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NRAGE promotes the malignant phenotype of hepatocellular carcinoma

DAI SHIMIZU[1], MITSURO KANDA[1], HIROYUKI SUGIMOTO[1], SATOSHI SUEOKA[1], HIDEKI TAKAMI[1], KAZUHIRO EZAKA[1], YURI TANAKA[1], RYOJI HASHIMOTO[1], YUKIYASU OKAMURA[2], NAOKI IWATA[1], CHIE TANAKA[1], SUGURU YAMADA[1], TSUTOMU FUJII[1], GORO NAKAYAMA[1], MASAHIKO KOIKE[1], SHUJI NOMOTO[3], MICHITAKA FUJIWARA[1] and YASUHIRO KODERA[1]

[1]Department of Gastroenterological Surgery (Surgery II), Nagoya University Graduate School of Medicine, Nagoya, Aichi 466-8550; [2]Department of Hepatobiliary-Pancreatic Surgery, Shizuoka Cancer Center, Shizuoka 411-8777; [3]Department of Surgery, Aichi-Gakuin University School of Dentistry, Nagoya, Aichi 464-8651, Japan

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Abstract. Hepatocellular carcinoma (HCC) is a fatal disease, primarily due to the limited effective therapies available for patients with advanced or recurrent stages of the disease. Therefore, in order to improve patient prognosis, it is important to identify an informative biomarker for HCC progression, as well as a molecular target for therapy. Neurotrophin receptor-interacting melanoma antigen-encoding protein (*NRAGE*), a member of the type II melanoma-associated antigen family, mediates apoptosis and cell death through interactions with a wide range of proteins, and is implicated as a tumor suppressor or oncoprotein depending on cell type. However, the role of *NRAGE* in HCC is currently unknown, therefore, the present study aimed to identify the underlying function of *NRAGE* in HCC tumorigenesis. Resected tumor and non-cancerous liver tissues from 151 patients with HCC, alongside HCC cell lines, were analyzed by polymerase chain reaction and immunohistochemical techniques to determine NRAGE expression levels, as well as the expression levels of potential genes encoding interacting proteins. It was demonstrated that the expression levels of *NRAGE* mRNA correlated significantly with those of apoptosis-antagonizing transcription factor (*AATF*), and were not affected by cirrhosis in non-cancerous liver tissues when compared to elevated levels in HCC tissues. The expression patterns of NRAGE protein and mRNA were consistent among 30 representative specimen pairs. Furthermore, increased *NRAGE* expression in patients with HCC correlated significantly with a shorter disease-specific survival time, and was identified as an independent prognostic factor via multivariate analysis (hazard ratio, 2.23; 95% confidence interval, 1.06-3.83; P=0.020). Therefore, the results of the present study indicated that increased *NRAGE* expression affects HCC progression via its interaction with *AATF*, and may represent a novel biomarker and molecular target for the treatment of HCC.

Correspondence to: Dr Mitsuro Kanda, Department of Gastroenterological Surgery (Surgery II), Nagoya University Graduate School of Medicine, 65 Tsurumai-cho, Showa-ku, Nagoya, Aichi 466-8550, Japan
E-mail: m-kanda@med.nagoya-u.ac.jp

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Introduction

Hepatocellular carcinoma (HCC) is one of the most prevalent malignant tumors, with high rates of mortality and morbidity (1,2). The worldwide incidence of HCC is ~1,000,000 cases per year and is equivalent to its mortality rate (3). Furthermore, HCC ranks as the third highest cause of cancer-associated mortality (4). Such statistics are largely explained by the difficulties faced when diagnosing HCC, with diagnosis often only confirmed once the disease is at a late stage and palliative care is the only treatment option available. An important strategy to improve patient outcome involves the identification of disease risk factors and the maintenance of vigilant surveillance of high-risk individuals to allow for recognition of the disease at a stage that is responsive to treatment with curative intent. Therefore, it is fundamental that high-risk groups are identified, followed by successful implementation of a surveillance program and recall protocol following any abnormal findings (5-8). Progress has been made in identifying molecular markers for the initiation and progression of HCC that will likely increase the rate of early and potentially life-saving diagnoses (9-12). The expanding amount of knowledge regarding the molecular foundations of HCC makes it increasingly clear that successful therapy requires treatment tailored to the individual patient (8).

