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Expression of Family A Melanoma Antigen in Human Gastric Carcinoma

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Abstract. *Background: Since the members of the MAGE (melanoma antigen) gene family have been reported to be expressed in tumor cells but not in normal tissues, they have been considered as targets for tumor-specific immunotherapy. Materials and Methods: The expression pattern of MAGE-A genes and their expression mechanisms were investigated in 10 gastric cancer cell lines and 1,097 gastric carcinoma specimens by RT-PCR, IHC, Western blot and MSP. Results: MAGE-A1, -A2 and -A3 gene transcripts were detected in 1, 3 and 4 of 10 gastric cancer cell lines, respectively. In those cases in which the mRNA expression of MAGE-A2 or -A3 was detected, the promoters of the corresponding genes were hypomethylated. MAGE-A protein expression was detected in 30% (3/10) of the cell lines and 15.8% (173 out of 1,097) of the carcinoma specimens. Promoter hypomethylation of the MAGE-A2 or -A3 genes correlated with their expression in primary gastric cancer tissue and gastric cancer cell lines. MAGE-A protein expression was associated with tumor invasiveness ($p=0.002$), lymph node metastasis ($p<0.001$), advanced pathologic stage ($p<0.001$) and a worse prognosis ($p<0.005$). Conclusion: MAGE-A protein expression occurred due to promoter hypomethylation in a minor subset of gastric cancers, and MAGE-A expression increased during the progression of the gastric cancer.*

The human MAGE (melanoma antigen) gene family consists of a large number of X-chromosome-linked genes, including MAGE-A genes at Xq28, MAGE-B genes at Xp21 and MAGE-C genes at Xp26-27 (1). MAGE genes are silent in normal tissues except in the testis and placenta (2). Although MAGE proteins are recognized by autologous cytotoxic T cells, they do not present targets for cytotoxic T lymphocytes (CTLS) in the testis or placenta (2, 3), because germ cells usually do not express classic MHC molecules.

MAGE genes have been reported to be tumor-specific antigens and ideal targets for cancer immunotherapy (4). Preliminary results of clinical trials suggest that tumor regression can be induced in a significant number of cancer patients by immunization with an antigen encoded by the MAGE-A3 gene (5-7). MAGE genes are most widely expressed in neoplasms such as breast cancer (8), laryngeal cancer, renal cell carcinoma (9), colon cancer (9), stomach cancer (9), esophageal carcinoma (10) and glioblastoma (11). Therefore, the identification of the mechanisms regulating MAGE gene expression in cancer cells may lead to the establishment of new methodologies that can circumvent its relatively reduced expression in the tumor lesions of individual patients and, eventually, to the designing of more effective vaccination strategies (12).

Epigenetic alterations, including the hypomethylation of promotor CpG islands and the histone deacetylation of tumor suppressors, have been recognized as important contributors to carcinogenesis in humans. Global DNA hypomethylation has been observed in carcinomas of the breast, liver and colon, and is thought to occur in the early stages of tumor development (4, 13-15). In fact, most MAGE-type genes have been found to have promoters with high CpG contents. (3, 16). Moreover, it has been suggested that there is good correlation between the mRNA expression of the MAGE-A1, -A3, -B2 genes and the hypomethylation of their promoters in lung cancer (17).

Thus, in this study, the methylation status of the promoter region in MAGE-A genes was studied and then compared with the protein and mRNA expression in gastric cancer cell lines and carcinoma specimens. The association between MAGE-A expression and the clinicopathological characteristics of the subjects in cases of gastric carcinoma was also investigated.

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Key Words: Tumor antigen, DNA methylation, reverse transcriptase polymerase chain reaction, stomach neoplasms, immunohistochemistry, survival analysis.

