大腸癌における MAGE gene の発現に関する検討

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Expression of MAGE genes in colorectal carcinomas

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【Summary】

The human genes MAGE-1 and MAGE-3 encode tumor rejection antigens recognized on melanoma cells by cytotoxic T lymphocytes (CTL). These antigens are potentially useful as targets for specific immunotherapy. Expression of MAGE genes in some malignant tumors has been reported, but MAGE gene expression in colorectal carcinomas has not been studied adequately. Therefore, we studied MAGE-1, 2, 3, and 4 a/4 b expression at the mRNA level, in 40 cases of surgery for colorectal carcinoma, using the reverse transcription polymerase chain reaction (RT-PCR). MAGE-1, 2, 3, and 4 a/4 b genes were expressed in 3 (7.5%), 6 (15.0%), 13 (32.5%), and 5 (12.5%), respectively, of these 40 cases. A total of 19 of the 40 samples (47.5%) expressed at least one of the MAGE genes. The relationships between clinicopathologic factors and MAGE gene expression were also examined. The frequency of lymph node metastasis was significantly higher in MAGE-3-positive than in MAGE-3-negative cases ($p < 0.05$). All the cases classified as Dukes' D expressed the MAGE-3 gene. This rate of expression was significantly higher than that for all other the Dukes' classifications together ($p < 0.05$). Our findings suggest that MAGE-specific immunotherapy against colorectal carcinomas may be feasible.

Key words : MAGE,
colorectal carcinoma,
RT-PCR,
specific immunotherapy

【概 要】

MAGE gene は 1991 年 Boon らによりメラノーマに発現する腫瘍退縮抗原遺伝子として同定された。12 の遺伝子ファミリーを形成し、MAGE-1 および MAGE-3 の遺伝子産物が HLA 拘束性に細胞障害性 T 細胞を誘導し得る。メラノーマ以外の悪性腫瘍にも発現の報告があり、精巣および胎盤以外の正常細胞には発現がないことより、その遺伝子産物は癌特異免疫療法の対象分子として注目されている。

これまで大腸癌における MAGE gene の発現に関し詳細に検討した報告はなく、今回我々は、MAGE-1, MAGE-2, MAGE-3, MAGE-4 a/4 b, それぞれに特異的なプライマーを用いその発現を RT-PCR を用い検討した。大腸癌 40 例において MAGE-1, 2, 3, 4 a/4 b はそれぞれ 3 例 (7.5%), 6 例 (15.0%), 13 例 (32.5%), 5 例 (12.5%) に発現を認めた。各種臨床病理学的因子と MAGE gene の発現との相関を検討した結果、リンパ節転移陽性症例において有意に MAGE-3 の発現率は高かった ($p < 0.05$)。また Dukes D 症例は全例 MAGE-3 の発現を認め、その発現率は他の Dukes 症例より有意に高かった ($p < 0.05$)。

大腸癌は他の消化器癌と比べ手術後の予後は良好であるが、進行癌症例における成績は未だ十分とはいえない。進行癌症例の予後の改善には補助療法を加える必要があると考えられる。しかしながら化学療法、放射線療法の効果は未だ十分とはいえず、その予後の改善には新たな治療法が必要とされている。今回我々の検討した結果において MAGE-3 は大腸癌において比較的高頻度に発現しており、進行癌症例においてその発現率が高くなることより、MAGE-3 特異免疫療法が新たな大腸癌治療になり得る可能性が示唆された。

I. Introduction

The overall prognosis associated with colorectal carcinoma is better than that with other digestive tract cancers, such as esophageal and gastric carcinomas, although the prognosis of patients with advanced colorectal carcinomas is still poor. The 5-year survival rates of patients with colonic carcinomas after surgery for Dukes' A, B, C and D disease were about 90%, 80%, 60%, and less than 10%, respectively^{1,2)}. Other therapy in addition to surgery is necessary to improve the prognosis of advanced cases, but chemotherapy and radiation therapy still appear to have relatively little effect on colorectal carcinomas, and, therefore, new types of therapy are needed to improve the prognosis of advanced colorectal carcinoma patients.

