NY-ESO-1 Expression and Immunogenicity Associated with Transitional Cell Carcinoma: Correlation with Tumor Grade

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Abstract

NY-ESO-1 mRNA expression in transitional cell carcinoma was investigated by reverse transcription-PCR and immunohistochemistry. NY-ESO-1 mRNA was detected in 20 of 62 (32%) tumor specimens. There was a correlation between NY-ESO-1 expression and tumor grade: 0 of 4 (0%) grade 1 (G1), 6 of 26 (23%) grade 2 (G2), and 14 of 32 (44%) grade 3 (G3) tumors were NY-ESO-1 mRNA positive. Immunohistochemical analysis using the murine monoclonal antibody ES121 showed that 2 of 14 NY-ESO-1 mRNA-expressing G3 tumors were positive for NY-ESO-1. No NY-ESO-1 staining was observed in the panel of 30 G1 or G2 tumor specimens, including 6 NY-ESO-1 mRNA-positive cases. Sera from an expanded panel of 124 patients with transitional cell carcinoma were tested for the presence of NY-ESO-1 antibody. Seropositivity was observed in 9 of 72 (12.5%) patients with G3 tumors, whereas none of 52 patients with G1 or G2 tumors produced antibody against NY-ESO-1. In the 9 positive patients with NY-ESO-1 antibody, 4 had muscular invasive tumors, and 5 had carcinoma in situ.

Introduction

The list of human tumor antigens recognized by human CD8 T cells or antibodies has grown rapidly over the past several years (1). Most of these antigens can be classified according to expression pattern or structural features into one of the following categories: (a) CT antigens, e.g., members of the MAGE gene family (2) and NY-ESO-1 (3); (b) differentiation antigens, e.g., tyrosinase (4), gp 100 (5), and Melan A/MART-1 (4, 7); (c) mutated gene products, e.g., β-catenin (8) and caspase-8 (9); and (d) overexpressed self-antigens, e.g., HER2/neu (10). Because of their broad representation in cancer and restricted expression in normal tissues, CT antigens have received much attention as potential targets for human cancer vaccine (11). Of these antigens, members of the CT antigens can be classified according to expression pattern or structural features into one of the following categories: (a) CT antigens, e.g., members of the MAGE gene family (2) and NY-ESO-1 (3); (b) differentiation antigens, e.g., tyrosinase (4), gp 100 (5), and Melan A/MART-1 (4, 7); (c) mutated gene products, e.g., β-catenin (8) and caspase-8 (9); and (d) overexpressed self-antigens, e.g., HER2/neu (10). Because of their broad representation in cancer and restricted expression in normal tissues, CT antigens have received much attention as potential targets for human cancer vaccine (11).

Materials and Methods

Patients. TCC specimens were obtained from 62 patients undergoing surgery. The specimens consisted of 54 bladder, 4 ureteral, and 4 renal pelvic TCCs from 49 males and 13 females with a mean age at diagnosis of 65.7 years (range, 25–86 years). Tumor grade was determined according to standard criteria (17), and TNM classification of TCC was assessed according to the consensus report (19). Sera from 124 patients (94 males and 30 females) with a mean age at diagnosis of 66.4 years (range, 25–87 years) were tested for serum antibody to NY-ESO-1. All patients, with the exception of patient TCC44, had tumors at the time of serum collection.

RT-PCR Analysis. mRNA was isolated from frozen tumor specimens using the QuickPrep Micro mRNA Purification kit (Pharmacia, Uppsala, Sweden). Isolated mRNA was subjected to cDNA synthesis using the First-Strand cDNA Synthesis kit (Pharmacia). Primers for RT-PCR were: ES01-1, 5'-AGTTCTACCTGCATGCTC-3' and ES01-2, 5'-TCCCTCCAGCGAACAAC-3'. The amplification program for NY-ESO-1 was 1 min at 94°C, 1 min at 60°C, and 1.5 min at 72°C for 35 cycles after denaturing at 94°C for 1 min. These cycles were followed by a 10-min elongation step at 72°C. The PCR products (385 bp) were analyzed on 0.8% agarose gel.