Table I. *Oligonucleotide primers used in reverse transcription-PCR (RT-PCR) and methylation-specific PCR (MSP).*

Gene	Sequence		Annealing temperature ˚C
MAGE-A1			
RT-PCR	AS	5'-TGTGGGCAGGAGCTGGGCAA-3'	61
	S	5'-GCCGAAGGAACCTGACCCAG-3'	
MAGE-A2			
RT-PCR	AS	5'-CATTGAAGGAGAAGATCTGCCT-3'	60
	S	5'-GAGTAGAAGAGGAAGAAGCGGT-3'	
MSP	U-AS	5'-GTTGTGAATTTAGGGAAGTTATGG-3'	62
	U-S	5'-ACATCAAACCATTACTCAAAACAAA-3'	
	M-AS	5'-TTTGTCGTGAATTTAGGGAAGTTAC-3'	62
	M-S	5'-GTCAAACCGTTACTCAAAACGA-3'	
MAGE-A3			
RT-PCR	AS	5'-AAGCCGGCCCAGGCTCGGT-3'	62
	S	5'-GCTGGGCAATGGAGACCCAC-3'	
MSP	U-AS	5'-TGTTAGGATGTGATGTTATTGATTTGT-3'	62
	U-S	5'-CCTCACCAAACCTAAACCAA-3'	
	M-AS	5'-CCATCTGACGTTATTGATTTGC-3'	64
	M-S	5'-CTCACCGAACCTAAACCGAC-3'	

AS, anti-sense; S, sense; U, for unmethylated DNA; M, for methylated DNA

Materials and Methods

Gastric cancer cell lines. Ten human gastric cancer cell lines, SNU-1, SNU-5, SNU-16, SNU-216, SNU-484, SNU-601, SNU-620, SNU-638, SNU-668 and SNU-719 were obtained from the Korean Cell Line Bank (Seoul, Korea) and were cultured in RPMI-1640 supplemented with 10% heat-inactivated FBS (Hyclone, Logan, UT, USA). Genomic DNA was extracted by a standard proteinase-K digestion and phenol/chloroform procedure.

Total RNA was isolated with the TRIZOL reagent (Gibco BRL, Gaithersberg, MD, USA). To generate cDNA, mRNA (5ng) was reverse-transcribed using MMLV-reverse transcriptase (Bioneer, Seoul, Korea) with oligo(dT) primer. PCR amplification was performed for 35 cycles each at 95˚C for 30 sec, 60 ~ 62˚C for 30 sec, 72˚C for 1 min, and final extension at 72˚C for 10 min in a thermal cycler (Bioneer). To verify the integrity of the cDNA, the β-actin gene was amplified for each sample. The primer sequences and annealing temperatures are listed in Table I.

For the methylation-specific PCR, 1 μg of genomic DNA was denatured with NaOH (final concentration 0.2 M), treated with 3 M sodium-bisulfite (Sigma, St. Louis, MO, USA) and 10 mM hydroquinone (pH 5.0, Sigma), and then incubated at 50˚C for 16 h. After incubation, DNA was purified using a Wizard DNA purification kit (Promega, Madison, WI, USA), and then treated with NaOH, recovered in ethanol and resuspended in 20 μl of distilled water. After the sodium bisulphate modification, PCR amplification was performed in a thermal cycler for one cycle at 95˚C for 5 min followed by 35 cycles each at 95˚C for 30 sec, 60~64˚C for 30 sec, 72˚C for 1 min, and final extension at 72˚C for 10 min. The sequences of the primers and their annealing temperatures are listed in Table I.

Cellular protein extracts from the gastric cancer cell lines were prepared by dissociation with lysis buffer (iNtRON Biotechnology, Seoul, Korea), measured using a BCA protein assay kit (Pierce, Rockford, IL, USA), and separated on 12% SDS-polyacrylamide gel. The proteins were then transferred onto a reinforced PVDF membrane (Millipore, Bedford, MA, USA). The non-specific sites on the blots were blocked by incubating them for 1 h in TBS containing 0.1% Tween 20 and 5% non-fat dried milk. Anti-MAGE-A (Zymed Laboratories, Carlsbed, CA, USA, 1:500) was used as the primary antibody. After overnight incubation at 4˚C and washing with TBS, the blots were incubated for 1 h at room temperature with anti-mouse HRP as the secondary antibody. After extensive washing, the antigen-antibody complexes were visualized by ECL staining (Pierce).

