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High mobility group box-1-inducible melanoma inhibitory activity is associated with nodal metastasis and lymphangiogenesis in oral squamous cell carcinoma

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Melanoma inhibitory activity (MIA) is an 11-kDa secretory protein isolated from malignant melanoma cells that is correlated with invasion and metastasis in various human malignancies. We examined MIA expression in 62 oral squamous cell carcinomas (OSCC) by immunohistochemistry. MIA expression was significantly associated with nodal metastasis ($P = 0.00018$). MIA expression was also associated with expression of high mobility group box-1 (HMGB1) ($P < 0.0001$) and lymph vessel density ($P < 0.0001$). Expression levels of MIA, HMGB1, nuclear factor kB (NFkB) p65 and HMGB1–NFkB p65 binding were significantly higher in a metastatic human OSCC cell line (HSC3) than those in a non-metastatic OSCC cell line (HSC4). Treatment with receptor for advanced glycation end products (RAGE) antisense or small interfering RNA and human recombinant HMGB1 (hrHMGB1) did not affect MIA expression, whereas HMGB1 antisense or siRNA treatment decreased MIA expression in HSC3 cells. Then HMGB1 enhanced MIA expression as an NFkB cofactor but not as a RAGE ligand. MIA neutralization by MIA antibodies increased extracellular signal-related kinase 1/2 phosphorylation, but decreased p38 phosphorylation and the expression of vascular epithelial growth factor (VEGF)-C and -D. Treatment with p38 inihibitor decreased VEGF-C and -D expression in HSC3 cells. These results suggest that MIA expression is enhanced by the interaction of intracellular HMGB1 and NFkBp65 and MIA is closely involved in tumor progression and nodal metastasis by the increments of VEGF-C and VEGF-D in OSCC. (*Cancer Sci* 2008; 99: 1806–1812)

Head and neck cancer is the sixth most common malignancy worldwide and the first leading cause of cancer death in South Asia.[1] About 300 000 patients develop OSCC every year in the world.[2,3] OSCC has a high potential for nodal metastasis and locoregional invasion,[4] from which over 50% of patients die.[5,6] To control lymph-node metastasis of OSCC, we need to study the molecular aspects of the mechanism of metastasis.

MIA is an 11-kDa secretory protein isolated from supernatants of HTZ-19 malignant melanoma cells,[7,8] the gene locus of which is mapped to chromosome 19q13.32–13.33.[9] Although previous reports indicated that MIA is correlated with invasion and metastasis in malignant melanoma,[10–12] breast cancer,[10] chondrosarcoma,[13] glioma,[14] and pancreatic cancer,[15] the definite functions of MIA for cancer cells are still unclear.

HMGB1 has a dual role as an extracellular secretory protein and a chromosomal structural protein.[16] HMGB1 works as a cytokine or a growth factor in neural ontogenesis, septic inflammation and neoplasm. HMGB1 is also considered an amphoterin, which is isolated as a motility factor in neurite outgrowth.[17] We previously reported coexpression of HMGB1 and receptor for advanced glycation end products (RAGE), which is a major membrane receptor for HMGB1 and is significantly associated with tumor progression and metastasis[18–24] and suppression of tumor-associated macrophages.[25,26] As a chromatin structural protein, HMGB1 participates in gene expression, DNA repair and functions of the p53 family.[27] Recently, HMGB1 was revealed to interact with NFkB p65 to accelerate MIA expression.[28,29] HMGB1 and NFkB p65 concurrently bind to a 30-bp region in the promoter region of the MIA gene designated as the highly conserved region (HCR).

MIA is suspected to play an important role as a pro-metastatic factor in HMGB1-overexpressing cancers. In the present study, we analyzed the relationship between MIA expression and nodal metastasis and HMGB1 expression in human OSCC.

Materials and Methods

Tumor specimens. Sixty-two formalin-fixed, paraffin-embedded specimens of primary OSCC were randomly selected at Nara Medical University Hospital, Kashihara, Japan. None of the samples was treated using neo-adjuvant therapy. Medical records and prognostic follow-up data were obtained from the patient database administered by the hospital. None of the patients was treated before surgery and sample preparation.

Immunohistochemistry. Consecutive 4-μm sections were cut from each block. Immunohistochemistry was performed as previously described.[24] The sections were subjected to antigen retrieval with pepsin (DAKO, Carpinteria, CA, USA) treatment for 20 min and the immunoperoxidase technique was used in immunostaining. After blockade of endogeneous peroxidase activity by incubation in 3% hydrogen peroxide–methanol for 15 min, the sections were rinsed with phosphate-buffered saline (PBS) and incubated with diluted primary antibodies: Anti-MIA antibody,[10] anti-HMGB1 antibody (Upstate biotechnology, Lake Placid, NY, USA; diluted to 0.5%) and anti-D2-40 antibody (a marker for lymph duct endothelial cells recognizing sialo-glycoprotein type O, Signet Laboratories Inc., Dedham, MA, USA; diluted to 0.5%). After 2 h incubation at room temperature, they were rinsed again with PBS and treated for an hour with the secondary antibody peroxidase-conjugated antigoat (Medical & Biological Laboratories, Nagoya, Japan) or antirabbit

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Abbreviation: OSCC, oral squamous cell carcinoma; MIA, melanoma inhibitory activity; HMGB1, high mobility group box-1; RAGE, receptor for advanced glycation end products; NFkB, nuclear factor kB.

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(Medical & Biological Laboratories) diluted to 0.5%. All sections were then rinsed with PBS, color-developed using diaminobenzidine (DAB) solution (DAKO), washed in water and counterstained with Meyer's hematoxylin (Sigma Chemical, St. Louis, MO, USA). Immunostaining of all samples was performed under the same conditions of antibody reaction and DAB exposure.

Evaluation of immunoreactivity. Immunoreactivity was classified according to Allred's score (AS).[30] We divided the immunoreactivity into four grades by AS: Grade 0, AS is 0; Grade 1, AS is 2–4; Grade 2, AS is 5–6; Grade 3, AS is 7–8. MIA positiveness was exhibited as grades 2 and 3. Labeling index for HMGB1 was calculated as follows: (the number of the HMGB1 positive nucleus/total number of the nucleus) × 100. Anti-D2-40 immunostained specimens were observed under 200× magnification microscopy and three maximum lymph vessel density (LVD) fields were selected from around of the tumor cells (the 'hot spot'). These fields were captured by digital imaging with charge coupled device camera (Olympus, Tokyo, Japan). LVD and mean lymph vessel area (MLA) were measured on the computer-captured image using NIH Image software (National Institutes of Health, Bethesda, MD, USA).

Cell culture. Human OSCC cell lines, HSC3 and HSC4, were studied. HSC3 cells are tongue squamous cell carcinoma-derived metastatic cell lines, which provide many sublines with high metastatic potential. In contrast, HSC4 cells show low metastatic potential. No metastatic sublines are derived from HSC4 cells.[31] Controlled cell line was used for U937 (monocytic leukemia cell line, purchased from Dainihon Pharmaceutical, Tokyo, Japan). All cells were maintained in Roswell Park Memorial Institute medium (RPMI)-1640 (Sigma Chemical) supplemented with 10% fetal bovine serum (Sigma Chemical Co) in 5% CO_2 and 95% air at 37°C. Anti-MIA antibody was used for neutralizing MIA (Santa Cruz Biotechnology) in cultured medium at 2 μL/mL concentration. Rabbit serum (DAKO) was used for control. The cells were also treated with p38 mitogen-activated protein kinase (MAPK) inhibitor SB239063 (Sigma Chemical) at 5 μM for 12 h.

Short interferent RNA. FlexiTube short interferent RNA (siRNA) for MIA, RAGE and HMGB1 were purchased from Qiagen Genomics (Bothell, WA, USA). AllStars Negative Control siRNA was used for control (Qiagen Genomics). These cells were transfected with 50-nM siRNA for each gene using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

Antisense phosphorothioate(S)-oligodeoxynucleotide assay. The 18-mer S-oligodeoxynucleotide (ODN) for antisense sequence from 6th to 23rd nucleotide of RAGE cDNA (GenBank AB036432) and the 18-mer S-ODN for antisense sequence from 1st to 18th nucleotide of HMGB1 cDNA (GenBank X12597) were synthesized and purified by reverse-phase high performance liquid chromatography (Sigma Genosys, Ishikari, Japan). The sequence of RAGE antisense was 5′-CTG CTT CCT TCC AGG GTC-3′; the sequence of HMGB1 antisense was 5′-AGG ATC TCC TTT GCC CAT-3′. The sense sequence of the antisense S-ODN was used for the control S-ODN. These cells were pretreated with 3 μM of antisense or sense S-ODN for 6 days with medium exchange and an addition of antisense or sense S-ODN every 2 days. After pretreatment, the cells were used for further experiments. Cytotoxicity of the antisense S-ODN were not relevant at the working concentration.[19]

Immunoblot analysis. Whole-cell lysates were prepared as described previously.[26] Fifty-microgram lysates were subjected to immunoblot analysis in 12.5% sodium dodecyl sulfate–polyacrylamide gels followed by electrotransfer to nitrocellulose filters. The filters were incubated with primary antibody and then with peroxidase-conjugated immunoglobulin G antibody (Medical and Biological Laboratories, Nagoya, Japan). A γ-tubulin antibody was used to assess the levels of protein loaded per lane (Oncogene Research Products, Cambridge, MA, USA). The immune complex was visualized using an enhanced chemiluminescence Western-blot detection system (Amersham, Aylesbury, UK). The primary antibodies used were anti-MIA, anti-NFkB p65, anti-VEGF-C, anti-VEGF-D, anti-ERK1/2, antiphospho-ERK1/2, antiphospho-p38 (Santa Cruz Biotechnology), anti-integrin α5β1 (Serotec Ltd, Oxford, UK) and anti-HMGB1 (Upstate Biotechnology).

