Expression of Melanoma Antigen-Encoding Genes (MAGE) by Common Primers for MAGE-A1 to -A6 in Colorectal Carcinomas Among Koreans

This study was to investigate Melanoma-antigen gene (MAGE) expression by reverse transcription-nested polymerase chain reaction (RT-nested PCR) with the original common primers of MAGE-A1 to -A6 and analysis of correlation between its expression and the well-known clinical parameters in addition to evaluate the clinical feasibility of the common primers. Surgical tumor and corresponding non-neoplastic tissue samples from 38 patients with colorectal cancer were studied. To confirm the identities of RT-PCR products, direct sequencing was done after in vitro subcloning. No expression of MAGE was observed in the non-neoplastic colorectal mucosal tissues. Sixteen (42.1%) of 38 carcinomas expressed at least one of MAGE A-1 to -6. The expression of the MAGE genes was not related to age, sex, histological grades, the depth of invasion, metastasis to lymph nodes, vessel, neural, or perineural invasion. The identities with the corresponding mRNAs were confirmed in 6 cases for MAGE-A2 (15.8%), 6 cases for MAGE-A4 (15.8%), 2 cases for MAGE-A3 (5.3%), and one case for MAGE A-6 (2.6%). These results suggest that MAGE expressions, except those of MAGE-A2 and -A4, seem to have a limited role in the molecular pathogenesis of colon cancer. However, the common primer sets to detect of expressions for MAGE-A1 to -A6 simultaneously appear to be feasible to differentiate malignant from benign lesions in colorectal diseases.

Key Words : *MAGE Gene; Colorectal Neoplasms, Polymerase Chain Reaction, RT-Nested; Common Primers*

Myeng-Sun Park, Jong-Wook Park*,
Chang-Ho Jeon[†]**, Kang-Dae Lee**[‡]**,**
Hee-Kyung Chang

Department of Pathology, Medical College of Kosin University, Busan; Department of Immunology of Medical College of Keimyung University*, Daegu; Department of Clinical Pathology of Medical College of Daegu-Catholic Univeristy[†], Daegu; Department of Otolaryngology[‡], Medical College of Kosin University, Busan, Korea

Address for correspondence
Hee-Kyung Chang, M.D.
Department of Pathology, Medical College of Kosin University, 34 Amnam-dong, Suh-gu, Busan 602-702, Korea
Tel : +82-51-990-6325, Fax : +82-51-241-7420
E-mail : changhkg@ns.kosinmed.or.kr

INTRODUCTION

In recent years, several tumor-specific shared antigen families, such as melanoma-antigen genes (MAGE), GAGE, BAGE, and LAGE/NY-ESO-1, which are recognized by autologous cytotoxic T lymphocytes (CTL) have been characterized at the molecular level (1, 2). These usually consist of peptides derived from intracellular proteins and are presented to CTL by HLA class I molecules (3-5). These antigens are of particular interest in tumor immunology, because their expression, with an exception of testis and fetal tissues, seems to be restricted to tumor cells (6).

The common features of these antigens (4-13) are the predominant mRNA expression in testis, but generally not in other normal tissues, the gene activation and high-level mRNA expression in certain malignancies, and the expression in malignancies in a lineage-nonspecific manner. Another intriguing feature of these antigens is they are mapped to the X chromosome. These antigens may serve useful targets for specific immunotherapy, since the expression of MAGE has not been recognized in normal tissues except for the testis. Among these, the MAGE gene products are currently under

clinical test as a potential new target for active specific immunotherapy (14, 15). In other words, peptides encoded by the MAGE genes serve targets for specific immunotherapy as they are presented in association with human leukocyte antigen class I molecules and are recognized by CTL (3-5). The MAGE genes comprise more than 4 subfamilies: MAGE-A, B, C, D, etc (4-13). The MAGE-A genes respresent a family of more than 12 closely related genes located on the long arm of the chromosome X (region Xq28) (4, 11, 12). In the majority of the tumors investigated only the expression of MAGE-A1, -A2, -A3, -A4, -A6 and -A12 has been mostly shown (4). However, there is a report that the subfamily MAGE-D was detected even in normal adult tissues (16). Moreover, a recent study reported that the cytologic specimens of former smokers without tumor mass expressed MAGE-A1, -A3, and -B2 (17).

