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ORIGINAL ARTICLE – TRANSLATIONAL RESEARCH AND BIOMARKERS

Overexpression of Renal Tumor Antigen Is Associated with Tumor Invasion and Poor Prognosis of Hepatocellular Carcinoma

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ABSTRACT

Purpose. The aim of this study was to investigate the roles of renal tumor antigen (RAGE) in the progression and clinical outcome of hepatocellular carcinoma (HCC).

Methods. RAGE mRNA levels in 350 cases of HCC were investigated by quantitative real-time reverse transcription polymerase chain reaction. We analyzed the relationship of RAGE mRNA level with clinicopathologic parameters and clinical outcome. To identify the possible role of RAGE on cellular invasion, we performed in vitro analyses using small interfering RNAs (siRNAs).

Results. RAGE mRNA level was significantly higher in HCC than in noncancerous hepatic tissues ($P < 0.001$). Overexpression of RAGE was significantly correlated with the presence of multiple tumors ($P = 0.021$), high alfa-fetoprotein level ($P = 0.042$), and advanced tumor stage ($P = 0.016$). Higher levels of RAGE expression were associated with significantly shorter overall survival time ($P = 0.029$). Knockdown of RAGE expression by siRNAs suppressed the invasive ability of HCC cells and the expression and secretion of matrix metalloproteinase-9 (MMP-9). We found that RAGE and MMP-9 expressions were correlated in HCCs, and furthermore, the combination of RAGE and MMP-9 expression was associated with the survival of patients ($P = 0.0066$).

Conclusions. Our results suggest that RAGE may be important in tumor invasion and could be a potential predictor for the prognosis of HCC patients.

Hepatocellular carcinoma (HCC) is the fifth most common cancer worldwide.[1] Its incidence is high in Asia and Africa and is increasing in Europe and the United States.[1,2] HCC is resistant to conventional chemotherapy, so this disease has a poor prognosis.[3] Surgical resection and liver transplantation provide an opportunity to cure HCC, but these approaches have limited applicability as a result of frequent tumor recurrence.[4,5] Therefore, it is necessary to identify important prognostic factors and to develop novel therapeutic strategies targeting HCC.

Renal tumor antigen (RAGE; GenBank accession no. NM_014226) was originally found in a renal cell carcinoma library.[6] RAGE is also known as a MAPK/MAK/MRK overlapping kinase.[7] Because the RAGE gene encodes tumor antigens recognized by cytotoxic T lymphocytes, RAGE has been investigated as a potential therapeutic target for cancer-specific immunotherapy.[6,8]

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RAGE expression levels have been reported in several types of tumors including renal cell carcinoma, acute myeloid leukemia, and mesothelioma.[8–10] However, the functional role of RAGE in the progression of cancer and the relationship between RAGE expression and clinical outcome of cancer patients remains largely unknown.

In this study, RAGE mRNA levels were found to be statistically significantly higher in 350 HCCs than in noncancerous hepatic samples as determined by quantitative reverse transcription-polymerase chain reaction (RT-PCR). Overexpression of RAGE gene was associated with several clinicopathologic features and poor prognosis of HCC patients. Inhibition of RAGE expression by small interfering RNA (siRNA) reduced the invasive ability of HCC cells, and at the same time decreased the expression and enzyme activity of matrix metalloproteinase-9 (MMP-9). Furthermore, simultaneous high expression of RAGE and MMP-9 was correlated with a poor prognosis. These results suggest that RAGE could be a useful prognostic marker and a potential therapeutic target for HCC.

MATERIALS AND METHODS

Patients and Tissue Samples

HCCs and surrounding noncancerous hepatic tissues were obtained with informed consent from 350 patients who underwent curative hepatectomy for primary HCC between the years 2001 and 2005 in the Department of Surgery, Samsung Medical Center, Korea. The study protocol was approved by the institutional review board of the Samsung Medical Center. Complete clinical data were available in all 350 cases (Table 1). The mean age of patients was 51 years (range 20–69 years). All patients had adequate liver function reserve, and had survived for at least 2 months after hepatectomy (median follow-up, 51.7 months; range 2.7–95.6 months). We defined recurrence as evidence of an overt new growing mass in the remaining liver, or as distant metastasis in radiologic studies. None of the patients had received treatment before surgery, such as transarterial chemoembolization or radiofrequency ablation. Immediately after hepatectomy, fresh tumors and background livers were partly snap-frozen in liquid nitrogen and stored at −80°C, and were partly embedded in paraffin after fixation in 10% formalin for histological diagnosis. All available slides stained with hematoxylin and eosin were reviewed. Tumor grading was based on the criteria of Edmondson and Steiner.[11] Tumor stages were identified according to the conventional tumor, node, metastasis system defined in the cancer staging manual of the American Joint Committee on Cancer (6th edition).

