

Expression Profile of Cancer-Testis Genes in 121 Human Colorectal Cancer Tissue and Adjacent Normal Tissue

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ABSTRACT

Purpose: Among tumor antigens identified to date, cancer-testis (CT) antigens, which are coded by CT genes, are identified as a group of highly attractive targets for cancer vaccines. This study is the first to analyze the mRNA expression and possible correlation with pathologic characteristics of multiple CT genes in a large cohort of colorectal cancer (CRC) patients.

Experimental Design: The expression of 10 individual CT genes in 121 CRC and adjacent tissues were analyzed by RT-PCR method. The presence of autologous antibodies against NY-ESO-1 was examined in serum samples by ELISA. To confirm the protein expression, immunohistochemistry was done for detecting the NY-ESO-1 antigen in mRNA-positive CRC tissues.

Results: The CT genes were detected with various frequencies in CRC tissue, SCP-1, 1.7%; SSX-2, 2.5%; SSX-4, 2.5%; SSX-1, 5.0%; CT10, 6.6%; NY-ESO-1, 9.9%; MAGE-1, 11.6%; LAGE-1, 15.7%; MAGE-4, 22.3%; and MAGE-3, 27.3%. In 56.2% of tumor tissues examined in this study, at least one CT gene was detected. In contrast, no CT gene expression was found in cancer adjacent tissues. Among 10 CT genes investigated, NY-ESO-1 and LAGE-1 are of particular interest because their mRNA expression in CRC was rarely reported before. In our study, NY-ESO-1 mRNA was found to express in 9.9% of the samples, and also correlated significantly with stages ($P = 0.041$) and local lymph node metastasis ($P = 0.002$). In addition, we also identified one NY-ESO-1 antibody-positive serum sample. MAGE-4 mRNA was expressed at a high frequency in tumor tissues with vessel emboli samples ($P = 0.025$).

Conclusions: These results suggested that CT genes, especially NY-ESO-1 and LAGE-1, do express in CRC. More than 50% of the CRC patients in this study express at least one CT gene, making them eligible for CT vaccination. NY-ESO-1 gene may serve as a marker for local metastasis and advanced disease. MAGE-4 gene is significantly associated with the vessel emboli.

INTRODUCTION

Colorectal cancer (CRC) is one of the most common tumor types in the world, with ~400,000 deaths annually (1, 2). In the United States, despite of a slight decrease in its incidence and mortality during the past two decades, CRC remains to be the third most common cancer, affecting ~140,000 people and counting for ~50,000 cancer-related deaths per year (3). In China, where incidence rate was initially low, due to the changes in lifestyle and nutritional habits, the CRC rate is increasing. The most alarming increase in CRC incidence was observed from 1972-1974 to 1987-1989, being 85% in males and 79% in females, increasing by 4.2% annually (4). Moreover, the prognosis of CRC is poor, with 5-year survival rate of only 50% (15-65%; ref. 5). Although 80% of CRC patients have a complete macroscopic clearance of the disease by surgical resection, 50% of them suffer recurrence presumably because of disseminated micrometastases present at the time of surgery (6). Several alternative therapeutic strategies are being actively pursued including immunotherapy.

The first step of active immunotherapy is to identify rational antigen targets for CRC. Among tumor antigens identified to date, cancer-testis (CT) antigens have been recognized as a group of highly attractive targets for cancer vaccine. CT antigens are encoded by CT genes. More than 40 CT genes have been categorized as CT genes, including the NY-ESO-1, MAGE, and SSX gene families and so on. These CT genes are widely expressed in a variety of human cancers, such as melanoma, breast cancer, and esophageal cancer (7). But their expression in normal tissues is restricted to testis. Furthermore, because testis is an immune-privileged organ (8), cancer vaccination of CT antigens is not expected to cause damages to normal tissues due to autoimmune responses.

