MAGE Expressions Mediated by Demethylation of *MAGE* Promoters Induce Progression of Non-small Cell Lung Cancer

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Abstract. *Background: The MAGE gene encodes cancer/testis antigens that are recognized on melanoma cells by autologous cytolytic T lymphocytes. These genes are expressed in various tumor cells, but not in healthy tissues except for the testis and placenta. MAGE expression is known to be activated by promoter demethylation. Materials and Methods: The expression of MAGE-A1 and -A3 and promoter methylation of MAGE-A1 and -A3 was investigated in 67 non-small cell lung cancer (NSCLC) specimens and their correlation with clinicopathological parameters was elucidated. Results: Expression of MAGE-A1 and -A3 was detected in 29.9% and 38.8% of the cases. Demethylation of MAGE-A1 and -A3 was detected in 41.8% and 46.3% of the cases. In 18 (of 20) cases, MAGE-A1 expression showed demethylation of MAGE-A1 and in 24 (of 26) cases MAGE-A3 expression showed demethylation of MAGE-A3. The patients with MAGE expression had a worse prognosis than those with no MAGE expression. Conclusion: MAGE expression mediated by demethylation of MAGE promoters is associated with aggressive progression of NSCLC.*

Cancer/testis antigens (CTAs) were the first human tumor-associated antigens to be molecularly featured (1). This group of antigens comprises products of different gene families expressed, under physiological conditions, predominantly in spermatogonia and in the placenta (2, 3). CTAs have been shown to be expressed in a large variety of tumors of different histological origin (4). Furthermore, since a number of them encompassed both HLA class I and class II restricted epitopes (5), they represent attractive targets for active specific immunotherapy in different areas of clinical oncology (6). CTAs can be classified into several super-families. The melanoma-associated antigen (MAGE) is one of the well-characterized members of the CTA family that includes six subfamilies, namely MAGE-A to -F (7). It is considered that MAGE family members play an important role physiologically and pathologically during embryogenesis, germ-cell development, cellcycle progression, apoptosis and neurogenetic disease (8-10). The *MAGE* genes are silent in normal tissues except for the testis and placenta (8, 11), but they are expressed in many types of cancer (12). Expression of *MAGE-A1* and *-A3* has been reported in human malignancies including melanoma (1), gastric cancer (13), colon cancer (14), hepatocellular carcinoma (15) and non-small cell lung cancer (NSCLC) (16).

Epigenetic alterations, including hypermethylation of promoter CpG islands and histone deacetylation of tumor suppressor and tumor-related genes, as well as global hypomethylation, have been recognized as important contributors to carcinogenesis in humans (17-19). *MAGE* expression is known to be activated by promoter demethylation in a similar manner to the oncogenes and growth-related genes (5, 13, 20). A correlation between *MAGE-A1* and *MAGE-A3* expression and demethylation of *MAGE-A1* and *MAGE-A3* promoter region has been observed in gastric cancer (13) and colorectal cancer (14) cell lines.

The present study investigated the expression status of *MAGE-A1* and *MAGE-A3* by reverse transcriptional-polymerase chain reaction (RT-PCR), the promoter methylation status of *MAGE-A1* and *MAGE-A3* by methylation-specific PCR (MSP) in 67 paired NSCLC and corresponding non-neoplastic lung tissue specimens and analyzed the correlation between the expression and the demethylation of these genes. In addition, the study also elucidated the correlation with clinicopathological parameters, thus including the survival rate in patients with NSCLC, following surgical resection.