ELISA. Recombinant NY-ESO-1 protein solution (100 μl/well) at a concentration of 1 μg/ml in coating buffer (15 mM Na2CO3, 35 mM NaHCO3, in distilled water (pH 9.6)) was added to 96-well plates (Nunc, Roskilde, Denmark) and incubated overnight at 4°C. Plates were washed with 0.05% Tween 20/PBS and blocked with 100 μl/well of 5% FCS/PBS for 1 h at room temperature. After washing, patients’ sera (100 μl/well) serially diluted with 5% FCS/PBS were added to the plate and incubated for 2 h at room temperature. After washing, diluted goat antihuman IgG (100 μl/well) labeled with peroxidase (MBL, Nagoya, Japan) was added and incubated for 1 h at room temperature. After washing, substrate solution [50 mM citric acid, 100 mM NaH2PO4, 0.03% ortho-phenylenediamine, 0.1% H2O2, in distilled water (pH 5.0)] was added in each well and incubated for 15 min at room temperature. After adding 3 μl H2SO4, plates were read by U-2001 spectrophotometer (TOSOH, Tokyo, Japan).

Immunohistostaining. Tumor specimens were fixed with buffered formalin and embedded in paraffin. Sections (5 μm) were placed on glass slides, heated at 60°C overnight, and then deparaffinized with xylene and ethanol. For antigen retrieval, tumor specimens mounted on glass slides were immersed into preheated target retrieval solution (DAKO, Carpenteria, CA) for 15 min and

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3 The abbreviations used are: CT, cancer/testis; TCC, transitional cell carcinoma; RT-PCR, reverse transcription PCR; CIS, carcinoma in situ; BCG, bacillus Calmette-Guérin; TNM, tumor-node-metastasis; mAb, monoclonal antibody.
allowed to cool for 20 min at room temperature. After the inactivation of endogenous peroxidase, specimens were incubated with 1.5% horse serum in PBS for 30 min at room temperature. mAb specific for NY-ESO-1 (clone ES121) was then added at a concentration of 2.5 μg/ml and incubated overnight at room temperature. After washing, diluted biotinylated antimouse IgG (Vector Laboratories, Burlingame, CA) was applied and incubated for 30 min at room temperature. Avidin labeled with peroxidase (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.

### Results

**Expression of NY-ESO-1 mRNA in TCC.** Expression of NY-ESO-1 mRNA in TCC specimens was investigated by RT-PCR. As shown in Table 1, NY-ESO-1 mRNA was detected in 20 of 62 (32%) tumor specimens. The size of PCR product in tumor was the same as in the T24 human bladder cancer cell line and in testis (Fig. 1A). The relationship between NY-ESO-1 mRNA expression and pathological and clinical features is shown in Table 1. NY-ESO-1 mRNA expression was correlated with tumor grade. Higher frequency of NY-ESO-1 mRNA expression was seen in higher grade TCC: 0 of 4 (0%) in G1; 6 of 26 (23%) in G2; and 14 of 32 (44%) in G3. No correlation could be established between NY-ESO-1 expression and age and sex of patients or with presence or absence of muscle invasion. TCC associated with lymph node or systemic metastases appeared to have a higher frequency of NY-ESO-1 expression than nonmetastatic tumors, but the numbers are too small to make a definitive statement.

**Immunohistochemistry of NY-ESO-1.** The panel of 62 TCC specimens was analyzed for NY-ESO-1 protein expression by immunohistochemistry using NY-ESO-1-specific mAb ES121. Positive staining was observed in 2 of 14 NY-ESO-1 mRNA-positive G3 tumors. The pattern of staining was heterogeneous rather than homogeneous (Fig. 1B). At higher magnification, staining could be seen to be predominantly cytoplasmic. One of 2 NY-ESO-1 protein positive specimens was from a primary tumor of a patient with systemic metastases; the other one was from a patient with localized and superficial tumors but without metastasis. No mAb ES121 staining was observed in 30 G1 and G2 specimens, including 6 NY-ESO-1 mRNA-positive tumors. The lower frequency of NY-ESO-1 detection by immunohistochemical staining as compared with RT-PCR typing is consistent with our previous experience with ES121 staining of other tumor types.

