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Expression of MAGE-1, -2, and -3 Genes in Gastric Carcinomas and Cancer Cell Lines Derived from Korean Patients

We investigated the expression of MAGE-1, -2, and -3 genes in tissues of 51 gastric carcinomas from Korean patients and in 11 gastric cancer cell lines established in Korea using reverse transcriptase-polymerase chain reaction along with immunohistochemical analyses and DNA sequencing. Among the 51 gastric carcinomas, MAGE-1, -2, and -3 genes were expressed in 16 (31%), 22 (43%), and 17 (33%), respectively, and 31 (60%) expressed at least one of the three genes. In contrast, none of the three MAGE genes were expressed in normal sites of gastric tissue from each cancer patient. Out of 11 gastric cancer cell lines, MAGE-1, -2, and -3 genes were expressed in two (18%), five (46%), and four (36%), respectively. According to the clinicopathological analysis, the expression of any of the three MAGE genes was not significantly correlated with several clinicopathological factors except histologic types (p= 0.067). Immunohistochemical analyses identified positive staining with monoclonal antibodies 77B and 57B specifically against MAGE-1 and -3 proteins, respectively, in nuclei and cytoplasms of cells in mRNA-positive tumor tissue. These findings suggest the possibility as a target for tumor-specific immunotherapy for Korean patients.

Key Words: *MAGE; Reverse Transcriptase-Polymerase Chain Reaction; Gene Expression; Immunohistochemistry*

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INTRODUCTION

Tumor-associated antigens (TAA) are expressed in tumors but not in normal adult tissues except for a few normal cell types that have been characterized and their corresponding genes have been cloned (1). The number of TAA is still growing. Since the cloning of the human melanoma antigen-1 (MAGE-1) gene by a gene transfection approach to identify antigens recognized by cytolytic T lymphocytes on a human melanoma cell line, 16 related genes have been cloned and characterized (2-7). Seventeen genes of the MAGE gene family can be categorized into three groups: MAGE-A, MAGE-B, and MAGE-C. MAGE-A, which consists of 12 members including MAGE-1 (also called MAGE-A1), is located in the Xq28 region (2, 3). Another group with 4 genes, MAGE-B, is located in the Xp21.3 region (4-6), and MAGE-C1 has been recently recognized on band Xq26 (7). Several members of the MAGE gene family are expressed in a number of human tumors of various histologic types but not in normal adult tissues except for the testis (2-7). Immunologically, both humoral and cell-mediated immune responses against MAGE antigens were detected in cancer patients and thus MAGE has been used as a model for cancer immunotherapy (2, 7-11). Since little is known about the expression of MAGE in gastric carcinomas in Korean population, we analyzed the expression of MAGE-1, -2, and -3 genes in 51 gastric carcinomas from Korean patients, 11 gastric cancer cell lines established in Korea, and analyzed the patients' data to reveal any correlation between expression of at least one of the three MAGE genes and clinicopathologic profile.

MATERIALS AND METHODS

Cell lines, which were established from pathologically-proven gastric carcinomas from Korean patients: SNU-1, -5, -16, -216, -484, -520, -601, -620, -638, -668, and -719 (12, 13), were purchased from the Korean Cell Line Bank (Cancer Research Center, Seoul National University, Seoul, Korea). Monoclonal antibodies (Ab) 77B and 57B,

produced against recombinant MAGE-1 and MAGE-3 proteins, respectively, were kindly provided by Spagnoli GC at Ludwig Institute for Cancer Research, Brussels, Belgium (14). The SNU cell lines were maintained in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum. Fifty-one gastric tumor biopsy specimens and their normal counterparts collected from each patient by preoperative endoscopy were frozen immediately in liquid nitrogen within 5 min and kept in a liquid nitrogen tank until RNA extraction. Of 51 gastric carcinoma specimens, 34 surgical resection specimens of primary gastric carcinoma were histologically classified according to the AJCC (15) and eight gastric carcinoma specimens with organ metastasis were classified as stage IV. The remaining nine gastric carcinoma specimens, from patients who refused surgical intervention, were excluded from any statistical analyses except expression of MAGE genes.