Materials and Methods

Study population. A total of 67 pairs of NSCLC and corresponding non-neoplastic lung tissue specimens were collected from the same patients, following surgical resection and histological diagnosis at

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Key Words: MAGE, non-small cell lung cancer NSCLC, expression, demethylation.

the Department of Surgery and Pathology, Yamagata University Faculty of Medicine, Japan. Written informed consent was obtained from all patients. Tissue samples were immediately frozen and stored at –80˚C until analysis. The patients had not undergone any chemo- or radiotherapy prior to surgical resection, thus avoiding up- or down-regulation of the cell-cycle proteins due to DNA damage. The patients included 48 males and 19 females who ranged in age from 39 to 82 years (average, 67.5 years) at the time of diagnosis. TNM staging was based on the TNM classification of the International Union Against Cancer (UICC) (21). Forty-two patients had stage I disease (Ia, 30; Ib, 12), six patients had stage II disease (IIa, 3; IIb, 3) and 19 patients had stage III disease (IIIa, 12; IIIb, 7). The histological classification was based on the World Health Organization classification of tumours (22). The histological subtypes included 37 adenocarcinomas (ACs), and 30 squamous cell carcinomas (SCCs). Most of the ACs were mixed subtypes. Forty-nine patients were smokers (including both current and former smokers), and 18 patients had never smoked. A follow-up evaluation was available for all patients and it ranged from 3.7 months to 76.9 months after surgery (average, 43.2 months).

DNA and RNA extraction. DNA was extracted from 67 primary NSCLC specimens and their corresponding non-neoplastic lung tissues with SepaGene (Sanko-Junyaku, Tokyo, Japan). RNA was extracted from 67 primary NSCLC specimens and their corresponding non-neoplastic lung tissues with GenElute Mammalian Total RNA Miniprep Kit (Sigma-Aldrich, Saint Louis, MO, USA).

RT-PCR. Isolated RNA was reverse-transcribed and amplified using a SuperScript One-Step RT-PCR (Invitrogen, Carlsbad, CA, USA). Primer sequences used were: MG1 forward (5'-TGT GGG CAG GAG CTG GGC AA-3') and MG1 reverse (5'-GCC GAA GGA ACC TGA CCC AG-3') for *MAGE-A1*; MG3 forward (5'-AAG CCG GCC CAG GCT CGG T-3') and MG3 reverse (5'-GCT GGG CAA TGG AGA CCC AC-3') for *MAGE-A3*; *β-actin* forward (5'-AAA TCT GGC ACC ACA CCT T-3') and *β-actin* reverse (5'-AGC ACT GTG TTG GCG TAG AG-3') for *β-actin*. Firstly, RT reaction (50˚C for 30 min and 94˚C for 2 min) was performed, followed by 35 PCR cycles of 94˚C for 30 s, annealing at 54˚C for 60 s and extension at 72˚C for 30 s, followed by a final 7 min extension at 72˚C. RT-PCR products were separated on 3% agarose gel.

MSP. DNA samples were treated with bisulfite to convert all unmethylated cytosines to uracils, while leaving methylated cytosines unaffected. Briefly, 2 μg of genomic DNA was denatured by treatment with NaOH and modified by sodium bisulfite. DNA samples were then purified using a Wizard DNA purification resin (Promega, Madison, WI, USA), treated with NaOH, precipitated with ethanol, and resuspended in 30 μl. Modified DNA was amplified in a total volume of 20 μl using GeneAmp PCR Gold Buffer (PE Applied Biosystems, Foster City, CA, USA) containing 1.0 mM $MgCl_2$, 20 μM of each primer, 0.2 mM dNTPs, and 1 unit of Taq polymerase (AmpliTaq Gold DNA Polymerase; PE Applied Biosystems). In order to examine the demethylation of *MAGE-A1* and *MAGE-A3*, after heating at 94˚C for 10 min, PCR was performed in a thermal cycler (GeneAmp 2400; PE Applied Biosystems) for 35 cycles, each of which consisted of denaturation at 94˚C for 30 s, annealing at 54˚C for 60 s and extension at 72˚C for 60 s, followed by a final 7 min extension at 72˚C. The following primer sets were used: MG1 M forward (5'-ATT TAG GTA GGA TTC GGT TTT C-3') and MG1 M reverse (5'-AAA CTA AAA CGT CTT CCC GCG-3') for the methylated *MAGE-A1* sequence; MG1U forward (5'-ATT TAG GTA GGA TTT GGT TTT T-3') and MG1U reverse (5'-AAA CTA AAA CAT CTT CCC ACA-3') for the unmethylated *MAGE-A1* sequence; MG3 M forward (5'-CGT TTT GAG TAA CGA GCG AC-3') and MG3 M reverse (5'-ACT AAA ACG ACG AAA ATC GAC G-3') for the methylated *MAGE-A3* sequence; MG3 U forward (5'-TGT TTT GAG TAA TGA GTG AT-3') and MG3 U reverse (5'-ACT AAA ACA ACA AAA ATC AAC A-3') for the unmethylated MAGE-A3 sequence. Methylated and unmethylated PCR products of *MAGE-A1* and *-A3* in NSCLC were sequenced. The PCR products were purified using OIA Quick PCR Purification Kit (Qiagen, Tokyo, Japan). The purified PCR products were sequenced with BigDye Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems). Gel electrophoresis, data collection and analysis were carried out with a Genetic Analyser (model 310; PE Appied Biosystems, Foster City, CA, USA).

