Fig. 2. (A) Integrin $\alpha 1$ and (B) Integrin $\beta 1$ mRNA expression in HO-1-u-1, SAS, and SCC-4 cells relative to HO-1-u-1 cells as determined by quantitative real-time polymerase chain reaction are shown. Data are the mean±SEM. **; $P<0.01$ vs. HO-1-u-1 cells.

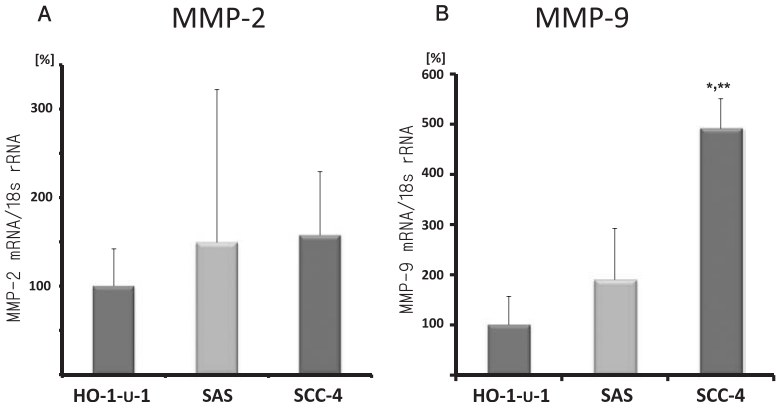

Fig. 3. (A) MMP-2 and (B) MMP-9 mRNA expression in HO-1-u-1, SAS, and SCC-4 cells relative to HO-1-u-1 cells as determined by quantitative real-time polymerase chain reaction are shown. Data are the mean±SEM. *; $P<0.05$ vs. SAS cells, **; $P<0.01$ vs. HO-1-u-1 cells.

of 10 or 100 μM significantly inhibited the migratory and invasion responses of OSCCs in a dose-dependent manner. The migratory response of HO-1-u-1 cells at 48 hours in the presence of 100 μM Marimastat was decreased by 86% ($P<0.01$; compared with control and 10 μM Marimastat), while in the presence of 10 μM Marimastat, migration was reduced by 49% ($P<0.05$; compared with control) after 34 hours (Fig. 4A). The migratory response of SAS cells in the presence of 100 μM Marimastat was decreased by 29% after 48 hours ($P<0.01$; compared with control and 10 μM Marimastat), and by 14% in the presence of 10 μM Marimastat ($P<0.01$; compared with control) (Fig. 4B). The migratory response of SCC-4 cells was decreased by 44% in the presence of 100 μM Marimastat after 48 hours ($P<0.01$; compared with control and 10 μM Marimastat), and by 19% in the presence of 10 μM Marimastat ($P<0.01$; compared with control) (Fig. 4C). The invasion response of HO-1-u-1 cells in the presence of 100 μM Marimastat was decreased by 71% ($P<0.01$; compared with control and 10 μM

Marimastat) after 48 hours, while invasion in the presence of 10 μM Marimastat was reduced by 86% ($P<0.05$; compared with control) after 24 hours (Fig. 5A). The invasion response of SAS cells was decreased by 92% in the presence of 100 μM Marimastat ($P<0.01$; compared with control and 10 μM Marimastat) after 48 hours, and by 36% in the presence of 10 μM Marimastat ($P<0.01$; compared with control) after the same time period (Fig. 5B). For SCC-4 cells, the invasion response was decreased by 75% in the presence of 100 μM Marimastat ($P<0.01$; compared with control and 10 μM Marimastat), and by 24% in the presence of 10 μM Marimastat ($P<0.01$; compared with control) after 48 hours (Fig. 5C). The invasion response of HO-1-u-1 cells was similar to the migratory response. Thus, Marimastat more markedly decreased the invasion responses of SAS and SCC-4 cells compared with the migratory responses.

Discussion

In this study, we demonstrated the migratory and invasion responses associated with MMP-9 in poorly differentiated OSCCs by RTCA. Prevention of cancer migration and invasion is an important strategy for improving a patient's prognosis. Cancer cell-matrix interaction is a critical step in promoting cell migration[25, 26], and proteolytic degradation of the ECM is a critical event during tumor invasion and metastasis. Cell migration across ECM tissue boundaries is required in many important biological processes and is dependent on the activities of proteases such as MMPs[27, 28]. MMP-2 and MMP-9 are involved in metastasis[29], making MMPs target enzymes for blocking migration and invasion. Our results demonstrated that SCC-4 cells, which strongly express MMP-9, have high migration and invasion abilities. This is consistent with a report by Lu *et al.*[30] that showed that downregulating MMP-9 production inhibited SCC-4 cell invasion and metastasis.