RNA extraction and RT-PCR

Total RNA was extracted using Tri reagent according to the manufacturer's guidelines (Molecular Research Center, Inc.). Two μg of RNA was reverse-transcribed (RT) in 20 μL of reaction mixture containing 2 μL of 10×PCR buffer (100 mM Tris-HCl, pH 8.3, 500 mM KCl), 4 μL of 25 mM $MgCl_2$, 8 μL of 10 mM dNTPs (Perkin-Elmer), 1 μL of 50 μM random hexamer (Perkin-Elmer), 14 units of RNasin (Promega), and 50 units of M-MLV reverse transcriptase (Promega) at 42℃ for 60 min. The RT products were subsequently amplified using primer sets specific for each MAGE gene (2), and for the β-actin gene which was used as an internal control. The primer sets and the expected sizes of the products were: 5'-CGGCCGAAGGAACCTGACCCAG-3' (CHO-14, sense) and 5'-GCTGGAACCCTCACTGGGTTGCC-3' (CHO-12, antisense) for MAGE-1 421 bp, 5'-AAGTAGGACCCGAGGCACTG-3' (CDS-9, sense) and 5'-GAAGAGGAAGAAGCGGTCTG-3' (CDS-7, antisense) for MAGE-2 230 bp, 5'-TGGAGGACCAGAGGCCCCC-3' (AB-5, sense) and 5'-GGACGATTATCAGGAGGCCTGC-3' (BLE-5, antisense) for MAGE-3 725 bp, and 5'-GGCATCGTGATGGACTCCG-3' (CHO-15, sense) and 5'-GCTGGAAGGTGGACAGCGA-3' (CHO-16, antisense) for β-actin with 615 bp. PCR was performed in a 20 μL reaction volume containing 2 μL of RT product, 10 pmol of each primer, and AccuPower PCR Premix solution (10 mM Tris-HCl, pH 9.0, 250 μM each dNTPs, 40 mM KCl, 1.5 mM $MgCl_2$, 1 unit of *Taq* DNA polymerase), purchased from Bioneer (Chungbuk, Korea). The mixture was initially incubated at 95℃ for 5 min and subject to 30 cycles of amplifications consisting of 3 steps: 94℃ for 1 min, 70℃ for 1 min, and 72℃ for 1 min for MAGE-1; 94℃ for 1 min, 66℃ for 2 min, and 70℃ for 2 min for MAGE-2; 94℃ for 1 min, 70℃ for 2 min, and 72℃ for 2 min for MAGE-3; and 94℃ for 1 min, 70℃ for 1 min, and 72℃ for 1 min for β-actin. The reaction mixture was finally incubated at 72℃ for 15 min. PCR products were size-fractionated on a 2% agarose gel and were visualized with ethidium bromide.

Southern blot analysis and DNA sequencing

PCR products were electrophoresed in a 2% agarose gel and transferred onto a Hybond-N+ membrane (Amersham). Membranes were pre-hybridized with 5× SSC containing 0.1% SDS, 5% dextran sulfate, and 100 μg of denatured salmon sperm DNA for 1 hr and were incubated at 39℃ or 41℃ (39℃ for MAGE-1, -2 and 41℃ for MAGE-3) overnight in the presence of probes labelled with fluorescein-11-dUTP using Gene Images 3'-Oligolabelling system (Amersham). The sequences of oligonucleotide probes were: 5'-CCCACAGGCAGATCTTCT-3' for MAGE-1 and -2, and 5'-CCCACTGGCAGATCTTCT-3' for MAGE-3 (16). After removing the unbound conjugate by washing in 0.3% Tween-20, Tris-HCl, pH 9.5, 300 mM NaCl for 10 min 3 times, the membrane was subject to detection reagent for 2 to 5 min, and exposed to an radiography film for 2 to 10 min with an intensifying screen on.

