[CANCER RESEARCH 61, 4671–4674, June 15, 2001]

*Advances in Brief*

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

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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 NY-ESO-1-specific 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[3] 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 (6, 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 the ten gene or gene families coding for CT antigens (12), the NY-ESO-1 product appears to be the most immunogenic, inducing a humoral immune response and specific CD8 T-cell reactivity in ~50% of patients with advanced NY-ESO-1-expressing tumors (13, 14). Immunity to NY-ESO-1 is clearly antigen driven, disappearing with tumor removal or tumor regression (15).

TCC comprises nearly 90% of primary malignant tumors of the bladder, displaying a broad biological spectrum ranging from superficial to invasive tumors (16). Tumor grade (G) is based on cellular dysplasia and architectural abnormalities in tumor tissue and is commonly used to classify TCC in terms of malignant potential (17). High-grade (G3) TCC progresses to muscle invasion more frequently than low-grade (G1 and G2) tumors. Although G1 and G2 tumors often recur, they are less likely to become invasive (18). Although CIS is a flat superficial tumor, it is classified as a high-grade lesion, which frequently progresses into invasive tumors (17, 18).

In the initial description for NY-ESO-1, we found a high frequency of *NY-ESO-1* mRNA expression in a small number of TCCs (3). In this study, we have evaluated NY-ESO-1 expression in a large number of TCCs and have found a correlation between *NY-ESO-1* expression and tumor grade. In addition, the sera of patients with TCCs have been screened for antibody against *NY-ESO-1*, and the antibody response has also been found to be restricted to patients with G3 tumors.

## 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: ESO1-1, 5′-AGTTCTACCTCGCCATGCCT-3′; and ESO1-2, 5′-TCCTCCTCCAGCGACAAACAA-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 $Na_2CO_3$, 35 mM $NaHCO_3$ 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 $Na_2HPO_4$, 0.03% *ortho*-phenylenediamine, 0.1% $H_2O_2$ in distilled water (pH 5.0)] was added in each well and incubated for 15 min at room temperature. After adding 3 M $H_2SO_4$, 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, Carpinteria, CA) for 15 min and

Received 2/12/01; accepted 5/1/01.

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[1] Supported in part by a Grant-in-Aid for Scientific Research on Priority Area (C) from the Ministry of Education, Science, Sports and Culture of Japan.

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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 $\mu$g/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. 1*A*). 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.

**Immunohistostaining 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. 1*B*). 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.

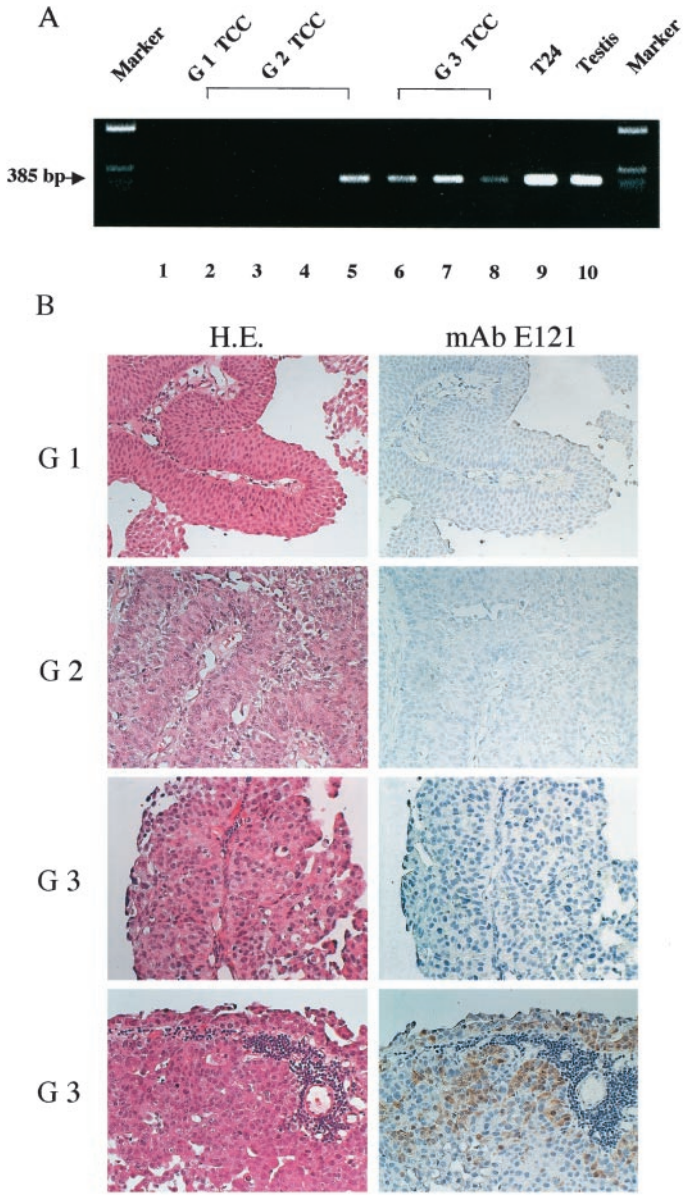


Fig. 1. *A*, RT-PCR analysis for *NY-ESO-1* mRNA expression. mRNA from G1 TCC (*Lane 1*), G2 TCC (*Lanes 2–5*), G3 TCC (*Lanes 6–8*), a human bladder cancer cell line, T24 (*Lane 9*), and testis (*Lane 10*) was analyzed for *NY-ESO-1* mRNA by RT-PCR. *B*, H&E staining (*left*) and immunohistostaining for NY-ESO-1 using mAb ES121 (*right*) in serial sections of G1, G2, or G3 TCC.