Melanoma-associated antigens (MAGEs), members of the cancer/testis antigen family that consists of >50 proteins, are divided into two types, MAGE-I and MAGE-II, dependent on varying gene structures and tissue-specific expression patterns (13,14). The MAGE-I subgroup consists of products yielded by numerous X chromosome clustered genes, including *MAGE-A, -B* and *-C*, which are typically expressed in cancer

cells of various origins, but not in adult tissues, with the exception of germ-line cells in the placenta, ovaries and testes (15,16). By contrast, the MAGE-II subgroup, including *MAGE-D* variants, do not have defined chromosome clustering and are cancer non-specific, with near universal expression in normal adult tissues and germ-line cells (14,16,17). MAGE proteins, as tumor-associated antigens, have attracted increasing attention regarding the development of vaccine-based immunotherapy for the treatment of cancer (18). Our strategy for addressing this issue involves detailed analysis of the literature to identify potential markers and targets for therapy of HCC (15). For example, a previous meta-analysis indicated that glypican-3, des-γ-carboxyprothrombin, α-L-fucosidase and vascular endothelial growth factor may serve as suitable serological markers for HCC (19). Meta-analyses are incisive and may potentially be more informative compared with omic surveys that are expensive, technology-intensive and arguably more time-consuming. This lead the current study to focus on the 86-kDa NRAGE protein, also known as MAGE-D1, which is encoded by the *NRAGE* gene located on the X chromosome (20-22). Cells of diverse embryonic and adult tissues, particularly those of the nervous system, express NRAGE, which subsequently interacts with proteins that regulate cell adhesion and migration, the cell cycle, cell differentiation, apoptosis and gene transcription (22-24). However, little is understood regarding the physiological relevance of these interactions.

NRAGE serves a role in the process of apoptosis through interactions with the p75 neurotrophin receptor (p75NTR) (25) and apoptosis-antagonizing transcription factor (AATF) (26,27). As NRAGE interacts with proteins with diverse functions, it is not unexpected that its effects are cell-type specific. For example, the downregulation of *NRAGE* transcription serves an important function in apoptosis, and when expressed ectopically, NRAGE inhibits the proliferation of breast cancer cells (27). Furthermore, downregulation of NRAGE in colorectal cancer is associated with a negative clinical course (24,28,29). By contrast, *NRAGE* functions as an oncogene in esophageal and lung cancers (22,30). Yang *et al* (22) demonstrated that the overexpression of *NRAGE* exerts tumor-promoting effects by interacting with the DNA polymerase III domain of proliferating cell nuclear antigen (*PCNA*) and consequently inhibits K48-polyubiquitin chain-mediated proteasome degradation of PCNA in esophageal cancer. However, to the best of our knowledge, there are currently no studies concerning *NRAGE* expression in HCC or its role in the pathogenesis of this disease. Therefore, the aim of the present study was to assess the clinical significance of *NRAGE* expression in HCC, as well as its relevance as a novel biomarker for tumor progression.