In this study, the expression frequency of 10 CT genes, including NY-ESO-1, LAGE-1, MAGE-1, MAGE-3, MAGE-4, CT-10, SCP-1, SSX-1, SSX-2, and SSX-4 was investigated in 121 CRC patients. The correlation between CT gene expression pattern and pathologic characteristics of CRC was also studied.

MATERIALS AND METHODS

Patients and Tumor Samples. One hundred twenty-one patients with a diagnosis of sporadic CRC were enrolled in this study including 50 (41.3%) women and 71 (58.7%) men. The female-to-male ratio was 1:1.42 with the median age of 61, ranging from 28 to 84 years old. All patients received surgical treatment between January and September 2003 in Beijing

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Cancer Hospital. The clinical data included demography (age, gender), tumor location (proximal, distal colon, and rectum), degree of histologic differentiation, and pathologic stage according to the tumor-node-metastasis system recommended by the American Joint Committee on Cancer (9). A criterion for exclusion was preoperative therapy.

All CRC and adjacent cancer tissue samples were freshly obtained from surgery. Tumor samples were taken from the luminal aspect of the malignancy, together with paired adjacent tissues taken from the luminal aspect of the bowel wall 2 cm away from tumor margin. All tissue samples were snap-frozen in liquid nitrogen within 30 minutes after resection and stored at -80°C . At the time of operation, 10 mL whole venous blood from 82 patients was obtained as previously described (10).

RNA Extraction and cDNA Preparation. Total RNA was isolated from ~ 100 mg of each tissue sample using Trizol reagent (Invitrogen, Carlsbad, CA), following the manufacturer's instructions. RNA samples were stored at -80°C until used. Reverse transcription was done with 1 μL of total RNA using SuperScript First-Strand Synthesis System (Invitrogen). GAPDH was used as internal quality control (11).

PCR. To analyze the expression of individual CT gene, 1 μL cDNA was amplified with 6.25 units AmpliTaq Gold (Roche, Basel, Switzerland), in a 25 μL reaction containing 0.5 mmol/L dATP, dCTP, dGTP, and dTTP and 1.5 mmol/L MgCl_2 . The PCR analysis was done on selected genes using the primers and the conditions shown in Table 1. The primers were designed to span different exons of each gene to prevent occasional false positives resulting from genomic DNA contamination in RNA preparations. All reactions were carried out in PTC-100 Peltier Thermal Cycler (MJ Research, Waltham, MA). Electrophoresis was done by loading 8 μL of each sample on a 1% agarose gel, and visualized by ethidium bromide staining using the Bio-imaging System (Ultra-Violet Products, UVP, Cambridge, United Kingdom).

ELISA. Autologous antibodies against NY-ESO-1 were done by ELISA as established by Stockert et al. (10), with

some modifications. Briefly, 30 μL /well of either 1 $\mu\text{g}/\text{mL}$ NY-ESO-1 recombinant protein or 3 $\mu\text{g}/\text{mL}$ bovine serum albumin (background control) in coating buffer [15 mmol/L Na_2CO_3 , 30 mmol/L NaHCO_3 (pH 9.6)] was absorbed to 96-well half-area plates (Costa, Acton, MA), respectively, overnight at 4°C . After blocking with 2% bovine serum albumin/PBS and washing, the plates were incubated for 1 hour with a 5-fold serial-diluted patient sera of 1:25, 1:125, and 1:625. Rabbit antihuman immunoglobulin (IgA, IgG, and IgM) peroxidase conjugated (Sigma, St. Louis, MO) was used as secondary antibody and reacted for 45 minutes. Then, the plates were incubated with substrate (*O*-phenylenediamine dihydrochloride) for 30 minutes and read by an ELISA reader (Bio-Rad Laboratories, Hercules, CA). One positive, eight negative (normal donors), and background (bovine serum albumin) controls were included in each test.

