ABSTRACT

NY-ESO-1 is a cancer/testis antigen expressed in normal adult tissues solely in the testicular germ cells of normal adults and in various cancers. It induces specific humoral and cellular immunity in patients with NY-ESO-1-expressing cancer. The aim of this study was to determine the frequency of NY-ESO-1 mRNA and protein expression in malignant and benign breast tumors. NY-ESO-1 mRNA expression was detected by conventional reverse transcription-PCR and real-time PCR, and that of the protein expression by immunohistochemistry and Western blot analysis. Expression of NY-ESO-1 mRNA was detected in 37 of 88 (42%) cancer specimens, whereas that of the NY-ESO-1 protein was detected only in 1 mRNA-positive specimen. In the latter case, expression level of NY-ESO-1 mRNA relative to that in the testis was relatively high (75% of testicular expression) and to the other among breast cancer specimens. In benign breast lesions, 21 of 31 (68%) specimens expressed low levels of NY-ESO-1 mRNA. In 1 case of fibroadenoma, NY-ESO-1 mRNA was 8% of the testicular level, and protein was detected by Western blot analysis. Only 1 breast cancer patient had detectable antibody at time of surgery, which disappeared within 2 years. Tumor specimen from this patient was both NY-ESO-1 mRNA and protein positive, and NY-ESO-1-specific CD8 T cells were detected in this patient by IFN-γ enzyme-linked immunospot assay using NY-ESO-1 recombinant adenov and vaccinia virus. A higher rate of NY-ESO-1 expression was noted in breast cancer with high histological grade and negative hormone receptor status, suggesting NY-ESO-1 as a potential tumor antigen for immunotherapy in patients with breast cancer and poor prognosis.

INTRODUCTION

A number of human tumor antigens have been identified since the MAGE-A antigen recognized by CTLs was first identified on malignant melanoma (1, 2). These antigens are categorized into various classes such as differentiation antigens (e.g., tyrosinase and MelanA/MART-1), antigens derived from gene overexpression/amplification (e.g., HER-2/neu), or mutation (e.g., p53), cancer/testis (CT) antigens (e.g., MAGE and NY-ESO-1), and viral antigens (e.g., human papillomavirus and EBV; Ref. 2). Among them, the CT antigens have received particular attention as potential vaccine targets because of their unique tissue expression (3, 4). To date, >15 genes or gene families encoding CT antigens have been identified (5). NY-ESO-1, one of the CT antigens, appears to be strongly immunogenic and is known to induce an efficient humoral immune response in patients with melanoma and other tumor types (6, 7). Approximately 50% of patients with advanced NY-ESO-1 mRNA-positive melanoma produce NY-ESO-1 antibody. In contrast to NY-ESO-1, only a few patients with melanomas expressing other CT antigens show antibody production. Furthermore, it has been demonstrated that NY-ESO-1 can elicit CD8 and CD4 T-cell response in seropositive patients (7–11).

Expression of several CT antigens has been investigated in breast cancer. MAGE-A1 is expressed in ~8%, MAGE-A3 in 14%, SSX-2 in 8%, and SCP-1 in 31% of breast cancers. Furthermore, NY-ESO-1 was reported to be expressed in 10–30% of breast cancers (6, 11–13). It has been noted that patients with benign breast lesions are at a relatively high risk for the development of breast cancer (14). For example, the risk of breast cancer in women with fibroadenoma and fibrocystic disease is 1.5–5.0 times that of age-matched women (14).

The aim of the present study was to determine the frequency of expression of NY-ESO-1 mRNA in malignant and benign breast tumors. The study also determined the rate of NY-ESO-1 protein expression by immunohistochemistry (IHC) and Western blot analysis, antibody response against NY-ESO-1 by ELISA using recombinant protein, and CD8 T-cell response against NY-ESO-1 by IFN-γ enzyme-linked immunospot assay. The results showed a high frequency of NY-ESO-1 mRNA expression in breast cancer in the Japanese population. Importantly, an even higher NY-ESO-1 mRNA expression rate was observed in benign breast lesions.
follows, ESO1-1, 5′-AGTCTTATCTGCGATGC-3′ and ESO1-2, 5′-TCCTCCCTCCGAGACACAAC-3′; ESO1-A, 5′-cagacagactcGCTTACGCTGACCC-3′ and ESO1-B, 5′-cagacagactcGCTTACGCTGACCC-3′ and ESO1-AN, 5′-GCCTGGCGTTGCCTGCT-3′. The integrity of each RNA sample was verified by performing RT-PCR for porphobilinogen deaminase. PCR products were visualized with ethidium bromide by electrophoresis on a 2% agarose gel.