Materials and methods

Sample collection. The HCC cell lines (Hep3B, HepG2, HLE, HLF, HuH1, HuH2, HuH7, PLC/PRF/5 and SK-Hep1) were obtained from the American Type Culture Collection (Manassas, VA, USA). All cell lines were cultured in Dulbecco's modified Eagle's Medium (Sigma-Aldrich, St. Louis, MO, USA), supplemented with 10% fetal bovine serum at 37˚C in an atmosphere containing 5% CO_2 (6). The primary HCC tissues and the corresponding non-cancerous tissues were collected from 151 patients with HCC who had undergone liver resection at the Nagoya University Hospital (Nagoya, Japan) between January 1998 and July 2012. The specimens were classified histologically according to the criteria published in the Classification of Malignant Tumours, Union for International Cancer Control (UICC) (31). Clinicopathological data were collected from medical records, and written informed consent for the use of clinical samples and data was obtained from all patients, as required by the Institutional Review Board of Nagoya University, Japan (32).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Expression levels of mRNA in all samples were analyzed using RT-qPCR, which was performed using an Applied Biosystems StepOne Plus (Thermo Fisher Scientific, Inc., Waltham, MA, USA) in triplicate. Total RNA (10 μg) isolated from the HCC cell lines, and the 151 primary HCC specimens and corresponding adjacent non-cancerous tissues, was used as the template for the synthesis of complementary DNA. RT-qPCR was performed using the SYBR® Green PCR Core Reagents kit (Applied Biosystems; Thermo Fisher Scientific, Inc.) and specific primers for NRAGE (Hokkaido System Science Co., Ltd., Tokyo, Japan) as follows: One cycle at 95˚C for 10 min, 40 cycles at 95˚C for 5 sec and 60˚C for 30 sec. For standardization, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA (TaqMan GAPDH Control Reagents; Applied Biosystems; Thermo Fisher Scientific, Inc.) was quantified in each sample. Expression levels are presented as the value of NRAGE mRNA divided by the value of GAPDH mRNA (33,34). Expression levels of mRNAs were normalized by the serially diluted standards (35). AATF, p75NTR and PCNA encode proteins that may interact with NRAGE, and the specific primers (Hokkaido System Science Co., Ltd.) used for each of these genes are listed in Table I.

Immunohistochemistry (IHC). IHC was performed to determine the expression and localization of NRAGE in 30 representative well-preserved HCC samples. Formalin-fixed, paraffin-embedded tissue samples were dewaxed twice in xylene for 5 min, rehydrated sequentially using a graded series of alcohol concentrations (100, 90 and 70%) for 2 min each and treated with 3% H_2O_2 to inhibit endogenous peroxidases. Antigen retrieval was performed by incubating the sections 5 times in citrate buffer (10 mM) at 95˚C for 5 min. The samples were then washed with phosphate-buffered saline, followed by a 10-min incubation with biotinylated goat anti-rabbit IgG secondary antibody (Histofine SAB PO(R) kit; Code 424032; Nichirei Corporation, Tokyo, Japan) for 5 min to limit non-specific reactivity, and incubated for 1 h with a rabbit polyclonal anti-human NRAGE antibody (catalog no., LS-C100414; LifeSpan BioSciences, Inc., Seattle, WA, USA) in a 1:200 dilution with ChemMateT antibody diluent (Dako Japan Co., Ltd., Tokyo, Japan). The samples were then washed with phosphate-buffered saline, followed by a 10-min incubation with biotinylated goat anti-rabbit IgG secondary antibody (Histofine SAB-PO(R) kit; Nichirei Corporation) in a 1:1,000 dilution with ChemMateT antibody dilutent. Subsequently, the sections were incubated for 1 min

Table I. Primers and annealing temperatures.

Gene	Oligo sequence (5'-3')	Product size, bp	Annealing temperature, ˚C
NRAGE			
Forward	GATTCCCTCAGACCTTTGC	170	60
Reverse	GAAGGAATCTGAGGCTTCAG		
AATF			
Forward	ACAAAGGTGGCCCAGAATTT	103	62
Reverse	TGGAAAAGCAACTCTTCCTGA		
p75NTR			
Forward	CTGCTGCTGTTGCTGCTTCT	98	60
Reverse	CAGGCTTTGCAGCACTCAC		
PCNA			
Forward	TGCAAGTGGAGAACTTGGAA	128	58
Reverse	TCAGGTACCTCAGTGCAAAAG		
GAPDH			
Forward	GAAGGTGAAGGTCGGAGTC	226	60
Probe	CAAGCTTCCCGTTCTCAGCC		
Reverse	GAAGATGGTGATGGGATTTC		

