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Expression of multiple cancer-testis antigen genes in gastrointestinal and breast carcinomas

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Summary Cancer-testis antigens (CTAs) such as *MAGE* are selectively expressed in various types of human neoplasms but not in normal tissues other than testis. This characteristic feature of CTAs makes them promising antigens for cancer-specific immunotherapy. A critical requirement for this therapy is identification of promising antigens. In this study, we investigated the expression of 6 genes recently identified by serological analysis of antigens by recombinant expression (SEREX) libraries: *NY-ESO-1*, *LAGE-1*, *SCP-1*, *SSX-1*, *SSX-2*, and *SSX-4*, in many surgical samples of gastrointestinal and breast carcinomas using reverse transcription-polymerase chain reaction. We found relatively high expression of *SCP-1* (23.5%) and *SSX-4* (20.6%) in gastric carcinoma, *LAGE-1* (39.1%) and *NY-ESO-1* (23.9%) in oesophageal carcinoma, and *SCP-1* (34.1%) in breast carcinoma. We also found frequent synchronous expression with *MAGE*, including *LAGE-1* (46.2%) in oesophageal carcinoma, *SSX-4* (46.7%) in gastric carcinoma, and *SCP-1* (38.3%) in breast carcinoma. Immunohistochemical analysis of the tumour samples expressing both *MAGE-4* and *NY-ESO-1* genes demonstrated differences in distribution between *MAGE-4* and *NY-ESO-1* in serial sections. We concluded that *NY-ESO-1*, *LAGE-1*, *SCP-1* and *SSX-4* genes may be promising candidates for cancer-specific immunotherapy in addition to *MAGE*, and that polyvalent cancer vaccines may be useful in cases of heterogeneous expressions of CTA genes in gastrointestinal and breast carcinomas. © 2001 Cancer Research Campaign   http://www.bjcancer.com

Keywords: *MAGE,* tumour-rejection antigens, cancer-testis antigen, immunotherapy, cancer vaccine

INTRODUCTION

Gastrointestinal and breast carcinomas are the most common cancers and are responsible for the largest numbers of cancer-related deaths in the world (Greenlee et al, 2000; Ries et al, 2000). However, therapeutic options for the treatment of patients with these tumours are limited to 3 fundamental modalities: surgical resection, radiation therapy and chemotherapy. Against advanced carcinomas, therapeutic options are limited to radiation therapy and chemotherapy, and these modalities do not yield results. Cancer-specific immunotherapy may be expected to become a novel treatment modality for gastrointestinal and breast carcinomas.

Many genes coding tumour rejection antigens such as *MAGE* (Van der Bruggen et al, 1991), *BAGE* (Boel et al, 1995) and *GAGE* (Van den Eynde et al, 1995) have been isolated from melanoma cell lines. These antigens are recognized by autologous cytotoxic T-lymphocytes (CTLs), which are restricted by human leukocyte antigen (HLA) class I molecules, and some antigenic peptides have been identified (Van den Eynde et al, 1997). Furthermore, these antigens are also reported to induce class II restricted T-cell responses (Chaux et al, 1999; Manici et al, 1999). Some of these antigens are expressed in various tumours of different histological origins, but not in normal tissues other than testis. Therefore, these antigens have been designated cancer-testis antigens (CTAs) (Gure et al, 1997) and their characteristics make them promising candidates for cancer-specific immunotherapy; clinical trials using

peptides such as MAGE-1 or MAGE-3 are in progress for malignant melanoma (Mukherji et al, 1995; Marchand et al, 1995).

We previously reported that *MAGE*-1 and *MAGE*-3 genes were expressed in 62% and 57% of oesophageal carcinoma (Inoue et al, 1997), 41% and 38% of gastric carcinomas (Inoue et al, 1995), 30% and 20% of colorectal carcinomas (Mori et al, 1996), 24% and 31% of breast carcinomas (Fujie et al, 1997), and more than 65% of hepatocellular carcinomas (Tahara et al, 1999). We also identified some MAGE gene-encoded peptides recognized by CTL (Tanaka et al, 1997; Fujie et al, 1999). Based on these findings, cancer-specific immunotherapy using the HLA class I restricted MAGE peptide has already begun in our institute for the treatment of gastrointestinal or breast carcinoma. Although this type of therapy appears promising and has thus far resulted in few side effects, application of this therapy is restricted by the tumour expression of the *MAGE* gene and patient HLA type.