In the NY-ESO-1 cDNA-specific real-time quantitative PCR assay, the reaction mixture contained 250 nM of ESO1-forward and 250 nM of ESO1-reverse primers, 200 nM TaqMan probe, 25 μl TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA), and 1 μl of cDNA, in a total reaction volume of 50 μl. After enzyme activation for 10 min at 95°C, 50 two-step cycles were performed (20 s at 95°C and 60 s at 64°C) by ABI PRISM 7700 Sequence Detection System (Applied Biosystems). Primers and TaqMan probe were designed as follows: ESO1-forward, 5′-GGCAAGATG-GATGCTGAGGA-3′; ESO1-reverse, 5′-CTGGAGGACAGCGTGTAGGA-3′; and TaqMan probe, 5′-FAM-TGGTGCCGCAAACATCTGACCT-CGA-TAMRA-3′. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was measured by TaqMan Human GAPDH Control Reagents (Applied Biosystems) for normalization. To transform the cycle threshold (Ct) values into absolute mRNA copy numbers, we used dilution series of linearized plasmid containing the NY-ESO-1 insert and constructed a calibration curve. Wells with no template were used for negative control.

IHC. Tissue specimens were deparaffinized and microwave-heated in an antigen retrieval buffer (10% citrate buffer [pH 6.0] for 20 min). After inactivation of endogenous peroxidase, and endogenous avidin and biotin activities (Avidin/Biotin Blocking Kit; Vector Laboratories, Burlingame, CA), specimens were incubated with 1.5% horse serum for 30 min at room temperature. Monoclonal antibody specific for NY-ESO-1, ESO1-A, ESO1-B, or ESO1-AN was then added at a concentration of 5 μg/ml and incubated overnight at room temperature. Isotype-matched controls were included in all of the assays (mouse IgG1; ICN Pharmaceuticals, Costa Mesa, CA). After washing, biotinylated horse-antimouse IgG (Vectastain Elite ABC kit; Vector Laboratories, CA) was applied and incubated for 30 min at room temperature. Avidin-labeled peroxidase (Vectastain Elite ABC kit, Vector Laboratories) was added after washing and incubated for 30 min at room temperature. Diaminobenzidine tetrahydrochloride was then added for development, followed by counterstaining with hematoxylin solution (16, 17). Specimens of testis were used as positive control IHC.

Western Blot. Fifty μg lysates of cells or tissues prepared with HEPES buffer (HEPES 50 mM, NaCl 150 mM, EDTA 2.5 mM, EGTA 1.0 mM, Tween 20 0.1%, Glycerol 10%, DTT 10 μM, leupeptin 10 μg/ml, and phenylmethylsulfonyl fluoride 1.0 μM) were subjected to 12.5% polyacrylamide gel (Readygels J; Bio-Rad Laboratories, Hercules, CA). After washing, biotinylated horse-antimouse IgG (Vectastain Elite ABC kit; Vector Laboratories, CA) was applied and incubated for 30 min at room temperature. Avidin-labeled peroxidase (Vectastain Elite ABC kit, Vector Laboratories) was added after washing and incubated for 30 min at room temperature. Diaminobenzidine tetrahydrochloride was then added for development, followed by counterstaining with hematoxylin solution (16, 17). Specimens of testis were used as positive control IHC.

Results

Expression of NY-ESO-1 mRNA in Malignant Breast Tumors.

Expression of NY-ESO-1 mRNA in breast cancer was analyzed by conventional RT-PCR using three different sets of primers. NY-ESO-1 mRNA was detected in 37 of 88 (42%) cancer specimens (Table 1; Fig. 1). No discrepancy was observed with the three sets of primers, ESO1-1 and ESO1-2, ESO1-A and ESO1-B, and ESO1-AN and ESO1-BN. The expression was observed in ID3391, ID3394, and ID3360. Normal tissues was used as positive control. In all, three benign breast lesions (B) ID2190, ID1725, and ID2091 and malignant (M) and normal (N) tissues from the same tumor specimens of ID3220 and ID3459 were examined. The expression was observed in ID1725, ID2091, and ID3459. No expression was observed in normal tissues.