Immunohistochemistry. Monoclonal antibody E978 (Zymed Laboratories, South San Francisco, CA) against NY-ESO-1 was used as the primary antibody. After heat-based antigen retrieval in EDTA buffer (1 mmol/L, pH 8.0), paraffin-embedded specimens were incubated overnight with E978 at a concentration of 1 $\mu\text{g}/\text{mL}$ at 4°C . Detection of the primary antibody was done with PowerVision two-step histostaining reagent (Zhongshan Biotechnology, Beijing, China).

Statistical Analysis. Statistical analysis was done with the SPSS program (SPSS Inc., Chicago, IL). Pearson χ^2 test was used to compare the correlation between disease stage distribution and CT gene expression. Statistical significance was accepted at $P < 0.05$ (two tailed).

RESULTS

Expression of Individual Cancer-Testis Genes. The most frequently expressed CT genes in CRC was *MAGE-3* (27.3%), followed by *MAGE-4* (22.3%), *LAGE-1* (15.7%), *MAGE-1* (11.6%), *NY-ESO-1* (9.9%), *CT-10* (6.6%), *SSX-1* (5%), *SSX-2* (2.5%), *SSX-4* (2.5%), and *SCP-1* (1.7%; Table 2).

Table 1 Primers and condition of RT-PCR analysis

Genes	Primers from 5' to 3'	Temperature and duration of			Cycle no.
		Denaturation	Annealing	Extension	
<i>NY-ESO-1</i>	f: CAG GGC TGA ATG GAT GCT GCA GA r: GCG CCT CTG CCC TGA GGG AGG	94°C, 1 min	60°C, 1 min	72°C, 1 min	35
<i>LAGE-1</i>	f: CTG CGC AGG ATG GAA GGT GCC CC r: GCG CCT CTG CCC TGA GGG AGC	94°C, 1 min	60°C, 1 min	72°C, 1 min	35
<i>MAGE-1</i>	f: CGG CCG AAG GAA CCT GAC CCA G r: GCT GGA ACC CTC ACT GGG TTG CC	94°C, 1 min	68°C, 1 min	72°C, 2 min	35
<i>MAGE-3</i>	f: TGG AGG ACC AGA GGC CCC C r: GGA CGA TTA TCA GGA GGC CTG C	94°C, 1 min	66°C, 1 min	72°C, 2 min	35
<i>MAGE-4</i>	f: GAG CAG ACA GGC CAA CCG r: AAG GAC TCT GCG TCA GGC	94°C, 30 s	68°C, 30 s	72°C, 30 s	35
<i>SCP-1</i>	f: GTA CAG CAG AAA GCA AGC AAC TGA ATG r: GAA GGA ACT GCT TTA GAA TCC AAT TTC C	94°C, 1 min	55°C, 1 min	72°C, 1 min	35
<i>SSX-1</i>	f: CTA AAG CAT CAG AGA AGA GAA GC r: AGA TCT CTT ATT AAT CTT CTC AGA AA	94°C, 1 min	55°C, 1 min	72°C, 1 min	35
<i>SSX-2</i>	f: GTG CTC AAA TAC CAG AGA AGA TC r: TTT TGG GTC CAG ATC TCT CGT G	94°C, 1 min	55°C, 1 min	72°C, 1 min	35
<i>SSX-4</i>	f: AAA TCG TCT ATG TGT ATA TGA AGC T r: GGG TCG CTG ATC TCT TCA TAA	94°C, 1 min	55°C, 1 min	72°C, 1 min	35
<i>CT10</i>	f: TGT ACT GCG CTG CCG TGA GAC TGG TG r: AAG CTC CAT GAA CTC ACG GGC TCT CTT G	94°C, 1 min	68°C, 1 min	72°C, 1 min	35