**Antibody Response to NY-ESO-1 in TCC Patients.** Sera from 124 TCC patients were analyzed for NY-ESO-1 antibody by ELISA using recombinant NY-ESO-1 protein. Fig. 2 shows titration curves of NY-ESO-1 antibody-positive and -negative sera, and Table 2 summarizes the results. NY-ESO-1 antibody was found in sera from 9 of 72 (12.5%) patients with G3 tumors. No antibody was detected in sera from 52 patients with G1 or G2 tumors or from 23 healthy volunteers. Table 3 lists TNM classification of TCC in patients with NY-ESO-1-positive antibody. Five of 9 seropositive patients had low-stage diseases, including the patients with CIS.

### Discussion

This study documents that TCC is a tumor type with a high frequency of NY-ESO-1 mRNA expression. NY-ESO-1 expression is correlated with the tumor grade, with 44% of G3 TCCs, in contrast to 20% of G1 and G2 TCCs, expressing NY-ESO-1. Although this frequency of NY-ESO-1 expression in TCCs is higher than reported in several other tumor types, there needs to be a more extensive study of

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Table 1 Correlation between NY-ESO-1 mRNA expression and pathological and clinical features in transitional cell carcinoma

<table>
<thead>
<tr>
<th>Pathological and clinical features</th>
<th>NY-ESO-1 positive/tumors examined</th>
</tr>
</thead>
<tbody>
<tr>
<td>All tumors</td>
<td>20/62 (32%)</td>
</tr>
<tr>
<td>Tumor grade&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>G1</td>
<td>0/4 (0%)</td>
</tr>
<tr>
<td>G2</td>
<td>6/26 (23%)</td>
</tr>
<tr>
<td>G3</td>
<td>14/32 (44%)</td>
</tr>
<tr>
<td>Tumor invasion</td>
<td></td>
</tr>
<tr>
<td>Superficial</td>
<td>10/36&lt;sup&gt;b&lt;/sup&gt; (28%)</td>
</tr>
<tr>
<td>Muscle invasion</td>
<td>10/26 (38%)</td>
</tr>
<tr>
<td>Regional lymph node metastasis</td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>16/54 (30%)</td>
</tr>
<tr>
<td>Positive</td>
<td>4/8 (50%)</td>
</tr>
<tr>
<td>Systemic metastasis</td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>16/57 (28%)</td>
</tr>
<tr>
<td>Positive</td>
<td>4/5 (80%)</td>
</tr>
</tbody>
</table>

<sup>a</sup> The difference in frequency of NY-ESO-1 mRNA expression between high-grade (G3: 14 of 32) and low-grade (G1 and G2; 6 of 30) tumors was statistically significant (P < 0.05). Statistical analysis was performed by χ² test.

<sup>b</sup> In this series of 36 superficial tumors, 3 were CIS, and 2 of the 3 CIS were NY-ESO-1 positive.
these other tumor types relating \textit{NY-ESO-1} expression to pathological and prognostic features. In addition, a survey of the \textit{NY-ESO-1} phenotype of primary versus metastatic disease and different metastatic deposits in the same patients needs to be determined for TCC as well as other types of tumors. Although there is a tendency for \textit{NY-ESO-1}-positive primary TCCs to be associated with a higher frequency of both local and systemic metastases, the number of samples needs to be increased before any significance can be attached to this observation.

Similar to the association of \textit{NY-ESO-1} mRNA expression and high tumor grade, \textit{NY-ESO-1} antibody responses appear to be restricted to patients with G3 TCC. Of the 9 patients with \textit{NY-ESO-1} antibody, 5 of the patients had CIS, a flat superficial tumor considered to be a progenitor for invasive tumors (17). BCG has been found to be an effective therapy for patients with CIS and superficial bladder cancer, with prolonged protection from tumor recurrence occurring in a significant proportion of BCG-treated patients (20). In fact, 3 of 5 \textit{NY-ESO-1}-antibody-positive CIS patients in our series received BCG therapy and have remained tumor free for 5 months to 5 years. One patient, TCC44, underwent transurethral resection of tumor followed by intravesical BCG instillation in 1995 and has been healthy without tumor recurrence since that time. A serum sample from this patient was first obtained in October 1997 and found to be \textit{NY-ESO-1} antibody positive, despite the long tumor-free interval. This observation of persistent \textit{NY-ESO-1} antibody in the absence of tumor stands in contrast to our experience with melanoma and other cancer types, where the presence of \textit{NY-ESO-1} antibody is clearly antigen driven and disappears with tumor removal or therapy-inducing tumor regression (15). The basis for: (a) the strong immunogenicity for \textit{NY-ESO-1} presented by CIS; (b) the persistence of \textit{NY-ESO-1} antibody in CIS patients after successful therapy; and (c) the possibility that \textit{NY-ESO-1} immunity might be involved in delaying tumor progression and mediate the therapeutic activity of BCG in superficial bladder cancer is an important topic requiring careful study.