Immunoprecipitation. For immunoprecipitation, the lysates were precleaned in a lysis buffer containing protein A/G agarose (Santa Cruz Biotechnology) for 1 h at 4°C and subsequently centrifuged. The supernatants were incubated with anti-HMGB1 antibody (Upstate Biotechnology), or anti NFkB p65 antibody (Santa Cruz Biotechnology) and protein A/G agarose for 3 h at 4°C. The precipitates were collected by centrifugation and washed 5 times with lysis buffer for sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE). For loading control, 5 μL of each preimmunoprecipitated sample (lysate diluted with buffer) was slot-blotted onto nitrocellulose membrane and stained with Coomassie blue.

Reverse transcriptase–polymerase chain reaction. Total RNA was extracted from cultured cells using the RNeasy Mini Kit (Qiagen Genomics). Total RNA (1 μg) was converted to cDNA with the First-Strand cDNA Synthesis Kit (Amersham Biosciences, Piscataway, NJ, USA). PCR were performed using Amplitaq Gold Kit (Applied Biosynthesis, Foster City, CA, USA) according to the manufacturer's instructions.

Primer pairs used are listed below: MIA (referred to GenBank NM006533), Sense: 5′-ACCCTA TCT CCA TGG CTG TG-3′, Antisense: 5′-AGG TTT CAG GGT CTG GTC CT-3′; RAGE (referred to GenBank NM172197), Sense: 5′-GCT GTC AGC ATC AGC ATC AT-3′, Antisense: 5′-ATT CAG TTC TGC ACG CTC CT-3′; HMGB1 (referred to GenBank NM002128), Sense: 5′-ATA TGG CAA AAG CGG ACA AG-3′, Antisense: 5′-GCA ACA TCA CCA ATG GAC AG-37; VEGF-C (referred to GenBank NM005429), Sense: 5′-GGA AAG AAG TTC CAC CAC CA-3′, Antisense: 5′-TTT GTT AGC ATG GAC CCA CA-3′; VEGF-D (referred to GenBank NM004469), Sense: 5′-AGG ACT GGA AGC TGT GGA GA-3′, Antisense: 5′-ATC GGA ACA CGT TCA CAC AA-3′; β-actin (referred to GenBank NM001101), Sense: 5′-CAA GAG ATG GCC ACG GCT GCT-3′, Antisense: 5′-TCC TTC TGC ATC CTG TCG GCA-3′. All primers were synthesized by Sigma Genosys (Ishikari, Japan).

Statistical analyses. Statistical differences in MIA expression were tested with the two-tailed χ^2 test. All statistics were calculated using StatView version 4.5 (SAS Institute, Cary, NC, USA) and a P-value of less than 0.05 was considered statistically significant.

Results

Relationship between MIA expression and clinical parameters. We examined MIA expression in 62 cases of OSCC (Table 1). Expression of MIA was observed in 48.4% of all cases (30/62). Immunoreactivity for MIA was found in the cell membrane and cytoplasm, except in the nuclei of the cancer cells, but was not found in normal epithelium of all cases (Fig. 1).

A significant association was found between MIA immunoreactivity and histological metastasis of lymph nodes. Four of 23 cases (17.4%) without nodal metastasis (n-) expressed MIA, whereas MIA expression was found in 26 of 39 (66.7%) cases with nodal metastasis (n+) ($P = 0.00018$). Representative cases showed significant MIA expression in the nodal metastatic foci (Fig. 1, MIA in metastasis). HMGB1 labeling indices were also high in the nodal metastatic foci (Fig. 1, HMGB1 in metastasis). However, no significant relationship was found between MIA grading and other parameters: age, sex, primary site, histological differentiation, T classification (extension of primary tumor), clinical stage, tumor recurrence and disease-free survival. To

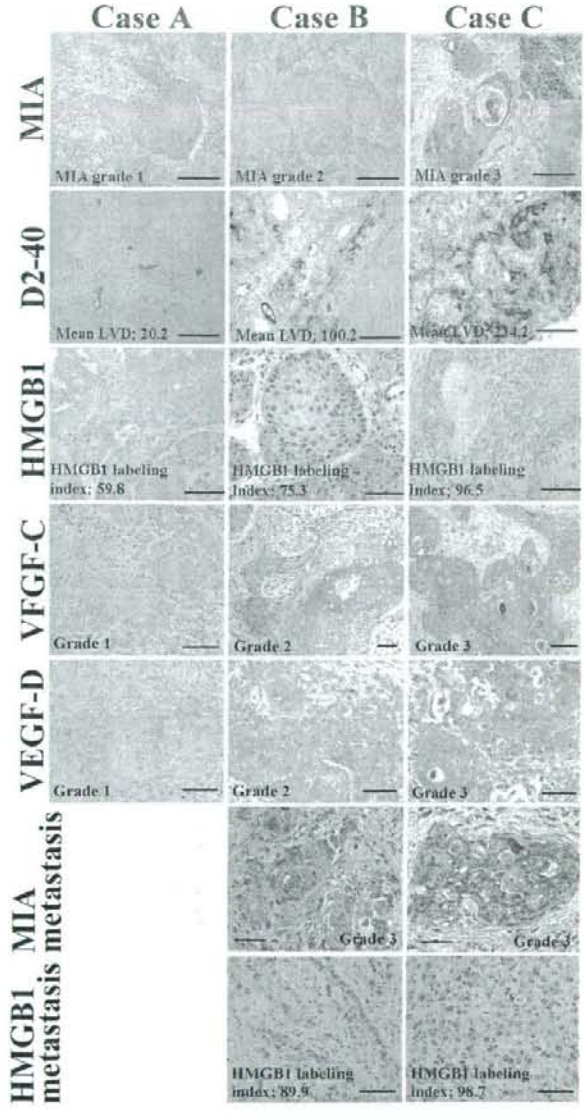

Fig. 1. Immunohistochemical staining of melanoma inhibitory activity (MIA), D2-40, high mobility group box-1 (HMGB1), vascular endothelial growth factor (VEGF)-C and VEGF-D in oral squamous cell carcinomas (OSCC). The expressions of D2-40, HMGB1, VEGF-C and VEGF-D were shown in case A (T2N0M0, stage II, well differentiated OSCC), case B (T2N1M0, stage III, well differentiated OSCC) and case C (T4N2M0, stage IV, well differentiated OSCC). Inset showed MIA localization at the cytoplasm and cytoplasmic membrane. Expressions of MIA and HMGB1 were examined in lymph node metastasis in case B and C. Bar, 100 μm.

confirm significance of MIA to lymph-node metastasis in OSCC, we examined LVD and MLA in tumor tissues (Fig. 2a,b). Lymph vessels around the tumor cells were detected by a lymph duct endothelial marker, D2-40 (Fig. 1). A significant correlation was observed between grading of MIA immunoreactivity and LVD ($P < 0.0001$) or MLA ($P < 0.0001$).

As a chromatin structural protein, HMGB1 is reported to participate in MIA transcription in association with NFkB.[28,29]

Table 1. Relationship between melanoma inhibitory activity (MIA) expression and clinicopathological parameters in oral squamous cell carcinoma (OSCC) patients

	n	MIA expression		*P*-value†
		Negative	Positive	
Age				
–60	43	25	18	
60–	19	7	12	NS
Sex				
Male	35	17	18	
Female	27	15	12	NS
Primary site				
Tongue	40	22	18	
Gingiva	14	7	7	
Buccal mucosa	5	2	3	
Hard palate	3	1	2	NS
Histological differentiation				
Well	33	16	17	
Moderately	26	13	13	NS
Poorly	3	3	0	
T classification (extension of primary tumor)[47]				
T1, 2	21	12	9	
T3	17	7	10	
T4	24	13	11	NS
Clinical stage[47]				
I, II	11	9	2	
III	25	11	14	
IV	26	12	14	NS
Histological nodal metastasis[47]				
n–	23	19	4	
n+	39	13	26	0.00018
Disease recurrence				
(–)	35	19	16	
(+)	27	13	14	NS

†Calculated χ^2 test.

To examine the role of HMGB1 in MIA expression in OSCC, we compared the expressions of MIA and HMGB1. HMGB1 immunoreactivity was found in cancer cell nuclei (Fig. 1, HMGB1). MIA grading index was significantly correlated with HMGB1 labeling ($P < 0.0001$) (Fig. 2c).

VEGF-C and VEGF-D were examined as the expressions of lymphangiogenesis -related growth factors in OSCC cases (Fig. 1). We showed comparison between expressions of VEGF-C or VEGF-D and MIA grade or HMGB1 labeling index (Fig. 2d–e). Immunostaining grades of VEGF-C and VEGF-D were correlated with MIA grade ($P < 0.0001$) and HMGB1 labeling index ($P < 0.001$). All above results suggest that MIA is associated with lymph-node metastasis of OSCC by up-regulation of lymphangiogenic factors, VEGF-C and VEGF-D.

Relation of MIA expression with HMGB1 or NFkB p65. We next compared the expression level of MIA, HMGB1 and NFkB p65 in metastatic HSC3 and non-metastatic HSC4 human OSCC cell lines by immunoblotting (Fig. 3). In HSC3 cells, expression levels of MIA, HMGB1 and NFkB p65 were higher than HSC4. U937 monocytic cells, which showed lower HMGB1 and higher NFkBp65 expression levels, expressed MIA at an untraceable level. A physical association between HMGB1 and NFkB p65 was also examined in order to be compared with HMGB1 expression (Fig. 3). The levels of NFkB p65 detected in HMGB1-immunoprecipitants of HSC3 cells were 4.5-times higher than that in HSC4 cells. In U937 cells, NFkB p65 binding with

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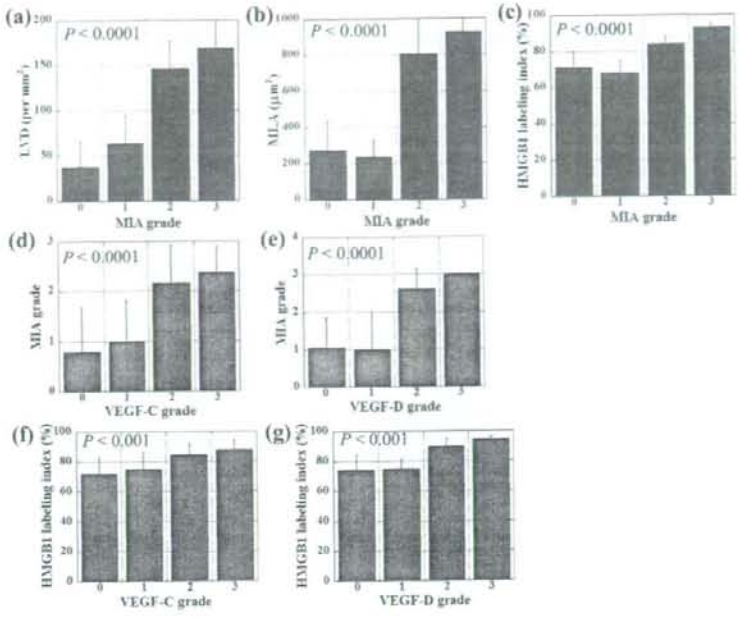

Fig. 2. Relationship between MIA expression and lymph vessels, and expressions of HMGB1, VEGF-C and VEGF-D. MIA grading was compared with lymph vessel density (LVD) (a), mean lymph vessel area (MLA) (b), HMGB1 labeling index (c), VEGF-C grading (d),and VEGF-D grading (e) in OSCC. HMGB1 labeling index was compared with VEGF-C grading (f), and VEGF-D grading (g) in OSCC. Expressions of MIA, VEGF-C, and VEGF-D were categorized according to Allred's score (AS).[20] Grade 0, AS is 0; Grade1, AS is 2-4; Grade 2, AS is 5-6; Grade 3, AS is 7-8. Error bar, SD.