Recently, a novel method has been established based on the specific recognition of autologous sera of some cancer patients, termed serological analysis of antigens by recombinant expression cloning (SEREX) (Sahin et al, 1995). This new method permits direct molecular determination of new tumour antigens that elicit an IgG antibody response in tumour patients. The method has enabled the discovery of several novel genes with tumour specificity, such as *NY-ESO-1* (Chen et al, 1997), *LAGE.* (Lethe et al, 1998), *SCP-1* (Türeci et al, 1998b), *SSX* (Türeci et al, 1996) and *CT7* (Chen et al, 1998). These antigens are also expected to become new candidates for cancer-specific immunotherapy, but little information is available on the comprehensive expression of CTAs in a large number of samples of gastrointestinal and breast carcinomas. In the present study, we compared the expression of newly found CTA genes with that of the *MAGE* gene in gastrointestinal and breast carcinomas, and investigated which CTAs are

Table 1 PCR amplification programs

Gene		Primers from 5′ to 3′	Temperature and duration Denaturation Annealing Extension	Cycle No.	Product. size
MAGE-1	f r	CGGCCGAAGGAACCTGACCCAG GCTGGAACCCTCACTGGGTTGCC	94˚C for 1 min 72˚C for 1 min 72˚C for 2 min	33	421 bp
MAGE-3	f r	TGGAGGACCAGAGGCCCCC GGACGATTATCAGGAGGCCTGC	94˚C for 1 min 72˚C for 1 min 72˚C for 2 min	33	725 bp
MAGE-4	f r	GAGCAGACAGGCCAACCG AAGGACTCTGCGTCAGGC	94˚C for 30 s 68˚C for 30 s 72 C for 30 s	30	446 bp
NY-ESO-1	f r	CGCCTGCTTGAGTTCTACCTC AGGGAAAGCTGCTGGAGACAG	94˚C for 1 min 59˚C for 1 min 72˚C for 1 min	30	218 bp
LAGE-1	f r	GCAGGATGGAAGGTGCCC CTGGCCACTCGTGCTGGGA	94˚C for 1 min 62˚C for 1 min 72˚C for 1 min	28	399 bp
SCP-1	f r	GAAGAAAGGATGTTGACACAAAT GTTTTTCCATAAGTGCTACCATT	94˚C for 1 min 54˚C for 1 min 72˚C for 1 min	42	487 bp
SSX-1	f r	CTAAAGCATCAGAGAAGAGAAGC AGATCTCTTATTAATCTTCTCAGAAA	94˚C for 1 min 54˚C for 1 min 72˚C for 1 min	32	422 bp
SSX-2	f r	GTGCTCAAATACCAGAGAAGATC TTTTGGGTCCAGATCTCTCGTG	94˚C for 1 min 54˚C for 1 min 72˚C for 1 min	32	435 bp
SSX-4	f r	AAATCGTCTATGTGTATATGAAGC GGGTCGCTGATCTCTTCATAAAC	94˚C for 1 min 56˚C for 1 min 72˚C for 1 min	32	415 bp
GAPDH	f r	GTCAACGGATTTGGTCGTATT AGTCTTCTGGGTGGCAGTGAT	94˚C for 1 min 56˚C for 1 min 72˚C for 1 min	24	540 bp

f: forward primer r: reverse primer.

useful candidates for cancer-specific immunotherapy for gastro-intestinal and breast carcinomas.

MATERIALS AND METHODS

Cell lines

The fibrosarcoma cell line HT1080 was kindly provided by the Institute of Development, Aging and Cancer, Tohoku University, Sendai, Japan. The gastric carcinoma cell line Ns-8, and the choriocarcinoma cell lines SCH, were supplied by the Japanese Cancer Research Bank, Tokyo, Japan. The melanoma cell lines MZ-2, and LB-373-MEL, were kindly provided by Dr Bernard Lethe, Ludwig Institute for Cancer Research Brussels Branch, Bruxelles, Belgium.

Tissue samples

Tumour samples (46 oesophageal squamous cell carcinomas, 102 gastric adenocarcinomas, 98 colorectal adenocarcinomas, and 129 breast adenocarcinomas) and matched control samples of the normal tissue located 5 cm away from the tumour edge were frozen immediately in liquid nitrogen less than 5 min after surgical resection and kept at –90˚C until RNA extraction. The surgical

samples were obtained from the Department of Surgery, Medical Institute of Bioregulation, Kyushu University, Beppu, Department of Surgery, Oita Prefectural Hospital, Oita, and the Department of Surgery, Saitama Cancer Center, Saitama, Japan.

Extraction of RNA and RT-PCR analysis

The acid guanidium thiocyanate-phenol-chloroform extraction procedure (Chomczynski and Sacchi, 1987) was used for extraction of total RNA. The cDNA was synthesized from 8.0 μg of total RNA as described previously (Inoue et al, 1995). The presence of *MAGE-1* and *MAGE-3* cDNA in the reverse transcription products was detected by polymerase chain reaction (PCR) amplification in separate reactions, using oligonucleotide primers located in the different exons of each gene (Weynants et al, 1994). The presence of *NY-ESO-1*, *LAGE-1*, *SSX-1*, *SSX-2*, *SSX-4* and *SCP-1* were determined by PCR amplification under the same conditions as reported previously (Lethe et al, 1998, Türeci et al, 1998a, Türeci et al, 1998b). Briefly a 1/100 aliquot of reverse transcription products were amplified in a 30 μl reaction mixture containing 10 nmoles of each dNTP (dATP, dTTP, dCTP, dGTP.), 1 μl of each oligonucleotide primer at 10 μM, 3 μl of 10 × PCR buffer (Perkin–Elmer, Branchburg, NJ, USA), and 1 U of AmpliTaq DNA polymerase (Perkin–Elmer). In the detection of *SCP-1*, 2 U