TABLE 1 Relations between mRNA levels of RAGE and clinicopathologic features in HCC

Clinicopathologic parameters	No. of patients ($N = 350$)		P value
	High RAGE ($n = 99$)	Low RAGE ($n = 251$)	
Age			0.653
<55 y	64	154	
≥55 y	35	97	
Sex			0.342
Male	74	201	
Female	25	50	
Hepatitis B virus			0.347
Absent	18	59	
Present	81	192	
Hepatitis C virus			0.307
Absent	97	238	
Present	2	13	
Liver cirrhosis			0.943
Absent	52	129	
Present	47	122	
Tumor stage			0.016
I	30	98	
II	39	111	
III and IV	30	42	
AFP level			0.042
<100 ng/ml	45	146	
≥100 ng/ml	54	105	
Vascular invasion			0.094
Absent	30	102	
Present	69	149	
Tumor number			0.021
Single	69	205	
Multiple	30	46	
Tumor size			0.250
<5 cm	53	153	
≥5 cm	46	98	
Edmondson grade			0.546
I	6	23	
II	83	208	
III and IV	10	20	
Child-Pugh class			0.851
A	96	246	
B	3	5	

[a] Cutoff value for copy number ratio of the RAGE mRNA was 0.022

RNA Extraction and cDNA Synthesis

RNA extraction and cDNA synthesis were conducted as described previously.[12,13] Briefly, total RNAs were extracted from cancerous and surrounding noncancerous frozen tissues with the RNeasy Mini kit (Qiagen) with DNase I

treatment. The integrity of all tested total RNA samples was verified with a Bioanalyzer 2100 (Agilent Technologies). Samples containing 4 μg of total RNA were incubated with 2 μl of 10 μM oligo $d(T)_{18}$ primer (Genotech) at 70°C for 7 min then cooled on ice for 5 min. After adding the enzyme mix to the annealed total RNA sample, the reaction mixture was incubated at 42°C for 90 min before heat inactivation of reverse-transcriptase at 80°C for 10 min.

Quantitative Real-time PCR

Real-time PCR amplifications were conducted as described previously.[12,13] Briefly, the real-time PCR analysis was performed in a total volume of 10 μl with the ABI PRISM 7900HT (Applied Biosystems) with amplification steps as follows: an initial denaturation step at 95°C for 10 min, followed by 45 cycles of denaturation at 95°C for 15 s, and elongation at 60°C for 1 min. The primer and probe sequences were designed with Primer Express 3.0 (Applied Biosystems) and all probe sequences were labeled with FAM at the 5' end and with TAMRA at the 3' end (Supplementary Table 1). The expression levels of RAGE and MMP-9 were assessed as the threshold cycle (C_T) in triplicate and then normalized relative to those of reference genes (B2M, GAPDH, HMBS, HPRT1, and SDHA) as the internal control.[14] Using the ΔC_T values (target gene C_T − average C_T of reference genes), the mRNA copy number ratio was calculated as $2^{-\Delta Ct}$. Standard curves were constructed from the results of simultaneous amplifications of serially-diluted cDNA samples.

Cell Culture and siRNA Transfection

Human HCC cell lines (Hep3B, HepG2, Huh7, and SK-Hep1) were purchased from ATCC (Manassas, VA); SH-J1 was obtained from Dr. Dae-Ghon Kim (Chonbuk University, Korea).[15] All cell lines were maintained in Dulbecco modified Eagle medium, supplemented with 10% fetal bovine serum and 1% penicillin–streptomycin at 37°C under 5% CO_2. For siRNA transfection, RAGE-specific siRNA and negative control siRNA were purchased from Bioneer (Korea). The target sequence of RAGE siRNA was 5′-GAAACUACUAUGCAUGUAA-3′. Cells were transfected with RAGE siRNA or negative control siRNA at a final concentration of 100 nM by use of Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Cells were collected 48 h after transfection for protein extraction and subsequent analyses.