HMGB1 is 1/10 of that in HSC4 cells. HMGB1–NFkB p65 binding levels in HSC3, HSC4 and U937 cells were well correlated with those of MIA expression levels.

Role of HMGB1 on MIA expression in HSC3 OSCC cells. HMGB1 has a dual role as a chromatin structural protein and as a cytokine accelerating macrophage-associated inflammation in cancer growth and invasion. We examined which form of HMGB1 participated in MIA expression (Fig. 4a). Exposure to antisense S-ODN for HMGB1 receptor RAGE did not affect MIA expression levels. The addition of hrHMGB1 into the culture medium also did not affect MIA expression. In contrast, reduction of intracellular protein by HMGB1 antisense S-ODN treatment decreased MIA expression. We confirmed the effects of RAGE and HMGB1 on decreasing the MIA expression by using siRNA for HMGB1 (Fig. 4b,c). RAGE siRNA decreased RAGE mRNA expression, but not MIA mRNA, whereas HMGB1 siRNA decreased HMGB1 and MIA mRNA expression in HSC3 cells. These results suggest that HMGB1 is significant in MIA induction as a transcriptional cofactor with NFkBp65 but not as a RAGE ligand.

MIA intracellular signals and expressions of VEGF-C and VEGF-D. Finally, we examined the MIA effects on HSC3 intracellular signals (Fig. 5). Expressions of VEGF-C and VEGF-D were examined by RT-PCR in HSC3, HSC4 and U937 cells (Fig. 5a). Metastasis-derived HSC3 cells expressed VEGF-C and VEGF-D, whereas original tumor-derived HSC4 cells expressed only VEGF-C. U937 monocytes did not express VEGF-C nor VEGF-D. As shown in Fig. 5(b), inhibiting the expression of MIA secreted by HSC3 cells with anti-MIA antibody increased the phosphorylated form of ERK1/2, whereas phosphorylated p38 was decreased. Expressions of pro-lymphangiogenic growth factors and VEGF-C and VEGF-D were inhibited by MIA neutralization in the antibody treatment. To confirm the relationship of p38 phosphorylation level with VEGF-C or VEGF-D expression, we examined the effect of p38 inihibitor on expression (Fig. 5c). HSC3 cells treated with p38 inhibitor (SB239063) showed decreased expressions of VEGF-C and VEGF-D by RT-PCR examination. Thus, MIA up-regulates VEGF-C and VEGF-D through p38 activation.

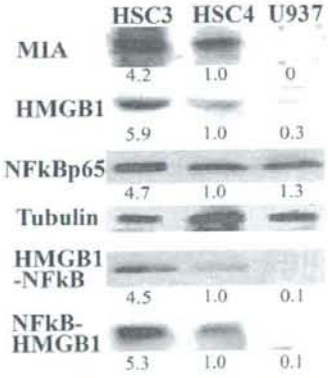

Fig. 3. Expression of melanoma inhibitory activity (MIA), high mobility group box-1 (HMGB1) and nuclear factor kB (NFkB) in human oral squamous cell carcinoma (OSCC) cells. MIA expression was compared with the expressions of HMGB1, NFkBp65 and coprecipitation between NFkBp65 and HMGB1 in HSC3, HSC4 human OSCC cells and U937 monocytic cells. Expressions of MIA, HMGB1 and NFkBp65 were examined by immunoblotting. Tubulin was served as a loading control. Co-precipitation of HMGB1 and NFkB was examined by immunoprecipitation. A precipitant with anti-HMGB1 antibody was detected with anti-NFkBp65 antibody by immunoblotting. Reverse immunoprecipitation was also examined. A precipitant with anti-NFkBp65 antibody was detected with anti-HMGB1 antibody.

Discussion

In the present study, we showed immunoreactivity of MIA was significantly correlated with nodal metastasis, LVD and HMGB1 labeling index in OSCC specimens. In *in vitro* examinations, MIA expression was associated with the expression levels of HMGB1 (not as a cytokine but as an intracellular form) and HMGB1–NFkB p65 binding. In a metastatic OSCC cell line,

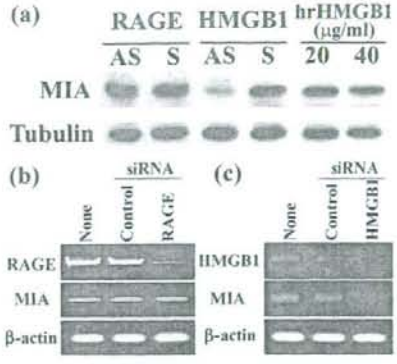

Fig. 4. Effects of antisense S-ODN and siRNA for receptor for advanced glycation end products (RAGE) and high mobility group box-1 (HMGB1) and hrHMGB1 on melanoma inhibitory activity (MIA) expression in HSC3 human oral squamous cell carcinoma (OSCC) cells. (a) MIA expression was examined by immunoblotting in HSC3 human OSCC cells treated with antisense (AS) or sense (S) S-ODN for RAGE and HMGB1 and human recombinant HMGB1 (hrHMGB1). Tubulin was served as a loading control. (b, c) MIA expression was examined by reverse transcriptase–polymerase chain reaction in HSC3 cells with or without treatment with siRNA for RAGE and HMGB1 or control siRNA. β-actin was served as a loading control.

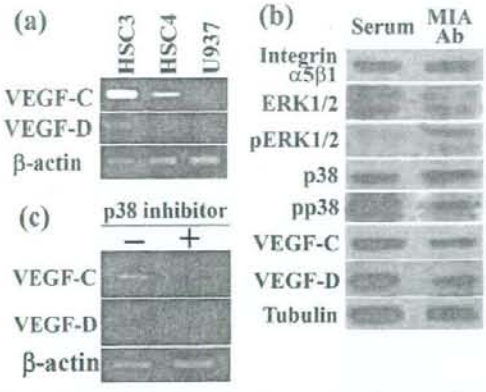

Fig. 5. Melanoma inhibitory activity (MIA) intracellular signaling and vascular endothelial growth factor (VEGF)-C and VEGF-D expression in HSC3 cells. (a) Expression of VEGF-C and VEGF-D was examined by reverse transcriptase–polymerase chain reaction (RT-PCR) in HSC3, HSC4 and U937 cells. β-actin was served as a loading control. (b) Effects of anti-MIA antibody on phosphorylation of extracellular signal-related kinase (ERK)1/2 and p38 and expressions of integrin α5β1, VEGF-C and VEGF-D in HSC3 human oral squamous cell carcinoma (OSCC) cells. Protein levels of MIA, ERK1/2, phosphorylated ERK1/2 (pERK1/2), p38, phosphorylated p38 (pp38), VEGF-C and VEGF-D were examined by immunoblotting in HSC3 human OSCC cells treated with MIA antibody. Tubulin was served as a loading control. (c) The effect of inhibition of p38 on expression of VEGF-C and VEGF-D. HSC3 cells were treated with p38 inhibitor (SB239063). Expression of VEGF-C and VEGF-D was examined by RT-PCR. β-actin was served as a loading control.

HSC3 showed higher MIA expression, which was associated with higher expression levels of HMGB1, NFkB p65 and HMGB1–NFkB p65 binding than those in a non-metastatic OSCC cell line (HSC4). Moreover, MIA expression was associated with VEGF-C and VEGF-D expression in HSC3 cells.

HMGB1 is a chromatin structural protein and also acts as a cytokine or growth factor.[32,33] Nuclear HMGB1 is recruited in gene replication, repair and transcription, whereas secreted HMGB1 worsens endotoxemic inflammation as a late mediator.[33] Secreted HMGB1 is also known as a pro-tumoral factor, as is a RAGE ligand.[34] We reported that co-overexpression of HMGB1 and RAGE is significantly associated with tumor progression and metastasis in gastric cancer,[24] colon cancer,[21,23] prostate cancer[22] and malignant transformation of colorectal adenoma.[35] Further, we found that HMGB1 induces apoptosis of macrophages, which is associated with colon cancer metastasis[25,26] and enhanced extracellular secretion of HMGB1 in colon cancer.[36] We also reported that a high expression level of RAGE is correlated with tumor progression and recurrence, but not with lymph-node metastasis in OSCC.[18–20] RAGE activation with HMGB1 as a ligand induced VEGF expression, but not VEGF-C in HCS3 and HCS4 OSCC cells.[19] In the present study, HMGB1 treatment did not alter MIA expression, whereas HMGB1 antisense S-ODN treatment decreased MIA expression. HMGB1 is able to bind to HCR located in the promoter region of the *MIA* gene where HMGB1 interacts with NFkB p65 to enhance MIA expression transcriptionally.[28,29] In the present study, we confirmed that physical association between HMGB1 and NFkB p65 regulates induction of MIA in OSCC. From these findings, HMGB1 might induce MIA expression acting as a chromosomal protein.

