

Cancer-Testis Antigens: Expression and Correlation with Survival in Human Urothelial Carcinoma

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Abstract Purpose: Vaccination against human cancer is a promising therapeutic approach but the optimal antigen or antigens remain undefined. Cancer-testis antigens (CTA), a family of tumor-associated antigens, have both potent immunogenicity and restricted expression patterns in normal adult tissues, highly desirable characteristics for targets of anticancer vaccines. These antigens were evaluated for both the degree of expression and prognostic value in cancer of the urothelium.

Experimental Design: The expression patterns of nine CTAs (NY-ESO-1, LAGE-1, MAGE-A1, MAGE-A3, MAGE-A4, MAGE-A10, CT7, CT10, and GAGE) were examined by immunohistochemistry and reverse transcription-PCR in a panel of high-grade urothelial carcinomas of the urinary bladder. Also assessed were correlations between the expression of CTAs by immunohistochemistry and both disease-free and overall survival.

Results: At least one CTA was expressed in 77% of samples and 61% of these tumors expressed more than one CTA. Additionally, patients with CT10-positive tumors had an improved disease-free survival ($P = 0.008$) and overall survival ($P = 0.037$) compared with patients with CT10-negative tumors.

Conclusions: These findings establish CTAs as potential prognostic markers and as target candidates for vaccine development for patients with urothelial carcinoma.

Cancer-testis antigens (CTA) are named after their pattern of expression, as they are found in various types of cancers but only in testicular germ cells of normal adult tissues (1). Because of this tumor-associated expression pattern, CTAs have been the focus of attention as potential targets in immunotherapy for cancer (2). CTAs were first identified by T-cell epitope cloning based on their ability to elicit autologous T-cell responses in a patient with melanoma (3). Subsequent studies using serologic analysis of recombinant expression libraries (SEREX) and database mining (4–6) identified multiple families of CTAs. To date, >44 distinct CTA gene or antigen families such as

MAGE, GAGE, BAGE, and NY-ESO-1 have been identified (7, 8). Several CTAs, including NY-ESO-1, MAGE-A3, and MAGE-A4, have been or are being studied as target antigens in vaccine clinical trials for various types of tumors, including non-small-cell lung cancer, ovarian cancer, gastrointestinal carcinoma, and urothelial carcinoma (7, 9–13). In prior studies of urothelial carcinoma of the bladder, we previously documented that NY-ESO-1 was highly expressed in high-grade carcinoma, forming the basis of a vaccine clinical trial in which the NY-ESO-1 protein was used as adjuvant treatment after complete resection of urothelial carcinoma (14).

In the present study, we analyzed both the individual and composite expression of nine CTAs in a large series of urothelial carcinoma samples. We also sought to assess for correlations between antigen expression of each CTA with both disease-free and overall survival to determine if any of these antigens had potential prognostic value. This information is crucial to establish potential new markers of disease outcome and to determine the proportion of patients with urothelial carcinoma who may be eligible for future vaccine clinical trials with these antigens. Our findings provide the foundation for the use of multiantigenic vaccines or sequential vaccination with two or more antigens for patients with urothelial carcinoma to potentially reduce or prevent the *in vivo* selection of antigen-loss tumor cell variants.

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Received 3/3/06; revised 5/16/06; accepted 7/10/06.

Grant support: The University of Texas M.D. Anderson Cancer Center Bladder Cancer Specialized Program of Research Excellence Career Development Award 5 P50 CA091846 04, PP-CDP4 (P. Sharma, Y. Shen, and S. Wen), The University of Texas M.D. Anderson Cancer Center Physician Scientist Program Award, The University of Texas M.D. Anderson Cancer Center Institutional Research Grant, and the American Society of Clinical Oncology Young Investigator Award (P. Sharma). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Note: Supplementary data for this article are available at Clinical Cancer Research Online (<http://clincancerres.aacrjournals.org/>).

