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# Expression of MAGE-1 Gene by Esophageal Carcinomas

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**Expression of the MAGE genes encoding tumor-rejection antigens on HLA-A1 and -Cw1601 recognized by cytotoxic T lymphocytes was investigated in esophageal carcinomas at the mRNA level by the semiquantitative reverse transcription-polymerase chain reaction method. MAGE-1 and -2 genes, but not MAGE-3, -3/-6 and -4a/-4b genes, were expressed in substantial proportions of the primary esophageal carcinomas and their metastatic lymph nodes. The proportion of MAGE-positive samples in the primary esophageal carcinomas correlated with the T factor of the TNM classification (pT1: 2 of 12 tumors, pT2: 1 of 6, pT3: 12 of 29, and pT4: 7 of 18). These results have important implications for specific immunotherapy of esophageal carcinomas using MAGE-1 gene product.**

Key words: MAGE-1 gene — Esophageal carcinoma — Specific immunotherapy

Esophageal carcinoma is an extremely lethal malignant tumor.[1,2] It occurs frequently within the so-called Asian esophageal cancer belt extending from the southern shore of the Caspian Sea in the west to China and Japan in the east. The five-year survival rate of patients with esophageal carcinoma who received curative operation ranged from 26 to 39% in Japan.[2] The prognosis of patients with advanced stages of esophageal carcinoma is poor, although recent therapeutic efforts have been directed at utilizing combined chemotherapy and radiotherapy before or after surgical excision.[1,2] Therefore, the specific immunotherapy of esophageal carcinoma is of great interest.

MAGE-1 or -3 gene codes for tumor antigens on HLA-A1 and -Cw1601 or -A1 and -A2 recognized by cytotoxic T lymphocytes, respectively.[3–6] Nomenclature of MAGE genes follows the recommendation by Gaugler *et al.*[4] The other MAGE genes (MAGE-4a, -4b and -6), with the exception of the MAGE-2 and -12 genes, also encode a potential tumor antigen on HLA-A1.[4–7] A family of 12 closely related genes (the MAGE gene family) is located on chromosome X.[7] Among them, the MAGE-1, -2, -3, -4a, -6, and -12 genes are preferentially expressed at the mRNA level in many different cancers, including melanomas, head and neck cancers, lung cancers and breast cancers.[3–10] In contrast, neither normal cells nor normal tissues other than testis and placenta express the MAGE gene. These results suggest that the MAGE gene products are appropriate target molecules for specific immunotherapy.

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In the present study, we investigated the expression of the MAGE-1, -2, -3, -3/-6, and -4a/-4b genes at the mRNA level in esophageal carcinomas using 65 primary thoracic esophageal carcinomas, 11 metastatic lymph nodes from 11 patients with esophageal carcinoma, and 5 non-tumorous esophageal tissues. This is the first report on the expression of the MAGE genes in esophageal cancers using relatively large numbers of samples. Samples were obtained at the time of surgery in our hospital from 1991 to 1994, and were cryopreserved at −130°C until use. The proportion of stromal or noncancerous cells in the primary tumors or metastatic lymph nodes used for the study was approximately 30 to 60% or 10 to 30%, respectively, as judged from pathological examinations. These 65 primary esophageal carcinomas were classified according to the UICC-TNM classification[11] into 12 pT1 (tumor invades lamina propria or submucosa), 6 pT2 (muscularis propria), 29 pT3 (adventitia) and 18 pT4 (adjacent structures) tumors. Sixty-five primary tumors were divided into 20 tumors without regional lymph node metastasis (pN0) and 45 tumors with regional lymph node metastasis (pN1), and also into 47 tumors without distant metastasis (pM0) and 18 tumors with distant metastasis to lymph nodes (pM1-LYM).

MAGE-1, -2, -3, -3/-6, and -4a/-4b genes were respectively expressed in 12 (18%), 17 (26%), 1 (2%), 3 (5%) and 5 (8%) of the 65 primary esophageal carcinomas, and 5 (45%), 2 (18%), 0, 0 and 0 of the 11 metastatic lymph nodes as evaluated by the reverse-transcription-polymerase chain reaction (RT-PCR) method (Table I). Representative results are shown in Fig. 1. These genes were respectively expressed in 6, 2, 3, 7 and 4 of the 9 esophageal tumor cell lines (KE3, KE3T, KE4, TE8,