In a recent study by Jäger \textit{et al.} (14) involving melanoma patients, a humoral immune response to \textit{NY-ESO-1} was predictive of a strong CD8 T cell response to \textit{NY-ESO-1}-derived peptides, as measured by tetramer, enzyme-linked immunospot, and cytotoxicity. We are now conducting a comparable analysis of patients with TCC to determine whether there is a link between the humoral and cellular immune response to \textit{NY-ESO-1} in this patient population. Because of the strong immunogenicity of \textit{NY-ESO-1}, there is considerable interest in vaccine strategies targeting this antigen, and a variety of \textit{NY-ESO-1} constructs, including \textit{NY-ESO-1} peptides, protein, DNA, and viral and bacterial vectors, are being prepared for clinical evaluation. A recent clinical vaccine trial with HLA-A2-restricted \textit{NY-ESO-1}-peptides has shown that these peptides can elicit a strong CD8 T-cell response in \textit{NY-ESO-1}-immunized patients (21). Because of the high frequency of \textit{NY-ESO-1} expression in TCC and the increasing awareness of the limitation of current chemotherapy against TCC, TCC represents a challenging tumor type to test the effectiveness of \textit{NY-ESO-1} vaccines.

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References


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**Table 3 TNM classification of TCC in patients with \textit{NY-ESO-1} antibody**

<table>
<thead>
<tr>
<th>Patient</th>
<th>Primary tumor</th>
<th>Regional lymph node</th>
<th>Systemic</th>
</tr>
</thead>
<tbody>
<tr>
<td>TCC 11</td>
<td>CIS</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>TCC 18⁴</td>
<td>T1 + CIS</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>TCC 21</td>
<td>T3³</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>TCC 22⁴</td>
<td>T4</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>TCC 25</td>
<td>CIS</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>TCC 38</td>
<td>T2</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>TCC 44²</td>
<td>CIS</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>TCC 84⁴</td>
<td>T1 + CIS</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>TCC 91</td>
<td>T3³</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

⁴Tumor specimens with \textit{NY-ESO-1} expression confirmed by immunohistostaining. Frozen specimens from TCC18 for RT-PCR were not available.

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**Table 2 \textit{NY-ESO-1} antibody response in 124 patients with transitional cell carcinoma**

<table>
<thead>
<tr>
<th>Tumor grade</th>
<th>Antibody positive/sera tested</th>
</tr>
</thead>
<tbody>
<tr>
<td>G1</td>
<td>0/5 (0%)</td>
</tr>
<tr>
<td>G2</td>
<td>0/47 (0%)</td>
</tr>
<tr>
<td>G3</td>
<td>9/72 (12.5%)</td>
</tr>
</tbody>
</table>

⁴In this series of 124 patients with TCC, both frozen tumors and sera were available from 45 patients for \textit{NY-ESO-1} RT-PCR and antibody typing. Thirteen of 45 tumors were positive for \textit{NY-ESO-1} mRNA, and 2 of the 13 patients with \textit{NY-ESO-1}-positive tumors had \textit{NY-ESO-1} antibody. No \textit{NY-ESO-1} antibody was found in the 32 patients with \textit{NY-ESO-1}-negative tumors.

⁵The difference in \textit{NY-ESO-1} antibody frequency in patients with high-grade (G3; 9 of 72) and low-grade (G1 and G2; 0 of 52) tumors was statistically significant (\(P < 0.05\)). Statistical analyses were performed by \(\chi^2\) test.

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**Fig. 2. \textit{NY-ESO-1} antibody in patients with G3 TCC. Serially diluted sera from 5 patients with G3 TCC (○), serum from an \textit{NY-ESO-1}-positive patient (○), and sera from 5 healthy volunteers (□) are shown. ELISA tests with recombinant \textit{NY-ESO-1}-protein are also shown.**
new gene coding for a differentiation antigen recognized by autologous cytolytic T lymphocytes on HLA-A2 melanomas. J. Exp. Med., 180: 35–42, 1994.


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