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doi:10.1158/1078-0432.CCR-06-0527

Materials and Methods

Patients and tissue samples. Tumor samples from 95 patients with high-grade (HG) disease (carcinoma *in situ*, pT_{aHG}, pT₁, pT₂, pT₃, and pT₄) were collected according to a tissue acquisition protocol approved

by the Memorial Sloan-Kettering Cancer Center institutional review board. All patients gave informed consent to participate. Tumor samples were collected either retrospectively as paraffin-embedded tissues from the archives of the Department of Pathology of Memorial Sloan-Kettering or prospectively as both fresh and paraffin-embedded tissues. Immediately after surgical removal, fresh tumor samples were divided into two pieces, one of which was processed as a formalin-fixed paraffin-embedded sample and the other was embedded in optimum cutting temperature compound, snap-frozen in isopentane that had been cooled in dry ice, and stored at -70°C for RNA extraction.

Immunohistochemical analyses. Formalin-fixed paraffin-embedded tissues were used for immunohistochemical analyses. A total of 94 specimens were available for immunohistochemistry [sample no. 95 was only available for reverse transcription-PCR (RT-PCR) analyses]. The primary antibodies, their corresponding antigens, and the working conditions are listed in Supplementary Table S1. Immunohistochemical analysis was done as previously described (15, 16). Briefly, slides were deparaffinized in xylene and rehydrated in a series of graded alcohols. Slides were incubated with primary antibodies overnight at 4°C . A biotinylated horse anti-mouse secondary antibody (1:200; Vector Laboratories, Burlingame, CA) followed by an avidin-biotin system (Vectastain Elite ABC kit, Vector Laboratories) was used to detect monoclonal antibodies (mAb) MA454, M3H67, 57B, CT7-33, and GAGE. An EnVision Plus system (DakoCytomation, Carpinteria, CA) was used to detect mAb E978. Liquid 3,3-diaminobenzidine tetrahydrochloride (Biogenex, San Ramon, CA) was used as the chromogen. Antigens were retrieved by heating the slides in a household vegetable steamer (Black & Decker, Miramar, FL) at 95°C for 30 minutes; antigen retrieval buffer solutions and working concentrations are listed in Supplementary Table S1. Normal adult testis with intact spermatogenesis served as positive controls. Appropriate negative controls were included for each case. The extent of tumor staining was estimated and graded as follows: focal, $<5\%$ of tumor cells stained; +, 5% to 25% of cells stained; ++, $>25\%$ to 50% of cells stained; +++, $>50\%$ to 75% of cells stained; and +++++, $>75\%$ of cells stained.

RT-PCR. Thirty-eight samples were available for RT-PCR analysis and, with the exception of case no. 95, all samples were additionally assessed for antigen expression by immunohistochemical staining. PCR analysis was limited by availability of specimens: NY-ESO-1, LAGE-1, and MAGE-A3 mRNA expressions were assessed in all 38 samples; MAGE-A10 expression was assessed in 32 cases; MAGE-A4 in 31 cases; and MAGE-A1 in 23 cases. mRNA was isolated with the TriReagent LS kit (Molecular Research Center, Cincinnati, OH). The mRNA preps were quantified by measuring the absorbance at 260 nm. Reverse transcription was done with $4\ \mu\text{g}$ of mRNA, and PCR was carried out with 200 ng of cDNA. Primers for NY-ESO-1 and LAGE-1 were as previously published (14); other primers are as follows: MAGE-1, 5'-GCTGGAACCCCTCACTGGTTGCC-3' (forward) and 5'-CGGCCGAA-GGAACCTGACCCAG-3' (reverse); MAGE-3, 5'-GAAGCCGGCC-CAGGCTCG-3' (forward) and 5'-GGAGTCCTCATAGGATTGGCT-3' (reverse); MAGE-4, 5'-GAGCAGACAGGCCAACCG-3' (forward) and 5'-AAGGACTCTGCGTCAGGC-3' (reverse); and MAGE-10, 5'-GGAACC-CCTCTTTTCTACAGAC-3' (forward) and 5'-TCCTCTGGGGTGCTTGGT-ATTA-3' (reverse).

Statistical analyses. Statistical analyses were based on information from 72 cases in which at least one CTA was expressed by immunohistochemical analyses. The primary objectives of the statistical analyses were to examine the correlations between the seven antigens for which mAbs are available (E978, MA454, M3H67, 57B, CT7.33, CT10.5, and GAGE) and disease stage, and to explore the association between the antigens and disease-free survival and overall survival. Of the 72 patients included in this analysis, 27 experienced recurrent disease and 20 died during the follow-up period (median of 36 months and minimum of 1.1 months).