Table 2 Stage and CT genes expression

	Numbers of patients in different stages (frequency)				Total number (frequency)
	I	II	III	IV	
<i>NY-ESO-1</i>	0	1 (0.8%)	9 (7.4%)	2 (1.7%)	12 (9.9%)
<i>LAGE-1</i>	2 (1.7%)	7 (5.8%)	9 (7.4%)	1 (0.8%)	19 (15.7%)
<i>MAGE-1</i>	2 (1.7%)	8 (6.6%)	4 (3.3%)	0	14 (11.6%)
<i>MAGE-3</i>	2 (1.7%)	11 (9.1%)	15 (12.4%)	5 (4.1%)	33 (27.3%)
<i>MAGE-4</i>	1 (0.8%)	12 (9.9%)	11 (9.1%)	3 (2.5%)	27 (22.3%)
<i>CT-10</i>	0	2 (1.7%)	6 (4.9%)	0	8 (6.6%)
<i>SCP-1</i>	0	2 (1.7%)	0	0	2 (1.7%)
<i>SSX-1</i>	2 (1.7%)	3 (2.5%)	1 (0.8%)	0	6 (5%)
<i>SSX-2</i>	0	1 (0.8%)	2 (1.7%)	0	3 (2.5%)
<i>SSX-4</i>	0	0	3 (2.5%)	0	3 (2.5%)
Total	11	46	50	14	121

Examples of positive RT-PCR results on each CT gene were shown in Fig. 1. In our study, some amplicon bands seemed faint. These cases were independently investigated by another author and were scored positive only if these amplicons could be reproducibly amplified.

No CT gene expression was found in the adjacent tissue.

Coexpression of Multiple Cancer-Testis Genes in Colorectal Cancer. In all 121 CRC patients, 68 patients (56.2%) expressed at least one CT gene. Among them, 35 patients (28.9%) expressed one CT gene. The other 33 patients (27.3%) expressed more than two CT genes (Fig. 2).

Antibody Responded to NY-ESO-1. Sera from 82 CRC patients were assayed for the presence of NY-ESO-1 antibody by ELISA using recombinant NY-ESO-1 protein as the antigen. Positive cutoff for ELISA was the mean of eight negative controls plus three SDs. The criterion for a sample to be serum positive was two positive out of three serial dilutions. All data were analyzed after subtraction of the bovine serum albumin background. One serum sample from a stage III CRC patient was determined as NY-ESO-1 antibody positive (Fig. 3). Its corresponding CRC tissue was NY-ESO-1, LAGE-1, MAGE-3, and MAGE-4 positive by RT-PCR.

Detection of Protein Expression in NY-ESO-1 Messenger RNA-Positive Colorectal Cancer Samples. Twelve NY-ESO-1 mRNA-positive CRC tissues were immunohistochemically stained by monoclonal antibody E978. One tumor sample was strongly stained by the antibody (Fig. 4). The expression of NY-ESO-1 in this case showed a heterogeneous pattern, which is in accordance with previously observed CT antigen expression pattern reported by Jungbluth et al. (12).

Relationship Between Cancer-Testis Gene Expression and Tumor Characteristics. The number of patients in each stage and their status of CT gene expression were summarized in Table 2. There were 11 cases of stage I, 46 of stage II, 50 patients of stage III, and 14 in stage IV. NY-ESO-1 mRNA-positive cases were primarily found in stage III patients in a statistically

significant fashion ($P = 0.041$; Table 3). Similarly, only *NY-ESO-1* gene was significantly associated with lymph node metastasis ($P = 0.002$; Table 4). The other CT genes studied did not show statistically significant different distribution between stages.

After reviewing the pathologic slides of all cases, we identified vessel emboli in 41 of 121 tumor tissue samples. Among CT genes investigated in this study, *MAGE-4* expression was found to be associated with the presence of vessel emboli. Of tumor tissues with vessel emboli, 34.1% were positive for *MAGE-4* expression; on the other hand, only 16.3% were *MAGE-4* positive in those samples without vessel emboli ($P = 0.025$; Table 5).

