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Expression of *MAGE-B* Genes in Esophageal Squamous Cell Carcinoma

Hideki Nagashima,[1] Noriaki Sadanaga,[1] Kohjiro Mashino,[1] Keishi Yamashita,[1] Hiroshi Inoue,[1] Masaki Mori[1,3] and Keizo Sugimachi[2]

[1]Department of Surgery, Medical Institute of Bioregulation, Kyushu University, 4546 Tsurumihara, Beppu 874-0838 and [2]Department of Surgery and Science, Graduate School of Medical Sciences, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582

The *MAGE-B* (*MAGE-B1*, *-B2*, *-B3*, and *-B4*) genes share strong homology with the *MAGE-A* gene family. MAGE-B1 and -B2 encode common tumor-specific peptide antigens. There is, however, still very little information about the expression of these genes in human gastro-intestinal carcinomas. We investigated the expression of *MAGE-B1* and *-B2* genes in 29 cell lines and 53 clinical tumor samples of esophageal squamous cell carcinoma by reverse transcription polymerase chain reaction (RT-PCR). *MAGE-B1* and *-B2* gene transcripts were detected by RT-PCR in 1 (3%) and 6 (21%) cell lines, and in 9 (17%) and 17 (32%) clinical samples, respectively. Among them, 7/29 (24%) cell lines and 19/53 (36%) clinical samples expressed at least either MAGE-B1 or -B2. A significant correlation was found between negative *MAGE-B* gene expression and vascular invasion (P=0.008). In 45 out of 53 esophageal carcinoma RNA samples, the *MAGE-A1*, *-A2*, and *-A3* genes were detected in 27 (60%), 23 (51%), and 30 (67%) samples, respectively, while the *MAGE-B* genes were detected in 18 (40%) samples. The frequency of *MAGE-B* gene expression in esophageal carcinoma was relatively higher than that observed for gastric or colorectal carcinomas (12% and 2%, respectively). Therefore, the *MAGE-B* genes could be used as targets in specific immunotherapy of esophageal squamous cell carcinomas.

Key words: MAGE-B — Esophageal carcinoma — Antigenic peptide — Specific immunotherapy

The gene *MAGE-A1* was identified by analyzing a cytotoxic T lymphocyte clone which demonstrated specificity for the autologous melanoma cell line MZ2-MEL.[1)] When a MAGE-A1 fragment was used as the probe on Southern blots, it revealed twelve hybridizing bands of different intensities. These proteins constitute the *MAGE-A* gene family and are located in Xq27-qter.[2)]

A key finding regarding the expression of the *MAGE-A* genes is that their expression was limited to tumor tissue and has not been observed in any normal tissue with the exception of testis and placenta.[2, 3)] *MAGE-A* genes are expressed not only in melanoma, but also in some other tumors, such as lung carcinoma,[4)] breast carcinoma,[5)] neuroectodermal tumors,[6)] lymphocytic leukemia,[7)] and bladder carcinomas.[8)] As we reported earlier, these genes are also expressed in gastric carcinoma,[9)] colorectal carcinomas,[10)] hepatocellular carcinoma (HCC)[11)] and esophageal carcinomas.[12)]

The *MAGE-Xp* gene, which has strong homology to the *MAGE-A* gene family, was isolated using exon-trapping of cosmids through the Xp21.3 region. MAGE-Xp transcripts were identified in testis, but were not found in any of 12 different tumor tissues tested, which included seven melanoma and four lung tumor samples.[13)] Dabovic *et al.* also identified the *MAGE-Xp* gene and named it *DAM10*. They also reported another homologous gene nearby, which they named *DAM6*. Moreover, they showed that DAM10 and DAM6 are expressed not only in adult testis, but also in certain lung tumors.[14)] In a later study, it was revealed that the complete sequence of a 42-kb stretch in the region of Xp21.3 contained four MAGE-related genes, which were named *MAGE-B1*(*DAM10/MAGE-Xp*), *-B2*(*DAM6*), *-B3*, and *-B4*. The *MAGE-B* genes were silent in normal tissues with the exception of testis. The *MAGE-B1* and *MAGE-B2* genes were expressed in a significant fraction of tumors of various histological types, such as melanoma, non-small cell lung carcinoma, sarcoma, and mammary carcinoma.[15)]

Several *MAGE-A* genes have become candidates for tumor-specific immunotherapy, because antigenic peptides have been identified as being encoded in their genes. For example, the MAGE-A1-encoded peptide is presented by major histocompatibility molecules HLA-A1[16)] and -A24,[17)] forming a tumor antigen. Similarly, the *MAGE-A3* gene encodes a tumor-specific antigen presented by HLA-A1,[3)] -A2,[18)] and -A24.[19)] Some of these peptides are already being applied in tumor-specific immunotherapy.