Western Blot Analysis

Western blotting was performed as described previously.[16] Anti-RAGE antibody was purchased from Abcam. Anti-β-actin was obtained from MP Biomedicals. Horseradish peroxidase–conjugated secondary antibodies were obtained from Zymed Laboratories.

Invasion Assay

Cell invasion was assessed by using a 24-well invasion chamber coated with Matrigel (BD Biosciences). Cells (1×10^4/well) were seeded onto the upper chamber with a serum-free medium. The lower chamber contained a medium with 10% fetal bovine serum. After incubation for 24 h, noninvasive cells were removed with a cotton swab and the membrane was fixed with 3.5% paraformaldehyde and stained with 0.1% crystal violet. Invasive cells were counted at 400× magnification under a microscope.

Gelatin Zymography

The sample was loaded onto an 8% polyacrylamide gel containing 0.1% gelatin for sodium dodecyl sulfate–polyacrylamide gel electrophoresis. The gel was washed with 2.5% Triton X-100 for 30 min to remove sodium dodecyl sulfate and incubated at 37°C in 50 mM Tris–HCl, 5 mM $CaCl_2$ (pH 7.6) for 24 h. The gel was then stained with 0.25% Coomassie Blue for 30 min and destained until bands became clear.

Statistical Analysis

All statistical analyses were conducted with the open source statistical programming environment R. Significant differences between gene expression levels were evaluated by Student's *t*-test. For determination of cutoff value, a walking cutoff was run from the first quartile (25th percentile) to the third quartile (75th percentile) of RAGE mRNA levels. For each cutoff value, the patient samples were subdivided into two groups, with values below or above the particular cutoff. The log rank test was performed to compare the length of overall survival (OS) in the respective groups, and the cutoff value yielding the largest difference in OS was selected. Correlation between gene expression and clinicopathologic variables was evaluated by the χ^2 test. The differences between Kaplan–Meier survival curves for OS or disease-free survival (DFS) were examined by the log rank test. The Cox proportional hazard regression model was used to identify independent prognostic factors for OS. P values of < 0.05 were considered statistically significant.

RESULTS

Overexpression of RAGE in HCC

Real-time RT-PCR analysis for RAGE mRNA was performed for a total of 350 HCCs and 115 noncancerous

hepatic samples derived from 350 HCC patients. The RAGE mRNA levels were measured in triplicate and then normalized relative to the mRNA levels of a set of five reference genes as the internal control.[14] The RAGE mRNA level was significantly higher in HCC than in noncancerous hepatic tissues ($P < 0.001$; Fig. 1a), and increased according to tumor stage (stage I vs. III and IV, $P = 0.012$; stage II vs. III and IV, $P = 0.018$; Supplementary Fig. 1a) and tumor differentiation (Edmondson grade I vs. II, $P = 0.042$; Supplementary Fig. 1b). RAGE mRNA levels were significantly higher in tumors with portal vein invasion than in those without it ($P = 0.029$; Fig. 1b) and tended to be higher in tumors with vascular invasion than in those without it ($P = 0.055$; Fig. 1c).

Correlation between mRNA levels of RAGE and Clinicopathologic Features

The correlation between mRNA levels of RAGE and major clinicopathologic features was investigated to better understand the significance of RAGE gene expression in HCC. The most statistically significant cutoff value of the RAGE mRNA level was used to discriminate patients with a good prognosis from those with the poor prognosis. A high RAGE mRNA level was correlated with the occurrence of multiple tumors ($P = 0.021$), high alfa-fetoprotein (AFP) level ($P = 0.042$), and advanced tumor stage ($P = 0.016$) but not with other clinicopathologic parameters by the χ^2 test (Table 1).

Impact of mRNA levels of RAGE on OS and DFS

During the follow-up observation period of up to 95.6 months, locoregional recurrence or distant metastases occurred in 209 patients (59.7%) and death was confirmed in 104 patients (29.7%). To assess the prognostic significance of RAGE expression, Kaplan–Meier curves for OS and DFS were analyzed. At the five-year and seven-year follow-ups, approximately 77% and 66% of the patients with low RAGE expression (< 0.022; copy number ratio) survived, whereas 62% and 51% of the patients with high RAGE expression (≥ 0.022; copy number ratio) survived, respectively (Fig. 2a). The log rank test showed that patients with high RAGE mRNA levels had a significantly shorter OS ($P = 0.029$) but no significantly different DFS compared to those with low RAGE mRNA levels (Fig. 2b). Subgroup analysis indicated that significant differences in OS time were found between high and low RAGE mRNA groups, based on categorization by the following variables: single tumor ($P = 0.040$; Supplementary Fig. 2a), and high AFP level ($P = 0.021$; Supplementary Fig. 2b).