of AmpliTaq Gold (Perkin–Elmer) were substituted for AmpliTaq DNA polymerase. The reaction mixtures were then subjected to the appropriate PCR programmes as listed in Table 1. To confirm the specificity of the PCR products of the genes, we cloned the PCR product into pCRII vector (Invitrogen, San Diego, CA, USA) and then sequenced the cDNA by using the chain-termination DNA sequencing method. We then determined the nucleotide sequence of representative samples of PCR products and

confirmed them to be identical to the expected fragments of cDNA in each CTA gene. An 8 μl aliquot of each PCR product was separated on a 1.5% agarose gel and visualized with ethidium bromide staining. The pattern of expression of the CTA genes was established with reverse transcription-PCR (RT-PCR) assays by evaluating the intensity of a band in agarose gels; if the band was recognized, the case was positive. The integrity of the RNA was confirmed by performing PCR amplification of each cDNA with
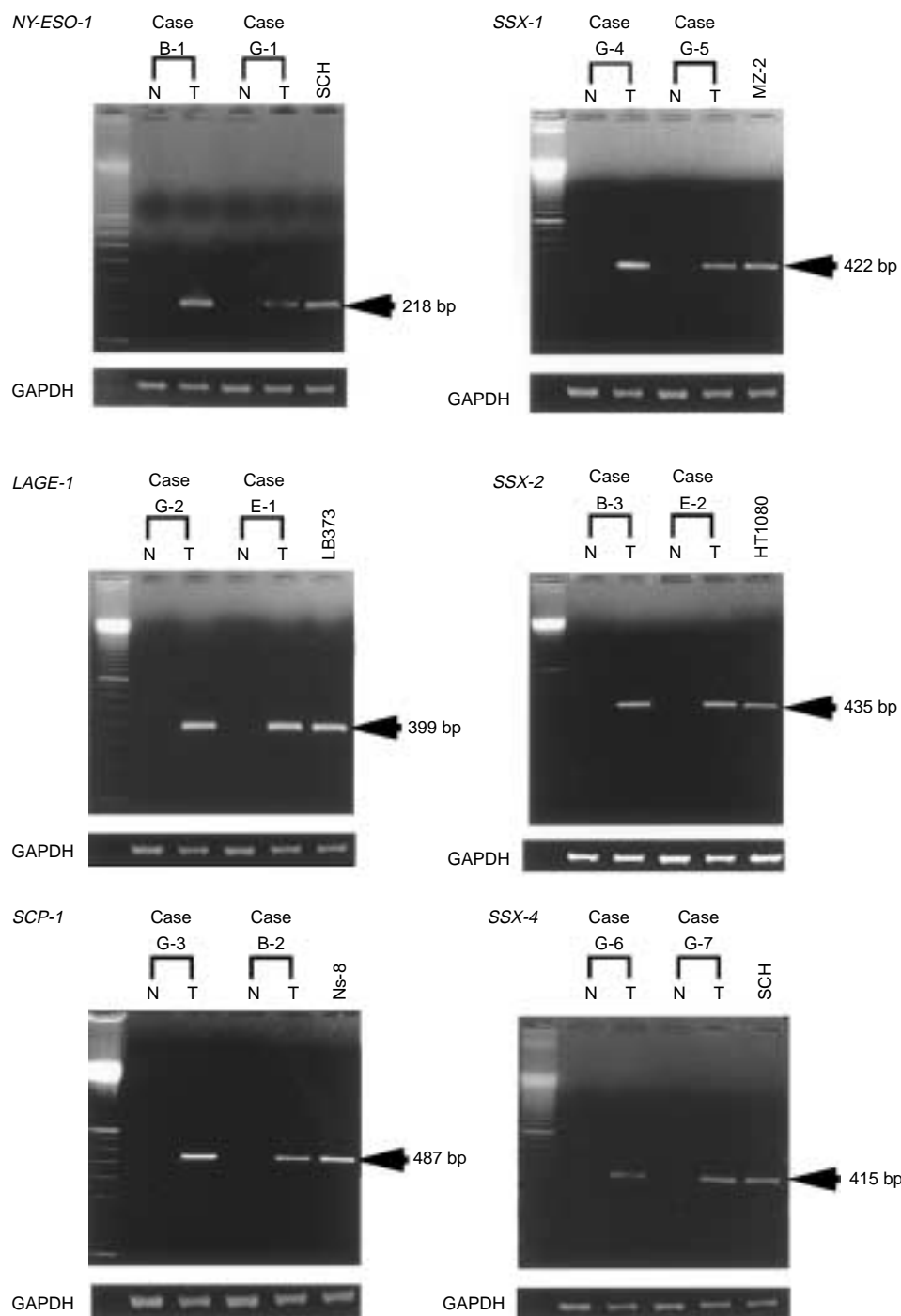
Figure 1   Detection of *NY-ESO-1*, *LAGE-1*, *SCP-1*, *SSX-1*, *SSX-2* and *SSX-4* mRNA expression by RT-PCR in 2 representative cases of oesophageal carcinoma (Cases E-1, 2), 7 cases of gastric carcinoma (Cases G-1–7), and 3 cases of breast carcinoma (Cases B-1–3). cDNA extracted from tumour tissue (T) and paired normal tissue (N) was amplified in each case. Only tumour sites expressed CTA genes and there was no sample with expression of CTA genes in a normal site. GAPDH mRNA expression is shown as the corresponding control in the lower panel. Only positive cases were chosen to be presented