It has been shown that peptide D10/6-271, derived from codons 271–279 of MAGE-B1 (DAM10) and MAGE-B2 (DAM6), is recognized by HLA-A2-restricted cytotoxic T lymphocytes (CTL).[20)] Therefore, it has been suggested that the *MAGE-B* genes might encode a new group of tumor-specific antigens that could be included in the list of possible target antigens for specific immunotherapy of

[3] To whom correspondence should be addressed.
E-mail: mmori@tsurumi.beppu.kyushu-u.ac.jp

neoplastic disorders. In the present study, we investigated the expression of *MAGE-B* genes in gastro-intestinal carcinomas, and we suggest that the *MAGE-B* genes may indeed present possible targets for tumor specific immunotherapy for patients with esophageal squamous cell carcinoma.

MATERIALS AND METHODS

Cell lines The cell lines derived from esophageal carcinoma, TE-1, -2, -3, -4, -5, -6, -7, -8, -9, -10, -11, -12, -13, -14, and -15, were kindly provided by the Institute of Development, Aging and Cancer, Tohoku University (Dr. T. Nishihara and Dr. T. Kudo), Sendai. The cell lines derived from esophageal carcinoma, KYSE-30, -50, -70, -110, -140, -150, -170, -180, -190, -200, -220, -270, -410, and -510 were kindly provided by Dr. Shimada, Kyoto University, Kyoto. The melanoma cell line MZ-2-MEL (MZ-2) was kindly provided by Dr. F. Brasseur, Ludwig Institute for Cancer Research, Brussels, Belgium. All cell lines were maintained in an RPMI 1640 medium containing 10% fetal bovine serum (FBS) and antibiotics.

Treatment of cells with 5-aza-2′-deoxycytidine MZ-2 was maintained in an RPMI medium in a 5% CO_2 incubator at 37°C, and 1 μM 5-aza-2′-deoxycytidine (DAC) (Sigma Chemical Corp., St. Louis, MO) was added for 48 h. The cells were harvested and washed with phosphate-buffered saline prior to RNA extraction.

Tissue samples The tumor samples and the matched control samples taken from normal tissue located far from the tumor site of esophageal, gastric, and colorectal carcinomas were frozen in liquid nitrogen immediately after surgical resection, and kept at −90°C until RNA extraction. The surgical samples were obtained at the Department of Surgery, Medical Institute of Bioregulation, Kyushu University, Beppu, and the Department of Surgery, Saitama Cancer Center, Saitama. Histological verification showed that all of the esophageal carcinomas studied were squamous cell carcinomas, and all of the gastric and colorectal carcinomas were adenocarcinomas.

RNA preparation and reverse transcription The total RNA isolated by the acid guanidinium-phenol-chloroform (AGPC) procedure was treated with DNase.[21] cDNA was synthesized from 2.5 μg of total RNA as described previously.[11]

Oligonucleotide primers of *MAGE-A* and *MAGE-B* genes The presence of MAGE-B1, -B2, -A1, -A2, and -A3 cDNA in the reverse transcription products was detected by polymerase chain reaction (PCR) amplification in a separate reaction, using oligonucleotide primers located at different exons in the *MAGE* genes. The primer sequences were: MAGE-B1, 5′-CCCGAGCGAGCTTAAGGAGT-3′ and 5′-GTCAGATTCGGTACATGACACAG-3′[15]; MAGE-B2, 5′-AGCGAGTGTAGGGGGTGCG-3′ and 5′-TGAGGCCCTCAGAGGCTTTC-3′[15]; MAGE-A1, 5′-CGGCCGAAGGAACCTGACCCAG-3′ and 5′-GCTGGAACCCTCACTGGGTTGCC-3′[5]; MAGE-A2, 5′-AAGTAGGACCCGAGGCACTG-3′ and 5′-GAAGAGGAAGAAGCGGTCTG-3′[22]; MAGE-A3, 5′-TGGAGGACCAGAGGCCCCC-3′ and 5′-GGACGATTATCAGGAGGCCTGC-3′[2].