NRAGE, neurotrophin receptor-interacting melanoma antigen-encoding protein; AATF, apoptosis-antagonizing transcription factor; p75NTR, p75 neurotrophin receptor; PCNA, proliferating cell nuclear antigen; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

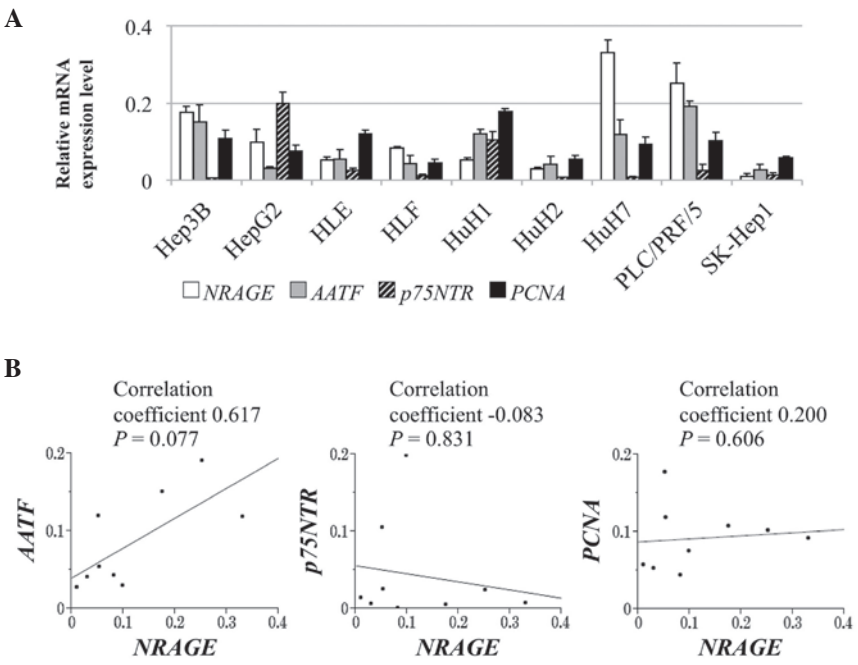

Figure 1. Analysis of *NRAGE* mRNA expression in the HCC cell lines. (A) Levels of *NRAGE* mRNA and its putative interacting partners in the HCC cell lines. Error bars indicate the standard deviation of three biological replicates. (B) Analysis of the correlation between *NRAGE* mRNA level and those of its putative interacting partners *AATF*, *p75NTR* and *PCNA*. *NRAGE*, neurotrophin receptor-interacting melanoma antigen-encoding protein; HCC, hepatocellular carcinoma; *AATF*, apoptosis-antagonizing transcription factor; *p75NTR*, p75 neurotrophin receptor; *PCNA*, proliferating cell nuclear antigen.

with liquid 3,3'-diaminobenzidine (Nichirei Corporation) to detect antigen-antibody complexes. Staining of NRAGE was evaluated using vessels as internal controls. To avoid bias when interpreting data, specimens were randomized and coded prior to analysis by two independent observers who were uninformed of the status of the samples. Each observer evaluated all specimens at least twice within a given time interval to minimize intraobserver variation (36,37).

Statistical analysis. The significance of the association between the levels of *NRAGE* mRNA and the clinicopathological features was evaluated using the χ^2 test, and differences between

Table II. Association between *NRAGE* mRNA levels and clinicopathological parameters of 151 hepatocellular carcinoma patients.

