Melanoma antigen gene family A as a molecular marker of gastric and colorectal cancers

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Abstract. The present study aimed to evaluate the role of melanoma antigen family A (MAGEA) in gastric and colorectal cancer cell lines and clinical tissue samples. We used 10 gastric and 9 colorectal cancer cell lines, 20 early-stage and 21 advanced-stage gastric cancer tissues, 20 colon adenomas and 19 colorectal cancer tissues. Real-time RT-PCR assay was used for the determination of MAGEA mRNA levels. Western blot analysis and immunohistochemistry were used for the determination of MAGEA protein levels in cell lines and tissues, respectively. Gastric and colorectal cancer cell lines showed variable mRNA expression levels of MAGEA. The MAGEA protein was detected in 30% of gastric cancer cell lines and in 22.2% of colorectal cancer cell lines. There was a high correlation between mRNA and protein expression. Regarding the clinical samples, MAGEA expression was noted in 25, 28.6 and 31.6%, respectively in early-stage, advanced-stage gastric cancer tissues and colon adenocarcinoma, but was negative in the adjacent normal tissues of the stomach and colon as well as colon adenoma. These results indicate that MAGEA is involved in the carcinogenesis of gastric and colorectal cancer and, therefore, can be used as a diagnostic marker to predict these cancers.

Introduction

Cancer is one of the leading causes of death worldwide. In Korea, gastric cancer (GC) is the most common cause of cancer-related death in women and the second most common in men (1). Moreover, colorectal cancer (CRC) is the fourth leading cause of cancer-related mortality (2). Recently, in Korea, CRC has shown the most sharply increasing tendency of all malignancies. In spite of improvements in cancer diagnosis and therapy, many patients are still diagnosed at the late stages of the disease, and this often occurs only after curative surgery.

Cancer develops as a result of multiple genetic and epigenetic alterations (3,4). Better knowledge of the molecular changes in the gene expression during gastric and colorectal carcinogenesis could lead to improvements at several levels, including diagnosis, treatment and prevention. In order to identify potential molecular markers for GC and CRC carcinogenesis and to better understand the development of GC and CRC at the molecular level, comprehensive analyses of gene expression are useful (5,6).

To date, many researchers have studied the classification and the diagnostic prediction of cancers using gene expression. These molecular markers were consequently correlated with patient prognosis and survival. Thus, if GC and CRC are diagnosed at an early stage, patients may have a highly favorable prognosis and avoid extensive surgery.

Many human melanomas express antigens that are specific targets of the cytotoxic T lymphocytes of tumor-bearing patients. Melanoma antigen gene family A (MAGEA) is one of them and it has been studied for cancer diagnosis and immunotherapy (7). The MAGEA family consists of 12 subtypes, including MAGEA1 to MAGEA12 (8). MAGEA genes are highly expressed in different types of cancer, such as melanoma, lymphocytic leukemia, and various cancers of the lung, head and neck, esophagus, bladder, stomach, colorectum, breast, liver and ovary (7,9). Furthermore, it is known that MAGEA is activated by demethylation of the promoter region in most cancer cells (10). Since MAGEA genes are expressed in many types of cancers, MAGEA has been assessed as an important marker for cancer diagnosis (11-13). Although many studies have reported that MAGEA genes function as oncogenes, evidence of the role played by MAGEA in the carcinogenesis of GC and CRC is still lacking.

In the present study, we examined the expression of MAGEA genes in GC and CRC cancer cell lines and related clinical tissues to evaluate their role in carcinogenesis. In the present study, we report that the expression of MAGEA plays an important role in gastric and colorectal carcinogenesis.

Furthermore, our results suggest that MAGEA can be used as a diagnostic marker to predict gastric and colorectal carcinogenesis.

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Table I. Primers, probes and thermal cycling conditions of the RT-PCR.