MIA takes a pivotal role for progression and metastasis in melanoma.[12] MIA promotes cell detachment, migration and invasion and inhibits apoptosis of the cancer cells and infiltration of lymphokine activated killer cells (LAK). MIA binds to fibronectin via SH3 domain-like structures, which inhibits cell-to-stromal attachment.[37,38] Further, MIA is able to bind to cell surface integrin α4β1 and α5β1, which suggests MIA might play a role as a ligand for selected integrins.[39]

In the present study, we found a significant relationship between MIA expression and LVD in OSCC tumors. Expression of integrin α5β1 in lymph vessel endothelial cells is associated with outgrowth of new lymphatic vessels.[40] MIA might stimulate lymphatic endothelial cells directly to induce lymphangiogenesis. We also found a relationship between MIA expression and VEGF-C and VEGF-D expression in HSC-3 OSCC cells, which expressed integrin α5β1. MAPK activity is reported to be affected by MIA,[39] which we confirmed in the present study. ERK1/2 phosphorylation was recovered by MIA neutralization by the antibody treatment. In contrast, p38 phosphorylation levels were decreased by the antibody treatment. VEGF-C expression is inhibited by p38 inhibitor but not by ERK1/2 inhibitor.[41] We also confirmed the significance of p38 activation on up-regulation of VEGF-C and -D in HSC3 cells. The alteration of the signal balance between ERK1/2 and p38 might be associated with up-regulation of VEGF-C and -D expression by MIA. Further examination will reveal the details of the mechanism of MIA-dependent VEGF-C and -D induction. VEGF-C and -D are known as strong lymphangiogenic factors in various cancers.[42] Increased VEGF-C expression is associated with cervical lymph-node metastasis in head and neck cancer.[43] Although there are still controversies about the role of VEGF-D in lymph-node metastasis, VEGF-D expression is also associated with lymph-node metastasis in the animal model.[44] Our data suggest that up-regulation of VEGF-C and -D might explain the relationship between MIA expression and lymph-node metastasis in OSCC.

HSC3 cells are tongue squamous cell carcinoma-derived metastatic cell lines, which provide many sublines with high metastatic potential. In contrast, HSC4 cells show low metastatic potential. No metastatic sublines are derived from HSC4 cells.[45] Metastatic HSC3 cells show colony formation in the type-I collagen matrix, adherence to type-IV collagen,[45] high heparanase activity[31] and reduced nm23H1 expression and up-regulated matrix metalloproteinase (MMP)-2 and -9[46] in

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comparison with HSC4 cells. In the present study, HSC3 cells showed overexpression of MIA, HMGB1, NFkBp65, VEGF-C and VEGF-D in comparison with HSC4 cells. Thus the increase of lymphangiogenic capacity might be associated with high potential of lymph-node metastasis in HSC3. Further examination of the lymphangiogenic capacity might control lymph-node metastasis in OSCC. In the present study, MIA expression was associated with a high incidence of lymph-node metastasis, whereas MIA expression did not correlate with recurrence nor with disease-free survival. Our previous data show that RAGE–HMGB1 coexpression is associated with T classification (extension of primary tumor) but not nodal metastasis in OSCC; however, RAGE expression is closely associated with recurrence and disease-free survival.[18–20] Many OSCC recurred at the local sites but not from nodal metastasis (data not shown). Local recurrence of OSCC depends on aggressiveness of cancer invasion and anatomical difficulties in the head to obtain sufficient surgical margins. In OSCC, both metastatic potential and local aggressiveness are significant factors to determine the disease outcome.

We showed that MIA was expressed in more than 60% of metastasized OSCC. Considering MIA is a secretory protein, MIA might be a useful marker for metastasis of OSCC, which is detectable in the serum and saliva of OSCC patients. Further examination of MIA might be expected to provide a new target for suppression of lymph-node metastasis.

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Positive immunohistochemical staining of γH2AX is associated with tumor progression in gastric cancers from radiation-exposed patients

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Abstract. To elucidate the mechanism of radiation-induced cancers, molecular analysis of cancers in atomic bomb (A-bomb) exposure is important. DNA double-strand breaks (DSBs) are thought to be caused by the deleterious effects of ionizing radiation, and γH2AX (serine 139 phosphorylated form of histone H2AX) is reported to be a significant marker for DSBs. In the present study, we performed immunohistochemical analysis of γH2AX in gastric cancers (GCs) from 66 exposed and 47 non-exposed patients who developed GC after the bombing. Of the 47 GCs from non-exposed patients, 6 (13%) cases showed nuclear positive staining for γH2AX, whereas of the 66 GCs from exposed patients, 20 (30%) cases were positive (P=0.0405). However, among stage I GC, there was no significant difference in γH2AX expression frequency between exposed patients and non-exposed patients. Among exposed patients, stage II-IV cases were more frequently positive for γH2AX than stage I cases (P=0.0197). Among GCs from non-exposed patients, γH2AX staining showed no significant association with Lauren's classification, depth of invasion, lymph node metastasis or TNM stage. These results suggest that the characteristics of tumor cells differ between GCs from exposed and non-exposed patients. DSBs may be involved in progression of GC in exposed patients.

Introduction

More than 60 years have passed since atomic bomb (A-bombs) exposure in Hiroshima and Nagasaki, Japan. A prospective cohort study (Life Span Study, LSS) of 120,000 subjects is being conducted by the Radiation Effects Research Foundation (RERF) (1). It was reported that exposure to ionizing radiation (IR) increases the risk of leukemia and other cancers (2), and damage to nuclear DNA likely represents an initiating event for carcinogenesis. Increases in cancer risk due to exposure to IR are based on epidemiologic studies of exposed human populations, mainly the A-bomb survivors of Hiroshima and Nagasaki (3). Solid cancers, including breast, colon, lung and stomach cancers, have a long latency period, and the excess relative risks (RRs) of solid cancers remain high, specifically among those exposed when young (1). Although approximately half of the LSS members are now deceased, cancer mortality in the LSS has continued to increase as this population ages, and it is anticipated to peak in 2015.

According to the World Health Organization, gastric cancer (GC) is the fourth most common malignancy worldwide, with approximately 870,000 new cases occurring yearly. Cancer develops as a result of multiple genetic and epigenetic alterations (4,5). Although several genetic alterations, including mutations in *TP53* and *BRAF*, have been reported in selected cancers of A-bomb survivors (6-8), specific mutations for radiation-associated cancers have not been reported.

DNA double-strand breaks (DSBs) are thought to be caused by the deleterious effects of IR (9,10). DSBs can induce chromosomal aberrations that cause cells to malfunction, resulting in cell death or tumorigenesis (10). One of the earliest steps in the cellular response to DSBs is the phosphorylation of histone H2AX at serine 139 (γH2AX), the site of γ-phosphorylation (11). H2AX can be phosphorylated by several phosphoinositide-3 (PI3) kinases including ataxia telangiectasia mutated (ATM), DNA-dependent protein kinase (DNA-PK) and ataxia telangiectasia and Rad3 related (ATR) (12). The number of resulting γH2AX foci has been correlated directly with the number of DSBs produced by IR (13,14). Therefore, the number of γH2AX foci is a significant marker for DSBs. Immunohistochemical analyses of γH2AX have been reported for human cancers of the urinary bladder,

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Key words: γH2AX, gastric cancer, radiation, atomic bomb, DNA double-strand break

breast, lung, colon and prostate (15-17). It was also reported that γH2AX-positive cells are present in colorectal cancer (CRC) and precursor lesions, such as adenoma, but not in normal colonic epithelium (15). Furthermore, invasive CRCs were reported to show less γH2AX staining than adenomas (15). These results suggest that staining of γH2AX correlates with DNA damage checkpoint activation in premalignant lesions. Therefore, the existence of γH2AX foci might be a useful and sensitive marker of cancer, especially for detecting cancers or precursor lesions in A-bomb survivor, because IR induces DSBs. However, there are no reports of immunohistochemical analyses of γH2AX in GCs from either IR-exposed patients or -non-exposed patients. Therefore, in the present study, we performed immunohistochemical analysis of γH2AX in 113 GCs derived from A-bomb survivors.

Patients and methods

Patients and tumor specimens. For immunohistochemical analysis, we used formalin-fixed, paraffin-embedded archival tissues from 113 patients with GC who underwent surgery between 1975 and 2005 at Hiroshima University Hospital (Hiroshima, Japan) or an affiliated hospital. Only patients who did not undergo preoperative radio- or chemotherapy were enrolled in the study. All 113 patients were A-bomb survivors (LSS cohort members) in Hiroshima, Japan. Although these patients were survivors who developed GC after the bombing, they were further classified according to their level of radiation exposure (i.e., ≥5 mGy and <5 mGy were defined as 'exposed' and 'non-exposed', respectively). Our population comprised 66 exposed (median dose, 51 mGy; range, 5-2601 mGy) and 47 non-exposed patients (median dose, 0 mGy; range, 0-4 mGy).

Tumor staging was performed according to the Union Internationale Contre le Cancer (UICC) system (18). Histologic classification was carried out according to the Lauren classification system (19). The detailed procedures for acquiring informed consent from study patients and collecting tissue specimens were described previously (20). In accordance with the Ethical Guidelines For Human Genome/Gene Research enacted by the Japanese Government, tissue specimens were collected and used after approval from the Ethical Review Committee of the Hiroshima University School of Medicine and from the ethical review committees of collaborating organizations.

Radiation dose. A-bomb radiation doses were estimated with the DS02 system (21).

Immunohistochemistry. From each patient, one representative tumor block, including the tumor center and invasive front as well as tumor-associated non-neoplastic mucosa, was examined by immunohistochemistry. In cases of large, late-stage tumors, different sections were examined to include representative areas of the tumor center as well as of the lateral and deep tumor invasive fronts.

Immunohistochemical detection of γH2AX was performed with a mouse monoclonal antibody (Upstate Biotechnology, Chicago, IL, USA) and Dako Envision Kit (Dako, Carpinteria, CA). In brief, sections were pretreated by microwaving (500 W) in citrate buffer (pH 6.0) for 15 min to retrieve antigenicity. After endogenous peroxidase activity was blocked with 3% H_2O_2-methanol for 10 min, sections were incubated with normal goat serum (Dako) for 20 min to block non-specific antibody binding sites. Sections were then incubated with anti-γH2AX (diluted 1:200) for 1 h at room temperature followed by incubation with peroxidase-labelled anti-mouse IgG for 60 min. Staining was completed with a 10-min incubation with the substrate-chromogen solution. Sections were counterstained with 0.1% hematoxylin. Appropriate negative controls were created by omission of the primary antibody. All slices were evaluated without knowledge of the clinical data.