In recent studies, some tumor-specific antigens of human cancers were elucidated. Most such antigens are melanoma-specific, such as tyrosinase, MART-1 and Pmel 17/gp 100^{3,4,5,6)}. However MAGE genes are expressed in some malignant tumors besides melanomas. The MAGE gene family consists of 12 closely related genes⁷⁾. MAGE-1 and -3 encode tumor rejection antigens that are

recognized by cytotoxic T lymphocytes (CTL) in the context of HLA class I^{8,9,10,11,12)}. These antigens are potentially useful as targets for specific immunotherapy, as MAGE genes are expressed in no normal tissues except the testis and placenta⁷⁾. MAGE gene expression in colorectal carcinomas has not been studied adequately and, therefore, we studied MAGE gene expression in such tumors using the reverse transcription polymerase chain reaction (RT-PCR) and discuss the possibility of specific immunotherapy against colorectal carcinoma.

II. Materials and methods

1) Sample collection

Both tumor samples and normal colonic mucosal tissues located far from the tumor site from the same patient were frozen immediately after surgical resection and stored in liquid nitrogen.

2) RNA isolation

Total RNA was isolated by the guanidinium-isothiocyanate/acid-phenol extraction procedure¹³⁾, using TRIZOL™ Reagent (GIBCO BRL, Gaithersburg, MD). Tissue samples were homogenized in TRIZOL™ Reagent (a mono-phasic solution of

phenol and guanidine isothiocyanate). Following centrifugation after addition of chloroform, the mixture was separated into a lower phenol-chloroform phase, an interphase, and an upper aqueous phase in which RNA remains exclusively. After transfer of the aqueous phase, the RNA was recovered by precipitation with isopropyl alcohol. The RNA pellet was washed once with 75% ethanol, and was dissolved in water.

3) cDNA synthesis

cDNA was synthesized from total RNA by reverse transcription. A 5- μ g aliquot of total RNA was diluted with 13 μ l water, heated for 10 min at 70°C with 1 μ l (50 ng) random hexamers (GIBCO BRL) and placed on ice for 1 min. Then, the mixture was incubated at 42°C for 50 min, followed by 70°C for 15 min, with 2 μ l 10 $\times$ synthesis buffer (200 mM Tris-HCl pH 8.4, 500 mM KCl, 25 mM MgCl₂, 1 μ g/ μ l BAS), 1 μ l 10 mM dNTP mix, 2 μ l of 0.1 M DTT, and 1 μ l (200 U) of superscript reverse transcriptase (GIBCO BRL).

4) MAGE gene expression assay

Expression of MAGE-1, MAGE-2, MAGE-3, and MAGE-4 a/-4 b was detected by PCR amplification using MAGE gene-specific primers located in different exons, which enabled false positives due to DNA contamination of the RNA preparations to be eliminated. The primers used in this study were: CHO-14 (5'-CGGCCGAAGGAACCTGACCCAG-3') and CHO-12 (5'-GCTGGAACCCCTCACTGGGTTGCC-3') for MAGE-1¹⁷⁾; CDS-9 (5'-AAGTAGGACCCGAGGCACTG-3') and CDS-7 (5'-GAAGAGGAAGAAGCGGTCTG-3') for MAGE-2¹⁴⁾; AB-1197 (5'-TGGAGGACCAGAGGCCCCC-3') and BLE-5 (5'-GGACGATTATCAGGAGGCCTGC-3') for MAGE-3⁹⁾, and MSFf-1 (5'-ACCAAGAGAAGATCTGCCAGTGGGTCTC-3') and M 41 Sr (5'-GTCCGCTCCATTGCAATTGTGC-3') for MAGE-4 a/4 b (both MAGE-4 a and 4 b)¹⁵⁾. Each gene was subjected to the PCR using 2 μ l of cDNA in 25 μ l reaction mixture containing 1.25 μ l of the primers (25 mM), 0.13 μ l of Taq DNA polymerase

(5 U/ μ l, Takara, Otsu, Japan), 2 μ l of 10 mM dNTP mix, and 2.5 μ l of 10 $\times$ synthesis buffer (200 mM Tris-HCl pH 8.4, 500 mM KCl, 25 mM MgCl₂, 1 μ g/ μ l BSA) (GIBCO BRL). Thirty-five amplification cycles (1 min at 94°C, 4 min at 72°C for MAGE-1, 3 and 4 a/4 b, 1 min at 94°C, 2 min at 68°C, and 2 min at 72°C for MAGE-2) were performed, after which the PCR products were separated electrophoretically in 2% agarose gel and visualized by ethidium bromide staining. K-562 (an erythroleukemia cell line) was used as a positive control for MAGE-1, 3 and 4 a/4 b expression, RPMI-1788 (a B-cell line) was used as that for the MAGE-2 expression, and NALM-1 (a B-cell line) was used as a negative control for the expression of all the MAGE genes. A PCR assay of β -actin was performed, using a forward primer (5'-AGGAGCACCAAGGAGAAGATCT-3') located in exon 2 and a reverse primer (5'-TTGCAGTGCAGACTCCTCTG-3') located in exon 3 to ensure the RNA had not degraded. Thirty amplification cycles (1 min at 94°C, 1 min at 55°C, and 2 min at 72°C) were performed for a PCR assay of β -actin.