Gastric cancer tissue. A total of 1,097 cases of formalin-fixed, paraffin-embedded gastric tumor specimens and 59 normal gastric tissues, which had been taken over a period of 2 years (Jan. 1995 ~ Dec. 1996), were collected from the files of the Department of Pathology, Seoul National University Hospital. The age, sex and pTNM (tumor, lymph node, metastasis) stage were evaluated by reviewing the medical charts and pathologic records. Glass slides were reviewed to determine the histological type (according to the WHO and Lauren classifications). Ninety-three percent of the patients had undergone curative resection (R0 according to the UICC guideline). The clinical outcome of the patients was followed from the date of surgery to either the date of death or up to 6 years, resulting in a follow-up period ranging from 1 ~ 72 months (mean: 48 months). Those cases lost to follow-up and those ending in death from any cause other than gastric cancer were regarded as censored data during the analysis of the survival rate.

To investigate the protein expression patterns of MAGE-A, we prepared 21 tissue array blocks containing both gastric cancer and normal mucosa tissue, and then performed serial sectioning (18). These 4-μm-thick tissue array sections were deparaffinized and dehydrated. Mouse anti-MAGE monoclonal antibody (Zymed Laboratories) was used at a dilution of 1:50 and immuno-

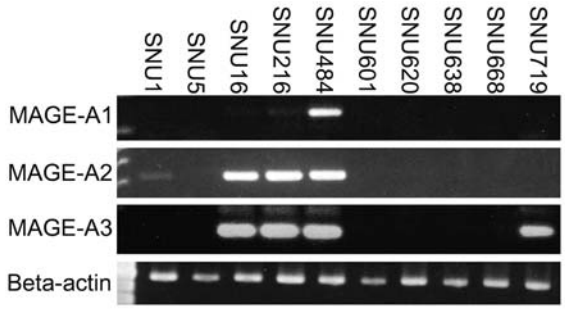

Figure 1. *Reverse-transcriptase PCR of MAGE-A1, -A2 and -A3 in gastric cancer cell lines. SNU16 and SNU216 expressed MAGE-A2 and -A3, SNU484 expressed MAGE-A1, -A2 and -A3, and SNU719 expressed MAGE-A3. β-Actin was used as a control.*

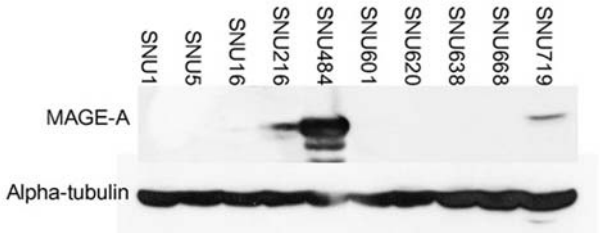

Figure 2. *Western blot of MAGE-A in gastric cancer cell lines. The MAGE-A protein was expressed in the SNU216, SNU484 and SNU719 cell lines. SNU16 which expressed MAGE-A2 and -A3 mRNA, did not express the MAGE-A protein. α-Tubulin was used as a control.*