Table I. Expression of MAGE Gene Family by Esophageal Carcinomas at the mRNA Level

| Esophageal samples | Proportion of positive samples | | | | |
|---|---|---|---|---|---|
| | MAGE-1 | MAGE-2 | MAGE-3 | MAGE-3/-6 | MAGE-4a/-4b |
| Primary tumors | 12/65 | 17/65 | 1/65 | 3/65 | 5/65 |
| Metastatic lymph nodes | 5/11 | 2/11 | 0/11 | 0/11 | 0/11 |
| Tumor cell lines | 6/9 | 2/9 | 3/9 | 7/9 | 4/9 |
| Non-tumorous tissues | 0/5 | 0/5 | 0/5 | 0/5 | 0/5 |
| Staging of primary tumors | | | | | |
| Tumor size (T) pT1 | 0/12 | 2/12 | 0/12 | 0/12 | 0/12 |
| pT2 | 0/6 | 1/6 | 0/6 | 0/6 | 0/6 |
| pT3 | 8/29 | 9/29 | 1/29 | 2/29 | 3/29 |
| pT4 | 4/18 | 5/18 | 0/18 | 1/18 | 2/18 |
| Lymph node involvement (N) | | | | | |
| pN0 | 5/20 | 4/20 | 1/20 | 1/20 | 3/20 |
| pN1 | 7/45 | 13/45 | 0/45 | 2/45 | 2/45 |
| Distant metastasis (M) | | | | | |
| pM0 | 9/47 | 14/47 | 1/47 | 2/47 | 4/47 |
| pM1-LYM | 3/18 | 3/18 | 0/18 | 1/18 | 1/18 |

TE9, TE10, TE11, YES1 and YES2). At least one of the MAGE genes was expressed in 22 (34%) of the 65 primary esophageal carcinomas, 6 (55%) of the 11 metastatic lymph nodes, and 7 (78%) of the 9 cell lines.

None of these genes was expressed in any of the 5 non-tumorous esophageal tissues. In contrast, the tumor counterparts from 2 of the 5 normal tissues expressed MAGE-1 gene.

MAGE-1 gene was expressed in 12 (18%) of the 65 primary esophageal carcinomas, and in 5 (45%) of the 11 metastatic lymph nodes. Five MAGE-1-positive metastatic lymph nodes were derived from 4 patients whose primary tumors did not express the MAGE-1 gene and one patient whose primary tumor did express it. Six MAGE-1-negative metastatic lymph nodes were obtained from 5 patients whose primary tumors did not express the MAGE-1 gene and one patient whose tumor did. These results suggest that the MAGE-1 gene became positive in metastatic lymph nodes in some patients whose primary esophageal carcinomas did not express it. The expression of MAGE-1 gene in the other carcinomas was 20% in breast cancers, 23% in laryngeal cancers, 33% in sarcomas, 35% in non-small lung cancers and 46% in metastatic melanomas.[4-10]

MAGE-2 gene was expressed in 17 (26%) of the 65 primary esophageal carcinomas and only 2 (18%) of the 11 metastatic lymph nodes. MAGE-2 gene was expressed in 9% of breast cancers, 33% of sarcomas, 46% of laryngeal cancers, and 47% of non-small lung cancers.[7, 12] In contrast, the proportion of samples positive for any of the MAGE-3, -3/-6 or -4a/-4b gene was below 10% in the primary esophageal carcinomas and undetectable in the metastatic lymph nodes. MAGE-3 gene encoding tumor-rejection antigens on HLA-A1 and -A2 was expressed in substantial proportions of many cancers except renal cell carcinomas.[4] MAGE-4a[7] and -4b genes[10] encoding a potential tumor antigen were also expressed in many different cancers. The reasons for the low or absent expression of the MAGE-3, -6, -4a, or -4b gene in esophageal carcinomas are unknown.

The correlation between the pTNM classification of the primary esophageal carcinomas and the proportion of MAGE gene expression was investigated (Table I). All MAGE genes except MAGE-2 were expressed in only pT3 and pT4 tumors. MAGE-2 gene was also preferentially expressed in pT3 and pT4 tumors. Proportions of tumors positive for at least one MAGE gene were 2 (17%) of 12 pT1 tumors, 1 (17%) of 6 pT2 tumors, 12 (41%) of 29 pT3 tumors and 7 (39%) of 18 pT4 tumors. These results indicated that the proportion of MAGE-positive samples in the primary esophageal carcinomas correlated with the T factor of the pTNM classification.