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

| Pathological and clinical features | *NY-ESO-1* positive/tumors examined |
|---|---|
| All tumors | 20/62 (32%) |
| Tumor grade[a] | |
| G1 | 0/4 (0%) |
| G2 | 6/26 (23%) |
| G3 | 14/32 (44%) |
| Tumor invasion | |
| Superficial | 10/36[b] (28%) |
| Muscle invasion | 10/26 (38%) |
| Regional lymph node metastasis | |
| Negative | 16/54 (30%) |
| Positive | 4/8 (50%) |
| Systemic metastasis | |
| Negative | 16/57 (28%) |
| Positive | 4/5 (80%) |

[a] 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 $\chi^2$ test.

[b] In this series of 36 superficial tumors, 3 were CIS, and 2 of the 3 CIS were *NY-ESO-1* positive.

## 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

these other tumor types relating *NY-ESO-1* expression to pathological and prognostic features. In addition, a survey of the *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 *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 *NY-ESO-1* mRNA expression and high tumor grade, NY-ESO-1 antibody responses appear to be restricted to patients with G3 TCC. Of the 9 patients with 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 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 NY-ESO-1 antibody positive, despite the long tumor-free interval. This observation of persistent 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 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 NY-ESO-1 presented by CIS; (*b*) the persistence of NY-ESO-1 antibody in CIS patients after successful therapy; and (*c*) the possibility that 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.

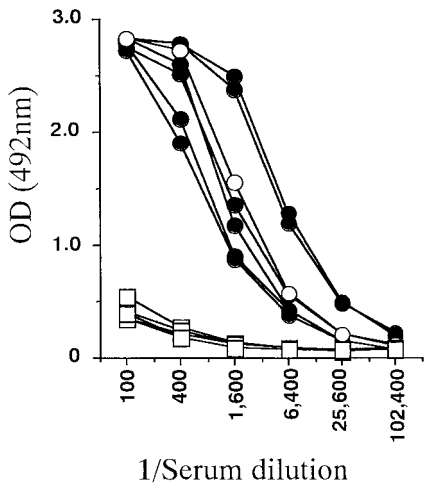


Fig. 2. NY-ESO-1 antibody in patients with G3 TCC. Serially diluted sera from 5 patients with G3 TCC (●), serum from an NY-ESO-1-positive patient (○), and sera from 5 healthy volunteers (□) are shown. ELISA tests with recombinant NY-ESO-1 protein are also shown.

Table 2 *NY-ESO-1 antibody response in 124 patients with transitional cell carcinoma*[a]

| Tumor grade | Antibody positive/sera tested |
|---|---|
| G1 | 0/5 (0%) |
| G2 | 0/47 (0%) |
| G3 | 9/72[b] (12.5%) |

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

[b] The difference in 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.

Table 3 *TNM classification of TCC in patients with NY-ESO-1 antibody*

| Patient | Primary tumor | Metastasis | |
|---|---|---|---|
| | | Regional lymph node | Systemic |
| TCC 11 | CIS | − | − |
| TCC 18[a] | T1 + CIS | − | − |
| TCC 21 | T3[b] | − | − |
| TCC 22[a] | T4 | + | + |
| TCC 25 | CIS | − | − |
| TCC 38 | T2 | − | − |
| TCC 44[b] | CIS | − | − |
| TCC 84[a] | T1 + CIS | − | − |
| TCC 91 | T3[b] | − | − |

[a] Tumor specimens with NY-ESO-1 expression confirmed by immunohistostaining. Frozen specimens from TCC18 for RT-PCR were not available.

[b] Patient TCC44 has been tumor free since September 1995 after transurethral resection of bladder tumor followed by BCG immunotherapy. Serum for NY-ESO-1 antibody test was obtained in October 1997. Sera from the 8 other patients were obtained at the time of surgical resection of their tumors.

In a recent study by Jäger *et al.* (14) involving melanoma patients, a humoral immune response to NY-ESO-1 was predictive of a strong CD8 T cell response to 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 NY-ESO-1 in this patient population. Because of the strong immunogenicity of NY-ESO-1, there is considerable interest in vaccine strategies targeting this antigen, and a variety of NY-ESO-1 constructs, including 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 NY-ESO-1 peptides has shown that these peptides can elicit a strong CD8 T-cell response in NY-ESO-1-immunized patients (21). Because of the high frequency of *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 NY-ESO-1 vaccines.

## Acknowledgments

We thank M. Isobe for excellent technical assistance and J. Mizuuchi for preparation of the manuscript.

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*Cancer Res* 2001;61:4671-4674.