primers for the gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Tokunaga et al, 1987).

Immunohistochemistry

The primary antibodies used in this study were 57B and B9.8 mAb. Both were kindly provided by Dr GC Spagnoli (Kocher et al, 1995, Schultz et al, 2000). 57B mAb stains tumour tissues that express *MAGE-4* (Landry et al, 2000), and B9.8 mAb stains tumour tissues that express *NY-ESO-1* (Schultz et al, 2000). Frozen tumour sections were thawed, washed in PBS, fixed in formalin and incubated overnight at 4°C in the presence of hybridoma supernatant from 57B or B9.8. For both preparations, bound antibodies were visualized using a Dako LSAB Peroxidase Kit (Dako), according to the manufacturer's recommendations, and cell nuclei were counterstained with hematoxylin.

Clinico-pathologic data

All data including sex, histology, tumour size, depth of tumour invasion, lymph node metastasis, lymph vessel permeation, vascular vessel permeation, and disease stage were obtained from the clinical and pathologic records. Disease stage was classified according to the criteria proposed by the Japanese Society of Oesophageal Disease (8th edn), the Japanese Research Society of Gastric Cancer (12th edn), the Japanese Research Society of Colon Cancer (5th edn), and the Japanese Breast Cancer Society (12th edn). Tumours with or without expression of the CTA genes were then compared.

Statistical analysis

Statistical analysis was performed by the chi-square or Fisher's exact test. The level of significance was set at $P < 0.05$.

RESULTS

Expression of CTA genes: cultured cell lines and clinical samples

The expression of 6 CTA genes, *NY-ESO-1*, *LAGE-1*, *SCP-1*, *SSX-1*, *SSX-2* and *SSX-4*, was determined by RT-PCR analysis. Each CTA was expressed in various types of carcinoma cell line. *NY-ESO-1* and *SSX-4* were expressed in SCH, *LAGE-1* in LB-373, *SCP-1* in Ns-8, *SSX-1* in MZ-2, and *SSX-2* in HT1080. These cell lines were used as positive controls in each analysis. In clinical samples, all 6 CTA genes were expressed exclusively in carcinoma

tissues. None of the 6 CTA genes were expressed in the paired normal tissues (Figure 1).

Frequency of expression of CTA genes in tumour samples

Expression of the 6 CTA genes was examined in 46 samples of oesophageal carcinoma, 102 samples of gastric carcinoma, 98 samples of colorectal carcinoma, and 129 samples of breast carcinoma. There was no CTA gene expressed more frequently than the *MAGE-1* and *MAGE-3* genes. We found relatively high expression of *LAGE-1* (39.1%) in oesophageal carcinoma, *SCP-1* (23.5%) and *SSX-4* (20.6%) in gastric carcinoma, and *SCP-1* (34.1%) in breast carcinoma, while we found high expression of none of the CTA genes including *MAGE-1* and *MAGE-3* in colorectal carcinoma. Table 2 summarizes the results of RT-PCR analysis of the CTA gene expression.

Expression of the 6 CTA genes correlated with none of the clinico-pathological parameters examined. There was no significant relationship between the expression of the 6 CTAs and clinical parameters for any type of carcinoma (data not shown).