Sequencing was performed on positive control (K-562, human leukemia cell line) and on two or three representative specimens from each tissue or cell line. PCR products were purified by agarose gel electrophoresis using QIAquick gel extraction kit (QIAGEN). Sequencing was performed by dideoxychain termination method using the T7 sequenase version 2.0 kit (Amersham).

Immunohistochemical analyses

Formalin-fixed, paraffin-embedded gastric cancer tissues were stained by the avidin-biotin complex (ABC) method. After antigen retrieval with microwave procedure in 0.01 M sodium citrate buffer (pH 6.0) (17), deparaffinised specimens were incubated at 4℃ overnight in anti-MAGE-1 mAb 77B, anti-MAGE-3 mAb 57B, or isotype-matched control mAb solution. The sections were then incubated with biotinylated goat anti-mouse IgG (Dako) for 2 hr, and allowed to react with peroxidase-conjugated streptavidin (Dako) for 45 min. Color development was performed with diaminobenzidine, followed by counterstaining with hematoxylin.

Statistical analysis

Statistical significance was evaluated in 42 of 51 gas-

tric carcinoma specimens using z-test or Fisher's exact test. The z-test was used for correlation analyses between expression of at least one of the three MAGE genes and histologic stage or depth of tumor invasion. Fisher's exact test was used for correlation analyses between expression of at least one of the three MAGE genes and 1) nodal metastasis, 2) organ metastasis, and 3) histologic types. Only 34 surgical resection specimens of primary gastric carcinoma were included for 1) and 3).

RESULTS

Expression of MAGE-1, -2, and -3 genes in gastric carcinoma tissues and gastric cancer cell lines

Tissues of 51 gastric carcinomas from Korean patients and 11 gastric cancer cell lines established in Korea were analyzed for MAGE-1, -2, and -3 gene expression by RT-PCR (Fig. 1) and the results were summarized in Table 1 and 2. Among 51 gastric carcinomas, MAGE-1, -2, and -3 genes were expressed in 16 (31%), 22 (43%), and 17 (33%), respectively, and 31 (61%) showed expression of at least one of the three genes (Table 1). None of the three MAGE genes were expressed in normal sites of gastric tissue from each cancer patient (data not shown). In 11 gastric cancer cell lines, MAGE-1, -2, and -3 genes were expressed in 2 (18%), 5 (46%), and 4 (36%), respectively, and 6 (55%) showed expression of at least one of the three genes (Table 1, 2). SNU-16 and -484 expressed all three MAGE genes, whereas SNU-5, -520, -620, -638, and -668 expressed none of the three genes. The expression patterns of MAGE-1, -2, and -3 were not related to the tissue origins, such as primary tumor, metastatic lymph node, or ascites. To verify that the amplified products were from authentic mRNA from MAGE gene, Southern blot analyses were performed using gene-specific probes. The MAGE-1, -2 or -3 probes