Statistical analysis. Statistical comparisons were performed using either the χ^2 test or Fisher's exact test, as appropriate. Overall survival was calculated from the date of surgery until death or the date of last follow-up and was analysed using the Kaplan-Meier method. Differences between groups were compared using the log-rank test. The Cox proportional hazard regression model was used for multivariate analysis. The factors were chosen by using a stepwise forward method. All data were analysed with using the StatView software program (Abacus Concepts, Inc., Berkeley, CA, USA). A *p*-value of less than 0.05 was considered to be statistically significant.

Results

Expression status of MAGE-A1 and MAGE-A3. Expression of *MAGE-A1* and *MAGE-A3* was detected in 29.9% (20 out of 67) and 38.8% (26 out of 67) of the NSCLC specimens respectively, while no expression was found in the corresponding non-neoplastic lung tissues. Both *MAGE-A1* and *MAGE-A3* expression was detected in 20.9% (14 out of 67), while one gene (either *MAGE-A1* or *MAGE-A3*) expression was detected in 26.9% (18 out of 67) of the NSCLC specimens; in total, expression of the *MAGE* gene was detected in 47.8% (32 out of 67) of the NSCLC specimens. Neither *MAGE-A1* nor *MAGE-A3* was expressed in 2.2% (35 out of 67) of the NSCLC specimens. These results are shown in Tables I and II and in Figure 1.

Demethylation status of MAGE-A1 and MAGE-A3. Demethylation of *MAGE-A1* and *MAGE-A3* was detected in 41.8% (28 out of 67) and 46.3% (31 out of 67) of the NSCLC samples and in 6% (4 out of 67) and 3% (2 out of 67) of their corresponding non-neoplastic lung tissues, respectively (Table I, Figure 2). Both promoters showed only methylation in 40.3% (27 out of 67) of the NSCLC samples. All samples showed methylation of *MAGE* promoters.

Table I. *Correlation between MAGE expression and demethylation of MAGE promoters in NSCLC.*

	Expression	No expression	*p*-Value
MAGE-A1	(20)	(47)	
Demethylation (28)	18	10	1.8×10^{-7}
Methylation (39)	2	37	
MAGE-A3	(26)	(41)	
Demethylation (31)	24	7	1.8×10^{-9}
Methylation (36)	2	34	

The respective number of specimens is shown in parentheses.

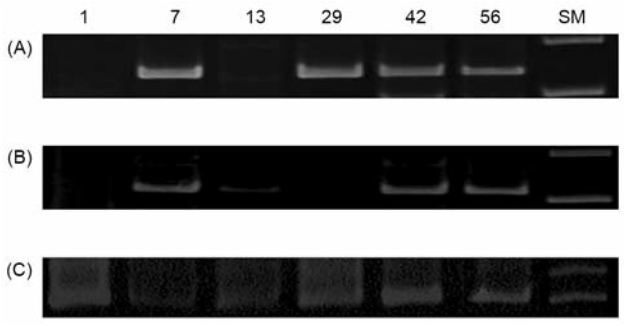

Figure 1. *RT-PCR of primary NSCLC. A: MAGE-A1, B: MAGE-A3 and C: β-actin, used as control. SM, Size marker.*

