

Expression of the *TRAG-3* gene in human esophageal cancer: The frequent synchronous expression of *MAGE-3* gene

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Abstract. We previously reported some cancer testis antigens, especially for MAGE genes, to be expressed in a relatively high population of gastro-intestinal and breast cancers. *TRAG-3* (Taxol resistant associated gene-3) may be another cancer testis antigen; however, its expression has still not been fully studied. The *TRAG-3* expression was evaluated in a total of 57 cancer cell lines and 322 cancer samples of gastrointestinal and breast cancers by RT-PCR. *TRAG-3* was expressed in 23/57 (40%) of the cell lines: the highest expression was found in gastric cancer (6/9: 67%), followed by esophageal (13/28: 46%), colon (3/11: 27%) and liver (1/4: 25%) cancers. In clinical samples, the expression was the highest in esophageal cancer (32/58: 55%), followed by liver (13/50: 26%), bile duct (5/27: 19%), gastric (5/50: 10%), breast (5/50: 10%) and colon (2/87: 2.3%) cancers. The *TRAG-3* expression significantly correlated with the expression of *MAGE-3* in esophageal cancer ($p < 0.05$). As the *TRAG-3* gene is located on Xq28, which is the same locus as the MAGE gene family, we found a frequent synchronous expression pattern with *TRAG-3* and *MAGE-3* in esophageal cancer.

Introduction

Cancer testis antigen (CTA) genes are expressed in a significant proportion of malignant tumors of various histological organs, whereas no such expression has been observed in normal tissue specimens except the testis. Therefore, CTAs have been recognized to be a promising target for cancer-

specific immunotherapy. In clinical trials, the vaccination of cancer patients with HLA-restricted peptides, which are produced by CTA genes, induced cancer-specific immune response and tumor regression (1,2). We previously reported the expression of such CTA genes as MAGE to have a relatively high rate in samples of gastrointestinal and breast cancers (3-5). In addition, we have reported that dendritic cell vaccination with MAGE peptide could be a novel therapy for gastro-intestinal cancer (6).

TRAG-3 (Taxol resistance associated gene-3), a novel 0.8 kb transcript, was isolated from a Taxol-resistant SKOV-3 daughter cell line (7). This transcript was identified by comparing the Taxol naïve SKOV-3 parent line and SKOV-3TR using differential display. Furthermore, the expression pattern of *TRAG-3* is considered to be CTA (8). However, the frequency of the expression of *TRAG-3* is still unknown in gastrointestinal and breast cancer.

In this study, we found the *TRAG-3* expression to be relatively high in gastrointestinal cancers, especially in esophageal cancer. Frequent synchronous expression of *TRAG-3* and *MAGE-3* was observed in the patients with esophageal cancer.

Materials and methods

Cell lines. Fifty-seven cancer cell lines (esophagus: KY30, KY50, KY70, KY110, KY140, KY150, KY170, KY180, KY190, KY200, KY220, KY270, KY410, KY510, TE1, TE2, TE3, TE4, TE6, TE7, TE8, TE9, TE10, TE11, TE12, TE13, TE14, TE15; stomach: MKN7, MKN45, NS8, NUGC3, NUGC4, AZ521, KATO3, SCH, GOTO; colon: LOVO, DLD1, CCK81, Colo201, Colo205, Colo320DM, Wdr, HT29, LS174T, RCM1, CaR1; liver: HepG2, Hep3B, HuH7, HuH28; pancreas: PK1, PK9, breast: MCF7, YMB1, YMB1B) were obtained from several sources including the Institute of Development, Aging and Cancer, Tohoku University, Sendai, Japan and Japanese Cancer Research Bank, Tokyo, Japan. All cell lines were maintained in an RPMI-1650 medium supplemented with 10% of fetal bovine serum and antibiotics. The cells were incubated in 5% CO₂ and air atmosphere at 37°C, and passaged twice every six days.

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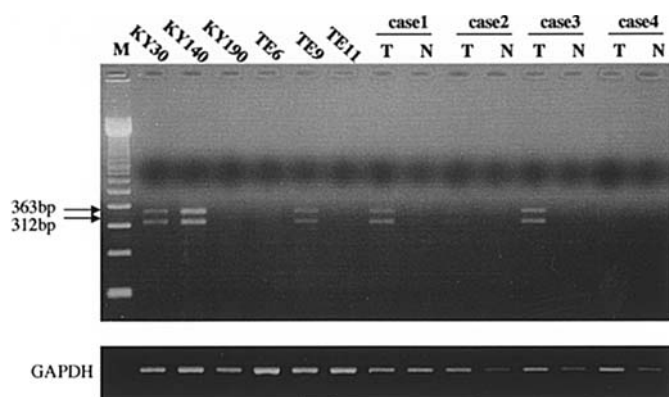


Figure 1. Representative RT-PCR analysis of TRAG-3 indicating two transcript sizes (312 and 363 bp) is shown in the upper panel. Lane 1: M, molecular weight marker; lanes 2-7: esophageal cancer cell lines (KY30, KY140, KY190, TE6, TE9, TE11); lanes 8-15: three clinical cases of esophageal cancer. cDNA extracted from tumor tissue (T) and paired normal tissue (N) was amplified in each case. GAPDH mRNA expression is shown as corresponding control in the lower panel.