Gene	Sense (5'→3') Antisense (5'→3')	Probe (5'→3')	Annealing extension
MAGEA1	GCCGAAGGAACCTGACC ACTGGGTTGCCTCTGTCG	TGTGTGCAGGCTGCCACCTCCT	90 sec, 65˚C
MAGEA2	AAGTAGGACCCGAGGCACTG GAAGAGGAAGAAGCGGTCTG	CATTGAAGGAGAAGATCTGCCTGTGGGTCTTC	1 min, 60˚C
MAGEA3	GTCGTCGGAAATTGGCAGTAT GCAGGTGGCAAAGATGTACAA	AAAGCTTCCAGTTCCTT	1 min, 62˚C
MAGEA4	CCACTACCATCAGCTTCACTTGC CTTCTCGGAACAAGGACTCTGC	AGGCAACCCAATGAGGGTTCCAGC	1 min, 63˚C
MAGEA6	GTCGTCGGAAATTGGCAGT GCAGGTGGCAAAGATGTACAC	TGCAAGGAATCGGAAGC	1 min, 65˚C
MAGEA10	TACTGCACCCCTGAGGAGGTC TGTGGTGGCAATTCTGTCCTG	AAATGGGAGTGATCCAAGATCCTTCCCAC	1 min, 64˚C
MAGEA12	GGTGGAAGTGGTCCGCATCG GCCCTCCACTGATCTTTAGCAA	AGGCATCTGATGGGAGG	1 min, 60˚C
β-actin	GGGAATCTGACGGATCGGA GGAATGGAACGCCTGGAAC	TGCTCCTGAAGAAGTCGTCATGCCTCC	1 min, 60˚C

Materials and methods

Cell culture. Ten human gastric adenocarcinoma cell lines (SNU-1, -5, -16, -216, -484, -601, -620, -638, -668 and -719) and 9 colorectal adenocarcinoma cell lines (SNU-C1, -C4, -C5, COLO320HSR, LoVo, DLD-1, HT-29, HCT-8 and HCT-116) were obtained from the Cancer Research Center at Seoul National University (Korea) and used in this study. All cells were cultured at 37˚C in a 5% CO_2 atmosphere using RPMI-1640 medium (Invitrogen, Carlsbad, CA, USA) with 10% heat inactivated fetal bovine serum (Sigma, St. Louis, MO, USA). The cells were maintained either as a suspension or as a monolayer culture and subcultured until they reached confluence.

Real-time RT-PCR. The total RNA was extracted using the MagExtractor® for the MFX-2100 (Toyobo, Osaka, Japan) auto-nucleic acid purification system, according to the manufacturer's instructions. The 1 μg RNA extracted from each sample was then reverse transcribed using 200 units of Moloney murine leukemia virus reverse transcriptase (Invitrogen) and an oligo (dT) primer for 1 h at 37˚C.

Real-time PCR was performed with the LightCycler 2.0 Instrument (Roche Diagnostics, Mannheim, Germany) using the TaqMan Master Mix (Roche Diagnostics). Each reaction (20 μl) contained 4 μl of 5-fold diluted cDNA, 10 pmol of each primer and probe, and 4 μl of Master Mix containing buffer, dNTPs, $MgCl_2$ and Taq polymerase. Primer, probe and cycling conditions, as presented in Table I, have been used in previous studies (14,15). Data were analyzed using the LightCycler software version 4.0 (Roche Diagnostics).

Protein extraction and western blot analysis. The cells were washed with phosphate-buffered saline (PBS) and lysed in 50 mM Tris-Cl (pH 7.4), 250 mM NaCl, 0.5% Triton X-100, 10% glycerol, 1 mM DTT, 1 mM PMSF and protease inhibitor cocktail (Pierce Biotechnology, Rockford, IL, USA). The cell lysates were then centrifuged and then fractionated by SDS-PAGE, and western blotting was performed using a slight modification of the method as previously described (16). The membrane was incubated with primary rabbit polyclonal antibodies for MAGEA (detection of MAGEA1, -A2, -A3, -A4, -A6, -A10 and -A12; 1:1,000; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and β-actin (1:2,500; Santa Cruz Biotechnology). The membrane was then washed and incubated with horseradish peroxidase-conjugated secondary antibody (1:2,000 for MAGEA; 1:5,000 for β-actin) against each IgG for hosts of primary antibodies for 1 h. The membrane was then stained using the detection reagent of the ECL detection kit (Amersham Pharmacia Biotech Inc., Piscataway, NJ, USA).