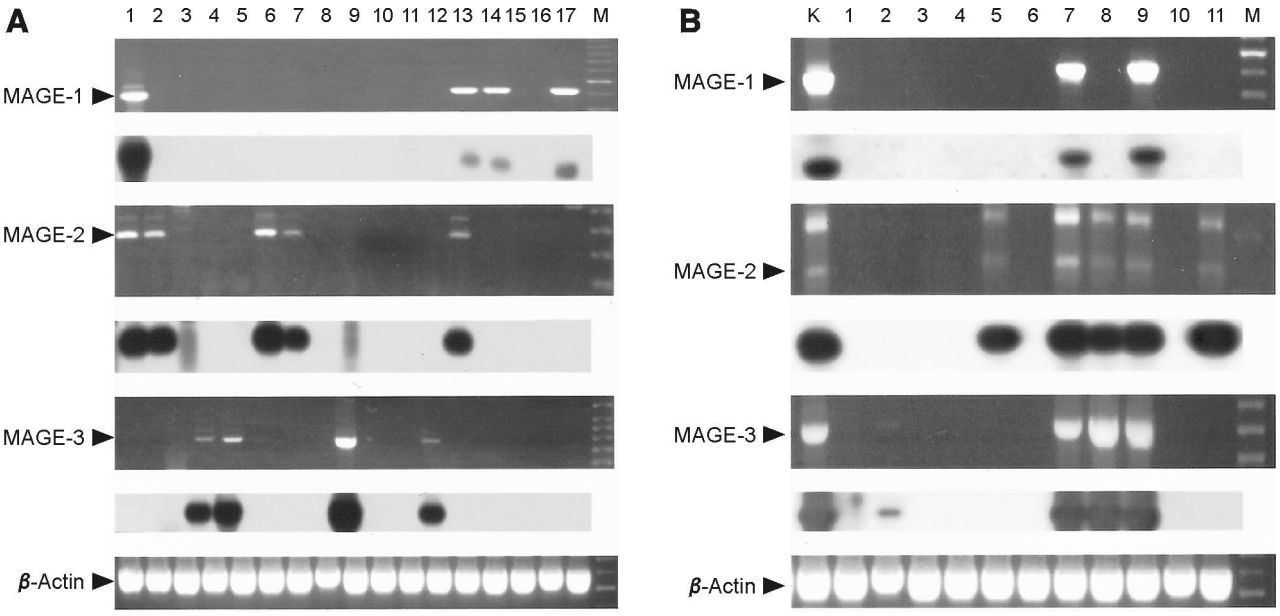

Fig. 1. Detection of MAGE-1, -2, and -3 genes in representative gastric carcinoma specimens from Korean patients **(A)** and in gastric cancer cell lines **(B)** by RT-PCR and Southern blot hybridization. The electrophoresed PCR products were visualized by ethidium bromide staining and hybridized with fluorescein-11-dUTP-labelled oligonucleotide probes specific for each of the MAGE-1, -2, and -3 genes. Amplification of cDNA for β-actin was also performed in parallel as a control. Lane "K" in each autoradiograph is the positive control (human leukemia cell line, K-562). M is DNA ladder marker for 100 bp.

Table 1. Expression of MAGE-1, -2, and -3 in 51 gastric carcinoma tissues and 11 gastric cancer cell lines (SNU cell lines)

Genes	Tissues	SNU cell lines*
MAGE-1	31% (16/51)	18% (2/11)
MAGE-2	43% (22/51)	46% (5/11)
MAGE-3	33% (17/51)	36% (4/11)
At least one of three MAGE genes	61% (31/51)	55% (6/11)

*established from gastric carcinomas of Korean patients; SNU-1, -5, -16, -216, -484, -520, -601, -620, -638, -668, and -719 were purchased from Korean Cell Line Bank (see Table 2)

Table 2. Expression of MAGE-1, -2, or -3 in 11 gastric cancer cell lines established from cancer patients in Korea

Name	Origin/Tissue	MAGE-1	MAGE-2	MAGE-3
SNU-1	Stomach	−	+	−
SNU-5	Stomach, ascites	−	−	−
SNU-16	Stomach, ascites	+	+	+
SNU-216	Stomach, lymph node	−	+	+
SNU-484	Stomach	+	+	+
SNU-520	Stomach	−	−	−
SNU-601	Stomach, ascites	−	+	−
SNU-620	Stomach, ascites	−	−	−
SNU-638	Stomach, ascites	−	−	−
SNU-668	Stomach, ascites	−	−	−
SNU-719	Stomach	−	−	+
% positive		18	46	36
K-562*		+	+	+

+, expressed; −, not-expressed
*Human leukemia cell line (positive control for the expression of MAGE -1, -2, and -3)

hybridized only to the cDNA PCR products of the three MAGE genes, not to those of genomic DNA (Fig. 1). We also confirmed by DNA sequencing that the PCR products produced from tumors, cancer cell lines, and positive control cell line (K562) corresponded to their original sequences (data not shown).