In recent years, the incidence of colorectal cancer in Korea has increased to the fifth most prevalent cancer. Therefore, an appropriate screening method is essential for the early detection and reduction of the death rate in colorectal cancer. Therefore we evaluated the feasibility of MAGE common primer sets as screening method for colorectal cancer. We found 42.1% of MAGE -A1 to -A6 expression by RT-PCR with common

primers without any significant relationship with clinical prognostic markers and also observed that MAGE-A2 and -A4 were most prevalent in colorectal carcinoma.

MATERIALS AND METHODS

Patients and Clinical Samples

Surgical specimens were obtained from a total of 38 consecutive patients with various stages of colorectal carcinoma who had undergone a surgical resection at the Department of Surgery of Kosin University Hospital in Pusan (Table 1). Fresh-frozen primary colorectal carcinoma samples and nonneoplastic colorectal mucosal tissues 2 cm from the tumor mass were subjected to mRNA extraction. Their pathologies were confirmed by frozen section, before mRNA extraction.

mRNA Isolation

Fresh-frozen colorectal cancer and corresponding non-neoplastic colorectal tissue blocks were cut into 4-μm sections using cryostat microtome at -20°C. The first and the last sections were immediately stained with methylene blue and examined under a microscope to confirm histologically normal tissues without tumor cell infiltration and tumor tissue consisting of at least 80% of tumor cells. Total RNA was isolated from each of the 37 samples after lysis in guanidinium isothiocyanate and phenol extraction using the commercial kit (Trizol, Invitrogen Laboratories, San Diego, U.S.A.).

RT-PCR and direct sequencing of PCR products

cDNA was synthesized from 4 μg of total RNA in a 25-μL reaction mixture containing 6 μL of $5\times$ reverse transcriptase reaction buffer, oligo (dT) (100 pmole/μL) 1 μL, 10 mM dNTP 4 μL, 40 units/μL of RNAsin, 0.5 μL (200 units/μL) of Moloney leukemia virus reverse transcriptase (MMLV RTase). The mixture was incubated at 42°C for 60 min, heated to 94°C for 3 min, and then chilled on ice. To test the cDNA integrity, GAPDH was amplified for each sample.

To detect the expression of MAGE-A1 to -A6 synchronously and to avoid amplification of genomic DNA of MAGE, we designed MAGE common primer sets MC1 sense (CTGAA GGAGAAGATCTGCC) and MC1 antisense (CTCCAGG TAGTTTTCCTGCAC) for first round PCR and MC2 sense (CTGAAGGAGAAGATCTGCCWGTG) and MC2 antisense (CCAGCATTTCTGCCTTTGTGA) for the second round PCR. These primer sets were designed in such a way that the 5′ (sense) and 3′ (antisense) primers span at least one intron in the genomic DNA. That is, each of sense and antisense primers were complementary to two exon sequences at either side of an intervening intron to prevent hybridization to the genomic DNA. These MAGE common primer

pairs were used for the nested PCR of the reverse-transcribed cDNAs. All oligonucleotide primers were synthesized by and purchased from the Bioneer Co. (Taejun, Korea). Oligonucleotide primers were solubilized in TE buffer to the concentration of 100 pmole/μL, aliquoted, and stored frozen at -75°C. Each aliquot was diluted to 10 pmole/μL before use. To ensure that RNA was not degraded, a PCR assay with primers specific for β-actin cDNA was carried out in each case, except that only 25 cycles were performed, under the following cycling conditions: 30 sec at 94°C, 30 sec at 60°C, and 30 sec at 72°C with pre-denaturation for 2 min at 94°C and post-extension at 72°C. A 10-μL aliquot of each reaction mixture was size-fractionated on 1% agarose gel and visualized with ethidium bromide staining.

Sequencing

Wizard plus SV minipreps kit was used for the template

Table 1. Relationship between clinicopathologic characteristics of 38 patients with colorectal carcinoma with mRNA expression of MAGE genes

