PO Box 2345, Beijing 100023, China
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Promoter hypomethylation and reactivation of *MAGE-A1* and *MAGE-A3* genes in colorectal cancer cell lines and cancer tissues

Kyung-Hee Kim, Jin-Sung Choi, Il-Jin Kim, Ja-Lok Ku, Jae-Gahb Park

Kyung-Hee Kim, Jin-Sung Choi, Il-Jin Kim, Ja-Lok Ku, Jae-Gahb Park, Laboratory of Cell Biology, Korean Cell Line Bank, Cancer Research Institute, Seoul National University College of Medicine, Seoul 110-744, Korea
Supported by the Korea Research Foundation Grant, No.KRF-2003-03-E00199
Correspondence to: Ja-Lok Ku, Laboratory of Cell Biology, Cancer Research Institute, Seoul National University College of Medicine, 28 Yongon-dong, Chongno-gu, Seoul 110-744, Korea. kujalok@snu.ac.kr
Telephone: +82-2-36687919 Fax: +82-2-7420021
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Abstract

AIM: To verify the expression and methylation status of the *MAGE-A1* and *MAGE-A3* genes in colorectal cancer tissues and cancer cell lines.

METHODS: We evaluated promoter demethylation status of the *MAGE-A1* and *MAGE-A3* genes by RT-PCR analysis and methylation-specific PCR (MS-PCR), as well as sequencing analysis, after sodium bisulfite modification in 32 colorectal cancer cell lines and 87 cancer tissues.

RESULTS: Of the 32 cell lines, *MAGE-A1* and *MAGE-A3* expressions were observed in 59% and 66%, respectively. Subsequent to sodium bisulfite modification and MS-PCR analysis, the promoter hypomethylation of *MAGE-A1* and *MAGE-A3* was confirmed in both at 81% each. Promoter hypomethylation of *MAGE-A1* and *MAGE-A3* in colorectal cancer tissues was observed in 43% and 77%, respectively. Hypomethylation of *MAGE-A1* and *MAGE-A3* genes in corresponding normal tissues were observed in 2% and 6%, respectively.

CONCLUSION: The promoter hypomethylation of *MAGE* genes up-regulates its expression in colorectal carcinomas as well as in gastric cancers and might play a significant role in the development and progression of human colorectal carcinomas.

Key words: *MAGE-A1*; *MAGE-A3*; Promoter; Hypomethylation; Colorectal cancer

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INTRODUCTION

Human tumors often display changes in DNA methylation, which include both genome-wide hypomethylation and site-specific hypermethylation. Global hypomethylation and CpG island hypermethylation have been recognized as important contributors to the development of carcinogenesis in humans. Hypermethylation of promoter CpG islands is the signature of transcriptional silencing of their downstream genes, including *RB, p16, VHL, BRCA1, E-cadherin, APC, hMLH1, FHIT, COX2,* and *CDX1* in various human cancers; and is as effective as inactivation by gene mutation or deletion[1-6]. Global DNA hypomethylation has been implicated in the activation of oncogenes such as *c-myc, k-ras*, and it may also contribute to tumor progression by the induction of genome instability[7,8].

The *MAGE* family of genes belongs to a group of germ line-specific genes that are activated in different types of tumors. This family of genes was reported to direct the expression of a tumor-specific antigen that was recognized in a melanoma cell by cytolytic T lymphocytes[9]. The *MAGE-A1* gene has a CpG-rich promoter, which, unlike classical CpG-rich promoters, is methylated in all normal somatic tissues, except for the placenta and testis. In contrast, the promoter region of *MAGE-A1* is completely unmethylated in testicular germ cells and in tumor cells that express the gene[10]. Demethylation, and therefore, activation of *MAGE-A1* in tumors appears to be a consequence of the genome-wide demethylation process, since the expression of this gene in tumor cells correlates with a decreased level of overall DNA methylation[11]. A correlation between *MAGE-A1* and *MAGE-A3* expression and genome-wide hypomethylation has been observed in some types of carcinomas[12,13]. The human *MAGE-A1* and *MAGE-A3* genes, which are located on chromosome X, are expressed in 29% and 66%, respectively, of human gastric cancer cells due to the hypomethylation of the promoter region[12]. However, it is unknown if this relationship is present in colorectal carcinomas.

In this study, we investigated the promoter methylation

status of *MAGE-A1* and *MAGE-A3* genes. A total of 32 colorectal cancer cell lines were tested for hypomethylation of the *MAGE-A1* and *MAGE-A3* genes promoter. In addition, we screened the methylation status of the *MAGE-A1* and *MAGE-A3* genes promoter in 87 paired colorectal cancers and normal mucosal tissue samples.