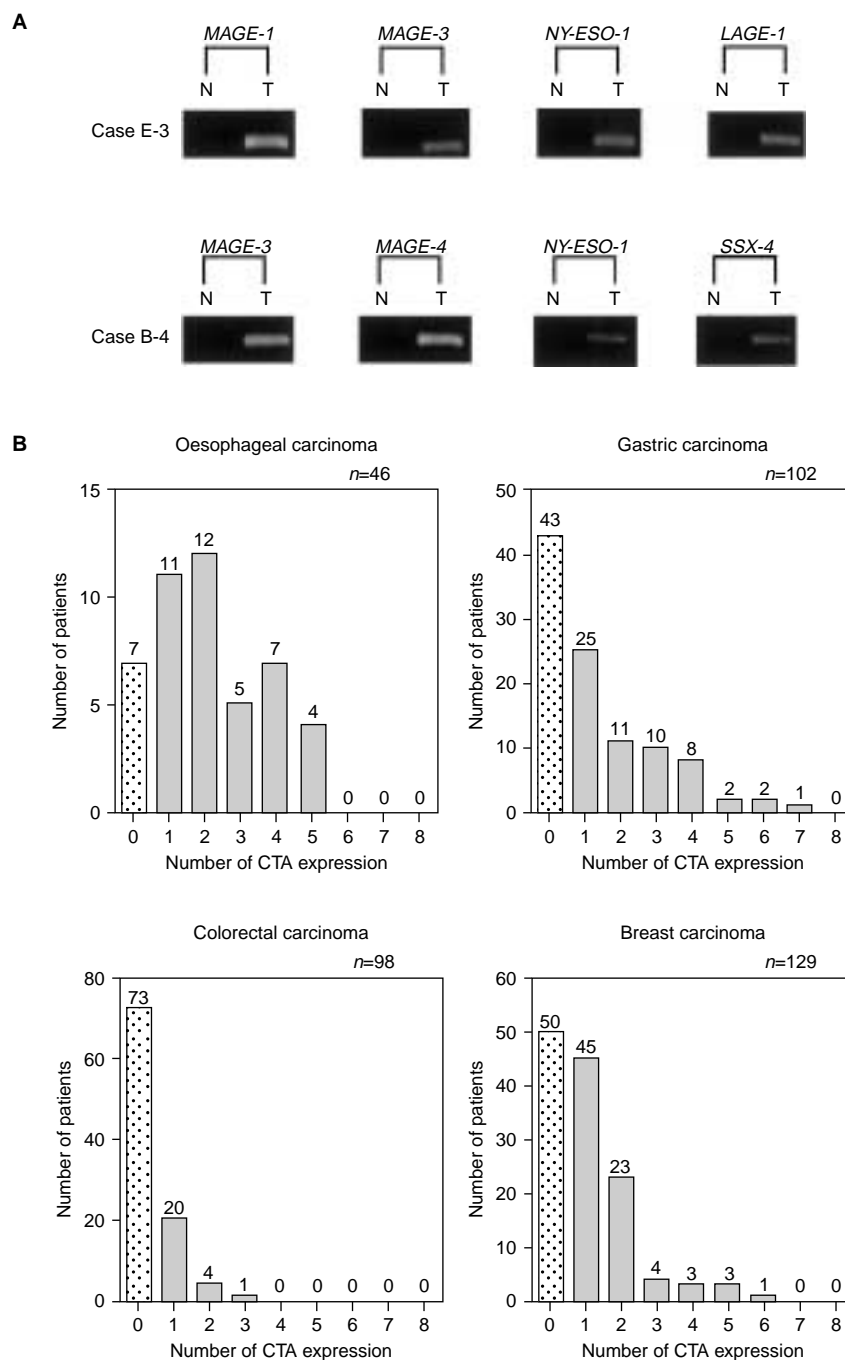## Synchronous expression of multiple CTA genes in the same tumour sample

Next, we analyzed multiple expression of the 8 CTA genes including *MAGE-1* and *MAGE-3* in each case. As shown in Figure 2A, synchronous expression of *MAGE-1*, *MAGE-3*, *NY-ESO-1*, and *LAGE-1* were detected in a clinical sample of oesophageal carcinoma (Case E-1). Oesophageal carcinoma expressed 2.1 CTA genes on average, the largest number among the 4 types of carcinomas examined in this study. In oesophageal carcinoma, 39 (84.7%) of 46 samples expressed at least 1 CTA gene and 28 (60.9%) of 46 samples expressed at least 2 CTA genes synchronously. In colorectal carcinoma, 25 (20.4%) of 98 samples expressed at least 1 CTA gene, and only 5 (5.1%) of 98 samples expressed multiple CTA genes synchronously. The results for gastric and breast carcinoma are summarized in Figure 2B.

Synchronous expression of CTA genes with *MAGE* genes

We analyzed the synchronous expression of CTA genes with *MAGE-1* or *MAGE-3* gene. A total of 18 (46.2%) of 39 oesophageal carcinoma samples expressed *LAGE-1* with synchronous expression of *MAGE-1* or *MAGE-3* genes, 21 (46.7%) of 45 gastric carcinomas synchronously expressed *SSX-4*, and 18

Table 2   Expression of CTA genes in gastrointestinal and breast carcinomas

|	NY-ESO-1 (%)	LAGE-1 (%)	SCP-1 (%)	SSX-1 (%)	SSX-2 (%)	SSX-4
Oesophageal carcinoma *n* = 46	11 (23.9)	18 (39.1)	1 (2.2)	0 (0)	0 (0)	6 (13.0)
Gastric carcinoma *n* = 102	8 (7.8)	12 (11.8)	24 (23.5)	3 (2.9)	3 (2.9)	21 (20.6)
Colorectal carcinoma *n* = 98	2 (2.0)	3 (3.1)	0 (0)	0 (0)	2 (2.0)	2 (2.0)
Breast carcinoma *n* = 129	13 (10.1)	4 (3.1)	44 (34.1)	1 (0.8)	5 (3.9)	12 (9.3)