Characteristics	No. of patients (N=38)	No.of MAGE positive cases (%)	*p*-value
Age (yrs±SD)			0.098
Mean	58±7.76		
<=60	23	7 (30.4)	
>60	15	9 (60.0)	
Gender			0.12
Male	18	7 (38.9)	
Female	20	9 (45.0)	
Histologic grade			0.072
1-2	30	14 (46.7)	
3	6	0 (0)	
4	2	2 (100)	
Depth of tumor invasion (pT)			0.203
1	2	1 (50.0)	
2	6	2 (33.3)	
3	26	12 (46.2)	
4	4	1 (25.0)	
Lymph node metastasis (pN)			0.10
Negative	16	7 (43.8)	
Positive	22	9 (40.9)	
N1	14	8 (571.)	
N2	6	1 (16.7)	
Stage			0.073
I	2	1 (50.0)	
II	14	6 (42.9)	
III	18	8 (44.4)	
IV	4	1 (25.0)	
Vessel Invasion			0.191
Negative	27	13 (48.1)	
Positive	11	3 (27.2)	
Perineural invasion			0.169
Negative	32	13 (40.6)	
Positive	6	3 (50.0)	

Pathological diagnoses and staging of colorectal cancer were made according to the UICC. NS; not-significant statistically (*p*>0.05).

DNA preparation for sequencing after subcloning of RT-PCR products and Automatic DNA sequencer (in Bukyung University) was used for sequencing. The sequence data were analysed by the NCBI (NIH, U.S.A.) Blast Search Program.

Statistical Analysis

The statistical analysis was performed using the χ^2 or Fisher's exact test as appropriate. The significance level was set at $p < 0.05$.

RESULTS

We initially analyzed 38 paired tissue samples of primary colorectal carcinoma to determine mRNA of the MAGE-A1 to -A6 genes by RT-PCR with the original common primers. We found 16 (42.1%) of 38 colorectal carcinomas expressed at least one of MAGE-A1 to -A6 (Fig. 1).

The expression of the MAGE genes was not related to age or sex (Table 1). There was no significant correlation between the expression and histological grades (Table 1), the depth of invasion, metastasis to the lymph nodes (Table 1), vessel and neural/perineural invasion (Table 1). On the other hand, non-neoplastic colorectal mucosal tissues did not express mRNA of MAGE-A1 to -A6 (Fig. 1).

RT-PCR fragments from each sample were recovered from gels and sequenced to confirm the identities with the corresponding mRNAs, and revealed 6 cases of MAGE-A2 (15.8%), 6 cases of MAGE-A4 (15.8%), 2 cases of MAGE-A3 (5.3%), and one case of MAGE-A6 (2.6%) (Table 2).

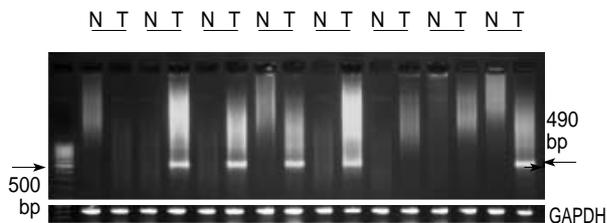None of the isotypes showed a significant correlation be-

Fig. 1. Electrophoretic analysis of nested RT-PCR amplification products with common MAGE primers from normal colorectal mucosa (N) and colorectal cancer (T) of sample No.17-24.

Table 2. Isotypes of positively detected cases by PCR with MAGE common primers in 38 cases of colorectal carcinoma

MAGE isotypes	No. of positive cases	% in 16 total positive cases)
A2	6	37.5
A4	5	31.25
-4b	1	6.25
A3	2	12.5
A6	1	6.25
Failed	1	6.25

tween its expression and clinicopathological parameters mentioned above.

DISCUSSION

Since the notion that the MAGE genes are expressed only in tumor tissues or tumor cells and are silent in normal tissues, except testis and placenta, has been widely accepted, some studies have investigated to the feasibility of MAGE as a tumor marker (1-10). Measuring mRNA was the common method to evaluate the expression patterns of the MAGE genes in most of the studies, because there are no commercially available antibodies. Thus, needless to say, the specific common primer for detecting these gene expressions by RT-PCR has been required, since the MAGE gene family consists of more than 12 genes and more than 4 subfamilies. Furthermore, there was a report that the subfamily MAGE-D was detected even in normal adult tissues (16).