Double immunofluorescence staining. Double immunofluorescence staining for dewaxed sections was performed with mouse monoclonal anti-γH2AX antibody (Upstate) with rabbit polyclonal anti-H2AX antibody (Upstate) or mouse monoclonal anti-γH2AX antibody with a rabbit polyclonal antibody against the activated form of caspase-3 (Promega, Madison, WI, USA). Microwave pretreatment in citrate buffer was performed for 15 min to retrieve antigenicity. Sections were then incubated with normal goat serum for 30 min to block non-specific antibody binding sites. Sections were treated consecutively at room temperature with primary antibodies for 60 min, and immunocomplexes were detected with Alexa Fluor 488-conjugated goat anti-mouse IgG and Alexa Fluor 546-conjugated goat anti-rabbit IgG (Molecular Probes, Eugene, OR, USA).

Statistical methods. Associations between clinicopathologic variables and immunostaining for γH2AX were analyzed by Fisher's exact test. A P-value <0.05 was considered statistically significant.

Results

Of 113 GC from A-bomb survivors, 48 (42%) showed nuclear staining of γH2AX (Fig. 1A). These 48 cases comprised 26 GC cases with diffuse staining for γH2AX and 22 GC cases with staining of γH2AX only in superficial portions (Fig. 1B) or in necrotic debris in the lumen (Fig. 1C). We confirmed that γH2AX yielded granular, nuclear staining (Fig. 1D). H2AX showed ubiquitous staining in GC (Fig. 1E). It was reported previously that γH2AX is expressed during early apoptosis triggered through the caspase-3/caspase-activated DNase (CAD) pathway (22,23). Double immunofluorescence staining revealed that γH2AX-positive tumor cells in superficial portions or necrotic debris were also positive for the activated form of caspase-3 (a marker of apoptosis) (Fig. 1F). Because we believed that γH2AX staining induced by apoptosis was not related to IR, cases with superficial staining and staining of necrotic debris were excluded from the positive cases. In contrast, the percentage of γH2AX-stained tumor cells was >5% in 26 GC cases showing diffuse staining; we considered these as positive cases. Twenty-four of 26 GC cases had from 5% to 10% of γH2AX-stained tumor cells. In particular, remaining two cases had >30% of γH2AX-stained tumor cells, both of which were α-fetoprotein (AFP)-positive GC.

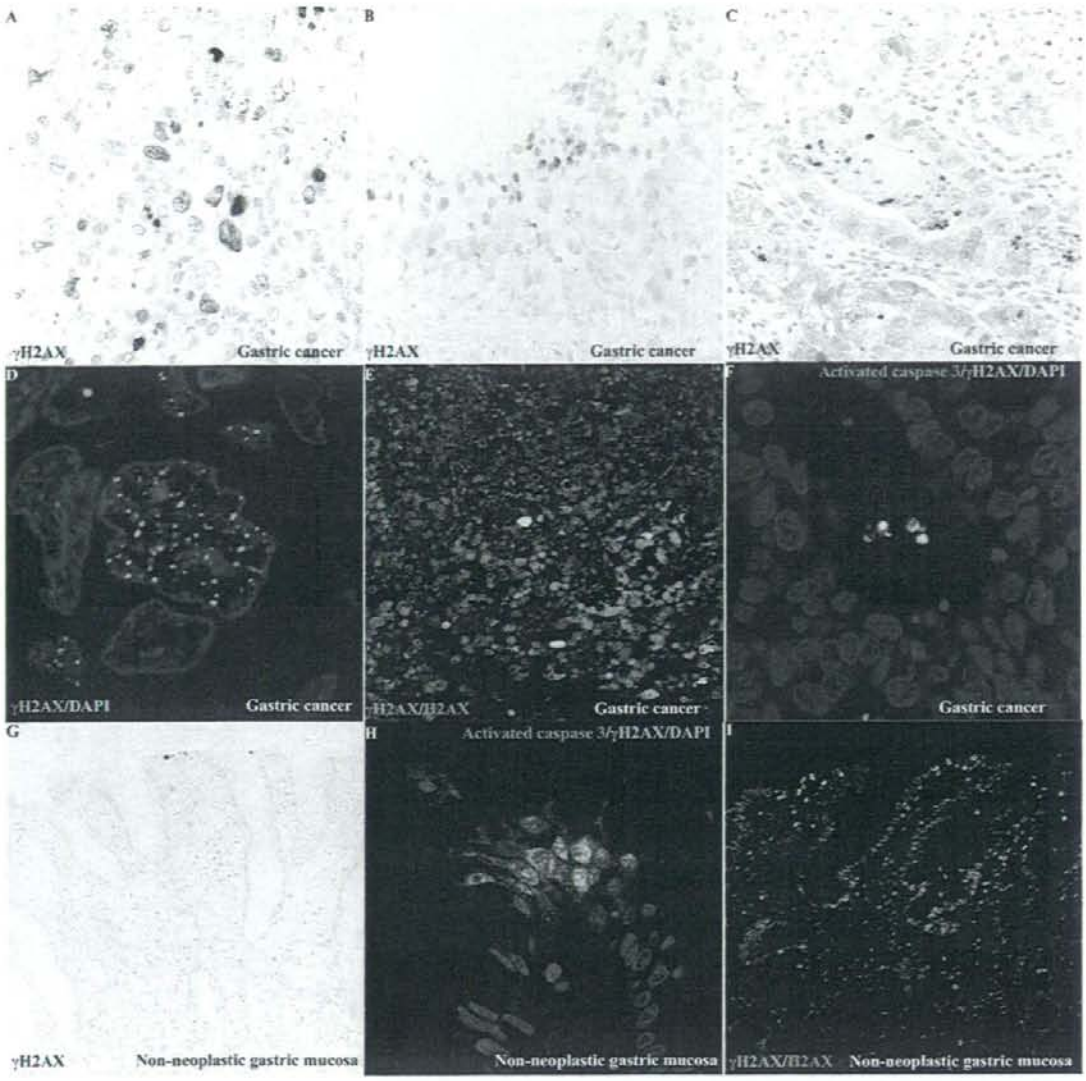

Figure 1. Immunohistochemical analysis of γH2AX in gastric carcinoma (GC) tissues (A-F) and non-neoplastic gastric mucosa (G-I). Staining for γH2AX in GC. Schematic representation of a positive case (A). Tumor cells or necrotic debris with γH2AX staining are located at the superficial portion of GCs (B) or in the lumen of the tumor gland (C). Granular staining of γH2AX is found in the nucleus (D). Staining for H2AX. H2AX shows ubiquitous staining both in GC (E) and non-neoplastic gastric mucosa (I). Some surface cells in non-neoplastic gastric mucosa also express γH2AX (G). Double immunofluorescence staining of the activated form of caspase-3 and γH2AX. Necrotic debris in the lumen of GCs (F) and superficial apoptotic cells in non-neoplastic gastric mucosa (H) are shown. Cells were imaged with a fluorescence microscope as described in Materials and methods.

We analyzed the association between γH2AX staining and clinicopathologic parameters in GCs from 66 IR exposed and 47 non-exposed patients (Table I). When all tumor stages were considered, γH2AX staining was detected in 20 (30%) of 66 exposed patients and 6 (13%) of 47 non-exposed patients (P=0.0405). Because IR is a carcinogen and can increase an individual's risk of tumor development, we analyzed immunohistochemical staining for γH2AX in early-stage GCs. In GCs showing T1 (tumor invades lamina propria or submucosa), N0 (no regional lymph node metastasis), or stage I, γH2AX positivity did not differ significantly between exposed and non-exposed patients. In contrast, among T2-4 GCs, γH2AX was expressed more often in exposed patients than in non-exposed patients (P=0.0236). In stage II-IV GCs, γH2AX expression showed a marginally significant difference between exposed patients and non-exposed patients (P=0.0602).

Of the 66 GCs from exposed patients, γH2AX was present in 20 (30%). γH2AX expression in GCs from exposed patients was associated significantly with depth of invasion (P=0.003) (Table II). Furthermore, γH2AX staining was observed more frequently in stage II-IV GCs than in stage I GCs (P=0.0197) (Table II). In contrast, the presence of γH2AX in GCs from non-exposed patients showed no signi-

Table I. Expression of γH2AX in GC and its association with clinicopathologic variables.

	No. of cases	γH2AX positive (%)	P-value
GC	113	26 (23)	
Exposed patients	66	20 (30)	
Non-exposed patients	47	6 (13)	0.0405
Intestinal type[a]			
Exposed patients	34	9 (26)	
Non-exposed patients	33	5 (15)	0.3689
Diffuse type[a]			
Exposed patients	32	11 (34)	
Non-exposed patients	14	1 (7)	0.073
Depth of invasion[b]			
T1			
Exposed patients	15	0 (0)	
Non-exposed patients	19	2 (11)	0.492
T2-4			
Exposed patients	51	20 (39)	
Non-exposed patients	28	4 (14)	0.0236
Lymph node metastasis[b]			
N0			
Exposed patients	25	5 (20)	
Non-exposed patients	24	2 (8)	0.4174
N1-4			
Exposed patients	41	15 (37)	
Non-exposed patients	23	4 (17)	0.1552
TNM stage[b]			
I			
Exposed patients	21	2 (10)	
Non-exposed patients	23	2 (9)	1.000
II-IV			
Exposed patients	45	18 (40)	
Non-exposed patients	24	4 (17)	0.0602

GC, gastric carcinoma. [a]Histologic classification of GC was according to the Lauren classification system. [b]Tumor stage was according to the Tumor-Node-Metastasis (TNM) staging system.

ficant correlation with Lauren's classification, depth of invasion, lymph node metastasis or TNM stage (Table II). There was no significant association between γH2AX staining and radiation dose at the time of A-bombing (data not shown).