Proportions of tumors positive for at least one MAGE gene were 6 (30%) of 20 pN0 tumors and 16 (36%) of 45 pN1 tumors, indicating that the N factor of the pTNM classification is not highly related to the expression of MAGE genes in primary esophageal carcinomas. Proportions of tumors positive for at least one MAGE gene were 18 (38%) of 47 at the pM0 stage and 4 (22%) of 18 at the pM1-LYM stage, indicating that the primary tumors at the pM1-LYM stage showed less expression of MAGE genes. The mechanism of this phenomenon is unclear. Only a few samples of pM1 stage were available for this study. It appears that these primary esophageal carcinomas were not associated with blood-borne metastasis at the time of surgery. Further studies are needed.

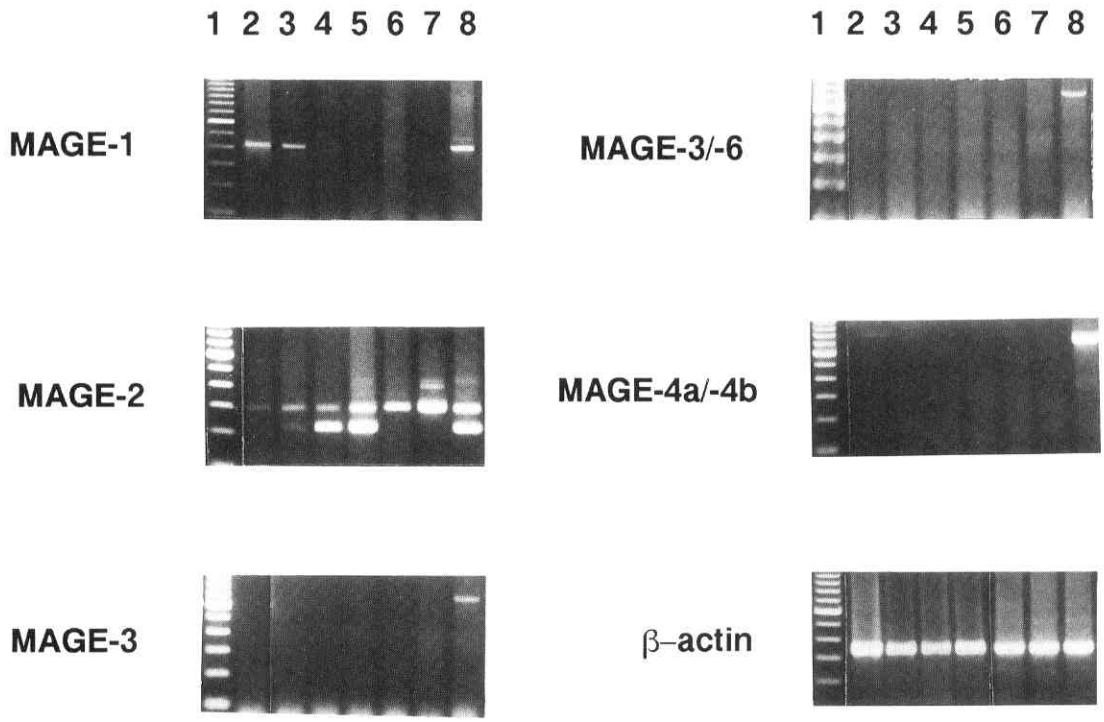


Fig. 1. Detection of MAGE genes at the mRNA level. Expression of MAGE genes at the mRNA level was investigated in 65 primary esophageal carcinomas and other samples as shown in Table I. Representative results are shown as follows: line 1, 100 bp ladder; lines 2 to 8, primary esophageal carcinomas (line 2, E93-17, MAGE-1$^+$, -4a/-4b$^+$; line 3, E91-10, MAGE-1$^+$, -2$^+$; lines 4 and 5, E91-13 and E92-11, MAGE-2$^+$; lines 6 and 7, E-93-18 and E-94-23, all negative; line 8, E91-02, all positive). The upper band in the gel of MAGE-2 was due to contaminating genomic DNA. Total cellular RNA was isolated from the various tissues and cell lines using the RNAzol (Biotecx Lab. Inc., Houston, TX) method according to the manufacturer's instructions. cDNA was prepared from 5 $\mu$l of total RNA in a 20 $\mu$l reaction mixture containing reaction buffer (pH 8.3), 0.5 m*M* triphosphate, 0.01 *M* DTT, and 200 U Super-Script reverse transcriptase (BRL, Gaithersburg, MD). The mixture was incubated at room temperature for 10 min, at 42°C for 50 min, and at 90°C for 5 min, and then chilled on ice. Two $\mu$l of the cDNA mixture was used for each assay in a 25 $\mu$l reaction mixture containing 0.1 $\mu$g of the primers and 0.13 $\mu$l of Taq DNA polymerase (5 U/$\mu$l, Promega, Madison, WI) in the buffer (50 m*M* KCl, 10 m*M* Tris-HCl [pH 9.0], 0.1% Triton X-100) (Promega), 1.5 m*M* $MgCl_2$, and 200 $\mu M$ each of dATP, dGTP, dCTP, dTTP. The primers used in this study were 5′-CGGCCGAAGGAACCTGACCCAG-3′ and 5′-GCTGGAACCCTCACTGGGTTGCC-3′ for MAGE-1 gene (a 421 bp band was expected)[7]; 5′-AAGTAGGACCCGAGGCACTG-3′ and 5′-GAAGAGGAAGAAGCGGTCTG-3′ for MAGE-2 gene (230 bp band)[11]; 5′-TGGAGGACCAGAGGCCCCC-3′ and 5′-GGACGATTATCAGGAGGCCTGC-3′ for MAGE-3 gene (725 bp band)[4]; 5′-ACCAAGGAGAAGATCTGCCAGTGGGTCTC-3′ and 5′-ACAGTCGCCCTCTTTTGCGATTATGG-3′ for MAGE-3 and -6 genes (726 bp band) (the accession number of MAGE-6 is D32076); 5′-ACCAAGGAGAAGATCTGCCAGTGGGTCTC-3′ and 