Univariate Cox regression analysis was conducted to identify important prognostic factors of OS. High RAGE expression ($P = 0.031$) as well as several clinical features were identified as important risk factors for OS (Table 2). However, in multivariate Cox analysis, vascular invasion ($P = 0.0008$), large tumor size ($P = 0.0200$), advanced tumor stage ($P = 0.0037$), and Child-Pugh class ($P = 0.0011$) were found to be independent poor prognostic factors for OS (Table 2).

Expression of RAGE and Its Effect on Tumor Cell Invasion In Vitro

Endogenous expression levels of RAGE in HCC cell lines were evaluated by real-time RT-PCR and Western blotting. RAGE mRNA expressions were higher in SH-J1 and SK-Hep1 cells than in HepG2, Huh7, and Hep3B cells (Fig. 3a). Similar results were obtained at the protein level (Fig. 3b). Because the expression of RAGE was higher in SH-J1 and SK-Hep1 cells, which have high invasive phenotypes, than it was in the other three cell types, we investigated whether RAGE could be involved in tumor invasion.[17,18] The siRNA-mediated RAGE knockdown was

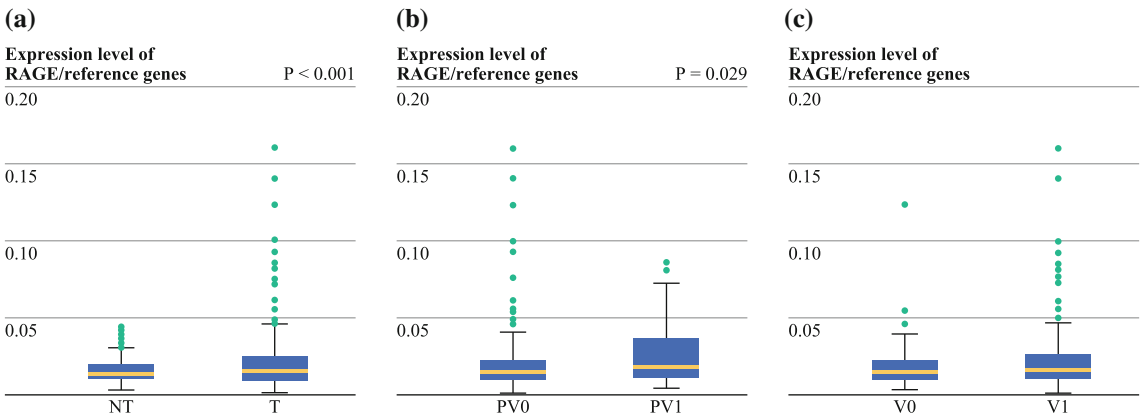

FIG. 1 Box and whiskers plot for mRNA levels of RAGE as determined by real-time RT-PCR. **a** *NT* noncancerous hepatic tissues, *T* HCC tissues. **b** *PV0* no portal vein invasion, *PV1* portal vein invasion. **c** *V0* no vascular invasion, *V1* vascular invasion