Immunonohistochemical analyses

Immunostaining was performed to confirm the results

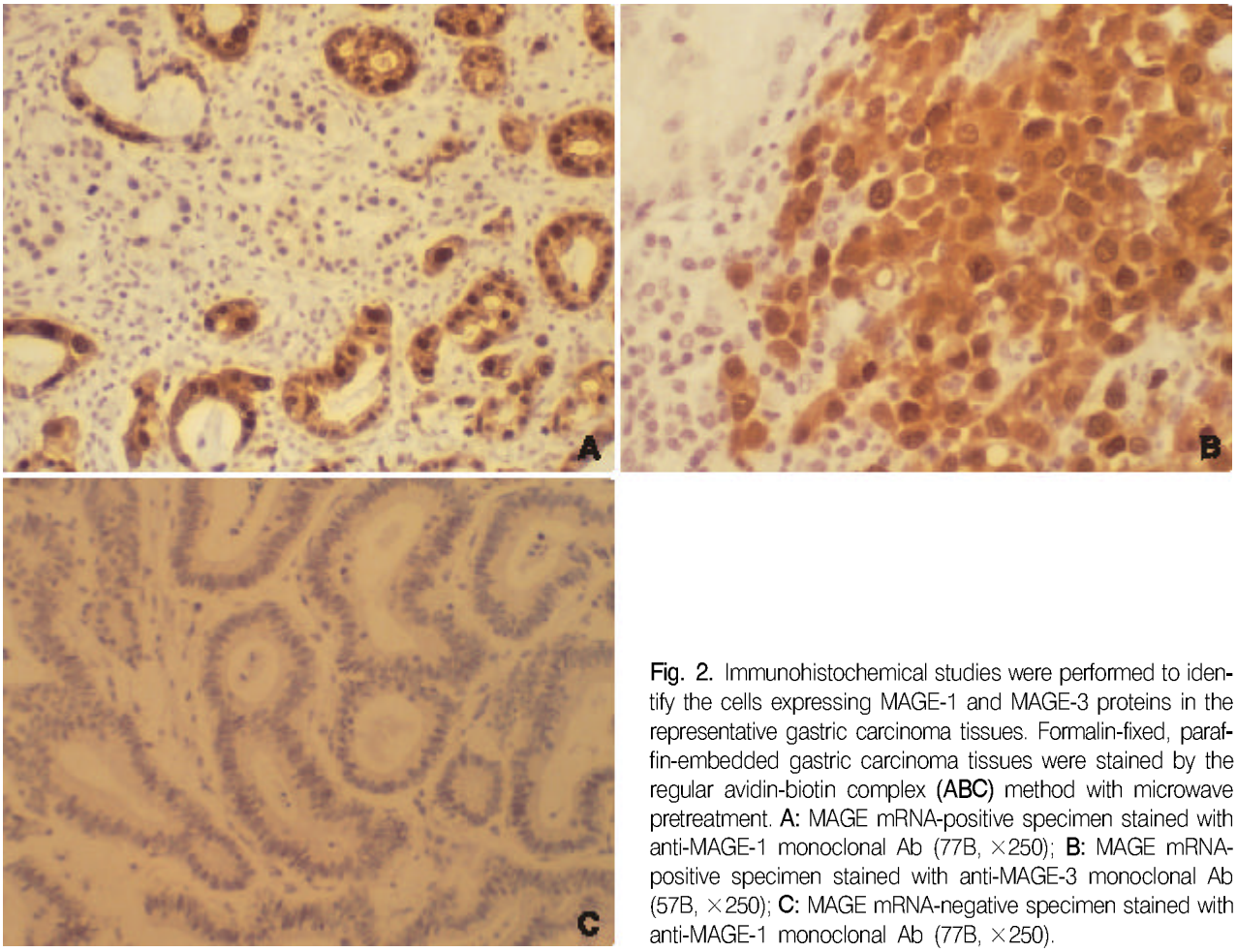

Fig. 2. Immunohistochemical studies were performed to identify the cells expressing MAGE-1 and MAGE-3 proteins in the representative gastric carcinoma tissues. Formalin-fixed, paraffin-embedded gastric carcinoma tissues were stained by the regular avidin-biotin complex (ABC) method with microwave pretreatment. **A:** MAGE mRNA-positive specimen stained with anti-MAGE-1 monoclonal Ab (77B, ×250); **B:** MAGE mRNA-positive specimen stained with anti-MAGE-3 monoclonal Ab (57B, ×250); **C:** MAGE mRNA-negative specimen stained with anti-MAGE-1 monoclonal Ab (77B, ×250).