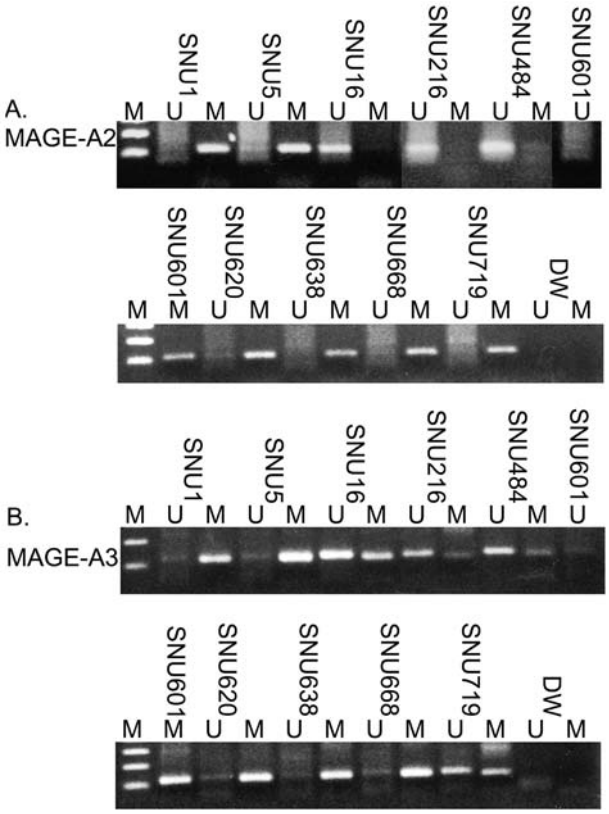

Figure 3. *Methylation-specific PCR of MAGE-A2, -A3 in gastric cancer cell lines. The hypomethylation of MAGE-A2 was observed in SNU16, SNU216 and SNU484. The hypomethylation of MAGE-A3 was observed in SNU16, SNU216, SNU484 and SNU719. DW, distilled water; M, methylated; U, unmethylated.*

peroxidase staining was performed with a Vector ABC kit (Vector Laboratories, Burlingane, CA, USA). For the comparison of this large scale data, the results of the immunostaining experiment were considered to be positive if 10% or more of the neoplastic cells were strongly stained. Forty-nine MAGE-A-negative cases and 52 MAGE-A-positive cases were selected and underwent methylation-specific PCR. Methylation-specific PCR was performed with the same primers as those used for the cancer cell lines.

Statistical analysis. The Chi-square test or Fisher exact test (2-sided) was performed to compare the clinicopathological characteristics of the patients. The results were considered to be statistically significant at a *p*-value of less than 0.05. Survival curves were estimated using the Kaplan-Meier product-limit method, and the significance of the differences between the survival curves was determined using the log-rank test. Multivariate survival analysis was performed using the Cox proportional hazards model. All statistical analyses were conducted using the SPSS 11.0 statistical software program (SPSS, Chicago, IL, USA).

Results

Expression and hypomethylation of MAGE-A in gastric cancer cell lines. MAGE-A1, -A2 and -A3 mRNA expressions in ten gastric cancer cell lines were investigated by RT-PCR. MAGE-A1 was found in one cell line (SNU216), MAGE-A2 in three cell lines (SNU16, SNU216, SNU484) and MAGE-A3 in four cell lines (SNU16, SNU216, SNU484, SNU719). SNU484 expressed all three types of gene, and four cell lines expressed at least one gene (Figure 1). All of the cell lines which expressed either MAGE-A1 or -A2 also expressed MAGE-A3. In the Western blot analysis, three cell lines (SNU216, SNU484, SNU719) demonstrated bands at 45-50 kDa, corresponding to MAGE-A1, -A2 and -A3. SNU216 and SNU719 showed a band at 50 kDa, and SNU484 showed two or more bands at 45-50 kDa (Figure 2). These three cell lines, which exhibited MAGE-A protein expression, showed positive mRNA expression for MAGE-A3.

Using the Methprimer program (http://www.ucsf.edu/urogene/methprimer/), the CpG island was located in the MAGE-A2 and -A3 promoters, but not in the MAGE-A1 promoter. All three cell lines (SNU16, SNU216, SNU484) with positive expression in the RT-PCR reaction of MAGE-A2 also had hypomethylated DNA in the promoter region of the corresponding gene (Figure 3). The remaining seven cell