In recent years, the incidence of colorectal cancer in Korea has increased approaching to the fifth most prevalent cancer. Therefore, an appropriate screening method has become essential for the early detection and reduction of the death rate in colorectal cancer. Therefore we evaluated the feasibility of MAGE common primer sets as screening method for colorectal cancer. We found 42.1% of MAGE-A1 to -A6 expression. Previous studies have shown various rates of expression of the MAGE genes in colorectal carcinoma from 30% to 88% (18-20) by RT-PCR. Our data seem to be in the middle of the previous data. This discrepancy may result from different primer sets and different clinical samples. Even when molecular biological techniques considered, we have already studied the MAGE expression in other organs such as head and neck (21), lung, uterine cervix, and stomach cancers and obtained results in line with those from others (data not shown) and also verified our techniques. Further studies on the MAGE expression in colorectal carcinoma among Koreans are needed to verify the results of this study.

In this study, we did not found any significant relationship the expression of MAGE to established prognostic markers by AJCC in colorectal carcinoma. There were two reports that MAGE, especially MAGE-3 expression was related to metastasis to lymph node or liver (18, 19). However, recently the study results that tumor stage was inversely related not to MAGE expression but GAGE expression have been reported in other gastrointestinal areas (22). The same study revealed MAGE expression correlated to good prognosis in esophageal and gastric cancer. This controversy appears to mean heterogenous expression patterns of MAGE as well as necessity of further studies to approach the established data.

One Jananese group (23) reported the expression rates were 7.5% of MAGE-A1, 15.0% of MAGE-A2, 32.5% of MAGE-A3, 12.5% of MAGE-A4 in colorectal cancer. In our study, 6 cases of MAGE-A2 (15.8%), 6 cases of MAGE-A4 (15.8%),

2 cases of MAGE-A3 (5.3%), and one case of MAGE-A6 (2.6%) were expressed. We observed no distinct co-expression of the MAGE isotypes in this study. Our results demonstrated only 2 cases of MAGE-A3 which is considered be the most promising target for immunotherapy in Koreans in the light of the HLA type patterns. Therefore, MAGE peptide-specific immunotherapy might have a limited role in colorectal carcinoma in Korean population.

Different patterns of the MAGE expressions between previous results and ours might have resulted from different primers and different research methods including RT-PCR conditions. We performed RT-PCR first with common primers and directly sequenced each RT-PCR product after subcloning. However, we followed previous RT-PCR conditions in the literature. Assessment of the utility of the common primer sets that we used need further studies.

The functional role of MAGE as well as the exact activation mechanism has not been well-established yet in colon cancer. DNA demethylation is widely accepted as the activation mechanism of MAGE. According to the molecular progression for the development of colorectal carcinoma (27), DNA methylation abnormalities may occur in the early stage (hyperproliferative epithelium). Our results that showed no relationship between MAGE expression and tumor stages might be interpreted in the context of the early MAGE expression during the tumor development. However, there is no hard evidence that genome-wide methylation abnormalities should be compatible with those of the MAGE genes.

ACKNOWLEDGMENT

REFERENCES

1. van der Bruggen P, Traversari C, Chomez P, Lurquin C, De Plaen E, Van den Eynde B, Knuth A, Boon T. *A gene encoding an antigen recognized by cytolytic T lymphocytes on a human melanoma. Science 1991; 254: 1643-7.*

2. van den Eynde BJ, van der Bruggen P. *T cell defined tumor antigens. Curr Opin Immunol 1997; 9: 684-93.*

3. van Pel A, van der Bruggen P, Coulie PG, Brichard VG, Lethe B, van den Eynde B, Uyttenhove C, Renauld JC, Boon T. *Genes coding for tumor antigens recognized by cytolytic T lymphocytes. Immunol Rev 1995; 145: 229-50.*

4. Kirkin AF, Dzhandzhungazyan K, Zeuthen J. *Melanoma-associated antigens recognized by cytotoxic T lymphocytes. APMIS 1998; 106: 663-79.*

5. Stockert E, Jager E, Chen YT, Scanlan MJ, Gout I, Karbach J, Arand M, Knuth A, Old LJ J A. *Survey of the humoral immune response of cancer patients to a panel of human tumor antigens. Exp Med 1998; 20; 187: 1349-54.*

6. Tureci O, Sahin U, Zwick C, Koslowski M, Seitz G, Pfreundschuh M. *Identification of a meiosis-specific protein as a member of the class of cancer/testis antigens. Proc Natl Acad Sci USA 1998; 28; 95: 5211-6.*

7. Boel P, Wildermann C, Sensi ML, Brausseur R, Renauld JC, Coulie P, Boon T, van der Bruggen P. *A new gene encoding an antigen recognized on human melanomas by cytolytic T lymphocytes. Immunity 1995; 2: 167-75.*