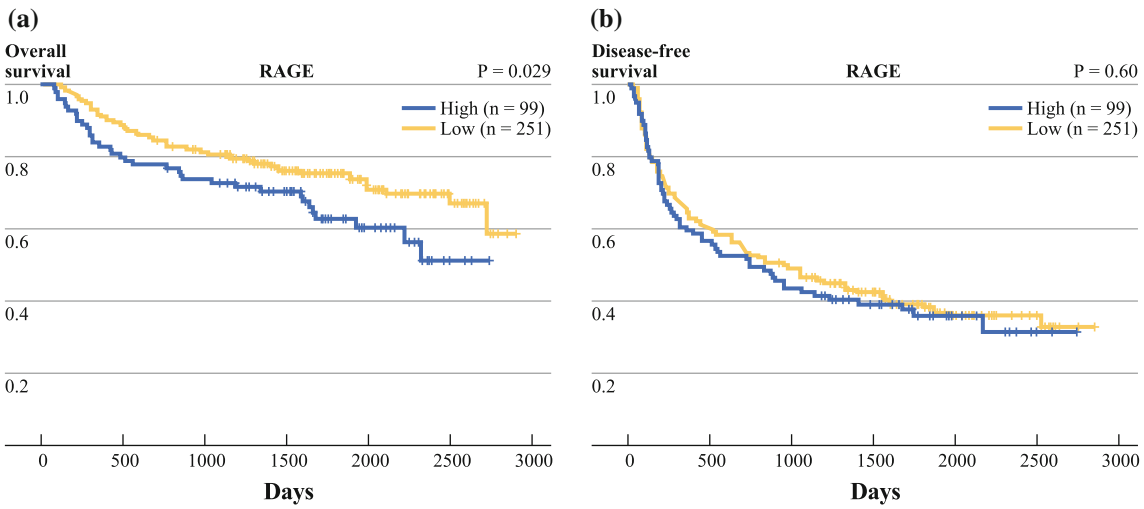

FIG. 2 Kaplan-Meier curves for OS and DFS. Patients with high RAGE mRNA levels exhibited a significantly shorter OS (**a**) but no significant difference in DFS (**b**) compared with those with low RAGE mRNA levels

TABLE 2 Univariate and multivariate Cox regression analysis for OS

Variable	OS			
	Univariate analysis		Multivariate analysis	
	HR (95% CI)	*P* value	HR (95% CI)	*P* value
Age (<55 y vs. ≥55 y)	0.77 (0.51–1.16)	0.20		
Sex (male vs. female)	0.80 (0.49–1.31)	0.38		
Edmondson grade (I–II vs. III–IV)	2.12 (1.23–3.67)	0.007	1.43 (0.81–2.50)	0.21
Hepatitis B virus (absent vs. present)	1.63 (0.97–2.74)	0.066		
Hepatitis C virus (absent vs. present)	1.57 (0.69–3.59)	0.28		
AFP level (<100 ng/ml vs. ≥100 ng/ml)	2.45 (1.64–3.65)	1.1×10^{-5}	1.24 (0.80–1.92)	0.33
Liver cirrhosis (absent vs. present)	1.39 (0.95–2.05)	0.093		
Vascular invasion (absent vs. present)	6.46 (3.46–12.1)	5.0×10^{-9}	3.20 (1.62–6.31)	0.0008
Tumor number (single vs. multiple)	4.29 (2.90–6.33)	$<1 \times 10^{-12}$	1.47 (0.84–2.59)	0.18
Tumor size (<5c m vs. ≥5 cm)	3.72 (2.47–5.61)	3.5×10^{-10}	1.76 (1.09–2.83)	0.020
Tumor stage (I–II vs. III–IV)	6.78 (4.59–10.0)	$<1 \times 10^{-12}$	2.46 (1.34–4.52)	0.0037
Child-Pugh class (A vs. B)	4.21 (1.84–9.64)	0.00066	4.26 (1.79–10.2)	0.0011
RAGE (low vs. high)	1.56 (1.04–2.33)	0.031	1.03 (0.68–1.57)	0.89

HR hazard ratio, *CI* confidence interval

efficient in SK-Hep1 cells (Fig. 3c). In the cell invasion assay, SK-Hep1 cells treated with RAGE siRNA exhibited a significantly reduced invasive activity compared with those with negative control siRNA ($P < 0.05$; Fig. 3d).

Effects of RAGE Knockdown on the Expression and Activity of MMP-9

To identify the possible role of RAGE during HCC cell invasion, we analyzed the effect of RAGE on the expression and activity of MMP-9. The mRNA level and enzyme activity of MMP-9 were significantly decreased in the cells transfected with RAGE siRNA (Fig. 4a, b). These in vitro data prompted us to further investigate the correlation between RAGE and MMP-9 expressions in HCC tissues. In the 350 cases of HCC, correlation between RAGE and MMP-9 expressions was positive and significant ($\rho = 0.18$, $P < 0.001$; Spearman's rank correlation). Furthermore, combined analysis of RAGE and MMP-9 expressions (with the median MMP-9 expression as a cutoff value) revealed a statistically significant association with OS of patients ($P = 0.0066$; Supplementary Fig. 3). Sixty patients with high expressions of both RAGE and MMP-9 had significantly shorter OS than 137 patients with low expressions of both RAGE and MMP-9 ($P = 0.00086$).