Correlation between MAGE expression and demethylation of MAGE promoters. A correlation between *MAGE* expression and demethylation of *MAGE* promoters in NSCLC is shown in Table I. A total of 18 out of 20 *MAGE-A1* samples showed demethylation of *MAGE-A1*, and 24 out of 26 MAGE-A3 samples showed demethylation of *MAGE-A3*. Correlation between expression and demethylation of *MAGE-A1,* and of *MAGE-A3* was statistically significant ($p<0.001$).

Correlation between MAGE expression and clinicopathological parameters. Correlation between *MAGE* expression and clinicopathological parameters are shown in Table II. Both *MAGE-A1* and *MAGE-A3* expression was frequently found in SCCs ($p=0.003$) and in advanced stage disease ($p=0.035$). The patients with *MAGE* expression had a worse prognosis than the patients with no expression (no expression *vs*. expression: $p=0.048$; no expression *vs*. of both expression: $p=0.047$) (Table III) (Figure 3). No statistically significant differences were found by multivariate analysis.

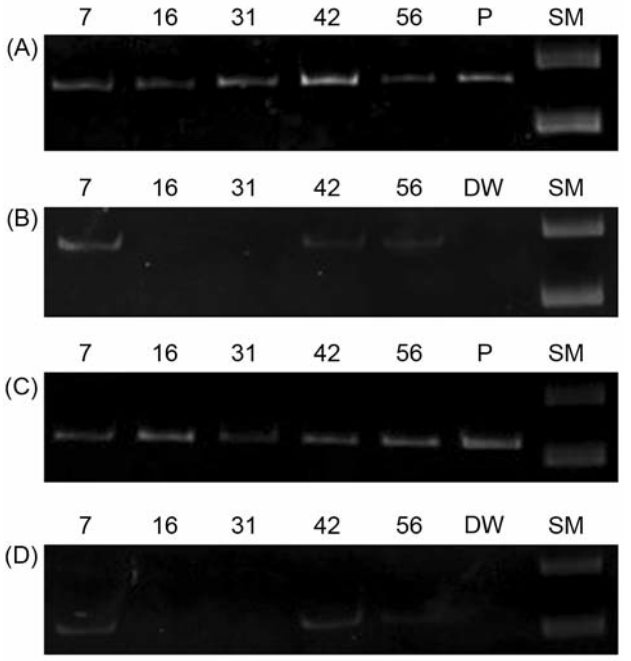

Figure 2. *Methylation-specific PCR of primary NSCLC. A: Methylated MAGE-A1; B: unmethylated MAGE-A1; C: methylated MAGE-A3 and D: unmethylated MAGE-A3. P, Positive control; DW, distilled water; SM, size marker. Methylated MAGE-A1 and -A3 is present in all lanes (A and C).*

Discussion

MAGE-A1 and *MAGE-A3* encode CTAs that are recognized on melanoma cells by autologous cytolytic T lymphocytes (1, 23). The frequency of *MAGE* expression is reported mostly as 30-50% in lung cancer (24-26). The results of the present study were in agreement with previous reports. Strong correlation between *MAGE-A1* or *MAGE-A3* expression and demethylation of *MAGE-A1* or *MAGE-A3* was found, suggesting that *MAGE-A* and *MAGE-A3* expression are mediated by demethylation of *MAGE-A1* and *MAGE-A3* in NSCLC similarly to other types of cancer. Although specific immunotherapy is currently performed for tumors having CTAs, this result suggests that there is also the possibility of epigenetic therapy. *MAGE* expression was strongly associated with advanced stage cancer and overall survival. The expression of CTAs has also been reported to be associated with progression of lung cancer, particularly with a poor prognosis (7, 16, 27, 28). The present results and previous reports support the theory that MAGE family members play a critical role during cell-cycle progression and apoptosis, and *MAGE* alterations drive aggressive progression in NSCLC. However, in this study, a few samples were discordant between expression and demethylation. Kim *et al*. showed that expression of MAGE and demethylation of *MAGE* promoter do not always correspond in colorectal cancer cell lines (14); *MAGE-A1* and *MAGE-A3* were expressed in 59% and 66%,