We did not detect any significant correlation between CT gene expression and other clinical factors such as age and gender of patients, tumor location, size, gross type, differentiation, and invasion depth.

DISCUSSION

Along with the first human tumor antigen identified by van der Bruggen et al. (13), it has been approved that cancer cells can be recognized and killed by immune system. CT gene products represent such attractive targets for cancer immunotherapy (7). However, only limited clinical data are available

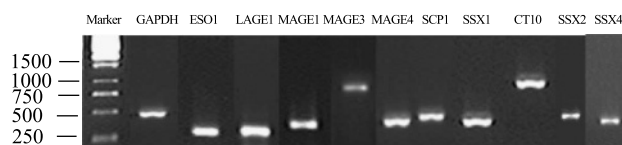


Fig. 1 Examples of positive CT genes in RT-PCR.

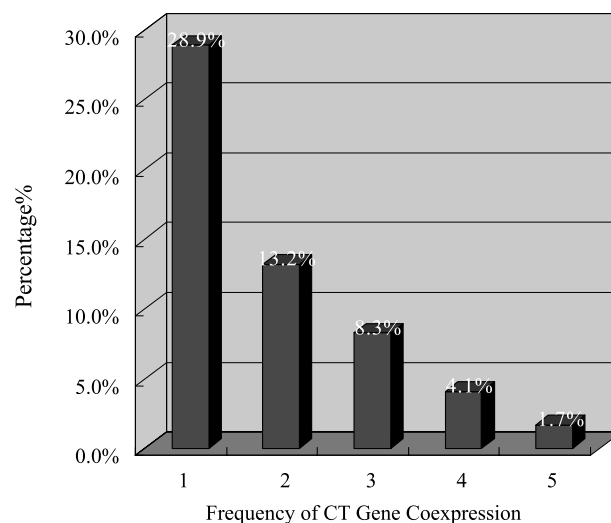


Fig. 2 Coexpression of CT genes in CRC. 1-5, number of coexpressed CT genes.

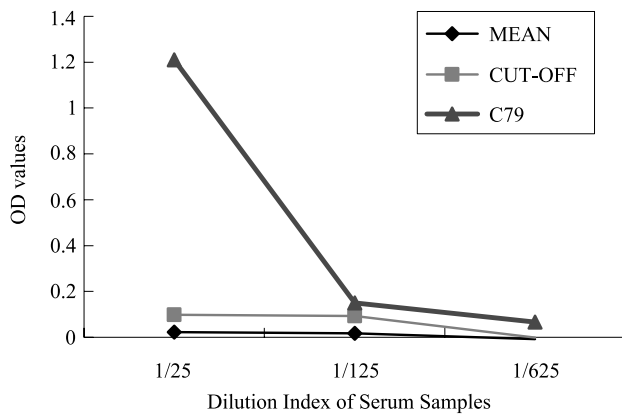


Fig. 3 Autologous antibody response detected by ELISA in one CRC serum sample (C79).

regarding the expression pattern of CT genes and its relationship with pathologic characteristics in CRC. CRC has not become an obvious target for immunotherapeutic inter-