The semiquantitative detection of mRNA In order to evaluate the amplified product quantitatively by PCR, preliminary experiments were carried out to determine a suitable number of cycles in the linear range of PCR amplification in representative cases (Fig. 1). Suitable numbers of PCR cycles for MAGE-A1, -A2, -A3, -B1, and -B2 were 33, 33, 33, 40, and 30, respectively.

PCR The amplification was performed for 30 cycles

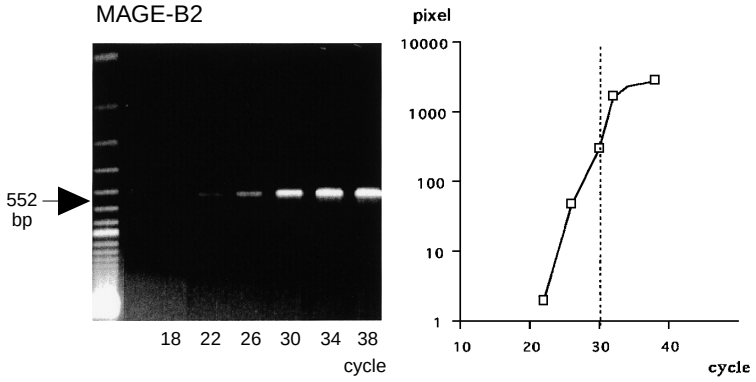

Fig. 1. The relationship between the number of polymerase chain reaction (PCR) cycles and the amount of PCR product of MAGE-B2. PCR products from each cycle were stained with ethidium bromide, imaged and quantified with NIH Image (version 1.57).[35] The PCR product increased linearly around the 30th cycle. A suitable number of cycles for the amplification of MAGE-B2 was therefore chosen as 30.

Table I. Clinicopathological Data and Expression of *MAGE-A* Genes with or without Expression of *MAGE-B* Genes in Esophageal Carcinoma

		Expression of *MAGE-B1* and/or *-B2*		*P* value
		Positive (n=19)	Negative (n=34)	
Histological type	well	6	8	NS
	moderately	9	18	
	poorly	3	6	
	others	1	2	
Depth of invasion	T1	4	2	NS
	T2	2	5	
	T3	11	25	
	T4	2	2	
Lymph node metastasis	N0	5	5	NS
	N1	14	29	
Lymphatic invasion	negative	5	4	NS
	positive	14	30	
Vascular invasion	negative	10	6	P=0.008
	positive	9	28	
Stage	I	1	3	NS
	IIA	2	2	
	IIB	1	2	
	III	15	27	
Expression of *MAGE-A* genes (*MAGE-A1*, *-A2* and/or *-A3*)	positive	16	22	NS
	negative	2	5	
	unknown	1	7	

NS: not significant.

(MAGE-B2) or 33 cycles (MAGE-A1, -A2, -A3) or 40 cycles (MAGE-B1) of 1 min at 94°C, 2 min at 68°C, 2 min at 72°C for MAGE-B1 and -B2; 30 s at 94°C, 40 s at 72°C for MAGE-A1 and -A3; and 30 s at 94°C, 30 s at 68°C, 20 s at 72°C for MAGE-A2. An 8 μl aliquot of each reaction mixture was size-fractionated in a 1.5% agarose gel and visualized by ethidium bromide staining. To ensure that the RNA was not degraded, a PCR assay with primers specific for the glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) gene was carried out in each case, except that only 24 cycles were performed under the following cycling conditions: 1 min at 94°C, 2 min at 56°C, and 2 min at 72°C.[23] The primer sequences for the GAPDH amplification were: 5′-GTCAACGGATTTGGTCGTATT-3′ and 5′-AGTCTTCTGGGTGGCAGTGAT-3′.

Clinicopathological data All data, including sex, age, stage of the disease, and pathologic factors, were obtained from the clinical and pathologic records. The TNM classification was used to determine the stage of the disease.[24] The tumors, either expressing or not expressing *MAGE-B* genes, were then compared and their properties are listed in Table I.

Statistical analysis Statistical analysis was performed using either the χ^2 method or Fisher's exact test. The level of significance was set at $P<0.05$.