Table II. *Correlation between MAGE expression and clinicopathological parameters.*

	Frequency of expression			*p*-Value	
	No expression (n=35)	One (n=18)	Both (n=14)	No expression *vs.* expression	No expression *vs.* of both expression
Age (years)	66.5	69.2	67.8	0.63	0.74
Gender					
Female (n=19)	9	7	3	0.62	0.75
Male (n=48)	26	11	11		
Smoking history					
No (n=18)	9	5	4	0.82	0.84
Yes (n=49)	26	13	10		
Histology					
AC (n=37)	25	9	3	0.005	0.003
SCC (n=30)	10	9	11		
TNM stage					
I (n=42)	24	13	5	0.30	0.035
II/III (n=25)	11	5	9		

AC: Adenocarcinoma; SCC: squamous cell carcinoma.

Table III. *Univariate and multivariate overall survival analyses.*

Variable	Log-rank *p*-value	Cox regression	
		Odds ratio (95% CI)	*p*-Value
Age (years) (<70 *vs.* >=70)	0.78	1.24 (0.71-1.30)	0.82
Gender (male *vs.* female)	0.91	1.13 (0.82-1.24)	0.73
Smoking history (no *vs.* yes)	0.97	1.17 (0.90-1.15)	0.80
Histology (AC *vs.* SCC)	0.19	1.43 (0.62-2.34)	0.26
TNM stage (I *vs.* II/III)	0.016	1.76 (0.22-14.08)	0.082
MAGE expression			
No expression *vs.* expression	0.048	2.02 (0.88-4.69)	0.095
No expression *vs.* of both expression	0.047	2.45 (0.92-5.31)	0.091

AC: Adenocarcinoma; SCC: squamous cell carcinoma; 95% CI: 95% confidence interval.

respectively, and demethylation of the *MAGE-A1* and *MAGE-A3* promoter region were recognized in 81% in colorectal cancer cell lines (14). For these reason, it is speculated that other trigger mechanisms may also affect the expression of *MAGE*. Wischnewski *et al.* suggested that not only hypermethylation but also histone deacetylation are responsible for the mechanism underlying *MAGE* gene silencing in human cancer cells (29). However, although histone acetylation was not examined in the present study, it will be needed in the near future.

In conclusion, *MAGE* expression was mediated by demethylation of *MAGE* promoters and was associated with aggressive progression of NSCLC.

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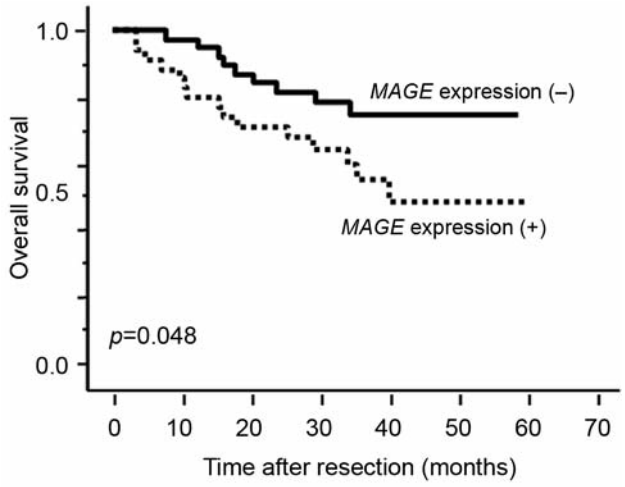

Figure 3. *Correlation between MAGE expression and overall survival of 67 NSCLC patients using the Kaplan-Meier method. The patients with any expression had poorer prognosis than those with no expression.*

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Received November 7, 2010
Revised November 26, 2010
Accepted November 29, 2010