Table 1

<table>
<thead>
<tr>
<th>NY-ESO-1 expression</th>
<th>Breast cancer</th>
<th>Benign breast lesion</th>
</tr>
</thead>
<tbody>
<tr>
<td>RT-PCR</td>
<td>IHC</td>
<td>Antibody positive/sera examined</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>1</td>
</tr>
<tr>
<td>+</td>
<td>-</td>
<td>23</td>
</tr>
<tr>
<td>+</td>
<td>ND</td>
<td>13</td>
</tr>
<tr>
<td>−</td>
<td>+</td>
<td>0</td>
</tr>
<tr>
<td>−</td>
<td>ND</td>
<td>31</td>
</tr>
<tr>
<td>Total</td>
<td>88</td>
<td>1/62</td>
</tr>
</tbody>
</table>

*RT-PCR, reverse-transcription PCR; IHC, immunohistochemistry; ND, not done.

μg/ml, and the ECL detection system (Amersham Pharmacia Biotech, Dübendorf, Switzerland) was used as the detection reagent.

ELISA. ELISA was performed using recombinant NY-ESO-1 protein according to the method described previously by Stockert et al. (11).

Generation of Viral Vectors. Adenoviral constructs encoding NY-ESO-1 were provided by Genzyme Corporation (Farmington, MA). Vaccinia virus constructs encoding NY-ESO-1 were provided by Theron Biologies (Cambridge, MA). These constructs have been described previously (10, 19).

Infection of Antigen-Presenting Cells or Target Cells with Recombinant Viruses. For antigen presenting cells (APC), CD8 T-cell-depleted PBLS were infected with adenovirus/NY-ESO-1 recombinant virus at 100 IU/cell for 20 h at 37°C in 300 μl X-VIVO-15 (Bio-Whittaker, Walkersville, MA). For target cells, 1 x 10^5 PBLS cultured with 200 units/ml interleukin (IL)-6 (Peprotech, London, United Kingdom) and 10 ng/ml IL-12 (Peprotech, London, United Kingdom) for 1 week were infected with vaccinia/NY-ESO-1 recombinant virus or vaccinia virus wild-type at 30 plaque-forming units/cell for 20 h at 37°C in 300 μl X-VIVO-15.

In Vitro Sensitization of CD8 T-Cells with Adenoviral Constructs. CD8 T cells were purified from PBLS using antibody-coated magnetic beads (Miltenyi Biotec, Alburn, CA). Five x 10^5 CD8 T-cells were cocultured with irradiated 2 x 10^5 APCs infected with adenovirus/NY-ESO-1 recombinant virus in 24-well plates (Becton Dickinson, Franklin Lakes, NJ) in RPMI 1640 containing 5% human AB serum (Sigma, St. Louis, MO), 2 mM l-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, and 1% nonessential amino acids for 10 days at 37°C in 5% CO_2 atmosphere. Ten units/ml IL-2 (Takeda, Osaka, Japan) and 20 ng/ml IL-7 (Peprotech) were added every 3 days.

Enzyme-linked immunospot Assay. Responding CD8 T-cells (2 x 10^5) and target cells (5 x 10^5 or 1 x 10^5) were cultured in 96-well nitrocellulose plates (Millipore, Bedford, MA) precoated with 2 μg/ml anti-human IFN-γ Monoclonal antibody (1-D1K; Mabtech, Stockholm, Sweden) for 20 h at 37°C in RPMI 1640 without IL-2 and human serum. After washing, rabbit anti-human IFN-γ serum diluted at 1:800 with PBS was added and incubated for 2 h at 37°C. After washing extensively, goat antirabbit IgG serum conjugated with alkaline-phosphatase (Southern Biotechnology, Birmingham, AL) diluted at 1:2000 was added and incubated for 1 h at 37°C. After washing, substrate (AP conjugate substrate kit; Bio-Rad Laboratories) was added and incubated for 15 min. In the final step, the plates were washed and the spots were counted under a microscope.

Results

Expression of NY-ESO-1 mRNA in Malignant Breast Tumors.