RESULTS

Expression of *MAGE-B* genes in cell lines and esophageal carcinomas The level of transcription of *MAGE-B* genes measured by semi-quantitative reverse transcription (RT)-PCR assays showed various patterns from high to low levels of expression in tumor samples. The intensity of a band in agarose gel was evaluated by comparison with the positive control and negative control, and if a band was recognized, the case was designated as positive. Among the 29 esophageal cancer cell lines, *MAGE-B1* and *-B2* gene expression was detected by RT-PCR in 1 (3%) and 6 (21%) cell lines, respectively. Thus, 7 (24%) out of 29 esophageal cancer cell lines expressed at least either MAGE-B1 or -B2. Among the 53 esophageal carcinoma samples, *MAGE-B1* and/or *-B2* gene expression was detected by RT-PCR in 9 (17%) and 17 (32%) clinical samples, respectively. Overall, 19 out of 53 (36%) tumors expressed at least either MAGE-B1 or -B2. No expression of these genes was found in any of the matched control samples consisting of normal tissue (Fig. 2).

Clinicopathological data and expression of MAGE-B Several pathologic factors were compared between cases of esophageal carcinoma with or without *MAGE-B* gene expression. A significant correlation was observed

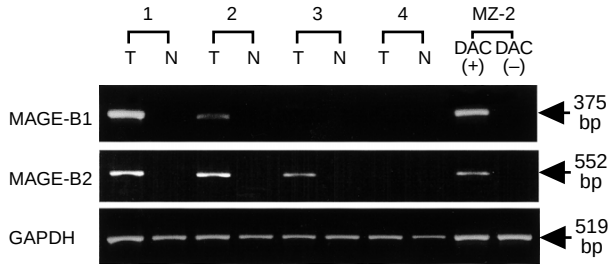

Fig. 2. Expression of *MAGE-B* genes shown by ethidium bromide staining of PCR products. T, tumor tissue; N, normal tissue. MZ-2-DAC(+), positive control; MZ-2-DAC(−), negative control.[15] Cases 1 and 2 express both *MAGE-B1* and *-B2* genes. Case 3 expresses only *MAGE-B2* gene. Case 4 expresses neither of the *MAGE-B* genes.

between negative MAGE-B expression and vascular invasion (P=0.008) (Table I).

Comparison of MAGE-B expression with the expression of MAGE-A1, -A2, and -A3 In 45 out of 53 esophageal carcinoma samples, the *MAGE-A1*, *-A2*, and *-A3* gene expression was investigated by RT-PCR and compared with *MAGE-B* gene expression. *MAGE-A1*, *-A2*, and *-A3* genes were expressed in 27/45 (60%), 23/45 (51%), and 30/45 (67%) samples, respectively, while *MAGE-B* genes were expressed in 18/45 (40%). Among the cases in which neither MAGE-A1, -A2, nor -A3 was detected (MAGE-A-negative cases), 2 cases (4%) showed MAGE-B-positive expression. When MAGE-A-positive cases were defined as the cases expressing at least one of MAGE-A1, -A2, and -A3, there was no significant correlation between MAGE-B expression and MAGE-A expression (Table I).

Expression of *MAGE-B* genes in gastric or colorectal carcinomas Out of 57 gastric carcinoma samples, *MAGE-B1* and/or *-B2* gene expression was detected by RT-PCR in 6 (11%) and 2 (3%) clinical samples, respectively. Overall, 7 (12%) of the 57 gastric carcinoma samples expressed at least either MAGE-B1 or -B2. On the other hand in the 49 colorectal carcinoma samples, *MAGE-B1* and/or *-B2* gene expression was detected by RT-PCR in 0 (0%) and 1 (2%) of the clinical samples, respectively. Overall, only 2 out of 49 (2%) samples expressed at least either MAGE-B1 or -B2 (Table II).