In non-neoplastic gastric mucosa or intestinal metaplasia adjacent to the tumor, only a few superficial cells in both exposed and non-exposed patients showed immunostaining of γH2AX (Fig. 1G) and activated form of caspase-3 (Fig. 1H). H2AX showed ubiquitous immunostaining (Fig. 1I).

Discussion

While the DNA damage response plays a major role in tumor suppression, how this response contributes to suppression of stomach tumorigenesis remains unclear. DSBs of chromosomal DNA are thought to be caused by the hazardous effects of IR and may result in chromosomal translocations, deletions or loss of genetic information, which are all causatively linked to tumorigenesis (15). Therefore, DSBs may be involved in radiation-associated gastric carcinogenesis among A-bomb survivors. In the present study, we provide immunohistochemical evidence that γH2AX is expressed in 13% of GCs from non-exposed patients and 30% of GCs from exposed patients. Because IR is a carcinogen and can increase an individual's risk of tumor development, DSBs appear to play a more important role in early-stage GC rather than late-stage GC. In fact, γH2AX is reported to be expressed commonly in early precursor lesions in urinary bladder, breast, lung, colon and prostate (15,17). However, in

Table II. Immunohistochemical analysis of γH2AX in GCs from exposed and non-exposed patients.

	No. of cases	γH2AX positive (%)	P-value
Exposed GC patients	66	20 (30)	
Histology[a]			
Intestinal type	34	9 (26)	
Diffuse type	32	11 (34)	0.5946
Depth of invasion[b]			
T1	15	0 (0)	
T2-4	51	20 (39)	0.003
Lymph node metastasis[b]			
N0	25	5 (20)	
N1-4	41	15 (37)	0.1792
TNM stage[b]			
I	21	2 (10)	
II-IV	45	18 (40)	0.0197
Non-exposed GC patients	47	6 (13)	
Histology[a]			
Intestinal type	33	5 (15)	
Diffuse type	14	1 (7)	0.6532
Depth of invasion[b]			
T1	19	2 (11)	
T2-4	28	4 (14)	1.000
Lymph node metastasis[b]			
N0	24	2 (8)	
N1-4	23	4 (17)	0.4158
TNM stage[b]			
I	23	2 (9)	
II-IV	24	4 (17)	0.6662

GC, gastric carcinoma. [a]Histologic classification of GC was according to the Lauren classification system. [b]Tumor stage was done according to be the Tumor-Node-Metastasis (TNM) staging system.

the present study, there was no significant difference in γH2AX staining between stage I GCs from exposed patients and non-exposed patients. Furthermore, in intestinal metaplasia adjacent to the tumor, which is considered to be a gastric precancerous lesion, staining of γH2AX was not observed in epithelial or stromal cells. These results suggest that DSBs are less likely to be involved in the genesis of GCs.

In contrast, in exposed patients, γH2AX-positive GC cases showed more advanced depth of invasion and higher TNM stage than γH2AX-negative GC cases, suggesting that DSBs may participate in progression of GC in exposed patients. It has been reported that deregulated c-myc expression induces DNA damage and the formation of γH2AX (24). However, mammalian SWI/SNF complexes facilitate DSBs repair by promoting γH2AX product (25), and we reported previously that increased expression of BRG1, a component of the SWI/SNF complex, is associated with advanced-stage GCs (26). It is possible that such signals may also contribute to γH2AX activation in GCs from exposed patients. Taken together, the molecular mechanisms that underlie phosphorylation of γH2AX may differ between IR-exposed patients and -non-exposed patients. It is also possible that because a single DSB can result in chromosomal translocations, deletions or loss of genetic information, several genes associated with tumor progression may be deleted in γH2AX-positive GC cases. Further studies are needed to identify these mechanisms.

In conclusion, DSBs do not appear to be characteristic alterations in stomach carcinogenesis in IR-exposed patients. However, immunohistochemical staining of γH2AX is increased with tumor progression in GCs from exposed patients. Although it is unclear whether all GCs from exposed patients in the present study were radiation-induced cancers, DSBs may serve as a marker for progression of GCs.

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RET/PTC Rearrangements Preferentially Occurred in Papillary Thyroid Cancer among Atomic Bomb Survivors Exposed to High Radiation Dose

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Abstract

A major early event in papillary thyroid carcinogenesis is constitutive activation of the mitogen-activated protein kinase signaling pathway caused by alterations of a single gene, typically rearrangements of the *RET* and *NTRK1* genes or point mutations in the *BRAF* and *RAS* genes. In childhood papillary thyroid cancer, regardless of history of radiation exposure, *RET/PTC* rearrangements are a major event. Conversely, in adult-onset papillary thyroid cancer among the general population, the most common molecular event is $BRAF^{V600E}$ point mutation, not *RET/PTC* rearrangements. To clarify which gene alteration, chromosome aberration, or point mutation preferentially occurs in radiation-associated adult-onset papillary thyroid cancer, we have performed molecular analyses on *RET/PTC* rearrangements and $BRAF^{V600E}$ mutation in 71 papillary thyroid cancer cases among atomic bomb survivors (including 21 cases not exposed to atomic bomb radiation), in relation to radiation dose as well as time elapsed since atomic bomb radiation exposure. *RET/PTC* rearrangements showed significantly increased frequency with increased radiation dose ($P_{trend} = 0.002$). In contrast, $BRAF^{V600E}$ mutation was less frequent in cases exposed to higher radiation dose ($P_{trend} < 0.001$). Papillary thyroid cancer subjects harboring *RET/PTC* rearrangements developed this cancer earlier than did cases with $BRAF^{V600E}$ mutation ($P = 0.03$). These findings were confirmed by multivariate logistic regression analysis. These results suggest that *RET/PTC* rearrangements play an important role in radiation-associated thyroid carcinogenesis. [Cancer Res 2008;68(17):7176–82]

Introduction

Thyroid cancer is, as is well-known, associated with exposure to external or internal ionizing radiation, such as from the atomic bombings (1) or the Chernobyl nuclear power plant accident (2, 3). The excess relative risk of thyroid cancer per Gy weighted thyroid dose was 1.15 in the Life Span Study (LSS) of atomic bomb (A-bomb) survivors (4), and a strong relationship between thyroid cancer and radiation exposure was indicated from the data of the Chernobyl accident (3). A histopathologic study has revealed that the thyroid cancers found in A-bomb survivors were largely conventional papillary in nature, and this is also the case of spontaneous thyroid cancer in the Japanese population at large. Solid variant papillary thyroid cancer (PTC) has not been found in A-bomb survivors yet, although this cancer has been frequently observed among post-Chernobyl children (5, 6).

Gene alterations that lead to constitutive activation of the mitogen-activated protein kinase (MAPK)-signaling pathway are frequently found in PTC. These alterations are mutually exclusive, nonoverlapping events that involve rearrangements of the *RET* and *neutrotrophic tyrosine kinase receptor 1* (*NTRK-1*) genes and point mutations in the *RAS* and *BRAF* genes (7–9). Alteration of one of these genes can be detected in >70% of PTC, suggesting that the constitutive activation of the MAPK-signaling pathway is a major early event in papillary thyroid carcinogenesis.

RET proto-oncogene is normally expressed in a subset of cells derived from the neural crest as well as from the kidney and the enteric nervous system (10, 11). In PTC, the *RET* proto-oncogene is activated by fusion of the *RET* TK domain with the 5′ terminal sequence of one of different heterologous genes via rearrangements that generate a series of chimeric-transforming oncogenes collectively described as *RET/PTCs*. To date, at least 12 rearranged forms of the *RET* gene have been isolated, of which *RET/PTC1* and *RET/PTC3* are by far the most common (12). *RET/PTC* rearrangements were commonly found in childhood PTC regardless of radiation history (13–15). Among the childhood PTC from areas contaminated by the Chernobyl nuclear accident in 1986, *RET/PTC3* rearrangement seemed to be strongly associated with solid variant-type PTC and with a short latency period after exposure (15, 16).

On the other hand, in the Japanese general adult population, typical frequency of *RET/PTC* seems to be of the magnitude of 10% to 40%, although a wide variation, ranging from 2.6% to 70%, has been observed in different geographic areas (17–19). *RET/PTC* rearrangements, especially *RET/PTC1*, was reported as being detected at higher frequency in PTC from adult patients with a

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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history of radiotherapy than in those without radiation history (20), but another report disputed such findings (21). Interestingly, we found that *RET/PTC1* rearrangements were induced in human thyroid cells by X-irradiation *in vitro* and *in vivo* as tissue transplants in severe combined immunodeficient mice (22). These findings may provide supporting evidence that activation of the *RET* oncogene via rearrangements plays a crucial role in radiation-associated papillary thyroid carcinogenesis.

The *BRAF* gene encodes a serine/threonine kinase responsible for transduction of signals in the MAP-kinase cascade (23). *BRAF* somatic mutations were first discovered in several types of human cancers, including malignant melanomas (24). Except for very rare instances, the *BRAF* mutation identified in thyroid cancer is thus far almost exclusively thymine-to-adenine transversion at nucleotide 1799, resulting in substitution of glutamate with valine at residue 600 (V600E; ref. 25). The V600E substitution is thought to convert BRAF inactive conformation into its active form by disrupting the residue-residue interaction between the activation loop and the ATP binding site (26).

$BRAF^{V600E}$ mutation has thus far been described as occurring with frequency ranging from 29% to 83% in PTC among an adult general population (25). Regarding the relationship with radiation exposure, the $BRAF^{V600E}$ gene mutation was studied in post-Chernobyl PTC, which is believed to have developed in those exposed to radiation in childhood. A very low frequency of $BRAF^{V600E}$ mutations in this PTC has been reported (range, 0–12%; refs. 27–31). However, prevalence of $BRAF^{V600E}$ mutation was originally low (range, 0–6%) in PTC among children, unrelated to their history of radiation exposure (27, 28, 31). Therefore, it may be difficult to assess the relationship between radiation exposure and childhood PTC in terms of $BRAF^{V600E}$ mutation. On the other hand, in adult-onset PTC among A-bomb survivors, we have previously reported that prevalence of $BRAF^{V600E}$ mutation was very low in adult-onset PTC among A-bomb survivors exposed to high radiation dose (>0.5 Gy), in contrast to high prevalence in nonexposed survivors or in the general population (32).