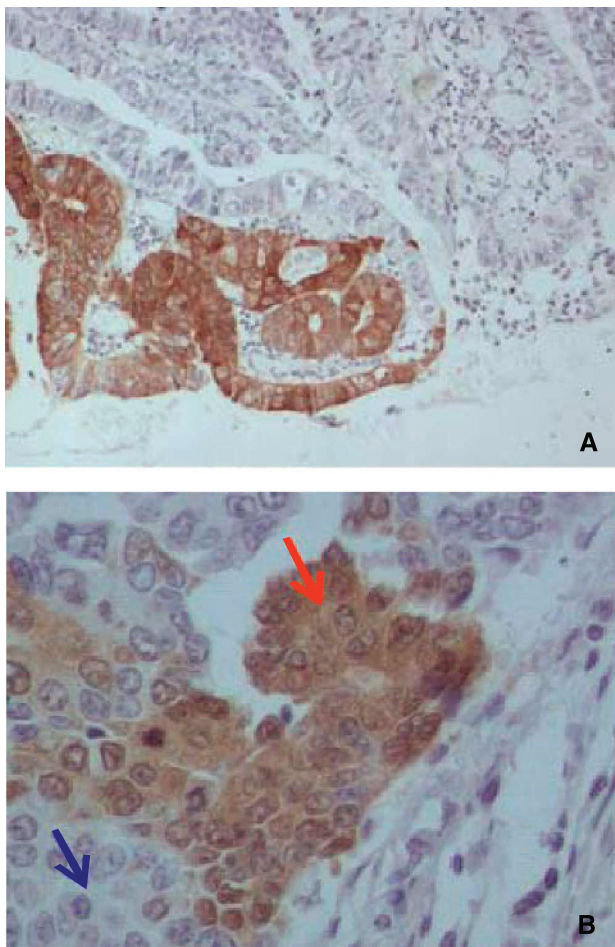


Fig. 4 Immunohistochemical staining of NY-ESO-1 protein in gastric cancer by monoclonal antibody E978. A, $\times 100$ photograph of the stained region; B, $\times 400$ photograph of the stained region, showing heterogeneously expression of NY-ESO-1. Red arrow, positive staining tumor cells; blue arrow, negative staining tumor cells.

Table 3 Correlation of the expression of NY-ESO-1 detected by RT-PCR and disease stages

Stage	NY-ESO-1		Total	P
	Negative	Positive		
Stage I	11	0	11	0.041
Stage II	45	1	46	
Stage III	41	9	50	
Stage IV	12	2	14	
Total	109	12	121	

NOTE. NY-ESO-1 expression is significantly higher in stage III patients ($P = 0.041$).

vention because of the lack of any tumor-specific antigen frequently expressed in CRC tumor tissue.

In this study, we determined the expression of 10 CT genes in 121 CRC samples and their adjacent normal tissue. We were able to detect relatively high expression frequency of MAGE-3 (27.3%) in CRC, which was similar to the results of Mori et al. (14). Over half of the CRC patients expressed at least one CT gene. There was a tendency for CT mRNA expression to be clustered [i.e., 33 (27.3%) tumor specimens were found to express multiple CT mRNAs simultaneously, whereas 53 specimens were negative for any CT genes]. This pattern was also found by Sahin et al. (15) in melanoma and breast cancer specimens and by Scanlan et al. (16) in non-small cell lung cancer. This clustering phenomenon is undoubtedly related to the activation/derepression process for CT genes in cancer. For instance, demethylation state in cancer might induce the activation of otherwise silent CT genes (8). It is also possible that activation of a single CT gene could be the switch for activating other CT genes.

Among 10 CT genes, we were particularly intrigued by the expression frequency of NY-ESO-1 (9.9%) and LAGE-1 (15.7%). The expression of these two genes has been reported in a wide variety of cancer types other than CRC (17). NY-ESO-1 and LAGE-1 were initially discovered by serologic analysis of recombinant cDNA expression library (SEREX) screening of an esophageal cancer cDNA library. These two genes are highly homologous (87% homology at amino acid level and 94% at nucleotide acid level) and reside within a 200 kb stretch on chromosome Xq28 (18). It is believed that they are among the most immunogenic antigens and are capable of inducing spontaneous humoral and cellular immune responses in up to 50% of patients with advanced NY-ESO-1-positive tumors (19, 20). In recent studies by Jäger et al. (21, 22), a humoral immune response to NY-ESO-1 was predictive of a strong CD8 T-cell response in melanoma patients. In our study, we were able to detect only one NY-ESO-1 antibody-positive serum in

Table 4 NY-ESO-1 expression correlated with lymph node metastasis ($P = 0.002$)