Expression of NY-ESO-1 mRNA in breast cancer was analyzed by conventional RT-PCR using three different sets of primers. NY-ESO-1 mRNA was detected in 37 of 88 (42%) cancer specimens (Table 1; Fig. 1). No discrepancy was observed with the three sets of primers. The size of PCR product was similar to that in the tests. The PCR product was amplified.
products were confirmed as NY-ESO-1 by nucleotide sequencing. NY-ESO-1 mRNA expression was observed at higher frequency in breast cancer of higher histological grade (P = 0.0975) or with negative estrogen receptor status (P = 0.0825; Table 2). The same tendency was also observed when relative expression level of NY-ESO-1 mRNA determined by TaqMan was used for nonparametric analysis (see below).

**Immunohistochemical Staining of NY-ESO-1 in Breast Cancer.** Twenty four NY-ESO-1 mRNA-positive and 31 NY-ESO-1 mRNA-negative breast cancer specimens were analyzed for NY-ESO-1 protein expression by IHC using monoclonal antibody ES121 or E978 (Table 1; Fig. 2). Positive staining was observed in 1 specimen. Staining was heterogeneous in the tissue and localized in the cytoplasm. This positively stained specimen, ID3394, was also positive for NY-ESO-1 mRNA expression.

**NY-ESO-1 Expression in Benign Breast Lesions and Normal Breast Tissues.** We then investigated NY-ESO-1 expression in benign breast lesions. Breast lesions that were resected with a provisional diagnosis of malignancy but proved histopathologically to be benign were examined for NY-ESO-1 expression by RT-PCR and IHC (Table 1; Figs. 1 and 2). Unexpectedly, 21 of 31 (68%) benign breast lesions (18 of 25 fibroadenomas, 2 of 4 fibrocystic disease, and 1 of 2 benign phyllodes tumors) showed NY-ESO-1 mRNA expression. However, no specimen of benign breast disease was positively stained by IHC. On the other hand, none of the 13 apparently normal tissue obtained from areas adjacent to breast cancer was positive for NY-ESO-1 mRNA or protein expression. We also observed MAGE-A1 (4 of 31; 13%) and MAGE-A3 (5 of 31; 16%) mRNA expression in benign breast lesions. There was no relationship between the expression of NY-ESO-1 and that of MAGE-A1 and MAGE-A3 (data not shown).

**Western Blot Analysis.** To confirm IHC results, Western blot analysis was performed using NY-ESO-1-specific antibody. Fig. 3 shows the results of Western blot analysis of samples from 2 breast cancers, 2 normal tissues, 2 benign lesions, 1 melanoma cell line, and recombinant NY-ESO-1 protein (lanes 1 and 10) showed bands specific to NY-ESO-1 at ~22 kDa. A

