IMMUNOHISTOCHEMICAL EXPRESSION OF TUMOR ANTIGENS MAGE-A1, MAGE-A3/4, AND NY-ESO-1 IN SQUAMOUS CELL CARCINOMA OF THE PENIS

TVRTKO HUDOLIN, ANTONIO JURETIC, JOSIP PASINI, DAVOR TOMAS, GIULIO CESARE SPAGNOLI, MICHAEL HEBERER, JORDAN DIMANOVSKI, AND BOZO KRUSLIN

ABSTRACT
Objectives. To investigate by immunohistochemistry the expression of MAGE-A and NY-ESO-1/LAGE-1, cancer testis antigens (CTAs), in squamous cell carcinoma of the penis.

Methods. A total of 30 penile carcinoma samples from patients undergoing penile amputation at the Urology Clinics at the Zagreb Clinical Hospital Center and University Hospital “Sestre milosrdnice” from 1997 to 2004 were investigated in this study. Three monoclonal antibodies were used for immunohistochemical staining: 77B specific for MAGE-A1, 57B recognizing multiple MAGE-A CTAs, and D8.38, specific for NY-ESO-1 antigen.

Results. The expression of MAGE-A1 was not observed in the carcinoma samples, but both multi-MAGE-A and NY-ESO-1-specific reagents stained 29 (97%) of 30 samples. Immunohistochemical staining was prevalingly detected in the cytoplasm. A significant heterogeneity was observed within the same specimen, in which areas with strong positivity coexisted with CTA-negative areas. The extent of CTA expression did not correlate significantly with tumor grade.

Conclusions. The results of this study have documented for the first time the expression of CTAs in squamous cell carcinoma of the penis. Additional research is warranted to explore the potential implications regarding both diagnosis and therapy.

Three monoclonal antibodies (mAbs) were used for IHC staining. 77B mAb\textsuperscript{10} was generated on immunization of mice with recombinant MAGE-A1. 57B mAb\textsuperscript{10} was generated on immunization of mice with recombinant MAGE-A3.\textsuperscript{11} However, studies from different laboratories have highlighted that it recognizes a variety of MAGE-A molecules and it is currently considered a multi-MAGE-A-specific reagent.\textsuperscript{9} D8.38 mAb, recognizing NY-ESO-1 and LAGE-1, CTA has been previously described.\textsuperscript{12}

Tissue sections of 3 to 5 \(\mu m\) thickness were cut from paraffin-embedded tissue blocks, placed on object slides (Menzel-Glaser, Germany), and incubated for 20 minutes in a thermostat at 60\(^\circ\)C. The sections were then deparaffinized and incubated for 3 to 5 minutes in 10 mmol/L of citrate buffer (pH 6.0) in a microwave oven at 800 W. Subsequently, the tissue slides were washed with phosphate-buffered saline (PBS) buffer (pH 7.2), and endogenous peroxidase activity was blocked by a 5-minute treatment with hydrogen peroxide (Dako, No. S2023). The slides were then washed with PBS buffer and incubated for 90 minutes with 77B, 57B, or D8.38 undiluted supernatants at room temperature.

After washing in PBS, the secondary biotinylated antibody (Dako, No. K0690) was added for 30 minutes of incubation. The slides were then washed with PBS and treated with streptavidin-horseradish peroxidase (Dako, No. K0690) for 30 minutes. The tissue sections were washed once more in PBS, and then Chromogen (Dako, No. K3468) was added for 5 minutes. The slides were washed in distilled water, stained with hemalum (Dako, No. S2020) for 1 minute, washed with water, dehydrated with alcohol (96\%), cleared with xylene, and mechanically covered.

Melanoma and testicular tissues expressing CTAs were used as positive controls throughout the study, and healthy penis tissue derived from the margins of the surgical excision and unstained tumor cells served as the negative controls. On average, six different slides were stained and evaluated for each tumor specimen.

IHC staining results were semiquantitatively expressed as follows: negative response, no staining in tumor cells; weakly positive response, up to 10\% of tumor cells positive; moderately positive response, 11\% to 50\% of tumor cells positive; and strongly positive response, more than 50\% of tumor cells positive.

**Statistical Analysis**

Statistical analysis was done using StatSoft Statistica data analysis software, version 6.1. The chi-square test and Spearman rank correlation coefficient were used throughout this work, and differences were considered statistically significant at 5\% (\(P < 0.05\)).

**RESULTS**

Expression of MAGE-A1 CTA, as detectable by 77B mAb staining, was not observed in the squamous cell carcinoma samples, although multi-MAGE-A 57B and NY-ESO-1/LAGE-1 specific D8.38 mAbs both stained 29 (97\%) of 30 samples. Representative examples are shown in Figure 1. In all cases, IHC staining was predominantly, although not exclusively, detectable in the cytoplasm. A significant heterogeneity was observed within the same tissue sample in which areas with strong positivity coexisted with CTA-negative areas. In particular, positivity areas involving more than 50\% of tumor cells were observed in 12 (40\%) and 21 (70\%) cases on staining with multi-MAGE-A 57B and NY-ESO-1/LAGE-1 specific D8.38 mAbs, respectively. The extent of CTA expression, however, did not correlate significantly with tumor grade.

**COMMENT**

Because CTAs are expressed in a large number of tumor types, we hypothesized that they might also be expressed in squamous cell carcinomas of the penis. We performed staining of serial sections from the whole excised part of the penis, permitting a thorough evaluation of CTA expression at the protein level. Our results have documented for the first time the expression of MAGE-A3/4 and NY-ESO-1 CTAs in squamous cell penile cancer specimens by IHC.
We found that CTAs are very frequently expressed in these tumors, with the typical prevalently cytoplasmic localization. Previously, studies that stained minute biopsies enclosed in tissue microarrays had shown negative results in penile cancer specimens. This discrepancy might be because CTAs are often focally expressed. Thus, relevant tumor areas might easily be missed, resulting in an underestimation of CTA expression. In contrast, we did not observe a correlation between tumor grade and the extent of CTA expression, possibly because of the small size of our study group.

Remarkably, we did not observe expression of MAGE-A1 CTA in any of the specimens investigated. These data should be interpreted cautiously, because we used only one specific mAb in this study. Therefore, we could not formally exclude that our negative results may have resulted from relatively weak staining by this reagent. However, because we had no access to fresh frozen material, but only paraffin-embedded material, we were unable to perform classic reverse transcriptase-polymerase chain reaction to confirm or disprove these data.

CONCLUSIONS

Penile cancer is uncommon in the Western world, and treatment options are limited. Surgery represents the conventional treatment, providing good disease control but obvious mutilating consequences. The disease can spread and result in distant metastases for which no effective therapy is available. Thus, additional treatment options are urgently required.

Immunotherapy focused on tumors cells expressing CTAs is currently under investigation in a number of clinical trials. In this study, we have shown different levels of expression of the CTAs MAGE-A3/4 and NY-ESO-1 in almost all our samples, suggesting a possible use of immunotherapy in patients with squamous cell penile carcinoma. Additional research is warranted to explore the potential implications regarding diagnosis and therapy.

REFERENCES