Figure 2 (**A**) Synchronous expression of multiple CTA genes in the oesophageal and breast carcinoma cases detected by RT-PCR. Case E-3, oesophageal carcinoma with expression of *MAGE-1*, *MAGE-3*, *NY-ESO-1*, and *LAGE-1*. Case B-4, breast carcinoma with expression of *MAGE-3*, *MAGE-4*, *NY-ESO-1*, and *SSX-4*. cDNA extracted from tumour tissue (T) and paired normal tissue (N) were amplified in each case. (**B**) Expression of 8 CTA genes (*MAGE-1*, *MAGE-3*, *NY-ESO-1*, *LAGE-1*, *SCP-1*, *SSX-1*, *SSX-2*, *SSX-4*) in 46 oesophageal carcinomas, 102 gastric carcinomas, 98 colorectal carcinomas, and 129 breast carcinomas. Number of CTA genes expressed per clinical sample is summarized. Synchronous expression of multiple CTA genes was detected in 28 (61%) of 46 oesophageal carcinoma samples, 34 (33%) of 102 gastric carcinoma samples and 34 (26%) of 129 breast carcinoma samples

(38.3%) of 47 breast carcinomas synchronously expressed *SCP-1*. We also analysed the cases with expression of neither *MAGE-1* nor *MAGE-3* genes to determine targets for cancer immunotherapy other than *MAGE* genes. A total of 26 (31.7%) of 82 samples of breast carcinoma and 12 (21.1%) of 57 samples of gastric carcinoma expressed the *SCP-1* gene, with expression of neither *MAGE-1* nor *MAGE-3*. These results are summarized in Figure 3.

Differential distribution of carcinoma cells staining with CTA protein-specific mAbs in carcinoma tissues

We investigated the distribution of carcinoma cells expressing CTA proteins in tumour tissues by immunohistochemical analysis for representative cases. The primary antibodies used in this study were 57B, a murine mAb against MAGE-4 protein (Landry et al,
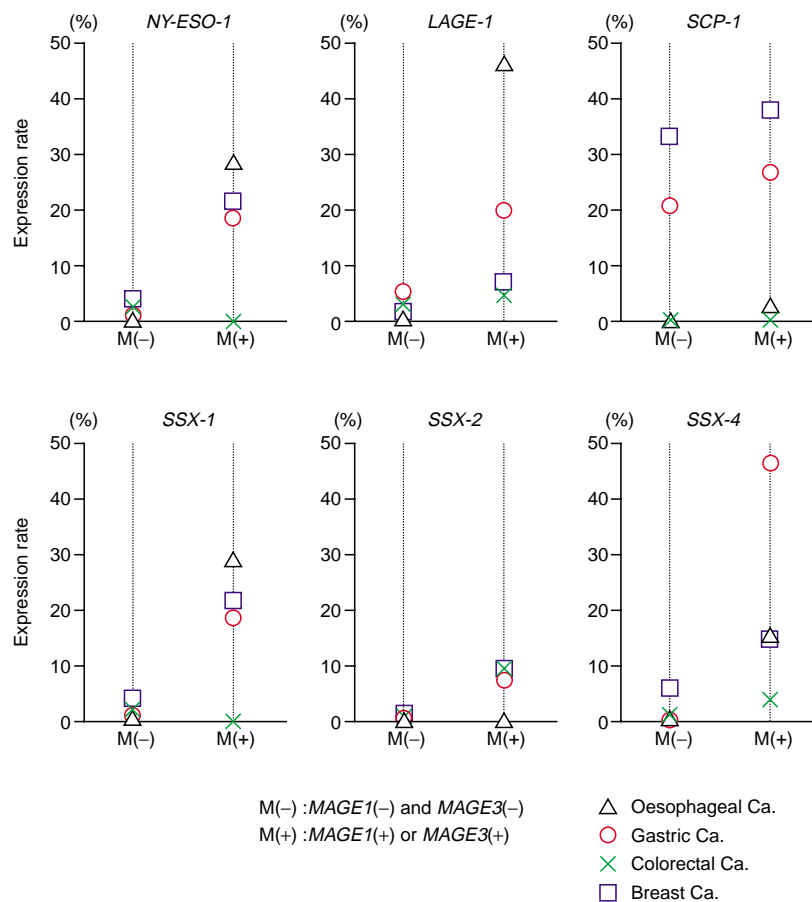
Figure 3 Comparison of CTA gene expression between *MAGE-1*-or *MAGE-3*-positive cases, M (+) and both the *MAGE-1*- and *MAGE-3*-negative cases, M (–). *MAGE*-positive cases tended to express the other CTAs frequently. *LAGE-1* in oesophageal carcinoma, *SSX-4* in gastric carcinoma and *SCP-1* in breast carcinoma were synchronously expressed with *MAGE* genes frequently. *SCP-1* tended to be expressed in both *MAGE*-positive and -negative cases