Clinicopathological parameters	Increased expression of *NRAGE* mRNA, n	Others, n	P-value
Age, years			0.014[a]
<65	34	33	
≥65	59	25	
Gender			0.096
Male	74	52	
Female	19	6	
Background of liver			0.628
Normal	6	4	
Chronic hepatitis	51	36	
Cirrhosis	36	18	
Child-Pugh classification			0.621
A	87	53	
B	6	5	
Hepatitis virus			0.728
Absent	17	13	
HBV	22	15	
HCV	54	30	
AFP, ng/ml			0.020[a]
≤20	43	38	
>20	50	20	
PIVKA II, mAU/ml			0.350
≤40	33	25	
>40	60	33	
Tumor multiplicity			0.707
Solitary	73	44	
Multiple	20	14	
Tumor size, cm			0.733
<3.0	28	19	
≥3.0	65	39	
Differentiation			0.314
Well	19	16	
Moderate to poor	74	42	
Growth type			0.208
Expansive	81	46	
Invasive	12	12	
Serosal infiltration			0.389
Absent	68	46	
Present	25	12	
Formation of capsule			0.289
Absent	26	21	
Present	67	37	
Infiltration to capsule			0.101
Absent	37	31	
Present	56	27	
Septum formation			0.407
Absent	35	18	
Present	58	40	
Vascular invasion			0.386
Absent	68	46	
Present	25	12	

Table II. Continued.

Clinicopathological parameters	Increased expression of *NRAGE* mRNA, n	Others, n	P-value
UICC pathological stage			0.919
I	59	35	
II	23	16	
III	11	7	

[a]P<0.05. HCC, hepatocellular carcinoma; NRAGE, neurotrophin receptor-interacting melanoma antigen-encoding protein; HBV, hepatitis B virus; HCV, hepatitis C virus; AFP, α-fetoprotein; PIVKA, protein induced by vitamin K antagonists; UICC, Union for International Cancer Control.

Table III. Prognostic factors of 151 patients with hepatocellular carcinoma.

		Univariate analysis			Multivariate analysis		
Variable	n	Hazard ratio	95% CI	P-value	Hazard ratio	95% CI	P-value
Age ≥65 years	84	1.92	1.07-3.57	0.030[a]	1.39	0.75-2.65	0.301
Male gender	126	1.27	0.60-3.13	0.553			
Cirrhosis of liver	4	1.58	0.88-2.81	0.123			
Child-Pugh classification B	11	0.93	0.28-2.32	0.889			
AFP >20 ng/ml	70	1.90	1.07-3.42	0.029[a]	1.30	0.70-2.44	0.790
PIVKA II >40 mAU/ml	93	2.10	1.14-4.07	0.016[a]	1.12	0.55-2.40	0.770
Tumor multiplicity	34	2.09	1.11-3.76	0.023[a]	1.32	0.67-2.53	0.416
Tumor size ≥3.0 cm	104	2.20	1.13-4.71	0.020[a]	1.87	0.82-4.64	0.140
Well-differentiated tumor	35	0.55	0.25-1.10	0.095			
Invasive growth	24	1.44	0.69-2.76	0.318			
Serosal infiltration	37	2.51	1.32-4.61	0.006[a]	1.53	0.76-2.95	0.225
Formation of capsule	104	1.05	0.57-2.02	0.884			
Infiltration to capsule	83	1.20	0.67-2.18	0.537			
Septum formation	98	0.87	0.49-1.60	0.651			
Vascular invasion	37	3.40	1.87-6.07	<0.001[a]	2.24	1.12-4.41	0.022[a]
Increased *NRAGE* expression	93	2.42	1.27-4.99	0.006[a]	2.23	1.06-3.83	0.020[a]

Univariate analysis was performed using the log-rank test. Multivariate analysis was performed using the Cox proportional hazards model. [a]P<0.05. CI, confidence interval; AFP, α-fetoprotein; PIVKA, protein induced by vitamin K antagonists; NRAGE, neurotrophin receptor-interacting melanoma antigen-encoding protein.

groups were evaluated using the Mann-Whitney U test. Correlations between the level of *NRAGE* mRNA and encoding *AATF*, *p75NTR* and *PCNA*, as well as those of pre-operative serum tumor markers, were analyzed using Spearman's rank correlation coefficient. Disease-specific survival rates were calculated using the Kaplan-Meier method, and the differences in survival curves were evaluated using the generalized Wilcoxon rank-sum test. P<0.05 was considered to indicate a statistically significant difference. All statistical analyses were performed using JMP® software, version 10 (SAS Institute Inc., Cary, NC, USA).