8. Van den Eynde B, Peeters O, De Backer O, Gaugler B, Lucas S, Boon T. *A new family of genes coding for an antigen recognized by autologous cytolytic T lymphocytes on a human melanomas. J Exp Med 1995; 182: 689-98.*

9. Lurquin C, De Smet C, Brasseur F, Muscatelli F, Martelange V, De Plaen E, Brasseur R, Monaco AP, Boon T. *Two members of the human MAGE B gene family located in Xp21.3 are expressed in tumors of various histological origins. Genomics 1997; 46: 397-408.*

10. Gillespie AM, Coleman RE. *The potential of melanoma antigen expression in cancer therapy. Cancer Treat Rev 1999; 25: 219-27.*

11. De Plaen E, Arden K, Traversari C, Gafori J, Szikora J P, De Smet C, Brasseur F, van der Bruggen P, Lethe B, Lurquin C. *Genome, structure, chromosomal localization and expression of twelve genes of the MAGE family. Immunogenetics 1994; 40; 360-9.*

12. Rogner UC, Wilke L, Steeck E, Korn B, Poustka A. *The melanoma antigen gene (MAGE) family is clustered in the chromosomal band Xq28. Genomics 1995; 29: 725-31.*

13. Muscatelli F, Walker AP, De Plaen E, Scafford AN, Monaco AP. *Isolation and characterization of a new MAGE gene family in the Xp 21.3 region. Proc Natl Acad Sci USA 1995; 92: 498-4991.*

14. Osanto S. *Vaccine trials for the Clinician: Prospects for Tumor Antigens. Oncologist 1997; 2: 284-99.*

15. Marchand M, Weynants P, Rankin E, Arienti F, Belli F, Parmiani G, Cascinelli N, Bourlond A, Vanwijck R, Humblet Y, Canon J-L, Laurent C, Naryaert J-M, Plagne R, Deraemarker R, Knuth A, Brasseur F, Herman J, Coulie PG, Boon T. *Tumor regression responses in melanoma patients treated with a peptide encoded by gene MAGE-3. Int J Cancer 1995; 63: 883-5.*

16. Jungbluth AA, Busam KJ, Kolb D, Iversen K, Coplan K, Chen YT, Spangnoli GC, Old LJ. *Expression of MAGE-antigens in normal tissues and cancer. Int J Cancer 2000; 85: 460-5.*

17. Jang SJ, Soria JC, Wang L, Hassen KA, Morice RC, Walsh GL, Hong WK, Mao L. *Activation of melanoma antigen gene tumor antigens occurs early in lung carcinogenesis Cancer Res 2001; 61: 7959-63.*

18. Hasegawa H, Mori M, Haraguchi M, Ueo H, Sugimachi K, Akiyoshi T. *Expression spectrum of melanoma antigen-encoding gene family members in colorectal carcinoma. Arch Pathol Lab Med 1998; 122: 551-4.*

19. Nishimura S, Fujita M, Terata N, Tani T, Kodama M, Itoh K. *Expression of MAGE genes in colorectal carcinomas. Nihon Rinsho Meneki Gakkai Kaishi 1997; 20: 95-101.*

20. Mori M, Inoue H, Mimori K, Shibuta K, Baba K, Nakashima H, Haraguchi M, Tsuji K, Ueo H, Barnard GF, Akiyoshi T. *Expression of MAGE genes in human colorectal carcinoma. Ann Surg 1996; 224; 183-8.*

21. Lee KD, Eura M, Ogi K, Nakano K, Chikamatsu K , Masuyama K, Ishikawa T. *Expression of the MAGE-1, -2, -3, -4 and -6 genes in non-squamous cell carcinoma lesions of the head and neck. Acta Otolaryngol 1996; 116: 633-9.*

22. Zambon A, Mandruzzato S, Parenti A, Macino B, Dalerba P, Ruol A, Merigliano S, Zaninotto G, Zanovello P. *MAGE, BAGE, and GAGE gene expression in patients with esophageal squamous cell carcinoma and adenocarcinoma of the gastric cardia. Cancer 2001; 91: 1882-8.*

23. Tahara K, Mori M, Sadanaga N, Sakamoto Y, Kitano S, Makuuchi M. *Expression of the MAGE gene family in human hepatocellular carcinoma. Cancer 1999; 15; 85: 1234-40.*