of MAGE mRNA expression in tumor tissues. MAGE mRNA-positive specimens were reactive against either anti-MAGE-1 monoclonal Ab (77B) (Fig. 2A) or anti-MAGE-3 monoclonal Ab (57B) (Fig. 2B). We also observed that MAGE mRNA-negative specimens were not reactive against 77B (Fig. 2C). MAGE-1 and -3 proteins were localized in the nuclei and the cytoplasms in immunoreactive cells (Fig. 2A&B). The staining patterns varied according to the tumor tissues: some tumor tissues had only small isolated clusters of positive cells while others were uniformly stained throughout the tumor tissue.

Statistic correlation between expression of at least one of the three MAGE genes and several clinicopathological factors

To investigate the potential role of MAGE antigen in tumor progression, analyses to reveal any correlation between expression of at least one of the three MAGE genes and several clinicopathological factors including histologic stage, depth of tumor invasion, lymph node metastasis, organ metastasis, and histologic type were performed (Table 3). The results showed that no significant correlations existed between expression of at least one of the three MAGE genes and histologic stage (p= 0.1423 by z-test), depth of tumor invasion (p=0.1736 by z-test), lymph node metastasis (p=0.307 by Fisher's exact test), and organ metastasis (p=0.099 by Fisher's exact test). However, weak but significant correlation was observed between expression of one of the three MAGE genes and histologic types (well-differentiated vs poorly-differentiated; p=0.067 by Fisher's exact test). In this study, well-differentiated types consisted of well-differentiated, papillary adenocarcinoma, and moderately-differentiated carcinomas. Poorly-differentiated types consisted of poorly differentiated and signet ring carcinomas.

DISCUSSION

We analyzed the expression of MAGE-1, -2, and -3 genes and the presence of their protein products in tissues of 51 gastric carcinomas from Korean patients and 11 gastric cancer cell lines established in Korea, using RT-

Table 3. Clinicopathological data from 42 cases of gastric carcinomas

	MAGE-1		MAGE-2		MAGE-3		One of three MAGE genes	
	+	−	+	−	+	−	+	−
No. of specimens	13	29	16	26	15	27	25	17
Age (mean; yr)	59.7	60.9	62.4	59.4	60.6	60.5	61.7	58.8
Sex								
Male	8	20	11	17	13	15	19	9
Female	5	9	5	9	2	12	6	8
Stage*								
I	2	6	2	6	2	6	4	4
II	0	3	0	3	1	2	2	1
III	3	10	6	7	5	8	7	6
IV	8	10	8	10	7	11	13	5
Histology†								
Well-differentiated	5	13	7	11	8	10	12	6
Poorly-differentiated	3	13	4	12	4	12	6	10
Lymph node metastasis‡								
n=0	2	6	2	6	3	5	4	4
n≥1	6	20	9	17	10	16	14	12
Depth of tumor invasion§								
t1	1	4	2	3	3	2	3	2
t2	3	8	3	8	2	9	5	6
t3	3	11	5	9	5	9	7	7
t4	6	6	5	7	5	7	10	2
Organ metastasis¶								
Present	5	5	6	4	4	6	8	2
Absent	8	24	9	23	11	21	17	15

+, expressed; −, not-expressed
*p=0.1423 by z-test
†Only 34 primary gastric carcinoma surgical resection specimens were analyzed by Fisher's exact test, p=0.067
‡Only 34 primary gastric carcinoma surgical resection specimens were analyzed by Fisher's exact test, p=0.307
§p=0.1736 by z-test
¶p=0.099 by Fisher's exact test