---

**Table 2. Correlation between NY-ESO-1 expression and clinicopathological features in breast cancer**

<table>
<thead>
<tr>
<th></th>
<th>mRNA positive/tumors examined</th>
<th>Expression level of mRNA</th>
<th>p*</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Median (10% Trimmed mean), Interquartile range</td>
<td></td>
</tr>
<tr>
<td>Stage</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>9/13 (69%)</td>
<td>5.243 (20.897), 36.112</td>
<td>0.3657</td>
</tr>
<tr>
<td>IIa,IIb,IIla</td>
<td>18/36 (50%)</td>
<td>0.000 (6.399), 10.233</td>
<td>0.5783</td>
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<tr>
<td>Tsize</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T1</td>
<td>10/18 (56%)</td>
<td>3.075 (15.561), 32.051</td>
<td></td>
</tr>
<tr>
<td>T2,T3</td>
<td>17/32 (53%)</td>
<td>0.000 (6.649), 10.233</td>
<td></td>
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<tr>
<td>Lymph node metastasis</td>
<td></td>
<td></td>
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<tr>
<td>-ve</td>
<td>21/48 (44%)</td>
<td>0.000 (6.199), 8.022</td>
<td></td>
</tr>
<tr>
<td>+ve</td>
<td>13/27 (48%)</td>
<td>0.000 (2.788), 7.085</td>
<td></td>
</tr>
<tr>
<td>Histological type</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Invasive ductal carcinoma</td>
<td>33/78 (42%) (0.7208)</td>
<td>0.000 (5.720), 8.093</td>
<td>0.3413</td>
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<tr>
<td>Special types of carcinoma</td>
<td>4/8 (50 %)</td>
<td>0.000 (1.497), 2.167</td>
<td>0.3759</td>
</tr>
<tr>
<td>Histological grade</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G1</td>
<td>5/20 (25%)</td>
<td>0.000 (2.234), 2.309</td>
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</tr>
<tr>
<td>G2,G3</td>
<td>25/54 (46%)</td>
<td>0.000 (6.453), 5.381</td>
<td></td>
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<tr>
<td>Estrogen receptor</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-ve</td>
<td>17/30 (57%)</td>
<td>0.000 (12.336), 9.767</td>
<td>0.2954</td>
</tr>
<tr>
<td>+ve</td>
<td>20/54 (37%)</td>
<td>0.000 (3.790), 7.717</td>
<td>0.9491</td>
</tr>
<tr>
<td>Progesteron receptor</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-ve</td>
<td>15/32 (47%)</td>
<td>0.000 (5.818), 7.312</td>
<td></td>
</tr>
<tr>
<td>+ve</td>
<td>22/52 (42%)</td>
<td>0.000 (5.268), 8.022</td>
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<tr>
<td>Recurrence</td>
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<tr>
<td>-ve</td>
<td>32/74 (43%)</td>
<td>0.000 (5.363), 8.093</td>
<td></td>
</tr>
<tr>
<td>+ve</td>
<td>4/11 (36%)</td>
<td>0.000 (1.189), 0.000</td>
<td></td>
</tr>
<tr>
<td>Conventional PCR</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-ve</td>
<td></td>
<td>0.000 (0.000), 0.000</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>+ve</td>
<td></td>
<td>9.767 (23.642), 33.949</td>
<td></td>
</tr>
</tbody>
</table>

* χ² test for independence.  
* Mann-Whitney’s U test.  
* Fisher’s exact probability test.
breast cancer sample from ID3394, which was positively stained on IHC, also showed a band of the same size by Western blot analysis (lane 5). Interestingly, a sample of fibroadenoma from ID1725, which expressed NY-ESO-1 mRNA by RT-PCR but did not stain by IHC, also showed a positive band (lane 8). No such band was observed in normal breast tissues (lanes 4 and 6), mRNA-negative breast cancer tissue ID3140 (lane 7), and fibroadenoma tissue ID2190 (lane 9).

**Quantification of NY-ESO-1 mRNA.** The relative amount of NY-ESO-1 mRNA in specimens of breast cancer that expressed the mRNA by conventional RT-PCR contained 3920 NY-ESO-1 mRNA copies. The relative amount of NY-ESO-1 mRNA in specimens of normal breast tissues and conventional benign breast lesions expressing fibroadenoma tissue, ID1725 (lane 8). However, no band was observed in samples of normal breast tissues (lanes 4 and 6), mRNA-negative breast cancer tissue ID3140 (lane 7), and fibroadenoma tissue ID2190 (lane 9).

**Antibody Response to NY-ESO-1.** Sera from 62 patients with breast cancer were examined for production of antibody against NY-ESO-1 by ELISA using recombinant NY-ESO-1 protein (Table 1). Only 1 patient, ID3394, showed NY-ESO-1 antibody production. The antibody was detected at the time of surgery, but disappeared within 2 years. Fig. 5 shows the titration curve. ID3394 tumor specimen showed strong NY-ESO-1 mRNA expression and positive staining with IHC. No NY-ESO-1 antibody was detected in the sera of 9 patients with benign breast lesions and of 19 healthy female volunteers (Table 1).

**CD8 T-Cell Response to NY-ESO-1 in a Seropositive Patient with Breast Cancer.** Previous studies showed that CD8 T cells specific for NY-ESO-1 were present among PBLs from seropositive patients with NY-ESO-1-expressing malignant melanomas by IFN-γ enzyme-linked immunospot assay using adeno and vaccinia NY-ESO-1 recombinant virus (10). We investigated CD8 T-cell response against NY-ESO-1 in the seropositive breast cancer patient ID3394 whose HLA type was A*2401, *2601, B7, 52, Cw07, *1202. Purified CD8 T-cells from the patient were cultured with autologous CD8-depleted PBLs infected with adeno/NY-ESO-1 recombinant virus for 10 days for sensitization. They were then cultured with autologous CD8-depleted PBLs infected with vaccinia/NY-ESO-1 recombinant virus for 24 h, followed by IFN-γ enzyme-linked immunospot assay.