Case selection and tissue sampling. Among the patients that underwent curative surgery for gastric adenocarcinoma [20 and 21 cases of early-stage gastric cancer (EGC) and advanced-stage gastric cancer (AGC), respectively], and colorectal adenocarcinoma (19 cases) at the Chosun University Hospital (Gwangju, Korea) from January 2008 to December 2009, non-consecutive patients were selected for this study, including relatively well-preserved paraffin-embedded tissues and complete medical records. Twenty cases of colorectal adenoma, with all samples obtained endoscopically, were subjected to analysis for a comparison study. Those patients who underwent preoperative chemo/radiotherapy and emergency surgery, and those who had evidence of hereditary non-polyposis colorectal cancer or familial adenomatous polyposis were excluded from the study. Informed consent was obtained from each subject according to the institutional

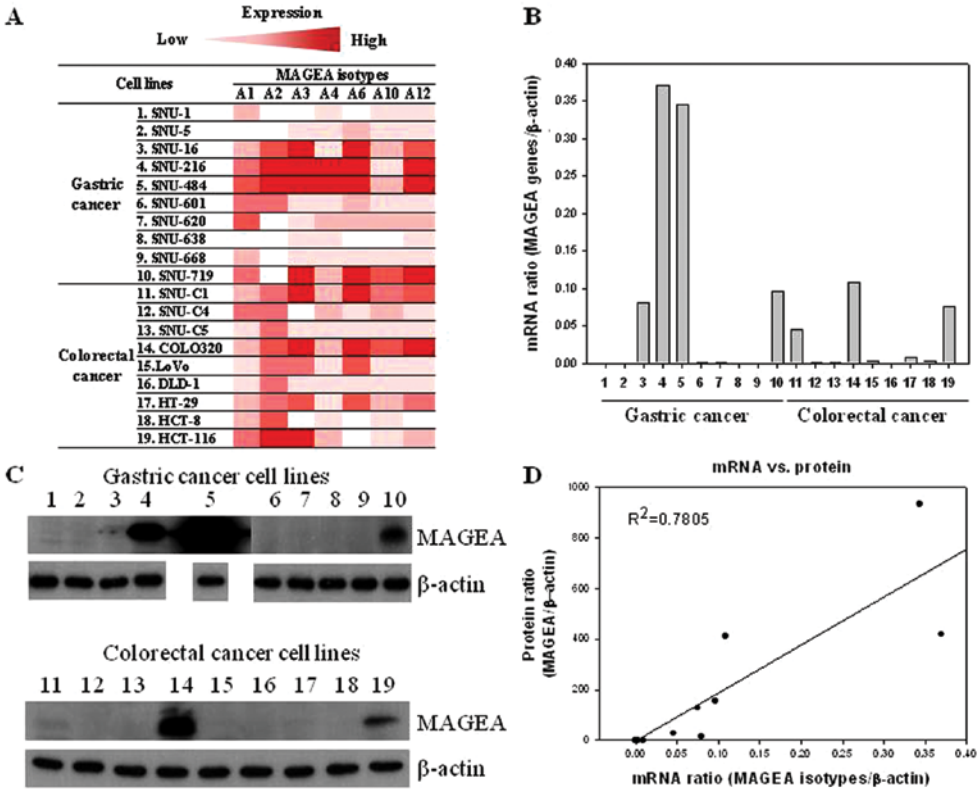

Figure 1. Expression of the MAGEA genes in 10 gastric and 9 colorectal cancer cell lines. (A) mRNA levels of the MAGEA genes, including MAGEA1, -A2, -A3, -A4, -A6, -A10 and -A12, are displayed using a color gradient, according to the level of expression and (B) as the sum of the mRNA ratio of each MAGEA gene. (C) Expression levels of mRNA were determined by real-time PCR assay. Protein expression of the MAGEA genes was determined by western blot analysis. (D) Correlation between the level of mRNA and the protein expression of the MAGEA genes.

guidelines, and the research protocols were approved by the IRB of our hospital.

Immunohistochemical staining. All tissues investigated in the study were tested for MAGEA mouse monoclonal antibody (1:2,000, Santa Cruz Biotechnology). Immunolocalization for MAGEA was performed using a Polink-2 HRP Plus Mouse DAB Detection system (Golden Bridge International, Inc., Mukilteo, WA, USA), according to the supplier's protocol. Briefly, 4-μm sections obtained after formalin fixation and paraffin embedding were deparaffinized in xylene and rehydrated with distilled water through graded concentrations of ethanol. After quenching the endogenous peroxidase activity in 0.3% hydrogen peroxide for 10 min, the slides were rinsed with distilled water. The sections were then placed in a glass jar with 10 mM citrate buffer (pH 6.0) and irradiated in a microwave oven for 15 min, and cooled down in the jar at room temperature for 20 min. The slides were then rinsed with Tris-buffered saline (TBS) and a blocking reagent was added for 10 min. After tapping off the excess blocking reagent, the specimen was carefully wiped around, and enough primary antibody to cover the specimen was applied for 1 h in a moist chamber at 37˚C. After washing with TBS, mouse antibody enhancer was applied for 10 min, followed by washing with TBS, as before. Then, polymer-HRP (horseradish peroxidase) for mouse was applied for 10 min to cover each section. After washing again with TBS, the localization of antibodies was visualized by incubating the sections for 5 min in DAB and counterstaining with Mayer's hematoxylin for 10 sec. An isotype matched control antibody was also used. The positive control for MAGEA used in the present study was early placental tissue. In contrast, instead of the primary antibody, normal goat serum was used as the negative control.