5′-GTCGCCCTCCATTGCAATTGTGC-3′ for MAGE-4a and -4b genes (726 bp band) (the accession numbers of MAGE-4a and -4b are D32075 and D32077); 5′-CTTCGCGGGGCGACGATGC-3′ and 5′-CGTACATGGCTGGGGTGTTG-3′ for $\beta$-actin (340 bp band). Because higher homology was found between MAGE-3 and -6 or between MAGE-4a and -4b genes (98% at the nucleotide level),[7] the primers shown above were designed to amplify both MAGE-3 and -6 (MAGE-3/-6) or both MAGE-4a and -4b genes (MAGE-4a/-4b), respectively. All these primers correspond to sequences located in different exons, and there was no risk of false-positivity due to small amounts of DNA contaminating the RNA preparation. Amplification was performed for 35 cycles (1 min at 94°C, 4 min at 72°C for all genes except for MAGE-2; 1 min at 94°C, 2 min at 68°C, 2 min at 72°C for MAGE-2 gene). The expression of MAGE genes was determined by the semiquantitative RT-PCR method. The PCR products were separated on agarose gel and visualized with ethidium bromide. The separated band corresponding to each amplified MAGE gene in the photograph was scanned by a ScanJet IIcx (Yokogawa Hewlett Packard, Tokyo) using the Adobe Photoshop 2.5.1 E software. The densitogram of each band was obtained with a Macintosh Quadra 650 using NIH image 1.55f, and was integrated to calculate the area. A band with an area more than 10% greater than that of the control (the band from TE10 for all MAGE genes) was considered positive in this study for the following reasons: 1) TE10 cell line expressed a 46 kDa MAGE-1 or 45 kDa MAGE-4 protein on immunoblot analysis with anti-MAGE-1 or -4 antibodies, respectively (data not shown), 2) tumor recognition by the anti-MAGE-1 cytotoxic T lymphocytes required a level of expression of MAGE-1 equal to at least 2 to 5% of that observed with the parental positive melanoma cell line (MZ2-MEL),[8,9] and 3) TE10 esophageal tumor cell line was used as the positive control in this study since MZ2-MEL was not available.

All these results suggest that the MAGE genes, particularly MAGE-1, became positive in relatively advanced stages of esophageal carcinomas. Alternatively, early stages of esophageal carcinomas expressing the MAGE proteins, tumor-rejection antigens, are eliminated by the host immune system. However, the mechanisms of MAGE gene expression in advanced stages of esophageal carcinomas are still unclear and remain to be fully addressed.

In summary, we have demonstrated that the MAGE-1 gene was expressed at the mRNA level in substantial proportions of primary esophageal carcinomas and their metastatic lymph nodes. These results should be helpful in developing a specific immunotherapy of esophageal carcinomas using the MAGE-1 gene product.

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