MATERIALS AND METHODS

Cell cultures

A total of 32 colorectal cancer cell lines (Table 1) and 2 gastric cancer cell lines (SNU-1 and SNU-5) were obtained from either the Korean Cell Line Bank (KCLB; Seoul, Korea) or the American Type Culture Collection (ATCC; Manassas, VA, USA). Sixteen SNU-colorectal cancer cell lines were established and were reported upon previously by this laboratory[14,15]. SNU-1 and SNU-5 gastric carcinoma cell lines were used as methylation positive (SNU-1) and negative (SNU-5) controls for *MAGE* gene expression[12]. All the cell lines were maintained in RPMI1640, which was supplemented with 10% FBS, 100 kU/L penicillin, and 0.1 g/L streptomycin. The cultures were maintained in humidified incubators at 37℃ in a 5% CO_2 and 95% ambient air atmosphere.

Nucleic acid isolation and cDNA synthesis from the cell lines

Genomic DNA and total RNA were isolated from washed-cell pellets. Total genomic DNA was extracted in accordance with the standard SDS-proteinase K procedure; and total cellular RNA was extracted based on the manufacturer's instructions (Intron Biotechnology; Seoul, Korea). For cDNA synthesis, 2 μg of total RNA was reverse transcribed with a random hexamer, dNTPs, and 1 μL (200 U) of Superscript™ II reverse transcriptase (Life Technologies; Gaithersburg, MD, USA) in a final volume of 20 μL for 1 h and 15 min at 42℃ after a 10-min denaturation at 70℃. Eighty microliters of distilled water were added subsequent to the reverse-transcription reaction.

Expression of MAGE-A1 and MAGE-A3 genes

For mRNA expression analysis, the cDNA was amplified in 25 μL of a PCR reaction mix with 1 μL of the reverse-transcription reaction, the primers and 0.5 U of Taq DNA polymerase. The PCR conditions consisted of 10 min at 94℃ for the initial denaturation, followed by 35 cycles of 94℃ for 30 s, 54℃ for 60 s, and 72℃ for 60 s, and a final elongation of 7 min at 72℃. The primer sequences are as follows. *MAGE-A1* cDNA was amplified by PCR with MG1 RT primers; MG1 RT sense, 5'-TGTGG GCAGGAGCTGGGCAA-3', MG1 RT antisense, 5'-GCCGAAGGAACCTGACCCAG -3'. For the *MAGE-A3* cDNA, the MG3 RT primers were used; MG3 RT sense, 5'-AAGCCGGCCCAGGCTCGGT-3', MG3 RT antisense, 5'-GCTGGGCAATGGAGACCCAC-3'. PCR amplification was performed in a programmable thermal cycler (PCR System 9700, Applied Biosystems; Foster City, CA, USA). Primers for *β-actin* were used to confirm RNA integrity. Both *MAGE-A1* and *MAGE-A3* and *β-actin* RT-PCR reactions used the same cDNA synthesis. The amplified DNA fragments were fractionated in 2% agarose gel and stained with ethidium bromide.

Tissue sample collection and DNA extraction

A total of 87 paired tumor and normal mucosal tissue samples were obtained from 87 patients, who had primary colorectal adenocarcinoma. The normal mucosal tissue specimens were collected from each patient 10 cm or more away from the tumor areas. Approximately 2 g of the surgically removed tissues were frozen immediately and then stored in liquid nitrogen. The remaining sections of the samples were fixed with formalin and used for further histological examination in order to confirm the diagnosis postoperatively. Genomic DNA was isolated from the frozen-tissue biopsies with the standard SDS-proteinase K procedure.

Methylation specific PCR

With respect to the MS-PCR, the sodium bisulfite modification of genomic DNA was performed as reported previously[16]. A total of 2 μg of genomic DNA obtained from colorectal cancer cell lines, was denatured with NaOH and hydroquinone. Then, 3 mmol/L sodium bisulfite was added and the mixture was incubated at 55℃ for 16 h. Following the bisulfite modification, the DNA was purified with a Wizard DNA purification system (Promega; Madison, WI, USA), ethanol precipitated, dried, and resuspended in 100 μL distilled water. The PCR was performed using the PCR primers that were described previously[12]. The amplified DNA fragments were fractionated in 2% agarose gel that was stained with ethidium bromide and visualized under UV light.

5-aza-2'-deoxycytidine treatment and RT-PCR

For 5-aza-2'-deoxycytidine treatment, the cells were seeded in two 2×10^5 cells/75 cm^2 culture flasks on d 0. The cells were treated with and without 1-5 μmol/L of 5-aza-2'-deoxycytidine (Sigma Chemical Co.) for 24 h on d 2 and 5, and the medium was changed 24 h after addition of 5-aza-2'-deoxycytidine. The cells were harvested on d 8 for the analysis of the *MAGE*s expression. Subsequently, the RNA was prepared, and RT-PCR was performed to detect *MAGE-A1* and *MAGE-A3* expression with the *MAGE-A1* and *MAGE-A3* RT-PCR primers as described above.