These findings lead us to a hypothesis that *RET/PTC* rearrangements in the MAPK-signaling pathway might play a major role in development of adult-onset radiation-associated PTC among A-bomb survivors. Therefore, to examine this hypothesis, this article analyzed pathologic and epidemiologic characteristics of adult-onset PTC in A-bomb survivors in terms of *RET/PTC* rearrangements and $BRAF^{V600E}$ mutation.

Materials and Methods

Patients and tissue specimens. Study patients comprised 71 adult-onset PTC cases diagnosed from 1956 to 1993, consisting of 50 exposed and 21 nonexposed patients found among A-bomb survivors in Hiroshima and Nagasaki; 54 of these 71 cases were those used in our previous study on $BRAF^{V600E}$ mutation (32). In the LSS (4), a total of about 250 PTC cases were identified in a cohort of LSS among A-bomb survivors during the aforementioned period. To date, we have obtained thyroid tissue specimens from 90 cases of these pathologically confirmed 250 cases. This number covered only about 36% of PTC found in the LSS cohort among A-bomb survivors during 1958 to 1993. After examining quality of RNA, 71 cases were analyzable for both *RET/PTC* and $BRAF^{V600E}$ in this study.

Classification of histology was done by one of the authors (T.H.) according to histopathologic typing established by the WHO (33). All study materials were formalin-fixed and paraffin-embedded PTC tissue specimens surgically resected during 1956 to 1993. This study was conducted under approval of the Human Investigation Committee and the Ethics Committee for Genome Research at the Radiation Effects Research Foundation (RERF).

RNA preparation and cDNA synthesis. RNA was extracted from microdissected noncancerous or cancerous regions using the High Pure RNA Paraffin kit (Roche Diagnostics GmbH), as described previously (34). Reverse transcription was performed with random primers (9 mer) using 100 ng total RNA as template, as described previously (34).

Identification of *RET/PTC* rearrangements and $BRAF^{V600E}$ mutation. Reverse transcription-PCR (RT-PCR) with *BCR* as internal control was

Table 1. Pathologic and epidemiologic characteristics of patients by radiation exposure status

		Exposed (dose > 0 mGy; n = 50)	Nonexposed* (n = 21)	P
Gender	Male (n)	6	2	1†
	Female (n)	44	19	
Histologic subtype	Conventional PTC (n)	47	21	0.6†
	Follicular variant (n)	3	0	
Median age ATB‡ (y, range)		22 (1–47)	20 (0–50)	0.3§
Median age at diagnosis (y, range)		50 (18–89)	48 (24–84)	0.9§
Median time after exposure (y, range)		24 (11–46)	—	—
Median radiation dose (mGy, range)		203 (0.4–2,758)	0	—
RET/PTC rearrangement	Absence (n)	39	20	0.09†
	Presence (n)	11	1	
	Frequency (%)	22	5	
$BRAF^{V600E}$ mutation	Absence (n)	22	4	0.06†
	Presence (n)	28	17	
	Frequency (%)	56	81	

*The nonexposed patients were either those with radiation dose estimated to be 0 mGy or those who were not in the city of Hiroshima or Nagasaki at the time of bombing.
† Fisher's exact test.
‡ ATB: at the time of atomic bombing.
§ Mann-Whitney's U test.

Table 2. Pathologic and epidemiologic characteristics of patients by *RET/PTC* rearrangement status

		All patients			Exposed patients (>0 mGy)		
		RET/PTC (n = 12)	Wild-type *RET* (n = 59)	P	*RET/PTC* (n = 11)	Wild-type *RET* (n = 39)	P
Gender	Male (n)	1	7	0.6*	1	5	0.6*
	Female (n)	11	52		10	34	
Histology	Conventional PTC (n)	11	57	0.9*	10	37	0.9*
	Follicular variant (n)	1	2		1	2	
Median age ATB†	Years	15	21	0.2‡	13	26	0.1‡
	Range	(3–41)	(0–52)		(3–41)	(1–47)	—
Median age at diagnosis	Years	39	51	0.1‡	39	54	0.05‡
	Range	(21–59)	(18–89)	—	(21–59)	(18–89)	—
Median time after exposure	Years	—	—	—	20	24	0.3‡
	Range	—	—	—	(15–36)	(11–46)	—
Median radiation dose	mGy	943	12	0.001‡	960	151	0.005‡
	Range	(0–2,304)	(0–2,758)	—	(67–2,304)	(0.4–2,758)	—

*Fisher's exact test.
†ATB: at the time of atomic bombing.
‡Mann-Whitney's U test.

conducted to confirm whether RNA extracted from archival tissue specimens was available for RT-PCR. The samples were examined for expression of *RET* TK domain by RT-PCR. RNA with detectable expression of the TK domain was further analyzed for determination of rearrangement types. cDNA derived from 10 ng of total RNA was used as an RT-PCR template. RT-PCR was performed with 0.5 U of Platinum Taq DNA polymerase (Invitrogen) for BCR, the TK domain, *RET/PTC1* and *RET/PTC3*, or 0.5 U of Platinum Taq DNA polymerase High Fidelity (Invitrogen) for *TRK-T2* and novel *RET/PTC* in 25 μL volume containing 1× PCR buffer, 200 μmol/L each of deoxynucleotide triphosphate mixture, and 0.4 μmol/L of each primer. RT-PCR conditions consisted of initial denaturation (95°C for 3 min), followed by 40 cycles (36 cycles for TK domain of *RET*) of denaturation at 95°C for 30 s, annealing for 30 s, extension at 72°C for 30 s, and a final extension at 72°C for 5 min. Primer sets, oligonucleotides, annealing temperature, and Mg^{2+} concentration are summarized in Supplementary Table S1.

For samples that showed expression of *RET* gene TK domain but not assigned as *RET/PTC1* or *RET/PTC3*, rearrangement types were examined by an improved SMART RACE method, which was developed by us.[11] Briefly, after completion of cDNA synthesis, the reaction solution was further incubated at 42°C for 60 min in the presence of SMART adaptor. This SMART-PCR was conducted using FastStart High Fidelity PCR system (Roche Diagnostics GmbH), and primers RET-Ex12PR9 and S-RACE 1, followed by nested RT-PCR using primer RET-Ex12A4 and SMART adaptor. SMART-PCR conditions were as described above, except for the cycle numbers (45 cycles for 1st PCR and 25 cycles for nested PCR). All target bands in RT-PCR were confirmed by digestion of restriction enzyme, *BamH* I (TaKaRa) for *RET/PTC1* and *RET/PTC3*, *Alu* I for *BCR*, and *Hae* III for the TK domain, which existed within each amplified target fragment. Other *RET/PTC* rearrangement types identified by improved SMART RACE were confirmed by sequencing using a CEQ8000 DNA sequencer (Beckman Coulter, Inc.).

BRAF gene mutation causing amino acid substitution of glutamic acid for valine at codon 600 ($BRAF^{V600E}$) was determined by RFLP using *TspR* I (New England Biolabs) and direct sequencing, as described previously (32).

[11] Submitted.

Statistical analysis. Mann-Whitney's U test was used for nonparametric two-sample comparisons of continuous variables. Fisher's exact test was used for categorical variables. The Cochran-Armitage test was used for nonparametric trend analysis. Logistic regression analysis was carried out among 39 A-bomb survivor exposed patients who had either *RET/PTC* rearrangement or $BRAF^{V600E}$ mutation, to assess differences between PTC patients with *RET/PTC* rearrangement and those with $BRAF^{V600E}$ mutation, in terms of pathologic and epidemiologic variables, including radiation dose, histology, gender, and time-related factors [Note that age at diagnosis = age at the time of A-bombing (ATB) + the time since exposure]. All statistical analyses were performed with SPSS software (version 12.0).

Radiation dose. A-bomb radiation doses used in this analysis were shielded organ dose to the thyroid estimated by the recently implemented DS02 system (35).

Results

Pathologic and epidemiologic characteristics of PTC among A-bomb survivors. Pathologic and epidemiologic characteristics of study patients are shown in Table 1. All tumors were well-differentiated PTC including three cases of follicular variant. When comparing exposed and nonexposed patients, no differences were found based in gender, histologic subtypes, age ATB, and age at diagnosis.

Of 71 patients, we detected *RET/PTC* rearrangements in 12 patients: 9 with only *RET/PTC1*, 1 with both *RET/PTC1* and *RET/PTC3*, 1 with *RET/PTC8*, and 1 with a novel *RET* rearrangement. This novel *RET/PTC* (*RET/PTCX*) was regarded as one *RET* rearrangement, whose partner gene, acyl-CoA binding domain containing 5 (*ACBD5*, located on chromosome 10p12.1), had at least one coiled-coil domain, expression of which was confirmed by RT-PCR (Supplementary Fig. S1). Although the exposed patients showed a higher frequency of *RET/PTC* rearrangements than did nonexposed ones, this difference was not statistically significant (Table 1). On the other hand, frequency of $BRAF^{V600E}$ mutation was marginally lower in exposed patients than that in nonexposed ones (P = 0.06; Table 1).

Pathologic and epidemiologic characteristics by *RET/PTC* rearrangement status. Pathologic and epidemiologic characteristics of study patients were shown by *RET/PTC* rearrangement status in Table 2, where nonexposed patients (0 mGy) were excluded ("exposed patients") or included ("all patients"). Significant difference was found in radiation dose between all patients with and without *RET/PTC* rearrangement ($P = 0.001$; median dose, 943 versus 12 mGy), and also between exposed patients with and without *RET/PTC* rearrangement ($P = 0.005$; median dose, 960 versus 151 mGy; Table 2). Presence or absence of *RET/PTC* rearrangement revealed marginal association with age at diagnosis in exposed patients ($P = 0.05$), although no significant association was found in all patients ($P = 0.1$). No significant relationship was observed between *RET/PTC* rearrangement status and age ATB, histologic subtype, or gender in both all patients and only exposed patients. Furthermore, no significant association was found in exposed patients with time elapsed since A-bomb exposure to diagnosis.