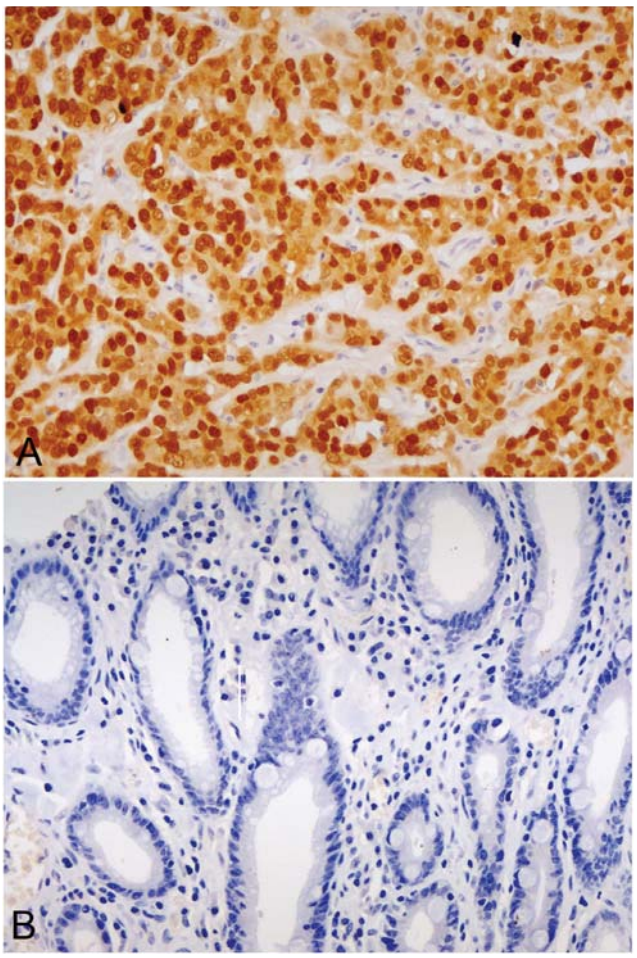

Figure 4. *Immunohistochemical staining of MAGE-A in gastric cancerous tissues and non-cancerous tissues. (A) In the positive cases, MAGE-A protein was homogenously stained in the cytoplasm as well as in the nucleus of all cancer cells. MAGE-A protein was positive in 15.8% of the cases. (B) MAGE-A protein was not stained in the normal or metaplastic gastric mucosa.*

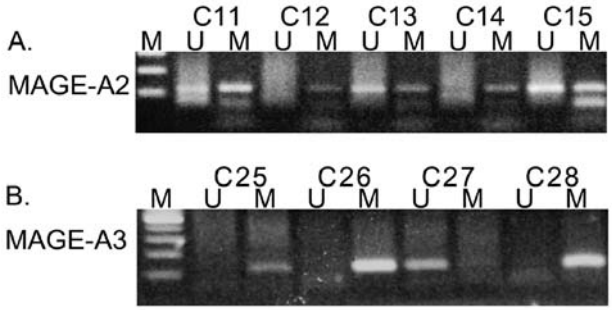

Figure 5. *Representative picture of methylation-specific PCR of MAGE-A2, -A3 in primary gastric cancer. (A) In MAGE-A2, the PCR product was demonstrated with primers of hypomethylated promoter in cases 11, 13 and 15. (B). In MAGE-A3, the PCR product was demonstrated with primers of hypomethylated promoter in case 27.*

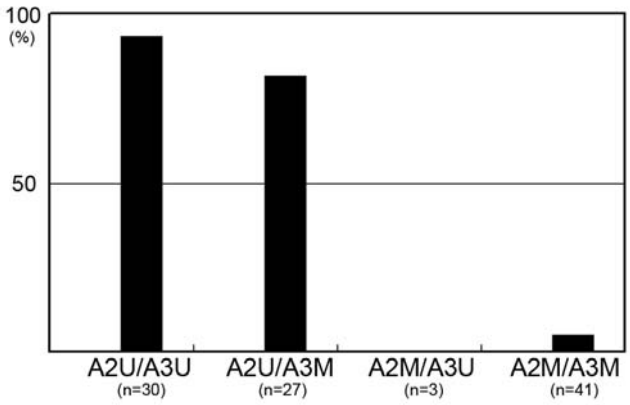

Figure 6. *Positive rate of MAGE-A immunostaining in cases with different methylation status of MAGE-A2 and MAGE-A3. Groups of gastric cancers with unmethylated MAGE-A2 promoter show a higher positive rate than that of methylated MAGE-A2 promoter. U, unmethylated; M, methylated*