We further confirmed the association of MMPs with the invasion and metastasis abilities of OSSCs using Marimastat, a broad-spectrum MMP inhibitor with pharmacokinetic properties appropriate for chronic oral administration. Marimastat shows potent inhibition of most of the major MMPs, with IC50s against MMP-2 and MMP-9 of 6 and 3 nM, respectively[31]. Indeed, Peterson *et al.*[32] showed reduced MMP-2 and MMP-9 activities in fibrosarcoma-derived HT-1080 cells by Marimastat. Marimastat was shown to inhibit tumor growth in a murine model of head and neck squamous cell carcinoma in combination with chemoradiation[33]. However, clinical trials involving Marimastat have been disappointing as many patients failed to benefit from the drug and also experienced significant side effects, such as musculoskeletal toxicity[34]. In this study, Marimastat was effective in blocking invasion, but not as effective in blocking migration in SCC-4 and SAS cells. Moreover, the effect required a high concentration of Marimastat (100 μM).

Many cell types constitutively express MMP-2, and its promoter does not contain a TATA box, as is the case for many housekeeping genes[35]. Our results showed that MMP-2 mRNA expression was not significantly different among the OSCC cell lines studied. On the other hand, MMP-9 gene transcription is inducible in most cell types[35], and can be induced in specific cell types by multiple polypeptide factors, including epidermal growth factor, platelet-derived growth factor, hepatocyte growth factor / scatter factor, basic fibroblast growth factor,

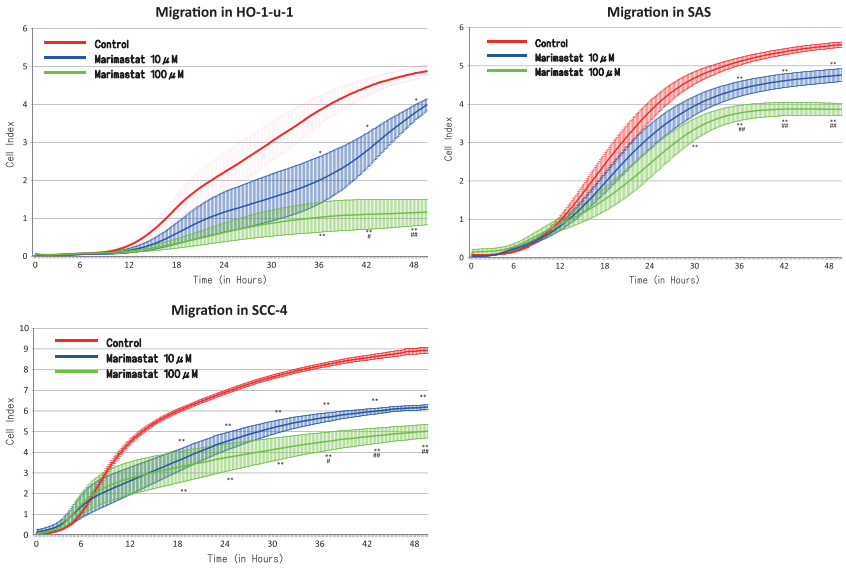

Fig. 4. This graph shows the migration of HO-1-u-1 (A), SAS (B), and SCC-4 (C) cell lines. Cells were resuspended in their respective growth media and seeded into the wells of a CIM-Plate 16 (SCC-4 ; 1.6×10^5/well, SAS and HO-1-u-1 ; 3.2×10^5/well) of the RTCA instrument. Cells were allowed to migrate in the presence (10 μM or 100 μM, added to the upper compartment of each well) or absence of Marimastat (Marimastat (-)) as indicated by the color code. Changes in impedance resulting from cells that had migrated to the bottom side of the membranes were recorded every 15 min and monitored for a total of 48 hours. Data are the mean values±SD of wells (A ; Control, n = 6 ; 10 μM, n = 6 ; 100 μM, n = 4, B ; Control, n = 18 ; 10 μM, n = 18 ; 100 μM, n = 7, C ; Control, n = 12 ; 10 μM, n = 9 ; 100 μM, n = 6). * ; $P<0.05$ vs. control, ** ; $P<0.01$ vs. control, # ; $P<0.05$ vs. 10 μM Marimastat, ## ; $P<0.01$ vs. 10 μM Marimastat.