Clinical samples. Tumor samples and matched control samples of normal tissue located far from the tumor site (esophagus 58, stomach 50, colon 87, liver 50, bile duct 27 and breast 50 paired samples) were frozen in liquid nitrogen less than 5 min after surgical resection and kept at -90°C until the extraction of RNA. The surgical samples were obtained at the Department of Surgery, Medical Institute of Bioregulation, Kyushu University, Beppu, and the Department of Surgery, Oita Prefectural Hospital, Oita, Japan.

Assay for TRAG-3 gene expression (RT-PCR). Total RNA isolated by the acid guanidium thiocyanate PhOH-chloroform (AGPC) procedure was treated with DNase. The cDNA was synthesized from 8 μl of total RNA as described previously. The presence of TRAG-3 in the reverse transcription products was detected by polymerase chain reaction amplification in separate reactions, using an oligonucleotide primer located in different exons of the TRAG-3 gene. The sequences were 5'-TGGGGGAGGCTTGGAGAGACC-3' and 5'-TGCCCTTG TGGTCCCGCTATG-3' for TRAG-3. The amplification was performed for 26 cycles (1 min at 94°C , 1 min at 58°C and 1 min at 72°C for TRAG-3). An 8- μl aliquot of each reaction was size-fractionated on a 1.5% agarose gel and visualized with ethidium bromide staining. To ensure that the RNA was not degraded, a polymerase chain reaction assay with primers specific for the gene glyceraldehyde-3-phosphate dehydrogenase cDNA was carried out in each case, except that only 24 cycles were performed under the following cycling conditions: 1 min at 94°C , 1 min at 56°C , and 1 min at 72°C .

Clinicopathologic data. All data including gender, age, histology, tumor size, depth of tumor invasion, lymph node metastasis, lymph vessel permeation, vascular vessel permeation, and stage of disease were obtained from the clinical and pathologic records. The disease stage was classified according to the criteria proposed by the Japanese Society of Esophageal Disease (8th edition), the Japanese Society of Gastric Cancer (12th edition), the Japanese Research Society of Colon Cancer (5th edition), and the Japanese Breast

Table I. The expression of TRAG-3 in cell lines and clinical cases.

Organ	Cell lines	Clinical cases
Esophagus	13/28 (46%)	32/58 (55%)
Stomach	6/9 (67%)	5/50 (10%)
Colon	3/11 (27%)	2/87 (2.3%)
Liver	1/4 (25%)	13/50 (26%)
Bile duct	ND	5/27 (19%)
Pancreas	0/2 (0%)	ND
Breast	0/3 (0%)	5/50 (10%)

Cancer Society (12th edition). The tumors with or without an expression of the TRAG-3 gene were then compared.

Comparison between TRAG-3 and other CTAs. We previously reported the expressions of CTA (MAGE-1, MAGE-3, LAGE-1, NY-ESO-1, SCP-1, SSX-1, SSX-2, SSX-4) in clinical samples of esophageal cancer (5). In this study, the expression of TRAG-3 was examined in the same available samples and it was compared with the expression of other CTAs from our database.

Statistical analysis. A statistical analysis was performed using the Chi-square or Fisher's exact test. The level of significance was set at $p < 0.05$.

Results

The TRAG-3 expression in cell lines and cases was evaluated by RT-PCR (Fig. 1). The two TRAG-3 transcript sizes (312 bp and 363 bp) were confirmed using direct sequencing. These transcripts demonstrated that larger transcript was added 51 bp-insert to the shorter transcript, splicing variant. This finding correlated with those of a previously report (8). The TRAG-3 expression frequency is summarized in Table I. In cell lines, TRAG-3 expression was observed in 13/28 (46%) esophageal, 6/9 (67%) gastric, 3/11 (27%) colorectal, 1/4 (25%) hepatocellular, 0/2 (0%) pancreatic, and 0/3 (0%) breast cancers. We observed a relatively high expression in esophageal and gastric cancer cell lines; however, the pancreas and breast cancer cell lines did not express TRAG-3, based on our findings. In clinical samples, TRAG-3 expression was observed in 32 (55%) samples of esophageal cancer. On the other hand, the expressions in other histological types of cancers were comparatively low. No expression was observed in any of the matched control samples of normal tissue.

Since the TRAG-3 gene was highly expressed in esophageal cancer cases, several pathological factors were compared between cases with or without TRAG-3 gene expression. In 50/58 esophageal cancer cases whose clinicopathological data were registered, no significant differences were observed between the cases with TRAG-3 gene expression and those without TRAG-3 gene expression regarding such factors as age, sex, lesion of main tumor, histology, depth of tumor invasion, lymphatic involvement,

Table II. The expression of TRAG-3 compared with that of other cancer testis antigens in esophageal cancer.