Analysis and interpretation of the staining. Staining for MAGEA was deemed positive when nuclear staining was identified under an optical microscope in >1% of the tumor cells in each tissue section. Positive expression of MAGEA was then classified into level 1 (weakly positive), when 1-25% of tumor cells were stained; level 2 (moderately positive), when 26-50% of tumor cells were stained; and level 3 (strongly positive), when >50% of tumor cells were stained.

Statistical analysis. Statistical analysis was performed using the Student's t-test. P-values <0.05 were considered to indicate statistically significant differences.

Results

Comparison of the MAGEA expression in gastric and colorectal cancer cell lines. mRNA and protein expression of the MAGEA genes, including MAGEA-1, -A2, -A3, -A4, -A6, -A10 and -A12, was analyzed using real-time PCR (RT-PCR) and western blot methods in 10 gastric and 9 colorectal cancer cell lines, respectively.

Table II. Comparison of protein expression of MAGEA in gastric and colorectal cancer cell lines.

Cell lines	Histopathology	MAGEA expression	Positive n (%)
Gastric			
SNU-1	Adenocarcinoma	-	3 (30)
SNU-5	Adenocarcinoma	-	
SNU-16	Adenocarcinoma	-	
SNU-216	Adenocarcinoma	+++	
SNU-484	Adenocarcinoma	++++++	
SNU-601	Adenocarcinoma	-	
SNU-620	Adenocarcinoma	-	
SNU-638	Adenocarcinoma	-	
SNU-668	Carcinoma	-	
SNU-719	Adenocarcinoma	++	
Colorectal			
SNU-C1	Adenocarcinoma	-	2 (22.2)
SNU-C4	Adenocarcinoma	-	
SNU-C5	Adenocarcinoma	-	
COLO320	Adenocarcinoma	+++	
LoVo	Adenocarcinoma	-	
DLD-1	Adenocarcinoma	-	
HT-29	Adenocarcinoma	-	
HCT-8	Adenocarcinoma	-	
HCT-116	Carcinoma	+	

Table III. MAGEA expression between EGC and AGC in gastric adenocarcinoma, and colorectal adenoma and adenocarcinoma.

Tissue	Classification	No. of cases	Positive n (%)	P-value
Gastric	EGC	20	5 (25.0)	0.5704
	AGC	21	6 (28.6)	
Colorectal	Adenoma	20	0 (0)	0.0157
	Adenocarcinoma	19	6 (31.6)	

EGC, early-stage gastric cancer; AGC, advanced-stage gastric cancer.

The color gradient from dark red to light red indicates the mRNA expression level of the MAGEA genes (Fig. 1A). Furthermore, the mRNA level of total MAGEA genes was determined as the sum of the mRNA levels found in each MAGEA gene. According to the MAGEA gene/β-actin ratio in the gastric cancer cell lines, the rank order was as follows: SNU-216 (0.37) > SNU-484 (0.34) > SNU-719 (0.1) > SNU-16 (0.08) > SNU-620 (0.002) > SNU-601 (0.0007) > SNU-1 (0.0001) > SNU-5 (0.00008) > SNU-668 (0.00005) > SNU-638 (0.00001) (Fig. 1B). Similarly, in the colorectal cancer cell lines, according to the MAGEA gene/β-actin ratio, the rank order was as follows: COLO320HSR (0.11) > HCT-116 (0.08) > SNU-C1 (0.04) > HT-29 (0.008) > LoVo = HCT-8 (0.003) > SNU-C4 = SNU-C5 = DLD-1 (0.001) (Fig. 1B).