lines, which did not exhibit MAGE-A2 mRNA expression, had hypermethylated promoters. As for MAGE-A3, all ten cell lines showed the positive PCR products with methylated primers irrespective of mRNA expression status. However, only four cell lines (SNU16, SNU216, SNU484, SNU719), which exhibited MAGE-A3 mRNA expression, had hypomethylated promoter regions in the corresponding genes. (Figure 3). It suggests that the partial unmethylation of MAGE-A3 can induce the mRNA in gastric cancer cell lines.

Expression and hypomethylation of MAGE-A genes in primary gastric carcinoma. MAGE-A protein expression was analyzed by immunochemistry in 1,097 cases of consecutive gastric carcinoma, as well as in the corresponding non-cancerous gastric mucosa. MAGE-A proteins were expressed in 15.8% of the cancer tissues (173 out of 1,097) with a cytoplasmic and nuclear staining pattern. In contrast, foveolar epithelium, gastric proper glands or metaplastic gastric mucosa did not express MAGE-A protein (Figure 4). Methylation-specific PCR of 52 randomly selected cases expressing MAGE-A revealed that 50 and 28 cases displayed hypomethylation of MAGE-A2 and -A3, respectively. In contrast, of the 49 cases in which MAGE-A protein expression was not exhibited, MAGE-A2 or -A3 hypomethylation was found in only 7 and 5 cases, respectively. Representative cases of methylation-specific

Table II. *Correlation between MAGE-A protein expression and clinicopathological characteristics in consecutive gastric cancer patients.*

	Negative (n=924) (%)	Positive (n=173) (%)	*P*-value
Age (years)	54.6±12.6	60.5±9.2	<0.001
Sex			0.001
Male	597 (64.6)	135 (78.0)	
Female	327 (35.4)	38 (22.0)	
WHO classification			<0.001
W/D	72 (7.8)	12 (6.9)	
M/D	273 (29.5)	91 (52.6)	
P/D	377 (40.8)	64 (37.0)	
Mucinous	51 (5.5)	5 (2.9)	
SRC	151 (16.3)	1 (0.6)	
Lauren classification			<0.001
Intestinal	343 (37.1)	99 (57.2)	
Diffuse	527 (57.0)	61 (35.3)	
Mixed	54 (5.8)	13 (7.5)	
Tumor invasion			0.002
AGC	645 (69.8)	141 (81.5)	
EGC	279 (30.2)	32 (18.5)	
Lymph node metastasis			<0.001
Absent	391 (42.3)	35 (20.2)	
Present	533 (57.7)	138 (79.8)	
pTNM stage			<0.001
Stage I	400 (43.3)	45 (26.0)	
Stage II	198 (21.4)	45 (26.0)	
Stage III	197 (21.3)	51 (29.5)	
Stage IV	129 (14.0)	32 (18.5)	
Lymphatic invasion			0.003
Absent	656 (71.0)	103 (59.5)	
Present	268 (29.0)	70 (40.5)	

W/D, well-differentiated adenocarcinoma; M/D, moderately-differentiated adenocarcinoma; P/D, poorly-differentiated adenocarcinoma; SRC, signet ring cell carcinoma; AGC, advanced gastric carcinoma; EGC, early gastric carcinoma