Pathologic and epidemiologic characteristics by *BRAF*^{V600E} mutation. Pathologic and epidemiologic characteristics of study patients were shown by *BRAF*^{V600E} mutation status (Table 3). Close association of *BRAF*^{V600E} mutation status with radiation dose and time since exposure remained unchanged from our previous results (32): PTC patients with *BRAF*^{V600E} mutation showed significantly lower radiation dose ($P = 0.0001$ or 0.0002 in all patients or exposed patients, respectively) and significantly longer time since exposure ($P = 0.0003$ in exposed patients), compared with those without *BRAF* mutation. Age at diagnosis was found to be significantly older in patients with *BRAF*^{V600E} mutation than those without *BRAF*^{V600E} mutation ($P = 0.001$ or 0.0002 in all patients or exposed patients, respectively), although this association did not reach significance in our previous study (32) based on a smaller number of patients. In addition, in only exposed patients, *BRAF*^{V600E} mutation status revealed a significant association with age ATB, but this was not significant in all patients. Furthermore, no significant association was found between *BRAF* mutation status and histology or gender as was also the case in our previous study (32).

Increased *RET/PTC* rearrangements and decreased *BRAF*^{V600E} mutation frequency with increased radiation dose. To examine the relationship between *RET/PTC* and *BRAF*^{V600E} mutation and radiation dose, exposed PTC patients were divided into three groups by dose tertiles. *RET/PTC* rearrangements were more frequently found in patients with increased radiation dose ($P_{trend} = 0.002$; Fig. 1*A*). Specifically, *RET/PTC* rearrangements were found in 50% (8 of 16) of PTC patients who were exposed to high doses (>0.5 Gy) in Fig. 1*A*: 5 with only *RET/PTC1*, 1 with both *RET/PTC1* and *RET/PTC3* (2.2 Gy), 1 with *RET/PTC8* (2.3 Gy), and 1 with *RET/PTCX* (1.5Gy).

On the other hand, prevalence of *BRAF*^{V600E} mutation significantly decreased with radiation dose ($P_{trend} = 0.00006$). In addition, PTC patients having wild-type *RET* and *BRAF* showed a marginally significant increasing trend with radiation dose ($P = 0.08$; Fig. 1*A*).

Frequency of *RET/PTC* and *BRAF*^{V600E} alterations in PTC patients grouped by time elapsed since atomic radiation exposure. *RET/PTC* rearrangements and *BRAF*^{V600E} mutation were further studied in relation to time since radiation exposure (Fig. 1*B*). *BRAF*^{V600E} mutation significantly increased with increased time since exposure ($P_{trend} = 0.001$), whereas unidentified alterations in PTC having wild-type *RET* and *BRAF* significantly decreased with increased time since exposure ($P_{trend} = 0.001$). In contrast, *RET/PTC* rearrangements showed a peak at time since exposure 18 to 27 years, suggesting that unidentified alterations other than *RET/PTC* may also play an important role in PTC occurred in relatively short time since the exposure.

Radiation-related factors underlying occurrence of *RET/PTC* rearrangements versus *BRAF*^{V600E} mutation. As was the case for PTC among the general population (7–9), *RET/PTC* rearrangements and *BRAF*^{V600E} mutation were found to be mutually exclusive among exposed PTC patients (Supplementary Table S2). On the basis of this result, pathologic and epidemiologic characteristics were compared between 11 PTC patients having

Table 3. Pathologic and epidemiologic characteristics of patients by *BRAF*^{V600E} mutation status

		All patients			Exposed patients (>0 mGy)		
		BRAF^{V600E} ($n = 45$)	Wild-type *BRAF* ($n = 26$)	*P*	BRAF^{V600E} ($n = 28$)	Wild-type BRAF ($n = 22$)	*P*
Gender	Male (n)	5	3	0.7*	3	3	0.5*
	Female (n)	40	23		25	19	
Histology	Conventional PTC (n)	44	24	0.3*	27	20	0.4*
	Follicular variant (n)	1	2		1	2	
Median age ATB†	Years	22	17	0.09‡	31	17	0.04‡
	Range	(0–52)	(1–47)	—	(1–47)	(1–47)	—
Median age at diagnosis	Years	54	39	0.001‡	55	38	0.0002‡
	Range	(20–89)	(18–62)	—	(20–89)	(18–59)	—
Median time after exposure	Years	—	—	—	29	18	0.0003‡
	Range	—	—	—	(15–46)	(11–36)	—
Median radiation dose	mGy	8	538	0.0001‡	69	859	0.0002‡
	Range	(0–2,758)	(0–2,304)		(0.4–2,758)	(12–2,304)	—

*Fisher's exact test.
†ATB: at the time of atomic bombing.
‡Mann-Whitney's *U* test.

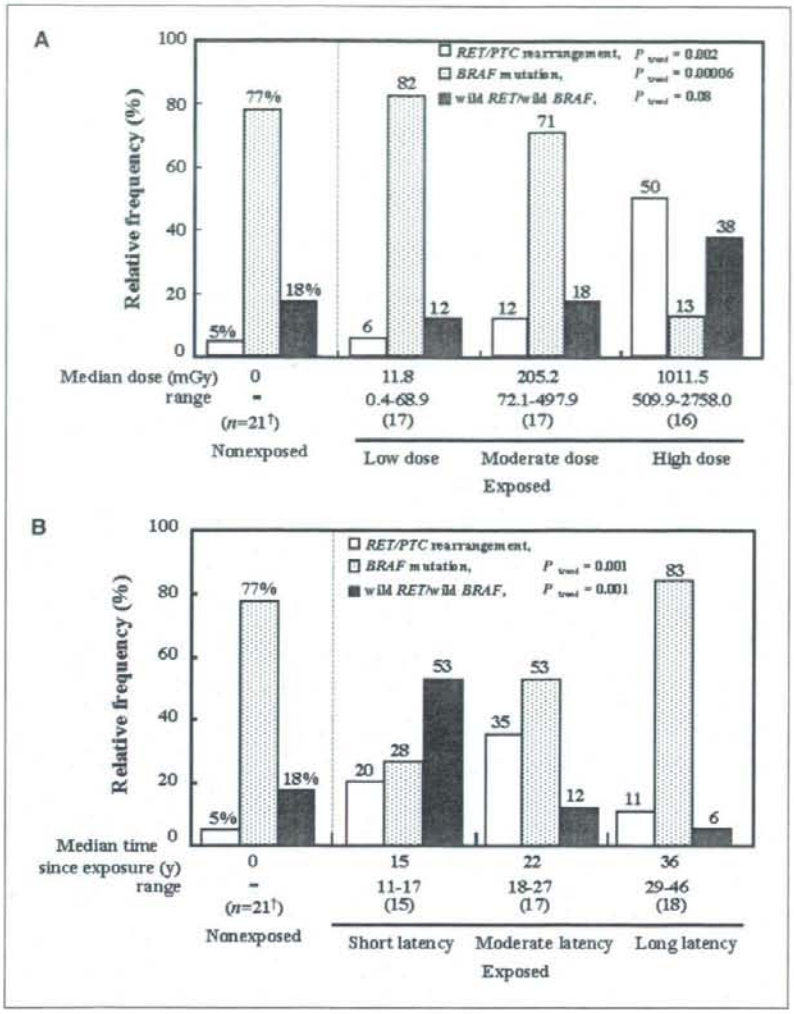

Figure 1. *A*, relative frequency of *RET/PTC* and *BRAF*^{V600E} alterations in PTC patients grouped by radiation exposure dose levels (nonexposed and dose tertiles). Exposed PTC patients were divided into three groups by dose tertiles. *B*, relative frequency of *RET/PTC* and *BRAF*^{V600E} alterations in PTC patients grouped by time elapsed since atomic radiation exposure (nonexposed and tertiles of time since exposure). Exposed PTC patients were divided into three groups by tertiles of time since exposure. †, one case in the nonexposed group had both *RET/PTC* and *BRAF*^{V600E}. Relative frequency of genes in the nonexposed group was calculated by using 22 for number of gene alterations. PTC with *RET/PTC* rearrangement (*open bars*), with *BRAF*^{V600E} mutation (*dotted bars*), or with other unknown alterations (*closed bars*), respectively, are shown.

RET/PTC rearrangements and 28 patients having *BRAF*^{V600E} mutation. PTC patients with *RET/PTC* rearrangements revealed past exposure to significantly higher radiation dose (P = 0.001; Fig. 2*A*), shorter time elapsed since radiation exposure (P = 0.03; Fig. 2*B*), and younger age ATB (P = 0.06; Fig. 2*C*), compared with the patients with *BRAF*^{V600E} mutation.

Subsequent logistic regression analysis for mutually exclusive occurrence of *RET/PTC* rearrangements or *BRAF*^{V600E} mutation confirmed these findings, using "age ATB" and "time since exposure" as independent time-related explanatory variables (Note that "age at diagnosis" = "age at exposure" + "time since exposure"). Radiation dose, age at exposure, and time elapsed since exposure were significantly associated with which alteration type of *RET/PTC* rearrangements or *BRAF*^{V600E} mutation occurred in the development of PTC among A-bomb survivors (P = 0.012, 0.031, and 0.034, respectively; Table 4).

Rearrangements of *NTRK1* and *BRAF* genes. *NTRK1* rearrangements and the *AKAP9-BRAF* fusion gene were also examined in the 71 cases. The *TRK-T2* gene was detected in only one exposed case with wild-type *RET* and *BRAF*. However, five *NTRK1*-derived nucleotides were deleted in this amplified fragment. On the other hand, no *AKAP9-BRAF* fusion gene was detected in these 71 cases.

Discussion

In papillary thyroid carcinogenesis, constitutive activation of the MAPK-signaling pathway, namely rearrangements of *RET* and *NTRK* genes and mutations in *RAS* and *BRAF* oncogenes, seems to be required for transformation (36). Recent *in vitro* and *in vivo* experiments have also shown the requirement of activation of the RET/PTC-RAS-BRAF-MAPK pathway in thyroid tumorigenesis (37–39). Interestingly, mutual exclusion of these genetic alterations in the MAPK-signaling pathway was reported; one event among *BRAF* mutation, *RAS* mutations, and *RET/PTC* rearrangements (7, 8, 29) or one among *BRAF* mutation, *RET/PTC*