RESULTS

Expression of MAGE-A1 and MAGE–A3 in colorectal cancer cell lines

Expression of *MAGE-A1* and *MAGE-A3* mRNA in 32 colorectal cancer cell lines was analyzed by RT-PCR, and *MAGE-A1* and *MAGE-A3* expressions were observed in 19 (59%) and 21 (66%) of the cell lines, respectively (Figure 1 and Table 1). PCR for *β-actin* confirmed the integrity of the RNA.

Clinico-pathological features

Of the 87 colorectal carcinomas, 57 (66%) were obtained from the proximal colon (cecum to splenic flexure), and 30 (34%) from the distal colorectum (splenic flexure to

Table 1 Methylation status of the promoter region of *MAGE-A1* and *MAGE-A3* genes in 32 colorectal cancer cell lines

	Cell line	*MAGEs* expression				MS-PCR			
		MAGE-A1		*MAGE-A3*		*MAGE-A1*		*MAGE-A3*	
		-5-aza/+5-aza		- 5-aza/+5-aza		M/U		M/U	
1	SNU-61	-	++	±	++	+	+	-	+
2	SNU-81	-	++	-	+++	+	-	+	-
3	SNU-175	++	++	+++	+++	+	+	+	+
4	SNU-283	+++	NT	+++	NT	+	+	+	-
5	SNU-407	+++	+	+++	++	+	-	+	+
6	SNU-503	++	NT	-	NT	+	+	+	+
7	SNU-769A	+++	++	+++	+++	-	+	-	+
8	SNU-769B	+++	+++	+++	+++	-	+	-	+
9	SNU-1033	++	+	++	+++	+	+	+	+
10	SNU-1040	-	-	±	++	+	-	+	-
11	SNU-1047	+++	++	++	++	+	-	-	+
12	SNU-1197	++	+++	++	+++	+	+	+	+
13	SNU-C1	±	NT	++	NT	+	+	-	+
14	SNU-C2A	++	NT	+++	NT	+	+	+	+
15	SNU-C4	-	NT	-	NT	+	-	+	+
16	SNU-C5	±	++	-	+++	+	+	+	-
17	Caco-2	-	++	++	+++	+	+	+	+
18	COLO201	-	++	-	+++	-	+	-	+
19	COLO205	-	-	-	++	+	+	+	+
20	COLO320	+++	NT	+++	NT	+	+	-	+
21	DLD-1	-	+++	-	+++	+	+	+	+
22	HCT-8	-	++	-	+++	+	-	+	-
23	HCT-15	-	++	-	+++	+	+	+	+
24	HCT-116	+++	NT	+++	NT	+	+	+	+
25	HT-29	+++	++	+++	+++	+	+	+	+
26	Lovo	+++	+++	+++	+++	+	+	+	+
27	LS174T	+++	NT	+++	NT	+	+	-	+
28	NCI-H716	+++	+++	+++	+++	+	+	-	+
29	SW403	+++	++	+++	+++	+	+	+	+
30	SW480	+++	NT	+++	NT	+	+	+	-
31	SW1116	-	NT	++	NT	+	+	-	+
32	WiDR	++	+++	+++	+++	+	+	-	+

5-aza: 5-aza-2′-deoxycytidine; NT: Not tested; M: The amplified product with primers recognizing methylated sequence; U: The amplified product with primers recognizing unmethylated sequence; +: Amplified product; -: Not amplified product.

rectum). Randomly selected patients aged 16-81 years, including 55 males and 32 females. Of the 32 colorectal cancer cell lines, 7 originated from the proximal colon and 8 from the distal colorectum. The origin of the remaining 17 colorectal cancer cell lines was unknown.