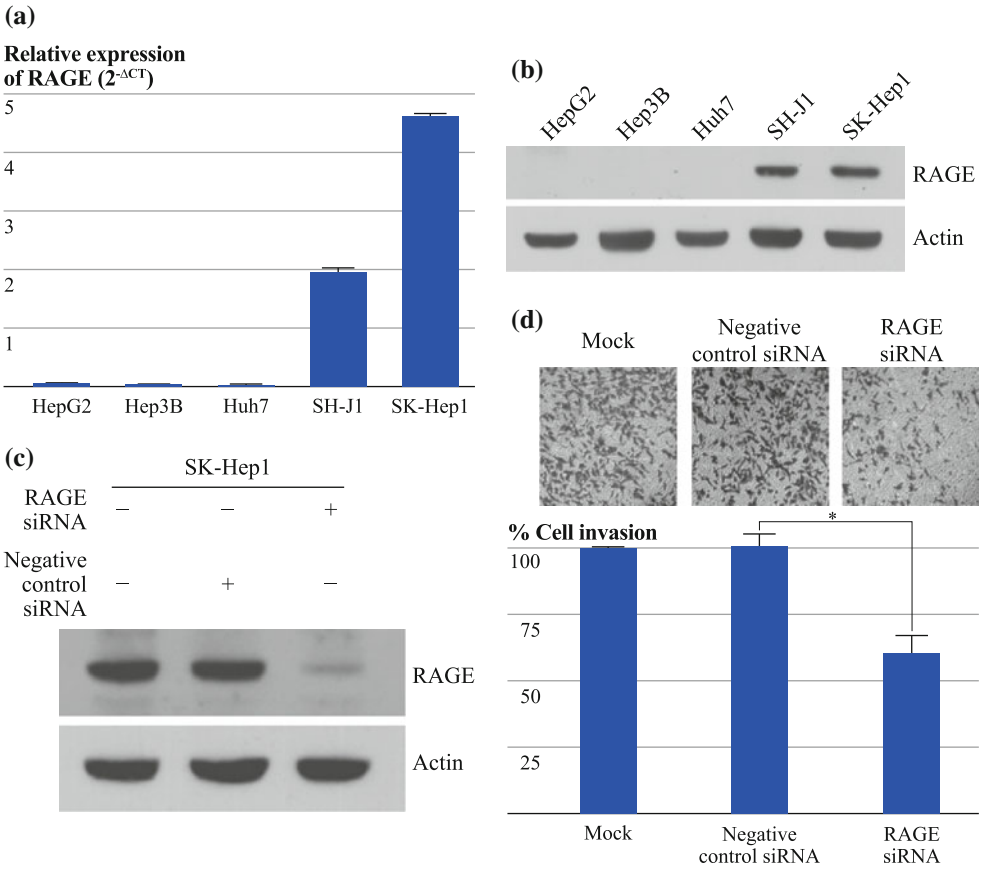

FIG. 3 Effect of the RAGE expression level on the cell invasion of HCC. Endogenous levels of RAGE mRNA and protein in five HCC cell lines were assessed by real-time RT-PCR (**a**) and Western blot test (**b**). Both mRNA and protein levels of RAGE in SK-Hep1 and SH-J1 were higher than those of other cell lines. **c** RAGE siRNA could effectively knock down endogenous RAGE expression in SK-Hep1. **d** Knockdown of RAGE expression decreased cell invasive ability of SK-Hep1 as determined by the Matrigel invasion assay

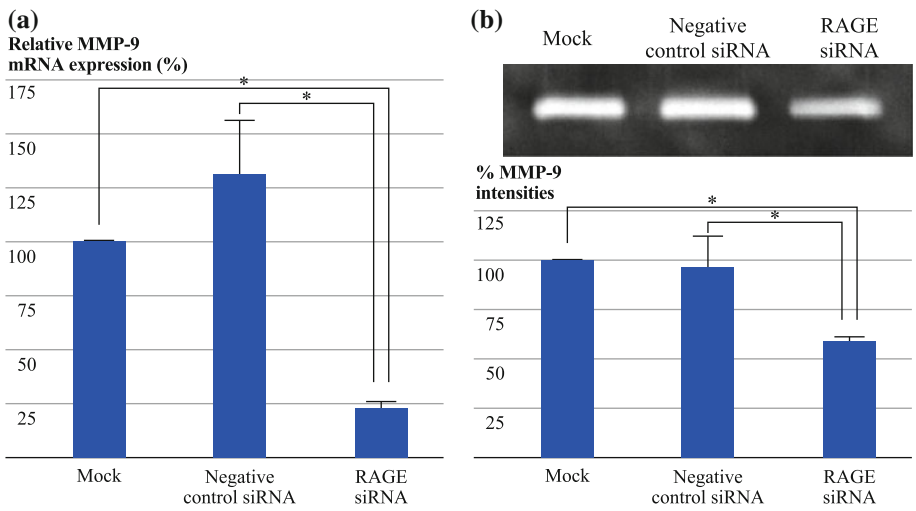

FIG. 4 Effects of siRNA for RAGE on expression and activity of MMP-9. **a** mRNA level of MMP-9 as determined by real-time RT-PCR. **b** Activity of MMP-9 in conditioned media as measured by gelatin zymography