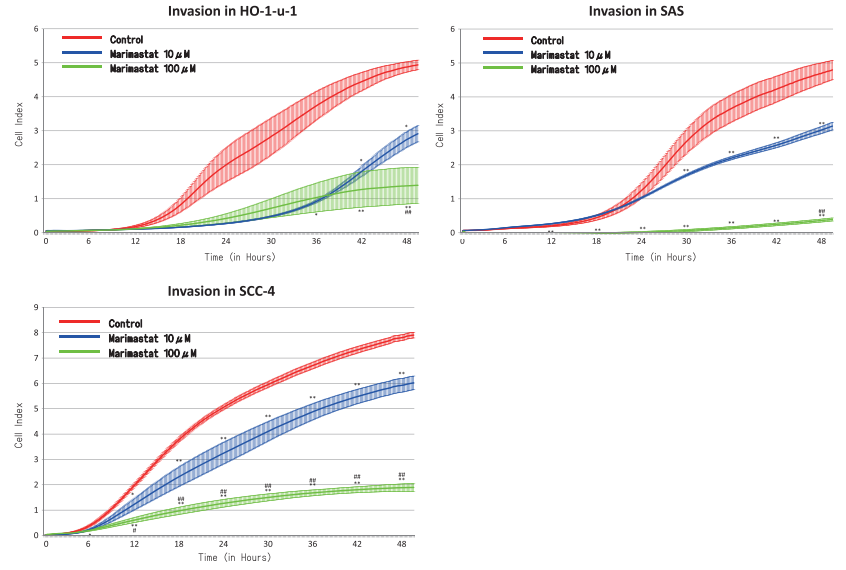

Fig. 5. This graph shows invasion of HO-1-u-1 (A), SAS (B), and SCC-4 (C) cell lines. Cells were resuspended in their respective growth media and seeded into the wells of a CIM-Plate 16 (SCC-4 ; 1.6×10^5/well, SAS and HO-1-u-1 ; 3.2×10^5/well) of the RTCA instrument. Cells were allowed to migrate in the presence (10 μM or 100 μM, added to the upper compartment of each well) or absence of Marimastat (Marimastat (−)) as indicated by the color code. Changes in impedance resulting from cells that had migrated to the bottom side of the membranes were recorded every 15 min and monitored for a total of 48 hours. Data are the mean values±SD of wells (A ; Control, n = 7 ; 10 μM, n = 4 ; 100 μM, n = 4, B ; Control, n = 10 ; 10 μM, n = 5 ; 100 μM, n = 10, C ; Control, n = 10, 10 μM, n = 10 ; 100 μM, n = 10). * ; $P<0.05$ vs. control, ** ; $P<0.01$ vs. control, $P<0.05$ vs. 10 μM Marimastat, ## ; $P<0.01$ vs. 10 μM Marimastat.

transforming growth factor-α, amphiregulin, tumor necrosis factor-α, interleukin-1 α, interleukin-1 β, interferon-α, interferon-γ, and transforming growth factor-β, as well as by phorbol ester stimulation[36]. However, poorly differentiated OSCCs, especially SCC-4 cells, may constitutively express MMP-9 mRNA. MMP-9 is closely linked to the metastatic potential of tumors, and enhanced production of MMP-9 has been observed in highly metastatic versus minimally metastatic cell lines[37].

Integrins consist of two transmembrane subunits, α and β, that form a non-covalent heterodimer. Both subunits contribute to the formation of the ligand binding pocket, but the α subunit is likely to play an important role in determining the binding selectivity because heterodimers sharing a common β subunit, but with distinct α chains, often bind different ligands[38]. The binding affinity state of many integrins can be regulated at the cell surface by several stimuli, allowing regulation of the intensity of adhesion during cellular interactions and migration[39]. Several reports have shown that integrin α1 β1 in particular is associated with migration and invasion of cancer cells[40, 41]. In this study, SCC-4 cells in which integrin α1 mRNA was strongly expressed showed high migration and invasion abilities.

In summary, we used the recently developed RTCA technology to further investigate the migratory and invasion responses associated with MMP-9 in poorly differentiated OSCCs in real time. By this strategy, we demonstrated that SCC-4 cells have high migration and invasion abilities, SAS cells have intermediate abilities, and HO-1-u-1 cells have low, but similar abilities in migration and invasion. Therefore, RTCA will be useful for analyzing the metastatic capability of OSCCs, potentially leading to the development of more effective new drugs for OSCC.

Conflict of interest

The authors have declared no conflict of interest.

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