5) Statistical analysis

Correlations between MAGE genes expression and several clinicopathologic factors were analyzed by the chi-squared, Fisher's exact or Student's t-tests.

III. Results

MAGE-1, 2, 3, and 4 a/4 b genes were expressed in 3 (7.5%), 6 (15.0%), 13 (32.5%), and 5 (12.5%), respectively, of the 40 cases of colorectal carcinomas. Nineteen (47.5%) of the cases expressed at least one MAGE gene (Figure 1, Table 1). No case expressed all these MAGE genes, one expressed three, 6 expressed two, and 12 expressed only one. No significant correlation between expression of the individual MAGE genes was found in this study. None of MAGE genes were expressed in any normal mucosal tissue sample (Figure 1). In each PCR assay, the positive control sample expressed the relevant MAGE gene, whereas the negative

Figure 1 The representative results of the expression of MAGE genes in colorectal carcinomas (A) and in normal mucosal tissue samples of the same patients (B) (lanes 1-12). The expected sizes are 421 bp (MAGE-1), 230 bp (MAGE-2), 725 bp (MAGE-3), 726 bp (MAGE-4 a/4 b), and 218 bp (β -actin). M is molecular marker lane. Clinicopathologic data (age, sex, histology* and Dukes' classification) of each cases are as follows.

lane 1 : 57 y.o., male, well, Dukes B.	lane 2 : 71 y.o., female, well, Dukes D.
lane 3 : 63 y.o., female, mod., Dukes B.	lane 4 : 59 y.o., male, mod., Dukes C.
lane 5 : 47 y.o., male, mod., Dukes C.	lane 6 : 57 y.o., female, mod., Dukes B.
lane 7 : 32 y.o., male, well, Dukes B.	lane 8 : 66 y.o., female, well, Dukes B.
lane 9 : 54 y.o., female, mod., Dukes A.	lane 10 : 63 y.o., female, well, Dukes A.
lane 11 : 51 y.o., male, well, Dukes C.	lane 12 : 74 y.o., female, mod, Dukes C.

*well : well differentiated adenocarcinoma mod. : moderately differentiated adenocarcinoma

Table 1 Frequency of MAGE genes expression in colorectal carcinomas

	Frequency of expression	
MAGE-1	3/40	(7.5%)
MAGE-2	6/40	(15.0%)
MAGE-3	13/40	(32.5%)
MAGE-4a/4b	5/40	(12.5%)
Total	19/40	(47.5%)

control samples expressed no MAGE genes.

The relationships between clinicopathologic factors and MAGE gene expression are listed in Table 2. The frequency of lymph node metastasis was significantly higher in MAGE-3-positive than in MAGE-3-negative cases ($p < 0.05$). The MAGE-3 expression frequency increased as the Dukes' classi-

Table 2 Relationship between clinicopathologic data and MAGE genes expression in colorectal carcinomas

	MAGE-1		MAGE-2		MAGE-3		MAGE4a/4b	
	+	-	+	-	+	-	+	-
Age (mean yr.)	70.3	60.4	67.0	60.2	62.8	61.1	60.7	61.7
±S.D.	±3.1	±11.1	±10.5	±11.5	±10.1	±12.3	±3.5	±12.3
Sex (number of cases)								
male	1	18	2	17	5	14	1	18
female	2	19	4	17	8	13	4	17
Depth of invasion (number of cases)								
within proper muscular layer	0	4	1	3	0	4	2	2
beyond proper muscular layer	3	33	5	31	13	23	3	33
Lymph node metastasis (number of cases)								
absent	1	18	2	17	2	17	4	15
present	2	19	4	17	11 *	10	1	20
Histology (number of cases)								
well ¹	2	20	4	18	10	12	2	20
moderately ²	1	16	2	15	3	14	3	14
signet ring cell ³	0	1	0	1	0	1	0	1
Dukes' classification (number of cases)								
A	0	4	1	3	0	4	2	2
B	1	13	0	14	2	12	2	12
C	1	16	2	15	6	11	1	16
D	1	4	3	2	5 **	0	0	5

+, presence of MAGE gene expression - , absence of MAGE gene expression

¹well differentiated adenocarcinoma, ²moderately differentiated adenocarcinoma, ³signet ring cell carcinoma,

*The frequency of lymph node metastasis in MAGE-3 positive cases was significantly higher than that in MAGE-3 negative cases ($p < 0.05$).