Table II. Expression of *MAGE-B* Genes in Gastric or Colorectal Carcinomas

	Positive expression (%)	
	Gastric carcinoma	Colorectal carcinoma
MAGE-B1	6/57 (11)	0/49 (0)
MAGE-B2	2/57 (3)	1/49 (2)
MAGE-B1 and/or *-B2*	7/57 (12)	1/49 (2)

DISCUSSION

The *MAGE-B* genes (*MAGE-B1* and *-B2*), as well as *MAGE-A* genes, have been shown to be expressed not only in melanoma, but also in other tumors.[15] However, there is still little information on the expression of *MAGE-B* genes in gastro-intestinal carcinomas. It has been reported that the frequency of the *MAGE-A* (especially *MAGE-A1*, *-A2*, and *-A3*) gene expression in gastric or colorectal carcinomas was approximately 40% or 30%, respectively.[10, 25] As shown in our previous report, we found that the *MAGE-A* genes were expressed in a high percentage of esophageal carcinomas (approximately 60%).[12, 26] Our present study demonstrated that the frequency of *MAGE-B* gene expression (36%) in esophageal carcinomas might be slightly lower than that of the *MAGE-A* genes, but relatively higher than in gastric or colorectal carcinomas (12% or 2%, respectively). Although the difference between squamous cell carcinoma and adenocarcinoma is interesting, no relationship between *MAGE-B* gene expression and the histology was found in another study.[15] However, the sample number was small in that study, so a further study should be performed to clarify the expression of *MAGE-B* genes in other carcinomas (squamous cell carcinoma and adenocarcinoma).

Several antigenic MAGE peptides have been identified, and tumor-specific immunotherapies using them have been started. An HLA-A1-restricted MAGE-A3 peptide has been injected as a vaccine into patients with far advanced melanoma, with[27] or without[28] dendritic cells (DCs). We have recently started tumor-specific immunotherapy for patients with far advanced gastro-intestinal carcinoma using HLA-restricted MAGE peptides. This therapy, however, has several problems. One is that candidates for this therapy are limited to patients who have both the matched HLA-allele and the MAGE-positive tumor. In addition, the heterogeneous expression of MAGE protein should also be considered when choosing antigen-specific immunotherapy.[29] This means that it will be necessary to study the expression of new tumor-specific antigens in order to expand the number of candidates. This was the reason why we studied the expression of *MAGE-B* genes in gastro-intestinal carcinomas. We found that MAGE-B was expressed at relatively high frequency, so much so that it may provide a new target for immunotherapy of esophageal carcinoma. Moreover, 2/45 investigated cases of esophageal carcinoma were MAGE-A1, -A2, and -A3-negative but MAGE-B-positive in this study. Thus, the newly identified HLA-A2-restricted MAGE-B peptide

(DAM10/6-271)[20] may prove valuable for the tumor-specific immunotherapy of esophageal carcinoma. However, the present study by semi-quantitative RT-PCR also suggested that heterogeneous expression of MAGE-B antigen may exist, and we should consider the amount of the antigen expression in relation to clinical application of cancer immunotherapy. Another possibility to expand the number of candidates for immunotherapy with the MAGE-B peptide is to induce *MAGE-B* gene expression in MAGE-B-negative tumors using a demethylation agent, such as DAC, as shown in Fig. 2.

In some reports, it has been shown that high *MAGE-A* gene expression correlates with disease progression in cases of melanoma,[30] gastric carcinoma,[31] breast carcinoma,[32] and bladder tumors.[8] In previous studies we demonstrated that *MAGE-A1*, *-A2*, and *-A3* gene expression was frequent in patients with liver metastasis of colorectal carcinoma,[10] and that significant correlations existed between MAGE-A expression status and some clinicopathologic factors in HCC.[11] However, there were no such correlations with regard to esophageal carcinoma.[12] Our present study discloses that MAGE-B-negative status is frequently correlated with pathologic vascular invasion by esophageal carcinoma. Correlations between pathologic vascular invasion and prognosis for patients with esophageal carcinoma have been reported before. For example, high VEGF levels[33] and a high tumor/normal (T/N) ratio of ornithine decarboxylase mRNA expression[34] in esophageal carcinoma were significantly associated with some clinicopathological factors, including venous invasion, and resulted in a poorer prognosis. Although our results seem to suggest that patients with MAGE-B-positive esophageal carcinoma will have a better prognosis, a larger number of cases still needs to be studied to confirm such a conclusion.

In summary, a relatively high incidence of *MAGE-B* gene expression was observed in clinical samples of human esophageal carcinoma, in contrast to gastric or colorectal carcinoma. We, therefore, suggest that analyzing the expression of *MAGE-B* genes is important if patients with esophageal carcinoma are to be treated with antigenic peptides, and this will result in widening the range of candidates for tumor-specific immunotherapy.

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