PCR, Southern blot, and immunohistochemical analyses. This is the first study on the expression of MAGE genes and their proteins in tissue specimens of gastric carcinoma in Korean population. The results are largely in accordance with those in Japanese patients with gastric carcinoma: in tissues of 68 gastric carcinomas from Japanese patients, MAGE-1, -2, and -3 mRNAs were detected in 28 (41%), 21 (31%), and 25 (38%), respectively (18), and MAGE-1 gene was expressed in 11 (28.9%) of 38 gastric tumor specimens and in two cases (5.3%) from adjacent non-tumorous tissue specimens (19). Of 11 gastric cancer cell lines, SNU-16 and -484 expressed all three MAGE genes, whereas SNU-5, -520, -620, -638, and -668 expressed none of the three genes. According to a recent study by Park et al. using 13 cancer cell lines including SNU-484, -638, and -668, MAGE-3 gene was expressed in SNU-484, but not in SNU-638 and -668 (20).

In immunohistochemical studies, the staining patterns of tumor tissues were either homogeneous or clustered as previously described in MAGE-3 protein in primary and metastatic melanomas (14).

No significant correlation between MAGE gene expression and clinicopathological factors was revealed in the present study although most of the tumor specimens were from advanced stage. This result is similar to that of Inoue et al. in which no significant correlation between MAGE-1, -2, or -3 gene expression and several clinicopathological factors was observed (18). However, Katano et al. demonstrated that MAGE-1 gene expression was significantly correlated with histologic stage of the disease, depth of tumor invasion, and lymph node metastasis (19). In addition, it was shown that in colorectal carcinomas from Japanese patients, the expression of MAGE-1, -2, or -3 was more frequent in cases with liver metastasis than in those without liver metastasis (21). The amino acid sequence of MAGE protein shows some homology to necdin, which is expressed in neurally differentiated embryonal carcinoma cells, in the developing mouse brain during the early stages of neuronal generation and differentiation, and in neurons at advanced stages of differentiation (22). It was reported that the expression of MAGE-1 was detected in skin during wound repair (23), suggesting that MAGE-1 is a cellular gene which is involved in cell proliferation and differentiation, or tumor progression as suggested by other studies (10). However, no evidence for the biological function of MAGE has been reported. Alternatively, the frequent expression of MAGE genes in tumors at later stages may result from aberrant genetic regulation in most tumors.

Immunotherapeutic approaches using MAGE proteins or relevant antigenic peptides have been developed and several successful results were released on a phase I clinical vaccination trial in melanoma patients using dendritic cells pulsed with antigenic peptides (8-11). MAGE-1 and MAGE-3 epitopes are presented to cytotoxic T lymphocytes in association with HLA-A1 or -A2 (10, 11). The incidence of HLA-A2-expressing individuals in Korean population is reported to be 29%, whereas that of HLA-A1 is very low (3.3%), according to the HLA haplotype analysis (24). The patterns of HLA allele frequencies in the two populations of Korea and Japan are quite similar (25). Since individuals expressing HLA-A2 represent a significant portion of Korean population and MAGE-3 gene is frequently expressed in gastric carcinomas from Korean patients (33% as shown in the present study), MAGE-3 protein or epitopes might be a good candidate for immunotherapy of gastric cancer in Korean population. In this regard, the results of this study may be useful as a basic data on the status of expression of MAGE-1, -2, or -3 gene for the development of antigen-specific immunotherapy of gastric carcinomas in Korean population.

In summary, our results indicate that MAGE genes are expressed in gastric carcinoma tissues as well as in cell lines established from gastric carcinomas of Korean patients. Further studies are needed to reveal the function of MAGE proteins, the type of tissue and cell which express MAGE genes in carcinogenesis, and the potential of MAGE protein as a tumor vaccine in gastric cancer in Korean population.

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