Results

Analysis of NRAGE, AATF, p75NTR and PCNA mRNA expression in HCC cell lines. The relative levels of *NRAGE* mRNA and those of its putative interacting partners, *AATF*, *p75NTR* and *PNCA*, in the HCC cell lines are presented in Fig. 1A. Heterogeneity was observed in the *NRAGE* mRNA levels between 9 HCC cell lines, which correlated significantly with those of *AATF* (correlation coefficient, 0.617), whereas no significant correlation was observed between the expression of *NRAGE* and *p75NTR* or *PCNA* (Fig. 1B).

Patient characteristics. The ages of the 151 patients ranged from 34-84 years (mean ± standard deviation, 64.7±9.8 years), and the male to female ratio was 5:1. A total of 37 and 84 patients were infected with hepatitis B and C virus, respectively. The numbers of patients with a normal liver, chronic hepatitis or cirrhosis were 10, 87 and 54, respectively. When classified according to the UICC's tumor-node-metastasis classification, 94, 39 and 18 patients were in stages I, II and III, respectively.

Analysis of NRAGE and AATF mRNA levels in surgically resected liver tissues. There were no significant differences

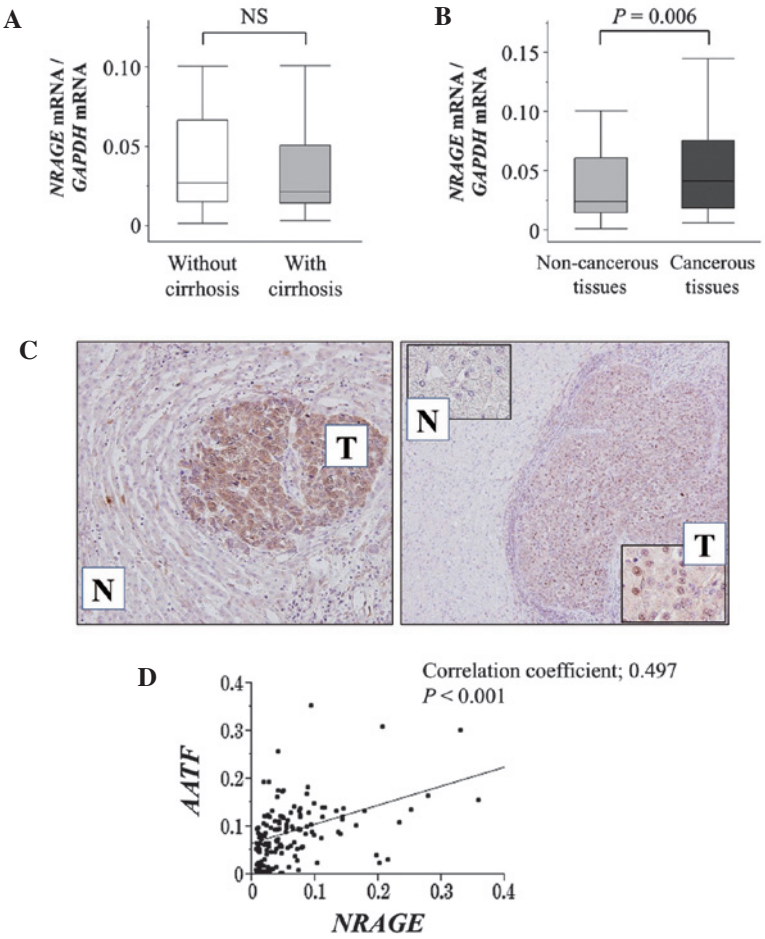

Figure 2. Analysis of *NRAGE* expression in the liver tissues. (A) *NRAGE* mRNA levels did not differ significantly between non-cancerous liver tissues of patients with and without cirrhosis. (B) NRAGE mRNA levels were significantly elevated in the HCC tissues compared with the corresponding non-cancerous liver tissues. (C) Two representative cases demonstrating strong immunoreactivity of *NRAGE* specific to the HCC tissues (magnification: Left, x200; right, x100; and inset, x400). (D) Analysis