![Fig. 3. Western blot analysis using ES121 monoclonal antibody was performed with lysates of cells and tissues, and recombinant NY-ESO-1 protein. ES121 reactive bands at 22 kDa, which were of the same size as that of recombinant protein (lanes 1 and 10), NY-ESO-1 protein-expressing cell line SK-MEL-35 (lane 2), and NY-ESO-1 protein-expressing esophageal cancer tissue (lane 3), were observed in immunohistochemistry-positive breast cancer tissue ID3394 (lane 5) and immunohistochemistry-negative but mRNA expressing fibroadenoma tissue. ID1725 (lane 8). However, no band was observed in normal breast tissues (lanes 4 and 6), mRNA-negative breast cancer tissue ID3140 (lane 7), and fibroadenoma tissue ID2190 (lane 9).](image)

![Fig. 4. Quantitative analysis of NY-ESO-1 mRNA by cDNA-specific real-time quantitative PCR assay. The regression equation of the calibration curve is: Ct = −1.7348Ln(NY-ESO-1 mRNA copies) + 42.792, (r² = 1.0). NY-ESO-1 mRNA copy numbers normalized by 10⁶ GAPDH mRNA were calculated in samples from 88 breast cancers (37 NY-ESO-1 mRNA-positive and 51 mRNA-negative by conventional reverse transcription-PCR), 31 benign breast diseases (21 positive and 10 negative), and 13 normal breast tissues (all negative). Testicular tissue had 3920 NY-ESO-1 mRNA copies.](image)

![Fig. 5. ELISA on sera from patient ID3394 using recombinant NY-ESO-1 protein. Serum obtained at the time of surgery. [] serum obtained at 2 years after surgery. - serum from a prostate cancer patient used as positive control.](image)

![Fig. 6. CD8 T-cell response in NY-ESO-1 seropositive patient ID3394. Purified CD8 T-cells were cultured with autologous CD8-depleted peripheral blood lymphocyte infected with adeno/NY-ESO-1 recombinant virus for 10 days for sensitization. Five × 10⁶ effector cells were then cultured with 5 × 10⁶ and 1 × 10⁷ autologous CD8-depleted peripheral blood lymphocyte infected with recombinant NY-ESO-1 (□) or wild-type (■) vaccinia virus for 24 h, and IFN-γ enzyme-linked immunospot assay was performed. Essentially similar results were obtained in three independent experiments during 2 months. ID3220 was a patient with a tumor that did not express NY-ESO-1 mRNA for control.](image)
As shown in Fig. 6, IFN-γ enzyme-linked immunospots were detected against cells infected with NY-ESO-1 recombinant but not wild-type vaccinia virus. Only a few spots were observed with CD8 T-cells from PBLs of the control patient ID3220 whose tumor did not express NY-ESO-1 mRNA (Fig. 1).

DISCUSSION

Using conventional RT-PCR, we found NY-ESO-1 mRNA in 37 of 88 (42%) breast cancer specimens. The assay was performed using 1 μg total RNA from frozen tissue for reverse transcription reaction followed by 35-cycle PCR with three different sets of primers in each specimen. No discrepancy was observed in the RT-PCR with the three sets of primers. The frequency of NY-ESO-1 mRNA expression in breast cancer observed in our study was higher than that in previous studies (12, 13). The reason for this difference in NY-ESO-1 expression frequency is unclear, although the genetic differences between Japanese and Caucasian populations or technical differences might be speculated as a possible explanation. Our study identified NY-ESO-1 protein expression only in 1 of 55 (2%) breast cancer specimens by IHC and by Western blot analysis. Although previous analysis showed discrepant results of RT-PCR versus IHC analysis (mRNA-positive/IHC-negative as well as mRNA-negative/IHC positive typing results) for NY-ESO-1 (15), the frequency of mRNA-positive/IHC-negative cases in our series is significantly higher than that observed in other tumor types. Surprisingly, our results showed a high frequency of expression of NY-ESO-1 mRNA in benign breast lesions (68%) compared with breast cancer. To our knowledge, NY-ESO-1 has not yet been reported in benign lesions previously. However, the presence of other CT antigens such as MAGE was found on a mRNA level in benign ovarian tumors (20). A number of reports suggested that some of the benign breast lesions were in fact early stage cancer (14, 21). In this regard, expression of cyclin D1 (22), loss of heterozygosity, and microsatellite instability (23) have been detected in some benign proliferative breast lesions like in breast cancer. Our finding is consistent with the results of these studies. None of our benign breast lesions stained positively by IHC, consistent with their low-level mRNA expression. One fibroadenoma, which expressed NY-ESO-1 mRNA by conventional RT-PCR, showed a positive band by Western blot analysis but was also IHC-negative.