Analysis of MAGE-A1 and MAGE-A3 methylation by MS-PCR

By using primers for unmethylated *MAGE-A1* DNA amplification on bisulfite modified DNA, amplified DNA fragments were observed in 26 (81%) cell lines (SNU-61, SNU-175, SNU-283, SNU-503, SNU-769A, SNU-769B, SNU-1033, SNU-1197A, SNU-C1, SNU-C2A, SNU-C5, Caco2, COLO201, COLO205, COLO320, DLD-1, HCT-15, HCT-116, HT-29, Lovo, LS174T, NCI-H716, SW403, SW480, SW1116, and WiDR) (Figure 2 and Table 1). And by using primers for unmethylated *MAGE-A3* DNA amplification on bisulfite modified DNA, amplified DNA fragments were found also in 26 (81%) cell lines (SNU-61, SNU-175, SNU-407, SNU-503, SNU-769A, SNU-769B, SNU-1033, SNU-1047, SNU-1197A, SNU-C1, SNU-C2A, SNU-C4, Caco2, COLO201, COLO205, COLO320, DLD-1, HCT-15, HCT-116, HT-29, Lovo, LS174T, NCI-H716, SW403, SW1116, and WiDR) (Figure 2 and Table 1). By using primers for amplification of unmethylated or methylated DNA, amplified DNA fragments were found in all 32 cell lines. *MAGE-A1* unmethylated DNA products were observed in 37 out of 87 tumor tissue samples (43%; Figure 3). In the normal tissue samples, the methylated DNA was amplified in all 87 samples. However, the unmethylated DNA was amplified in 2 normal tissues (2%). *MAGE-A3* unmethylated DNA products were observed in 67 out of 87 tumor tissue samples (77%; Figure 3). In the normal tissue samples, the methylated DNA was amplified in all 87 samples. However, the unmethylated DNA was amplified in 5 normal tissues (6%).

Reexpression of MAGE-A1 and MAGE-A3 after treatment with 5-aza-2′-deoxycytidine

We investigated whether *MAGE-A1* mRNA was re-expressed after 5-aza-2'-deoxycytidine treatment in 22 cell