Lymph node metastasis	NY-ESO-1		Total	P
	Positive	Negative		
Yes	11	49	60	0.002
No	1	60	61	
Total	12	109	121	

Table 5 MAGE-4 expression significantly correlated with vessel emboli ($P = 0.025$)

	MAGE-4		Total	<i>P</i>
	Positive	Negative		
Vessel emboli				
Positive	14	27	41	0.025
Negative	13	67	80	
Total	27	94	121	

12 patients with NY-ESO-1–positive tumors, whereas 11 were advanced (stage III and IV) CRC. The rather low frequency of NY-ESO-1 antibody in patients with NY-ESO-1–positive advanced tumors was in accordance with the concept that CRC was poorly immunogenic. However, in a recent study, Scanlan et al. (23) reported five autologous NY-ESO-1 antibody–positive CRC cases in a total number of 74 patients by serum antibody detection array method, without testing the mRNA expression. The much higher antibody responses may be due to the much higher sensitivity of serum antibody detection array method. To confirm the existence of NY-ESO-1 protein expression, the 12 NY-ESO-1 mRNA–positive CRC tissues were immunohistochemically stained by E978, the specific NY-ESO-1 monoclonal antibody. The protein was detected in only one CRC tissue, which was from the patient who exhibited positive autologous anti-NY-ESO-1 antibodies. This result indicated that high expression level of protein might be a prerequisite for inducing high titer of autologous NY-ESO-1–specific humoral immune as we described in gastric cancer (24). Taken together, the expression and the preexisting autologous humoral immunity of NY-ESO-1 and LAGE-1 in CRC patients indicated that NY-ESO-1/LAGE-1 antigen could be candidate vaccine antigens of CRC.

Among 12 positive NY-ESO-1 mRNA samples detected, nine cases were in stage III, accounting for nearly 20% (9 of 50) of all stage III patients, two in stage IV, and 1 in stage II. There was a statistically significant difference of NY-ESO-1 expression in different stages with an obviously high frequency in stage III ($P = 0.041$). Furthermore, in our separate study of CT genes expression in gastric cancer, we detected similar results that *NY-ESO-1* gene was expressed predominantly in stage III (24). The correlation between *NY-ESO-1* expression and tumor progression were also shown in other studies [i.e., NY-ESO-1 mRNA was detected in 2 of 20 (10%) primary melanomas and in 15 of 32 (47%) metastatic melanomas; ref. 25]. In another study of bladder cancer, NY-ESO-1 mRNA expression correlated with tumor grade (26). We also observed that *NY-ESO-1* gene expression significantly associated with local lymph node metastasis ($P = 0.002$). NY-ESO-1 is the most immunogenic CT antigen identified thus far. Further study on the correlation of its expression with local lymph node metastasis may give us some clues on its immunogenicity. In addition, the presence of lymph node metastases after curative intent resection is still one of the most important poor prognostic factors (27–29). The significant correlation of NY-ESO-1 expression with local lymph node metastasis may indicate that (a) NY-ESO-1 expression could be a poor prognostic factor and that (b) because of the antibody and T cell responses it induced, NY-ESO-1 expression may also favor the prognosis of the patients with lymph node metastasis. Fur-

ther study comparing NY-ESO-1 expression/immune responses–positive patients with negative cases will be interesting.

In the current study, we also found an interesting correlation between MAGE-4 expressions with the occurrence of vessel emboli. By multivariate analysis method, the presence of vessel emboli is well correlated with metastasis and can be an independent prognostic factor for poor prognosis of CRC (30, 31). So we speculate that MAGE-4 can possibly predict the potential of metastasis and poor prognosis on the molecular level. A similar finding was also obtained by Mori et al. (14) who found that *MAGE* genes were frequently expressed in CRC patients with liver metastasis. The definitive role of *MAGE-4* in predicting the prognosis needs to be further studied.

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