2000), and B9.8, a murine mAb against NY-ESO-1 protein (Schultz et al, 2000). Case B-4 was one of breast carcinoma, in which *MAGE-3*, *MAGE-4*, *NY-ESO-1*, and *SSX-4* were detected in RT-PCR analysis (Figure 2A). Heterogeneity of 57B and B9.8 staining was detected in the tumour specimens of Case B-4. The staining, which was mostly detectable in the cell cytoplasm, appeared to be limited to carcinoma cells. The tumour cells stained with 57B and B9.8 revealed the differential distribution of MAGE-4 and NY-ESO-1 in breast carcinoma tissues (Figure 4). We examined 5 cases expressing both *MAGE-4* and *NY-ESO-1* genes and revealed the differential distribution in 2 cases and the same distribution in 3 cases.

DISCUSSION

In our institute, clinical trials of cancer-specific immunotherapy have been ongoing on patients with gastrointestinal carcinomas, using antigen-presenting cells pulsed with MAGE peptides. Good results were observed in some clinical cases, but there are two problems that need to be solved. One of the problems is the restriction of candidates for immunotherapy. The prerequisite for therapy is *MAGE* gene expressions in tumour tissues and adaptation of patient HLA type to peptide-binding specificity. Another is the heterogeneous expression pattern of MAGE, which may be a strategy for evasion of immunosurveillance by malignant cells

(Jungbluth et al, 2000; Sadanaga et al, 1999). In the present study, we found 3 novel possibilities for a solution of these problems.

First, we found high expression of CTAs other than *MAGE-1* or *MAGE-3* in gastric and oesophageal carcinomas. We found high expression of *SCP-1* (23%) and *SSX-4* (20%) genes in gastric carcinoma and of *NY-ESO-1* (19%) and *LAGE-1* (38%) genes in oesophageal carcinoma. High expression of *SSX* genes was detected in a wide variety of neoplasms such as head and neck carcinomas, colorectal carcinoma, and breast carcinoma (Türeci et al, 1998a). We also found high expression of *SSX-4* in the gastric carcinoma, in which either the *MAGE-1* or *MAGE-3* gene was co-expressed. These findings suggest that these antigens might be targets for cancer-specific immunotherapy in addition to MAGE-1 and MAGE-3. *NY-ESO-1* and *LAGE-1* were also reported to be expressed in a large number of breast carcinomas (Chen et al, 1997; Lethe et al, 1998), but we could not detect a high rate of expression of either gene in breast carcinomas. These differences might potentially contribute to the disparity in survival rate after breast cancer among the various races (Dignam, 2000).

Secondly, we found high expression of *SCP-1* in cases expressing neither MAGE-1 nor MAGE-3 in gastric and breast carcinomas. The *SCP-1* gene was expressed in 26 (31.7%) of the 82 samples of breast carcinoma expressing neither *MAGE-1* nor *MAGE-3*, and was expressed in 12 (21.2%) of 57 samples of gastric carcinoma expressing neither *MAGE-1* nor *MAGE-3*. In
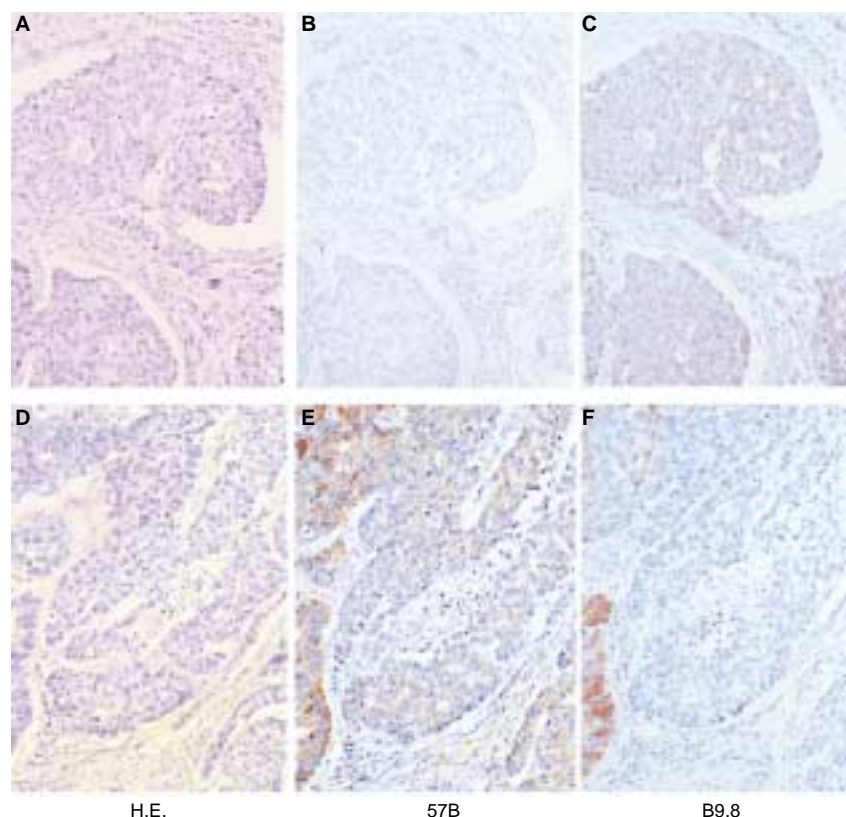
Figure 4 Immunohistochemical and RT-PCR analysis of cases expressing multiple CTAs. As shown in Figure 2A, RT-PCR analysis revealed synchronous expression of *MAGE-3*, *MAGE-4*, *NY-ESO-1*, and *SSX-4* in this breast carcinoma case, Case B-4. Immunohistochemical staining with 57B and B9.8, consecutive sections of Case B-4 (**A–F**). The upper panels show NY-ESO-1 protein immunoreactivity of B9.8 mAb (**C**) was detected in the carcinoma cells not expressing the MAGE-4 gene product (**B**). The lower panels show MAGE-4 protein immunoreactivity of 57B (**E**) was detected in the carcinoma cells not expressing NY-ESO-1 gene product (**F**). Haematoxylin-eosin staining of the sections is shown in (**A**) and (**D**). (Original magnification × 200)