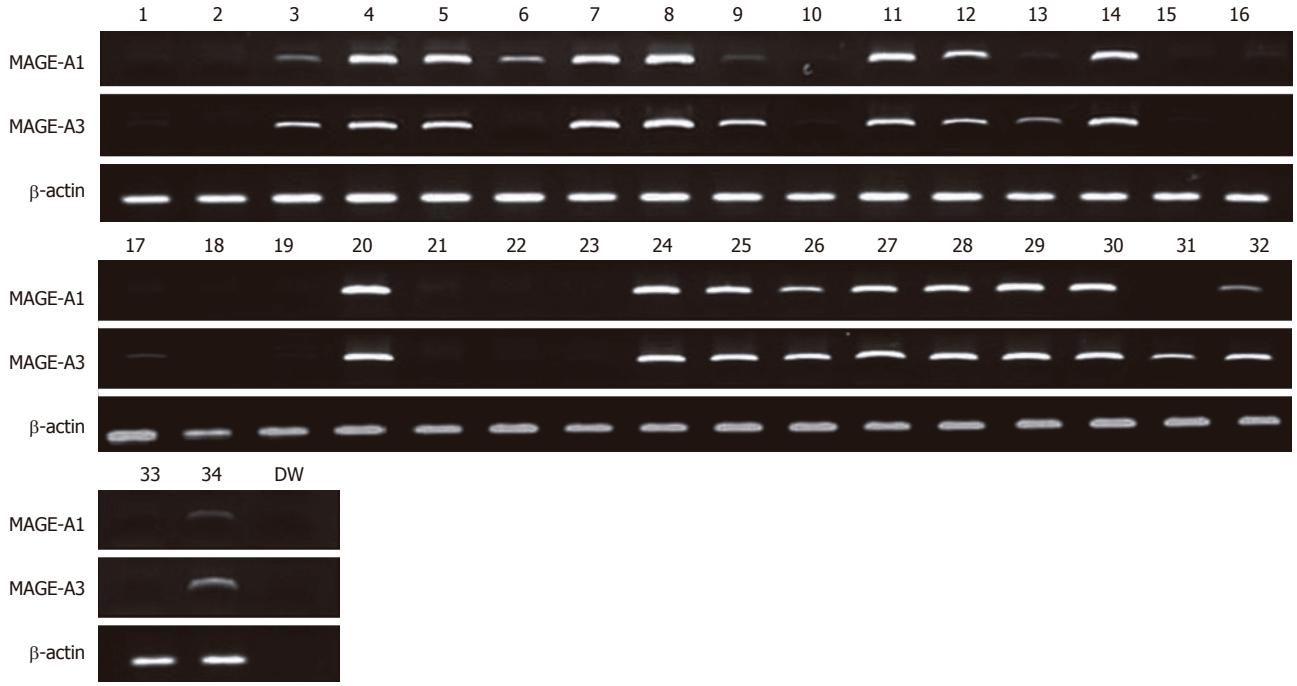

Figure 1 RT-PCR analysis of the *MAGE-A1* and *MAGE-A3* genes in 32 colorectal cancer cell lines. β-actin was amplified as an internal control. The *MAGE-A1* gene was significantly expressed in 19 colorectal cancer cell lines (Lanes, 3, 4, 5, 6, 7, 8, 9, 11, 12, 14, 20, 24, 25, 26, 27, 28, 29, 30 and 32). The *MAGE-A3* gene was expressed in 21 colorectal cancer cell lines (Lanes, 3, 4, 5, 7, 8, 9, 11, 12, 13, 14, 17, 20, 24, 25, 26, 27, 28, 29, 30, 31 and 32). Lane numbers 1 to 34 show cell lines SNU-61, SNU-81, SNU-175, SNU-283, SNU-407, SNU-503, SNU-769A, SNU-769B, SNU-1033, SNU-1040, SNU-1047, SNU-1197, SNU-C1, SNU-C2A, SNU-C4, SNU-C5, Caco-2, COLO201, COLO205, COLO320, DLD1, HCT-8, HCT-15, HCT-116, HT-29, LOVO, LS174T, NCI-H716, SW403, SW480, SW1116, WiDr, SNU-1, and SNU-5 respectively.

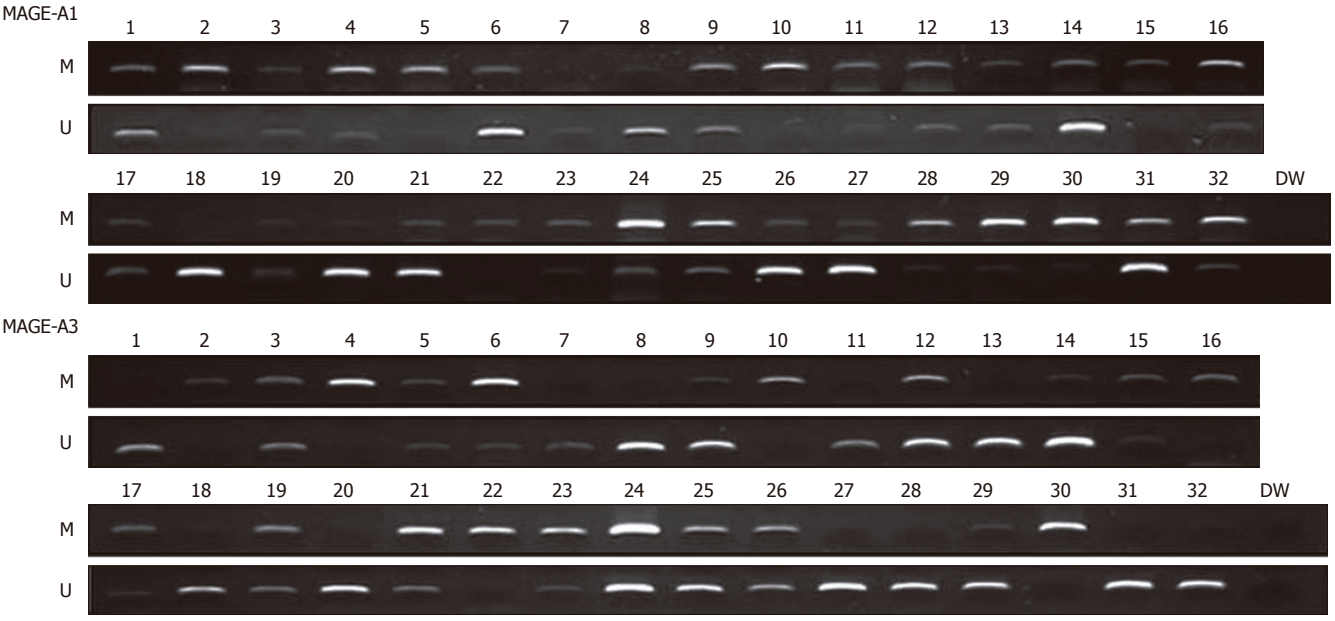

Figure 2 Methylation analysis of the *MAGE-A1* and *MAGE-A3* genes in colorectal cancer cell lines. The promoter region of the *MAGE-A1* gene was unmethylated in 26 cell lines (SNU-61, SNU-175, SNU-283, SNU-503, SNU-769A, SNU-769B, SNU-1033, SNU-1197A, SNU-C1, SNU-C2A, SNU-C5, Caco2, COLO201, COLO205, COLO320, DLD-1, HCT-15, HCT-116, HT-29, Lovo, LS174T, NCI-H716, SW403, SW1116, and WiDR cell lines). Unmethylated *MAGE-A3* DNA amplifications were found in 26 cell lines (SNU-61, SNU-175, SNU-407, SNU-503, SNU-769A, SNU-769B, SNU-1033, SNU-1047, SNU-1197A, SNU-C1, SNU-C2A, SNU-C4, Caco2, COLO201, COLO205, COLO320, DLD-1, HCT-15, HCT-116, HT-29, Lovo, LS174T, NCI-H716, SW403, SW1116, and WiDR cell lines). Lane M denotes product amplified by primers recognizing a methylated sequence and Lane U denotes the product amplified by primers recognizing an unmethylated sequence, respectively.