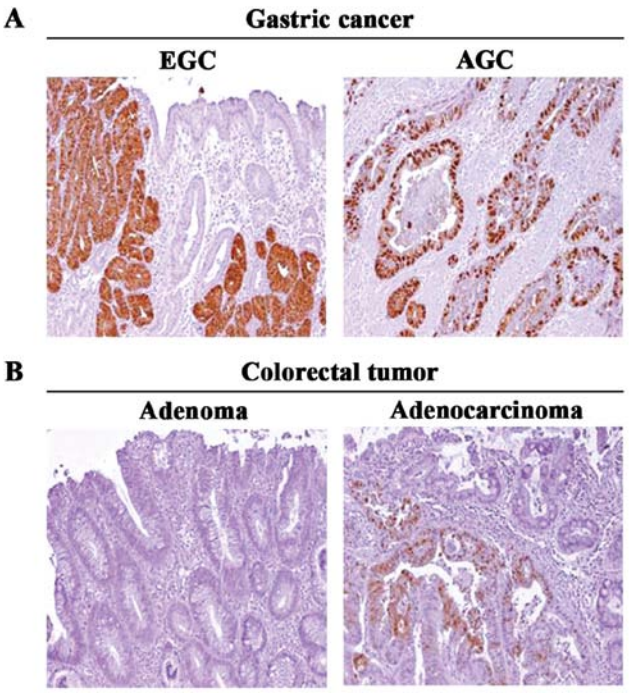

Figure 2. Immunohistochemical staining for the MAGEA genes in (A) gastric and (B) colorectal cancer tissues. Strong nuclear staining was identified in adenocarcinoma, but not in any adjacent normal tissues of the gastric and colorectal cancers. EGC, early-stage gastric cancer; AGC, advanced-stage gastric cancer.

We examined the protein level of MAGEA using western blot analysis to compare the mRNA levels obtained from real-time RT-PCR. Among the 10 investigated gastric cancer cell lines, the protein expression of MAGEA was be detected in 3 (30%) (SNU-484, SNU-216 and SNU-719) (Table II). Of the 9 investigated colorectal cancer cell lines, MAGEA was also detected in 2 (22.2%) (COLO320HSR and HCT-116) (Table II). MAGEA gene expression at the mRNA level was generally correlated with that at the protein level (Fig. 1D).

Clinicopathological significance of MAGEA in gastric and colorectal cancer tissues. Immunoreactivity of MAGEA by immunohistochemical staining was examined comparatively between EGC and AGC in the gastric adenocarcinoma, and between adenoma and adenocarcinoma in the colorectal cancers.

Among the 41 investigated gastric cancer cases, MAGEA expression was positive in 25% (5 cases) of EGC and 28.6% (6 cases) of AGC (Table III). However, among the 39 investigated colorectal cancer cases, MAGEA was detected only in adenocarcinoma, i.e. 3 1.6% (6 cases) (Table III).

Moreover, MAGEA was not detected in any adjacent normal tissues in either gastric or colorectal cases. Representative examples of the MAGEA immunohistochemical staining in both gastric and colorectal cancer tissues are shown in Fig. 2.

Discussion

In the present study, we examined the expression of MAGEA genes in gastric and colorectal cancer cell lines and in related clinical tissues to evaluate their role in carcinogenesis.

The inherited and acquired genetic and molecular alterations, leading to gastric and colorectal carcinogenesis, have been extensively studied over the past 20 years (17,18). However, the precise molecular alterations that might differentiate, for example EGC from AGC in gastric cancer, are not yet clear (19). In addition, the determinants of malignancy in colorectal cancer are lacking to date (20). Therefore, it is very critical to predict the carcinogenesis of gastric and colorectal cancers, allowing early diagnosis using biopsy samples, and to select the appropriate therapeutic regimens (19). It has been demonstrated that prognosis largely depends on whether gastric and colorectal cancers are diagnosed as EGC or AGC, and colorectal adenoma or adenocarcinoma, respectively (19,21).

Since the MAGEA genes are expressed exclusively in tumor cells, except for placental and normal testis tissues, they may be used as diagnostic markers for detecting malignancy, as previously suggested (9). However, the expression profile of the MAGEA genes as applied to gastric and colorectal carcinogenesis has been insufficiently studied. There was no significant difference in the MAGEA expression when comparing EGC and AGC. However, MAGEA expression was not detected in colorectal adenoma, nor in any adjacent normal colorectal tissues. In contrast, it was detected in several of the colorectal adenocarcinoma cases, thus suggesting the potential role of the MAGEA genes in the colorectal adenoma-adenocarcinoma sequence. However, the correlation between MAGEA expression and clinicopathological parameters, such as tumor stage and differentiation, was not statistically significant (data not shown).

In conclusion, expression of the MAGEA genes may play an important role in both gastric and colorectal carcinogenesis. Furthermore, MAGEA genes can be used as a diagnostic marker to predict gastric and colorectal carcinogenesis.

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