Case	TRAG-3	MAGE-3	LAGE-1	MAGE-1	NYESO-1	SSX-2	SSX-4
1	+	+	+	+	+	+	-
2	+	+	+	+	+	-	+
3	+	+	+	+	+	-	+
4	+	+	+	+	+	-	-
5	+	+	+	+	+	-	-
6	+	+	+	+	+	-	-
7	+	+	+	+	-	-	-
8	+	+	+	+	-	-	-
9	+	+	+	+	-	-	-
10	+	+	+	-	+	-	+
11	+	+	+	-	+	-	+
12	+	+	+	-	-	-	-
13	+	+	-	+	-	-	-
14	+	+	-	+	-	-	-
15	+	+	-	-	-	-	-
16	+	+	-	-	-	-	-
17	+	+	-	-	-	-	-
18	+	-	-	+	-	-	-
19	+	-	-	+	-	-	-
20	+	-	-	-	-	-	-
21	-	+	+	+	+	-	+
22	-	+	+	+	-	-	+
23	-	+	+	-	+	-	-
24	-	+	+	-	-	-	-
25	-	+	-	+	-	-	-
26	-	+	-	+	-	-	-
27	-	+	-	+	-	-	-
28	-	+	-	+	-	-	-
29	-	+	-	+	-	-	-
30	-	-	+	+	+	-	-
31	-	-	-	+	-	-	-
32	-	-	-	-	-	-	-
33	-	-	-	-	-	-	-
34	-	-	-	-	-	-	-
35	-	-	-	-	-	-	-
36	-	-	-	-	-	-	-
37	-	-	-	-	-	-	-

vascular involvement, lymph node metastasis, stage of disease or prognosis (data not shown).

In the 46 esophageal cancer cases, we already reported the expression of CTAs, such as MAGE-1, MAGE-3, NY-ESO-1, LAGE-1, SCP-1, SSX-1, SSX-2 and SSX-4 (5). In this study, we examined the expression of TRAG-3 in 37 of the 46 esophageal samples, which we have reported previously. The expression of TRAG-3 was compared with that of previously reported CTAs (Table II). The number of overlapping cases that expressed both TRAG-3 and some of the other CTAs was 20 of the 21 cases that expressed TRAG-3. In particular, the expression of TRAG-3 significantly correlated with that of MAGE-3 ($p<0.05$) (Table III).

Table III. Correlation between the expression of TRAG-3 and other CTAs in esophageal cancer.

CTA	expression	TRAG-3		p-values
		+	-	
MAGE-1	+	13	9	0.45
	-	7	8	
MAGE-3	+	17	9	0.03
	-	3	8	
NY-ESO-1	+	8	3	0.13
	-	12	14	
LAGE-1	+	12	5	0.06
	-	8	12	
SSX-2	+	1	0	0.35
	-	19	17	
SSX-4	+	4	2	0.49
	-	16	15	

Discussion

We previously reported the rate of MAGE gene expression to be 43-62% of esophageal and gastrointestinal cancer cases (3-5). At our institute, cancer-specific vaccination using the MAGE-3 peptide for patients with advanced esophageal cancer is performed, and both tumor regression and an immune response have been observed in some cases (6). Since the TRAG-3 gene expression is as high as the MAGE gene expression in esophageal cancer cases, the TRAG-3 gene may be a good candidate gene for immunotherapy. If TRAG-3 is the antigen that has immunogenicity recognized by cytotoxic T-lymphocytes (CTLs), it may be a promising target of immunotherapy. On the other hand, we and others have reported a heterogeneous expression of MAGE-3 in tumor tissue and this may be related to the phenomenon of tumor escape from T cell recognition (9,10). The heterogeneous expression of CTAs indicates that some parts of cancer cells might thus be enlarged despite the presence of a single peptide-targeted vaccination. Therefore, multiple-peptide vaccinations adding TRAG-3 may cause more immunological reactions and tumor regression than a single peptide-targeted vaccination.

The *MAGE-3* gene is located in X chromosome q28 forming the MAGE-A gene family (11,12). *TRAG-3* gene is also located in the area of X chromosome q28. Since the genes have no homology, the *TRAG-3* gene is not a member of the MAGE-A gene family. However, the simultaneous expression of TRAG-3 and MAGE-3 could often be observed. One reason for the simultaneous expression of these two genes may be due to the location of these genes. On the other hand, *TRAG-3* was initially isolated through an approach to identify the genes responsible for Taxol resistance. The expression of CTAs might be associated with drug resistance. The *MAGE-3* gene showed a higher expression in a doxorubicin-resistant colon cancer cell line than the sensitive

parental colon cancer cell line (13). In a separate study, PAGE-1 was also found to show a high expression in an androgen resistant cell line (14). However, the function of these CTAs is still not clear. If the roles and function of these CTAs can be elucidated, then more effective therapeutic approaches for cancer can be established.

In summary, a relatively high incidence of TRAG-3 expression was observed in clinical samples of esophageal cancer. Furthermore, the expression of TRAG-3 was significantly co-related with the expression of MAGE-3. TRAG-3 may therefore become the new candidate antigen for cancer-specific immunotherapy especially for esophageal cancer. We are presently trying to identify the HLA-restricted antigenic peptide of TRAG-3.

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