Disease stage was stratified as a binary variable based on the level of invasion and prognosis, with low-stage tumors without invasion of the muscularis propria (T_1 or T_a) distinguished from high-stage tumors

with muscle invasion (T_2 , T_3 , or T_4). Antigen expression was also stratified as a binary variable, with positive (+ or focal) versus negative. Fisher's exact test was used to assess relationships between categorical variables (disease stage and antigens). Kaplan-Meier curves were used to estimate disease-free and overall survival and the log-rank test was used to compare differences between the two survival curves. A multivariate Cox proportional hazards model was used to estimate hazard ratios while adjusting for other risk factors.

Results

The detailed results of the immunohistochemical analyses for individual patients are shown in Supplementary Table S2. Of the 94 samples subjected to immunohistochemical analysis, 72 (77%) cases showed immunoreactivity with at least one anti-CTA mAb, and 57 of the 94 (61%) samples showed immunostaining with at least two mAbs (Fig. 1). Five tumors were positive for all seven mAbs tested; representative staining patterns from one of those five cases (patient no. 36) are shown in Figs. 2 and 3. Among the reagents used, mAb 57B to MAGE-A4 showed the highest incidence of immunostaining (64%) followed by mAb M3H67 to MAGE-A3 (55%). Interestingly, in most of the 57B- or M3H67-positive tumors, the antigen was visualized in more than half of each tumor sample (corresponding to +++ or +++++ in our grading system). The mAbs MA454 (for MAGE-A1), E978 (for NY-ESO-1), GAGE (for GAGE), and CT7-33 (for CT7) produced staining in fewer cases, with $\sim 30\%$ of all cases positive. The mAb CT10.5 (for CT10) stained only $\sim 20\%$ of all specimens. Interestingly, these mAbs tended to stain less than half of tumor cells in each sample, thus displaying a heterogeneous expression pattern. On a cellular level, staining appeared mostly in the cytoplasm for mAbs MA454 (MAGE-A1) and E978 (NY-ESO-1); in both cytoplasm and nuclei for GAGE, M3H67 (MAGE-A3), and CT7-33 (CT7); and exclusively in the nuclei for mAb CT10.5 (CT10; Figs. 2 and 3).

Tumor samples that were available for analysis of mRNA expression (38 cases) were tested for NY-ESO-1, LAGE-1, MAGE-A1, MAGE-A3, MAGE-A4, and MAGE-A10 expression. Results of the RT-PCR analyses are shown in Supplementary Table S2. In summary, 13 of 38 (34%) samples were positive

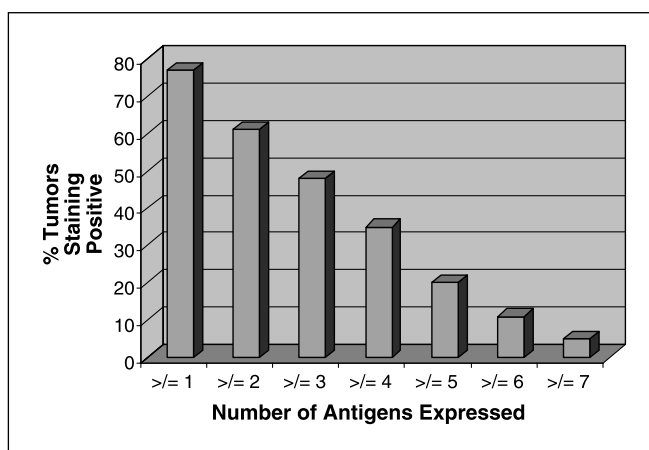


Fig. 1. Distribution of numbers of cancer-testis antigens expressed by transitional-cell carcinoma samples. Immunohistochemical analysis showed that 77% of the transitional-cell carcinoma samples tested expressed at least one CTA and some tumors expressed all seven CTAs studied.