addition, *SCP-1* was expressed more frequently than *MAGE-1* and *MAGE-3* in breast carcinoma. These results suggest that if *SCP-1* is a new target antigen in addition to *MAGE-1* and *MAGE-3*, the candidates for CTA-based cancer immunotherapy for gastric and breast carcinomas will be increased in number. *SCP-1* was identified as HOM-TES-14, during screening of a cDNA library enriched for testis-specific clones with serum from a renal cell carcinoma patient (Türeci et al, 1998b). High levels of expression of *SCP-1* in tumour tissue and normal testis are recognized at the mRNA and protein levels by RT-PCR and Western blot analysis, respectively. The antigenic peptides encoded by these CTAs will be identified in future studies. Patients' opportunities for cancer-specific immunotherapy can thus be expanded.

Thirdly, we found a high frequency of synchronous expression of multiple CTA genes in clinical tumour samples. At least 2 CTA genes were expressed in 28 (61%) of 46 oesophageal carcinomas, 34 (33%) of 102 gastric carcinomas, and 34 (26%) of 129 breast carcinomas (Figure 3). In the present study, we examined representative samples of breast carcinoma with immunohistochemical analysis of 57B or B9.8 antibodies. Antibody 57B stains the tumours that express the *MAGE-4* gene, and antibody B9.8 recognized tumours that expressed the *NY-ESO-1* gene (Landry et al, 2000; Schultz et al, 2000). As shown in Figure 2A and Figure 4, Case B-4 expressed both *MAGE-4* and *NY-ESO-1*, and immunohistochemical analysis revealed differential distribution of MAGE-4-or NY-ESO-1-positive carcinoma cells. We and others have reported heterogeneous expression of *MAGE* genes in tumour tissues using immunohistochemical analysis (Jungbluth et al, 2000; Sadanaga et al, 1999). In this study, we found the heterogeneous expression of MAGE-4 and NY-ESO-1 in carcinoma tissues, indicating that some parts of carcinoma cells were stained with 57B antibody, and others with B9.8 antibody (Figure 4). This differential distribution of staining of carcinoma cells indicates that the area of carcinoma cells expressing tumour rejection antigens might be enlarged. Therefore, in this case, a combined vaccination based on both MAGE-4 and NY-ESO-1 may cause immunological reactions in a larger area of tumour tissues than a single peptide-based vaccination. Taken together, our findings suggest that polyvalent vaccinations with multiple antigens might be necessary to obtain good clinical results in cancer immunotherapy.

Despite shared expression of multiple CTAs in tumour species, individual antigens exhibit distinct variation in quantity. Immunohistochemical analysis revealed heterogeneous expression, and RT-PCR analysis revealed distinct intensity of a band in agarose gels. It is important to evaluate the quantity of expression of each antigen when use of immunotherapy is decided.

In conclusion, the predominant expression of *NY-ESO-1* and *LAGE-1* in oesophageal carcinoma, *SSX-4* and *SCP-1* in gastric carcinoma and *SCP-1* in breast carcinoma suggest possible targets for cancer-specific immunotherapy in addition to *MAGE-1* and *MAGE-3*. Furthermore, *SCP-1* could be a novel target for patients

without expression of *MAGE-1* and *MAGE-3* genes in gastric and breast carcinomas. We also demonstrate the possibility that polyvalent vaccination with multiple CTA genes will yield effective clinical results for cancer-specific immunotherapy.

ACKNOWLEDGEMENTS

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