DISCUSSION

In this study, we provide the first evidence that overexpression of RAGE is frequent in HCC and is correlated with multiple tumors, high AFP level, and advanced tumor stage, indicating that RAGE might contribute to metastatic and aggressive phenotypes of HCC. Elevated expression of RAGE was associated with the poor prognosis of patients with HCC. Furthermore, siRNA-mediated knockdown of RAGE in SK-Hep1 cells resulted in suppression of invasive potential and inhibition of both expression and activity of MMP-9. Combined analysis of RAGE and MMP-9 identified patients with poor prognosis, suggesting that expression of RAGE together with MMP-9 might be useful for predicting prognosis of HCC patients.

Although various prognostic markers are under investigation for HCC, novel and better prognostic markers are greatly needed.[19,20] We examined the relationship between RAGE mRNA level and clinicopathologic characteristics as well as postoperative survival of the patients. RAGE mRNA level in HCC was significantly higher than those in the adjacent noncancerous lesions. HCC patients with high RAGE mRNA levels showed significantly shorter OS than those with low RAGE mRNA levels, although the RAGE mRNA level was not an independent prognostic factor as judged by multivariate survival analysis. We found that the RAGE mRNA level was correlated with high serum AFP levels. Previous studies suggested that high levels of AFP could be associated with metastasis and poor survival in patients with HCC.[21,22] Our data suggest that the overexpression of RAGE may be correlated with a metastatic phenotype and poor prognosis of HCC.

Metastasis and postsurgical recurrence are major causes of death and poor clinical outcomes in patients with HCC.[4,23,24] Cell invasion through the basement membrane is considered to be a critical step in the metastasis of HCC.[25] Therefore, we performed a reconstituted basement membrane invasion assay in vitro. Inhibition of RAGE by siRNA resulted in decreased invasion of SK-Hep1 cells, suggesting that RAGE could be a positive regulator of cellular invasion. Consistent with these findings, mRNA and protein levels of RAGE were elevated in SK-Hep1 and SH-J1, which are highly invasive HCC cell lines. Furthermore, HCC tissues with portal vein invasion showed significantly higher level of the RAGE expression than those without portal vein invasion, and HCCs with vascular invasion tended to show higher levels of RAGE than those without vascular invasion. Vascular invasion, especially portal vein invasion, is a significant predictor of intrahepatic metastasis and poor prognosis of HCC.[26,27] On the basis of our in vitro and clinical data, we suggest that the overexpression of RAGE should be an important factor related to the invasive potential of HCC and to poor clinical outcome.

MMP-9 has been shown to be important in the invasion of malignant tumors by degrading the extracellular matrix and the basement membrane.[28] Consistent with these findings, several studies showed that MMP-9 was highly expressed in HCC with invasive potential and might be involved in progression and invasion of HCC.[29,30] In addition, elevated plasma levels of MMP-9 were observed in HCC patients with portal vein invasion.[31] We observed that the siRNA-mediated RAGE knockdown in HCC cells decreased both mRNA level and enzymatic activity of MMP-9. Our results suggest the potential involvement of RAGE in regulating the expression of MMP-9. Consistent with in vitro findings, we found a positive correlation between RAGE and MMP-9 expressions in the clinical specimens of HCC. More importantly, combined analysis of RAGE and MMP-9 expressions could identify patients with poor prognosis. Taken together, our data indicate that RAGE can regulate the invasive potential of HCC, at least in part, by modulating MMP-9.

In conclusion, our study demonstrated overexpression of RAGE in HCC and its correlation with poor prognosis and aggressive clinicopathologic features of HCC. Moreover, downregulation of RAGE reduced the invasiveness of HCC cells by regulating the expression and activity of MMP-9. RAGE, especially in combination with MMP-9, merits further study as a prognostic marker and a novel therapeutic target for HCC.

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