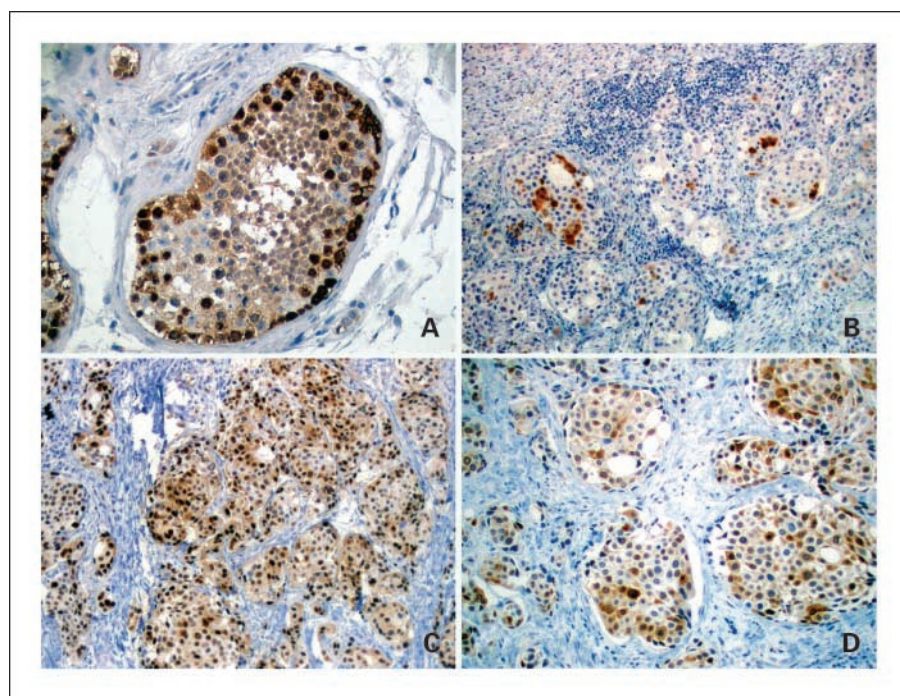


Fig. 2. Immunohistochemical staining patterns for CTAs in normal control testis tissue (A) and in an invasive area of a poorly differentiated transitional-cell carcinoma of the urinary bladder (patient 36) displaying typical staining patterns (B-D). A, germ cells of normal adult testis show strong staining with mAb M3H67 (MAGE-A3). B, tumor cells show patchy cytoplasmic staining with mAb MA454 (MAGE-A1). C, tumor cells show homogeneous nuclear and heterogeneous cytoplasmic reactivity for mAb M3H67. D, tumor cells show heterogeneous immunostaining for mAb 57B (MAGE-A4).

for NY-ESO-1, 18 of 38 (47%) were positive for LAGE-1, 10 of 23 (43%) were positive for MAGE-A1, 22 of 38 (58%) were positive for MAGE-A3, 18 of 31 (58%) samples were positive for MAGE-A4, and 12 of 32 (38%) tumors were positive for MAGE-A10 mRNA expression. In terms of the presence or absence of specific CTAs, RT-PCR and immunohistochemical analysis were both done on a total of 37 cases and produced similar results in the vast majority of tested cases. For example, for NY-ESO-1, in 29 of 37 (78%) cases, mRNA expression paralleled E978 immunoreactivity, but eight mRNA-positive tumors showed no immunostaining. For MAGE-A1 (mAb

MA454), congruent expression was present in 19 of 23 (83%) samples, and 4 cases were positive solely on the mRNA level. For MAGE-A3, 31 of 37 (84%) tumors were congruent in mRNA and M3H67 staining, 3 mRNA-positive samples were negative on immunostaining, and 3 mRNA-negative cases showed positive staining with mAb M3H67. For MAGE-A4, 25 of 31 (81%) specimens showed congruent expression of mRNA and protein and 6 cases were positive solely on the protein level.

Analysis of protein expression of CTAs in comparison with available survival data for 72 patients (Supplementary Table S3)