lines, including 10 cell lines (SNU-61, SNU-81, SNU-1040, SNU-C5, Caco-2, COLO201, COLO205, DLD1, HCT-8, and HCT-15) that did not express *MAGE-A1* mRNA. After an RT-PCR analysis, we observed that all of the *MAGE-A1* mRNAs were re-expressed, except for the SNU-1040 and COLO205 cell lines (Table 1). This suggested that the inactivation of *MAGE-A1* expression was caused by another mechanism. Further, we investigated whether *MAGE-A3* mRNA was re-expressed after 5-aza-2'-deoxycytidine treatment in 22 cell lines, including 9 cell lines (SNU-61, SNU-81, SNU-1040, SNU-C5, COLO201, COLO205, DLD1, HCT-8, and

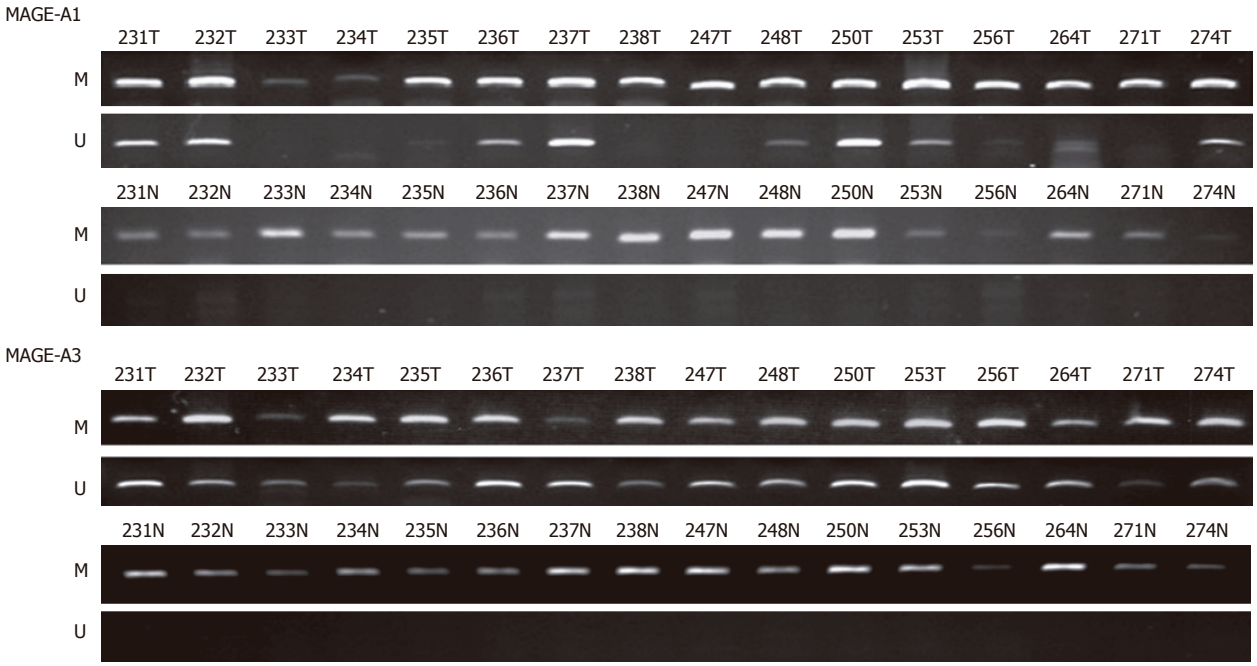

Figure 3 Methylation analysis of the *MAGE-A1* and *MAGE-A3* genes in colorectal cancer tissues and corresponding normal tissues. Methylation-specific PCR product amplified by primers recognizing methylated and unmethylated sequence. The promoter region of the *MAGE-A1* and *MAGE-A3* genes was unmethylated in colorectal cancer tissues, not normal tissues. Numbers represent each colorectal tissue and T denotes colorectal tumor tissues and N denotes corresponding normal tissues.