of the correlation between *NRAGE* and *AATF* mRNA levels in the HCC tissues. HCC, hepatocellular carcinoma; NRAGE, neurotrophin receptor-interacting melanoma antigen-encoding protein; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; N, normal; T, tumor; AATF, apoptosis-antagonizing transcription factor.

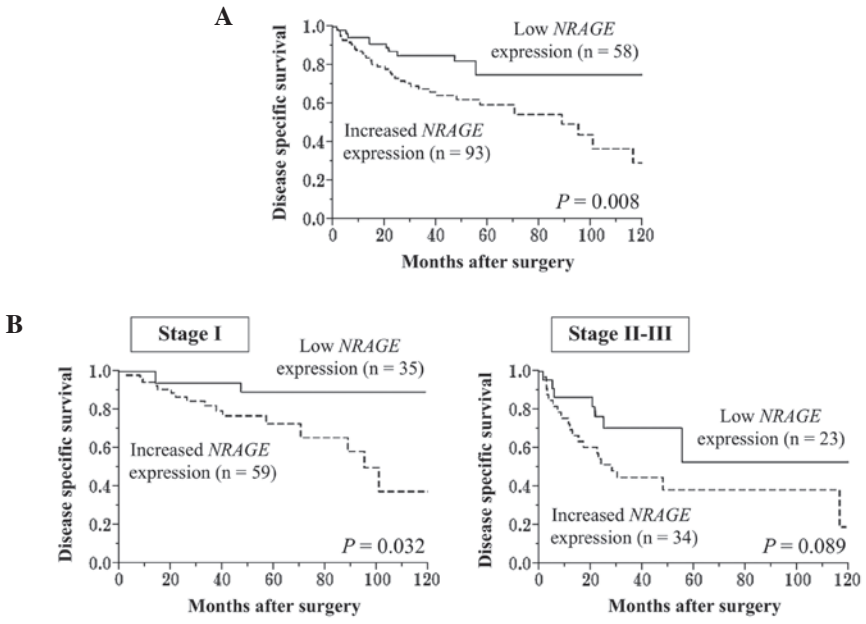

Figure 3. Patient survival analysis. (A) Patients with increased expression of *NRAGE* mRNA experienced significantly shorter disease-specific survival times. (B) Subgroup analysis as a function of Union for International Cancer Control tumor-node-metastasis staging. Increased levels of NRAGE mRNA were associated with shorter survival times in patients with stages I or II-III HCC. HCC, hepatocellular carcinoma; NRAGE, neurotrophin receptor-interacting melanoma antigen-encoding protein.

observed in the *NRAGE* mRNA levels in the non-cancerous tissue samples between patients with or without cirrhosis (Fig. 2A). However, the mean level of *NRAGE* mRNA in the HCC tissues was significantly higher compared with that in the corresponding normal tissues (P=0.006; Fig. 2B).

IHC analysis was conducted to determine whether NRAGE expression correlated with *NRAGE* mRNA levels in the tumor and non-cancerous tissues. Representative images showing strong staining intensities of NRAGE in cancer tissues are presented in Fig. 2C. The expression patterns of *NRAGE* protein and mRNA in the HCC and non-cancerous tissues were consistent among 30 representative pairs of specimens. It was observed that the level of *NRAGE* and *AATF* mRNA was directly correlated, consistent with results of the analysis of the HCC cell lines (Fig. 2C).