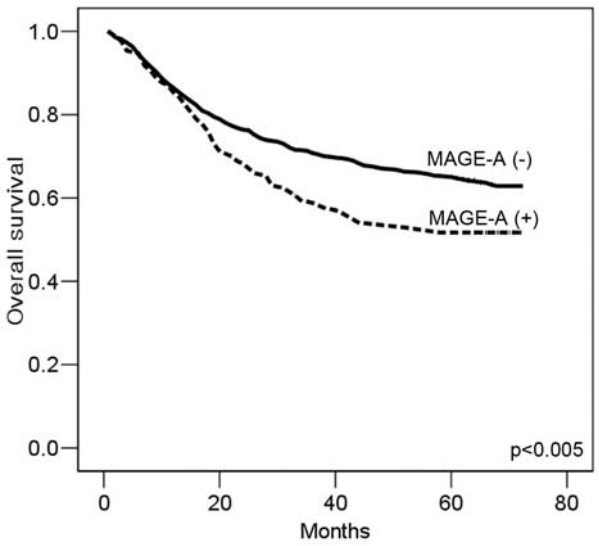

Figure 7. *Survival curve using Kaplan-Meier product-limit method for gastric cancer patients. Patients who expressed MAGE-A in immunohistochemistry showed a poorer prognosis compared to those who did not expressed MAGE-A (p<0.005).*

PCR are provided in Figure 5. The methylations of MAGE-A2 and -A3 were closely correlated, in that most of the cases (30/33) which presented MAGE-A3 promoter hypomethylation also presented MAGE-A2 promoter hypomethylation. Those cases with hypomethylated MAGE-A2 exhibited the higher rate of protein expression (Figure 6). The above results suggest that the hypomethylation of MAGE-A2 might be responsible for the expression of the MAGE-A in primary gastric cancer tissues. However, according to the cell line result, hypomethylation of MAGE-A3 may also contribute to the protein expression.

MAGE-A expression and clinicopathological parameters. MAGE-A protein expression was analyzed in 1,097 gastric cancer tissues by immunohistochemistry using the tissue array method, and the correlation between protein expression and patient clinicopathological parameters was investigated. The results are summarized in Table II. Those patients who exhibited MAGE-A expression tended to be older ($p<0.001$) and were more likely to be male ($p=0.001$), as compared with those patients who did not exhibit MAGE-A expression. Those cancers which exhibited MAGE-A expression were associated with the moderately-differentiated type of the WHO classification ($p<0.001$) and the intestinal type of the Lauren classification ($p<0.001$). They tended be deeper invasion ($p=0.002$), lymph node metastasis ($p<0.001$), advanced pTNM stage ($p<0.001$) and lymphatic invasion ($p=0.003$). However, there were no significant differences either in the tumor location or the presence of distant metastasis between the cases of MAGE-A-positive cancer and MAGE-A-negative cancer (data not shown). In the survival analysis, the survival rate of the patients with MAGE-A-positive gastric cancer, as determined by the log-rank test, was significantly lower than that of the patients with MAGE-A-negative gastric cancer ($p<0.005$) (Figure 7). However, in the analysis performed using the multivariate Cox proportional hazards model, MAGE-A was not significantly correlated with patient survival ($p>0.05$) when the pTNM stage was taken into consideration (data not shown).

Discussion

MAGE genes encode tumor-specific antigens, which are recognized by autologous cytolytic T lymphocytes, and are therefore considered to be ideal targets for cancer immunotherapy (14). Recently, it has been reported that

MAGE-A1 and -A3 expressions were demonstrated in seven and nine out of ten gastric cancer cell lines, respectively, and in 40% of gastric carcinomas (14). However, our results indicated much lower expression rates for the mRNA expression of MAGE-A1 (10%) and MAGE-A3 (40%). Our RT-PCR results were confirmed by the promoter hypomethylation experiment, which corresponded exactly to the mRNA expression results.

When the expression of MAGE-A proteins was examined in primary gastric carcinoma, 15.8% showed positive immunohistochemistry. In most cases, the positive cells exhibited intense staining in the cytoplasm and some cases showed both cytoplasmic and nuclear staining. None of the cases showed equivocal or faint staining. Furthermore, no cases of heterogeneous or irregular staining in the cancer tissues were encountered. This result suggests that MAGE-A hypomethylation is an on-off phenomenon, and that it occurs during the early stage of carcinogenesis.