The results of NY-ESO-1 mRNA expression detected by conventional RT-PCR were confirmed by quantification of NY-ESO-1 mRNA using real-time PCR. Breast cancers showed a wide variation of NY-ESO-1 mRNA levels by real-time PCR, and sample ID3394, which expressed NY-ESO-1 protein detected by both IHC and Western blot and showed a high NY-ESO-1 mRNA expression level (2940 copies; 75%) compared with other breast cancer tissues in our study. In comparison to breast cancers, the expression of NY-ESO-1 mRNA in benign breast lesions was weak, and all of the cases were IHC-negative. One fibroadenoma case, which was positive for NY-ESO-1 protein expression, as detected by Western blot but not by IHC, showed expression of 314 NY-ESO-1 mRNA copies. Although these findings suggest that tissues with a high level of NY-ESO-1 mRNA expression tend to express detectable expression levels of NY-ESO-1 protein, at least one breast cancer specimen with highest expression of mRNA level (4155 copies) and other samples expressing even higher mRNA levels, was IHC-negative. Discordance of the results between RT-PCR and IHC appeared to be mainly due to low mRNA expression in breast cancer. In our recent study on RT-PCR NY-ESO-1 mRNA-positive tumors, most of the breast cancers showed <100 copies of NY-ESO-1 mRNA, whereas most of the esophageal cancers showed >100 copies. In IHC, positive staining was observed in only 1 of 24 mRNA positive breast cancers but in 90% of mRNA positive esophageal cancers. Thus, the protein expression appeared to be generally correlated to the level of mRNA expression, although there were some exceptions. Additional study remains to be done. Interestingly, Western blot analysis appears to be more sensitive for detection of NY-ESO-1 protein than IHC.

Our results also showed that NY-ESO-1-specific CD8 T-cells were detected in the seropositive patient ID3394 with NY-ESO-1-expressing breast tumor. Detection of these cells using adeno and vaccinia NY-ESO-1 recombinant virus is useful because of no requirement of defining HLA-restricted epitopes. Previous studies indicated that CD8 T-cell response to NY-ESO-1 is found only in patients with antibodies to NY-ESO-1 (8). Although no NY-ESO-1 antibody was detected in the serum of patient ID3394 2 years after surgery, NY-ESO-1-specific CD8 T-cells were still detectable at that stage. This finding suggests that NY-ESO-1-specific CD8 T-cells remain long after the disappearance of the antibody.

CD8 T-cells play a pivotal role in in vivo tumor rejection (24). HLA class I binding peptides derived from the antigenic protein are assumed to be the most effective targets for immunotherapy, and several clinical trials using HLA class I binding peptide antigen have been reported (2, 25). Jager et al. (2) immunized cancer patients with NY-ESO-1 peptides and found specific T-cell responses that were in some cases associated with tumor regression. To augment tumor immune response, CD4 T-cell help will likely be needed (26, 27). CD4 helper T-cells recognize peptides presented on MHC class II molecules and secrete cytokines for CD8 T-cells and APC, increasing the expression of MHC class I, class II, and costimulatory molecules (e.g., ICAM-1, CD40, CD80, and CD86; Refs. 27, 28). Whole protein vaccination, containing HLA class I and II epitopes, would theoretically be much more effective in inducing CD8 effector cells (29, 30). Applying this strategy clinically, we are currently planning a clinical trial using NY-ESO-1 whole protein for vaccination in patients with breast cancer.

In conclusion, we have demonstrated in the present study that NY-ESO-1 is expressed in a high proportion of benign breast lesions and at a high proportion in high-grade, hormone receptor-negative breast cancer, suggesting that NY-ESO-1 could be a promising tumor antigen for immunotherapy of breast cancer with poor prognosis.

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