**The frequency of MAGE-3 expression for Dukes' D cases was significantly higher than that for all the other Dukes' cases ($p < 0.05$).

fication number increased : that for Dukes' D cases was significantly higher than that for all the other Dukes' cases together ($p < 0.05$). No significant correlations between MAGE gene expression and any other clinicopathologic factor were found in this study.

IV. Discussion

Recent studies have revealed MAGE gene expression in some digestive tract cancers. However, expression of each MAGE gene in colorectal carcinomas from the same samples has not been reported previously and, to our knowledge, the relationships between MAGE gene expression and clinicopathologic factors in colorectal carcinoma

patients have not been studied. In gastric carcinomas, the MAGE-1,2, and 3 expression frequencies were 41, 31, and 38%, respectively¹⁶⁾. In esophageal carcinomas, the MAGE-1,2,3, and 4 a/4 b expression frequencies were 29, 29, 50, and 21%, respectively (personal observations, data not shown). Therefore, in our present study, the MAGE gene expression frequencies in colorectal carcinomas were lower than those in esophageal and gastric carcinomas.

MAGE-1 encodes two different antigenic peptides recognized by autologous T lymphocytes in the context of HLA class I : one is presented by HLA-A 1⁸⁾ and the other by HLA-Cw 1601¹⁰⁾. MAGE-3 encodes three different antigenic peptides presented

by HLA-A 1, HLA-A 2 or HLA-B 44^{9,11,12)}. In this study, 7.5% of colorectal carcinomas expressed MAGE-1 and 32.5% expressed MAGE-3, showing the frequency of MAGE-1 expression was lower than that of MAGE-3. In general the incidence of HLA-A 1 in the Japanese is 1.3%¹⁷⁾ and the of HLA-Cw 1601 is suspected to be very low. Therefore, very few Japanese patients with colorectal carcinomas are likely to be candidates for MAGE-1-specific immunotherapy. However, the incidences of HLA-A 2 and HLA-B 44 in the Japanese are about 40 and 11%, respectively¹⁷⁾. Therefore, as 51% of the Japanese carry HLA-A 2 or HLA-B 44 and 32.5% of colorectal carcinomas express MAGE-3, about 16% of Japanese patients with colorectal carcinomas would be expected to have MAGE-3/HLA-A 2 or MAGE-3/HLA-B 44, and these patients may be candidates for MAGE-3-specific immunotherapy.

The prognosis of patients with colorectal carcinomas after surgery is better than for those with other digestive tract cancers. However, the 5-year survival rates of patients classified as Dukes' C and D were 60 and less than 10%, respectively^{1,2)}, and such patients require other therapy in addition to surgery to improve the prognosis. The clinicopathologic analysis in the present study revealed that the MAGE-3 expression frequency increased as the

Dukes' classification number increased : a total of 6 of 17 (35.3%) Dukes' C and 5 of 5 (100%) Dukes' D cases expressed MAGE-3. Accordingly, the frequency of lymph node metastasis in the MAGE-3-positive cases was significantly higher than that in the MAGE-3-negative cases. The reason for these findings is unclear, but, MAGE-3 expression in colorectal carcinomas may be related to lymph node or distant metastasis. Some workers pointed out that MAGE gene expression was related to metastasis in patients with esophageal carcinomas and melanomas^{15,18)}. These findings are hopeful for the development of MAGE-3-specific immunotherapy against colorectal carcinomas. Eleven of the 22 (50.0%) Dukes' C and D cases in our study expressed MAGE-3 and, therefore, about 26% of patients classified as Dukes' C and D should carry MAGE-3/HLA-A 2 or MAGE-3/HLA-B 44, suggesting that MAGE-3-specific immunotherapy against colorectal carcinomas may prove feasible for selected patients.

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