Most MAGE-type genes have been found to have promoters with high CpG contents (3, 16), and it has been reported that the hypomethylation of the promoter CpG island in MAGE genes triggers their expression in tumor cells (14). In lung cancer, good overall correlation between the mRNA expression of MAGE-A1, -A3 and -B2 genes and the hypomethylation of their promoters was reported (4). In this study, it was demonstrated that the SNU16, SNU216, SNU484 and SNU719 gastric cancer cell lines, which expressed either MAGE-A2 or -A3, had hypomethylated promoters. Good overall correlation between the protein expression of MAGE-A and the promoter hypomethylation of MAGE-A2 and -A3 was demonstrated for both gastric cancer and normal gastric tissues. Thus, we suggest that the promoter hypomethylation of the MAGE-A2 and -A3 genes may lead to protein expression in gastric cancer. The expression of MAGE-A1, which does not have any CpG islands in its promoter region, is not determined solely by the methylation status of the promoter region in hematological malignancies (12).

In this study, MAGE-A expression was investigated with anti-MAGE antibody 6C1, which can detect MAGE-1, 2, 3, 4, 6, 10 and 12. MAGE-10 has a molecular weight of 72kDa, while the remainder of the MAGE-A proteins have molecular weights in the range of 45-50 kDa. Among the four cell lines with hypomethylated MAGE-A2 or -A3 promoters, only three cell lines expressed the MAGE protein. Similarly, only 12 of the 49 cancer tissues in which MAGE was not expressed had a hypomethylated promoter in the MAGE-A2 or -A3 genes. Therefore, it can be concluded that the promoter hypomethylation of the MAGE-A2 or -A3 genes induces the expression of MAGE-A, but that other mechanisms may also be involved in the re-expression process during carcinogenesis.

In a previous study, it was found that the hypomethylation of the MAGE-A genes is not significantly correlated with the clinicopathological parameters, but that the hypomethylation of both the MAGE-A1 and A3 genes is significantly correlated with tumor invasiveness and lymph node metastasis (14). Also, those patients in whom both genes are hypomethylated tend to have a worse prognosis than those patients in whom none of the genes are hypomethylated, albeit with marginal statistical significance (14). In line with these data, we confirmed that MAGE protein expression was correlated with tumor invasiveness, lymph node metastasis and advanced pathologic stage. Those cancers expressing MAGE-A were associated with the moderately-differentiated type of the WHO classification and the intestinal type of the Lauren classification. In the survival analysis, the overall survival rate of the patients with MAGE-A-positive gastric cancer, as determined by the log-rank test, was significantly lower than that of those patients with MAGE-A-negative gastric cancer. In a current trial of a new anti-cancer strategy for patients with melanoma (11), the administration of 5'-aza-2'deoxycytidine, a demethylating agent, was used to increase the expression level of the MAGE genes and the antigenicity of the cancer. However, it should be pointed out that the function of the MAGE genes is as yet unknown, and that their expression is related to a worse prognosis. Therefore, if the expression of MAGE-A has a causative correlation with the patients' survival, the induction of MAGE-A with demethylating agents could be detrimental rather than beneficial to patients with gastric cancer.

Global DNA hypomethylation is thought to occur during the early stages of tumor development in gastric as well as other organ cancers. In pulmonary carcinogenesis, the hypomethylation of the promoter CpG islands of the MAGE genes has been observed, not only in tumors, but also in the adjacent non-neoplastic lung tissues and in the bronchial epithelia obtained from smokers (4). However, we did not find any positive staining in normal or metaplastic gastric mucosa, which is believed to be a precancerous lesion. Other types of gastric precancerous lesions also need to be investigated.

Herein, it has been demonstrated that the hypomethylation of the MAGE-A2 and -A3 promoters was correlated with their expression in gastric cancer tissues and cell lines. Moreover, MAGE protein expression was associated with tumor invasiveness, lymph node metastasis and advanced pathologic stage.

Acknowledgements

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