Clinical significance of NRAGE mRNA expression. Tissue sections were assigned to two groups (high and low expression) according to their level of *NRAGE* mRNA. The high and low expression groups included 93 and 58 patients, respectively. The high expression group was associated with increased age and an α-fetoprotein level of >20 ng/ml (P=0.014 and P=0.020, respectively; Table II). The disease-specific survival time of the patients was significantly shorter in the high expression group compared with the low expression group (5-year survival rate, 60 vs. 75%; P=0.008; Fig. 3A). Subgroup analysis according to UICC staging indicated that the disease-specific survival curves showed a similar tendency of differences between high and low NRAGE expression groups in each disease stage (Fig. 3B). Multivariate analysis identified vascular invasion and increased *NRAGE* expression as independent prognostic factors (hazard ratios, 2.24 and 2.23, respectively; Table III).

Discussion

Originally identified by Salehi *et al* (25) in a two-hybrid screen utilizing *p75NTR* as bait, *NRAGE* is a signaling cascade component that mediates apoptosis by interacting with *p75NTR* to antagonize its association with the nerve growth factor receptor tropomyosin receptor kinase A. Studies have indicated that *NRAGE* promotes apoptosis through the ubiquitination of *AATF* (26,27), therefore suggesting that *NRAGE* functions as a tumor suppressor by inducing tumor cell apoptosis. However, overexpression of *NRAGE* accelerates the proliferation and migration of esophageal cancer cells via interactions with *PCNA*. A genome-wide association study demonstrated that the initiation of *NRAGE* signals through the *JNK* pathway is associated with non-small cell lung cancer (22,30). Xue *et al* (38) identified an association between elevated *NRAGE* expression and the increased radioresistance of esophageal carcinoma cells. Such studies indicate that *NRAGE* functions to inhibit or promote oncogenesis depending on cell type, leading to the rationale behind the present study.

The current study demonstrated that *NRAGE* mRNA levels positively correlated with those of *AATF*, but not those of *p75NTR* or *PCNA*. The presence of cirrhosis had a minimal affect on *NRAGE* expression, and *NRAGE* mRNA levels were significantly higher in the HCC tissues compared with the corresponding non-cancerous tissues; this suggests that *NRAGE* is involved in hepatocarcinogenesis or the subsequent progression of HCC.

Multivariate analysis of disease-specific survival time following curative hepatectomy and subgroup analysis, according to UICC staging, identified that increased *NRAGE* mRNA expression in the HCC tissues functioned as an independent prognostic factor. These results indicate that *NRAGE* serves as a tumor promoting factor, and that the levels of *NRAGE* mRNA in resected primary lesions may function as a biomarker for the progression of HCC. Furthermore, the results of IHC analysis demonstrated that *NRAGE* expression correlated with mRNA level, subsequently indicating the physiological significance of the latter. Therefore, future investigation into the function of *NRAGE* in HCC may be facilitated using RT-qPCR analysis of *NRAGE* mRNA levels.

In conclusion, NRAGE mediates the progression of HCC and may serve as a novel biomarker for the malignant phenotype of the disease. The present study indicated that the analysis of *NRAGE* expression may enhance the clinical management of HCC. For example, the levels of *NRAGE* mRNA in liver biopsies or surgically resected tissues may facilitate risk stratification of patients with HCC, and may also serve as a criterion for determining the most appropriate therapy tailored to individual patients. Furthermore, the findings of the current study demonstrate promise for the development of novel therapies for HCC that employ small molecules or antibodies that target *NRAGE* and its interacting partners, including *AATF*. Research to further investigate the signaling pathways that are regulated by or function through *NRAGE* may expose alternative targets for the treatment of HCC.

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