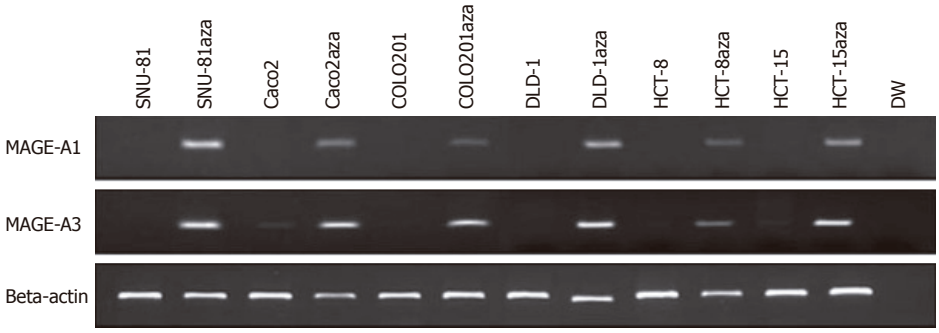

Figure 4 RT-PCR analysis after treatment with 5-aza-2'-deoxycytidine. The *MAGE-A1* and *MAGE-A3* genes were reactivated.

HCT-15) that did not express *MAGE-A3* mRNA. After an RT-PCR analysis, we observed that all of the *MAGE-A3* mRNAs were re-expressed (Table 1 and Figure 4).

DISCUSSION

Genome-wide hypomethylation and site-specific hypermethylation are common features of cancer cells. DNA hypomethylation in cancer cells is accompanied by the activation of germ line-specific genes, such as the *MAGE-A1* gene, the repression of which, in normal somatic tissues, is dependent upon DNA methylation[17]. Recent studies have reported the presence of very high *MAGE-A1* and *MAGE-A3* expressions in colorectal carcinomas[18,19]. Although previous reports have shown the expression of *MAGE* genes, the mechanism of *MAGE* genes expression in colorectal carcinomas was unclear. This led us to question whether it could be associated with decreased genomic methylation. It has been reported that *MAGE-A1* and *MAGE-A3* expression was related to gene hypomethylation in gastric carcinoma, hepatocarcinoma, and melanoma[12,20,21]. However, such a relationship has still not been confirmed in colorectal carcinoma. Accordingly, we analyzed the methylation status of the promoter region on the *MAGE-A1* and *MAGE-A3* genes in 32 colorectal cancer cell lines and estimated its association with *MAGE-A1* and *MAGE-A3* mRNA expression. We first examined the expression pattern of the *MAGE-A1* and *MAGE-A3* genes in these cell lines by an RT-PCR and observed that *MAGE-A1* and *MAGE-A3* were over-expressed significantly in 19 (59%) and 21 (66%) cell lines, respectively. This expression ratio of *MAGE-A1* and *MAGE-A3*, obtained by an RT-PCR in colorectal cancer cell lines, is similar to that observed in gastric cancer cell lines[12]. On the other hand, previous reports have revealed a much lower expression of MAGE in colorectal carcinomas ranging between 5%-39%[18,19,22]. In the literature, the expression of the *MAGE* genes was studied in colorectal carcinoma tissues; however, we have tested that in cancer cell lines. We assume that the major discrepancy of expression rates of *MAGE* genes between other reports and our findings might result from this. Since RNA or DNA was extracted from surgically removed frozen tissue biopsies, the tumor tissues may have been contaminated with normal stromal cells, therefore, masking the true levels of hypomethylation or expression

Table 2 Summary of expressions, promoter methylation status and clinical associations of the *MAGE-A1* and *MAGE-A3* genes in 32 colorectal cancer cell lines and 87 colorectal cancer tissues

	% of expression		% of hypomethylation	
	MAGE-A1	*MAGE-A3*	*MAGE-A1*	*MAGE-A3*
Cancer cell lines	59	66	81	81
Cancer tissues	NT[1]	NT	43	77
Normal tissues[2]	NT	NT	2	6

	MAGE-A1 hypomethylation			*MAGE-A3* hypomethylation		
	+	-	*P*	+	-	*P*
Location						
Proximal	14 (31.1%)	31(68.9%)		34 (75.6%)	11 (24.4%)	
Distal	17 (56.7%)	13(43.3%)	0.028	26 (86.7%)	4 (13.3%)	0.239
Sex						
Male	17 (30.9%)	38(69.1%)		38 (69.1%)	17 (30.9%)	
Female	20 (62.5%)	12(18.4%)	0.004	29 (90.6%)	3 (9.4%)	0.021

[1]Not tested; [2]Corresponding normal tissues of cancer tissues; +: Represent hypomethylation of *MAGE* genes; -: Represent no hypomethylation of *MAGE* genes.

of the *MAGE* genes in cancer tissues[23]. For further analysis of the methylation status of the *MAGE* genes in colorectal cancer tissues, laser capture microdissection techniques would allow more precise isolation of cancer cells and normal cells. It has already been reported that cancer cell lines have much higher levels of CpG island hypermethylation than corresponding malignant tissues, which may explain our lower incidence of hypomethylation in tissues *versus* cell lines. Moreover, cancer cells might be clonally selected with growth advantages over cancer cell lines. However, cancer cell lines often preserve hypermethylation or hypomethylation from the tumors they originate, thus they are indeed useful tools to study methylation status.

