Expression of MAGE-B Genes in Esophageal Squamous Cell Carcinoma

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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) MAGE-A genes are expressed not only in melanoma, but also in some other tumors, such as lung carcinoma, breast carcinoma, neuroectodermal tumors, lymphocytic leukemia, and bladder carcinomas. As we reported earlier, these genes are also expressed in gastric carcinoma, colorectal carcinomas, 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 and -A2, forming a tumor antigen. Similarly, the MAGE-A3 gene encodes a tumor-specific antigen presented by HLA-A1, -A2, and -A2.2) 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). 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...
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. 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% CO2 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. 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. cDNA was synthesized from 2.5 µg of total RNA as described previously.

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′-GTCAAGATTCCGTTACATGACACAG-3′; MAGE-B2, 5′-AGCGAGTGTAGGGGGTGCG-3′ and 5′-TGAGGCCCTCAGAGGCTTTC-3′; MAGE-A1, 5′-CGGCCGAAAGGAACCTGACCCAG-3′ and 5′-GCTTGGAACCTCGTGGGTGACC-3′; MAGE-A2, 5′-AAGTAGGACC-GAGGCTG-3′ and 5′-GAAGAGGAAGAAGCGGTC-3′; MAGE-A3, 5′-TGGAGGACCAGAGGCCC-3′ and 5′-GGACGATTATACGGAGGCCC-3′.

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.
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 | Positive (n=19) | Negative (n=34) | P value |
|----------------------------------|----------------|----------------|--------|
| 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.

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, 19 out of 29 (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...
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).

**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. 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. As shown in our previous report, we found that the MAGE-A genes were expressed in a high percentage of esophageal carcinomas (approximately 60%). 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. 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 or without 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. 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...
MAGE-B Expression in Esophageal Carcinoma

(DAM10/6-271) 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, gastric carcinoma, breast carcinoma, and bladder tumors. In previous studies we demonstrated that MAGE-A1, -A2, and -A3 gene expression was frequent in patients with liver metastasis of colorectal carcinoma, and that significant correlations existed between MAGE-A expression status and some clinicopathologic factors in HCC. However, there were no such correlations with regard to esophageal carcinoma. Our present study discloses that MAGE-B-negative status is not always associated with poor prognosis for patients with esophageal carcinoma have been reported before. For example, high VEGF levels and a high tumor/normal (T/N) ratio of ornithine decarboxylase mRNA expression in esophageal carcinoma were significantly associated with some clinicopathologic 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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