We analyzed promoter unmethylation of the *MAGE-A1* and *MAGE-A3* genes with a methylation-specific PCR after sodium-bisulfite modification and by direct sequencing analysis. Of the 32 cell lines analyzed, the promoter hypomethylation of *MAGE-A1* and *MAGE-A3* was observed in both at 26 cell lines each. Further, 23 cell lines (SNU-61, SNU-175, SNU-503, SNU-769A, SNU-769B, SNU-1033, SNU-1197A, SNU-C1, SNU-C2A, Caco2, COLO201, COLO205, COLO320, DLD-1, HCT-15, HCT-116, HT-29, Lovo, LS174T, NCI-H716, SW403, SW1116, and WiDR) were simultaneously unmethylated in both the *MAGE-A1* and *MAGE-A3* genes. With exception, there were two cell lines (SNU-61, COLO201) with negative gene expression for either *MAGE-A1* or *MAGE-A3*, but unmethylated *MAGE-A1* or *MAGE-A3* promoter was detected. On the contrary, there were four cell lines (SNU-283, SNU-407, SNU-1047 and SW480) in which *MAGE* genes were strongly expressed, although no unmethylated *MAGE* promoter could be detected, suggesting the activation or inactivation of *MAGE* expression by another mechanism.

In our study, the rates of hypomethylation of the *MAGE-A1* and *MAGE-A3* genes in colorectal cancer cell lines were 81% in both and those in colorectal cancer tissues were 43% and 77%, respectively. The DNA was extracted from the surgically removed frozen-tissues; however, the tumor tissues might have been contaminated with some normal stromal cells. Therefore, the levels of hypomethylation of *MAGE-A1* and *MAGE- A3* genes in cancer tissues might be affected by the DNA from normal cells. To obtain a better understanding of the promoter hypomethylation status of the *MAGE-A1* and *MAGE-A3* genes in colorectal cancer tissues, expression analysis of *MAGE-A1* and *MAGE-A3* (such as, *in situ* hybridization or immunostaining) and the more precise methylation analysis method (such as, laser capture microdissection techniques) to isolate cancer cells from normal cells need to be performed. The hypomethylation of the *MAGE-A1* and *MAGE-A3* genes in corresponding normal tissues was detected in only 2 and 5 samples (2% and 6%), respectively (Table 2).

To evaluate the association between the clinical parameters and *MAGE* expression, the Pearson χ^2 test was used to evaluate differences in tumor location (proximal or distal) or gender, and significance was determined using 95% confidence intervals. In our study, unmethylated *MAGE-A1* DNA expression was significantly different in respect of tumor location and gender. Unmethylated *MAGE-A1* DNA expression was significantly higher in distal location ($P = 0.028$) and in females ($P = 0.004$). However, unmethylated *MAGE-A3* DNA expression was not significantly associated with tumor location ($P = 0.239$), while it was only related to female gender ($P = 0.021$).

Our results supported the role of the promoter methylation in maintaining a silent phenotype of the *MAGE-A1* and *MAGE-A3* genes, as the *MAGE* gene was re-expressed after treatment with 5-aza-2'-deoxycytidine. This agent reactivates gene expression when methylation of CpG islands is the cause of reduced gene expression. We demonstrated that the *MAGE-A1* and *MAGE-A3* mRNAs were re-expressed after 5-aza-2'-deoxycytidine treatment in all 8 and 9 cell lines that did not express *MAGE-A1* and *MAGE-A3* mRNAs, respectively. However, the SNU-1040 and COLO 205 cell lines did not show re-expression, suggesting the inactivation of *MAGE-A1* expression by another mechanism.

In conclusion, we observed hypomethylation in the promoter region of both the *MAGE-A1* and *MAGE-A3* genes in 23 of 32 colorectal cancer cell lines. This methylation was confirmed by MS-PCR, treatment with 5-aza-2'-deoxycytidine, and bisulfite direct sequencing analysis. Hypomethylation of the promoter region appears to be a frequent phenomenon in human colorectal cancers and upregulates transcription of the *MAGE-A1 and MAGE-A3* genes in colorectal cancer cells. In addition, out of 87 colorectal cancer tissues, we observed hypomethylation in the promoter regions of the *MAGE-A1* and *MAGE-A3* genes in 37 (43%) and 67 (77%) tissues, respectively. This suggests that promoter hypomethylation of *MAGE-A1* and *MAGE-A3* genes up-regulates its expression in colorectal carcinomas as well as in gastric cancers, and might play a significant role in the development and progression of human colorectal carcinomas.

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