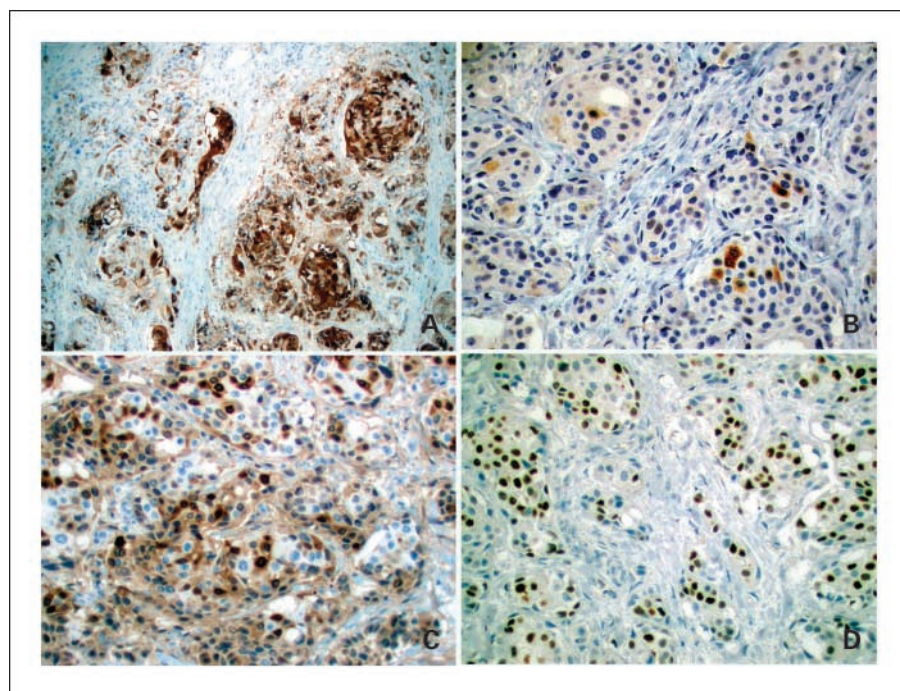


Fig. 3. Immunohistochemical staining patterns for CTAs. A, strong staining of most tumor cells with mAb CT7-33 (CT7). B, a single tumor cell shows immunoreactivity to mAb E978 (NY-ESO-1). C, tumor cells show heterogeneous cytoplasmic and nuclear staining for GAGE. D, tumor cells show exclusively nuclear immunoreactivity for mAb CT10.5 (CT10).

showed that immunopositivity with mAb CT10.5 (for CT10) correlated with better disease-free survival (Fig. 4A; $P = 0.008$) and overall survival (Fig. 4B; $P = 0.037$), and that patients whose tumors had positive staining with 57B (MAGE-A4) had a trend toward improved overall survival (Fig. 4C; $P = 0.094$). Protein expression of GAGE, NY-ESO-1, MAGE-A1, MAGE-A3, CT7, and MAGE-A4 was not associated with recurrence and survival outcomes in this cohort. As expected, pathologic stage correlated with overall survival; patients with pT₁ disease had better survival ($P = 0.01$) than patients with pT₂ or greater primary tumors (Fig. 4D).

A multivariate Cox regression model was used to assess simultaneously the effect of two or more antigens and stage on disease-free survival. Immunoreactivity with mAbs CT10.5 (CT10) and 57B (MAGE-A4) and disease stage were found to be independent predictors of both disease-free survival and overall survival (Table 1).

Associations between disease stage and the presence of a particular CTA, assessed with Fisher's exact test, are given with P values in Table 2. Disease stage was significantly associated with immunopositivity for mAb CT7.33 (CT7; $P = 0.04$); marginally significant relationships were found between disease stage and immunostaining with mAb E978 (NY-ESO-1; $P = 0.07$) or CT10.5 (CT10; $P = 0.09$). Associations between

six antigens were also examined based on Fisher's exact test; these tests revealed a P value of 0.001 between mAb E978 (NY-ESO-1) and mAb CT10.5 (CT10), which implies the existence of a strong association between these two mAbs. However, no association was found between mAb E978 (NY-ESO-1) and mAb M3H67 (MAGE-A3; $P = 0.78$).

Discussion

Successful development of cancer vaccines hinges on the identification of appropriate target antigens, the distribution of these antigens in target tumors, and effective immunization strategies that can induce tumoricidal immune responses (2). Because of their almost exclusive presence in cancer, CTAs are regarded as ideal targets for the immunotherapy of cancer and, hence, have received wide attention for vaccine development (17). Although the number of CTAs identified has been increasing, knowledge of the expression pattern of these antigens at the protein level is still limited due, in large part, to the availability of antigen isolation methods (6, 18–20). Protein analyses do, however, require the time-consuming generation of serologic reagents. We and others have now generated and studied mAbs to several CTAs, revealing that CTA

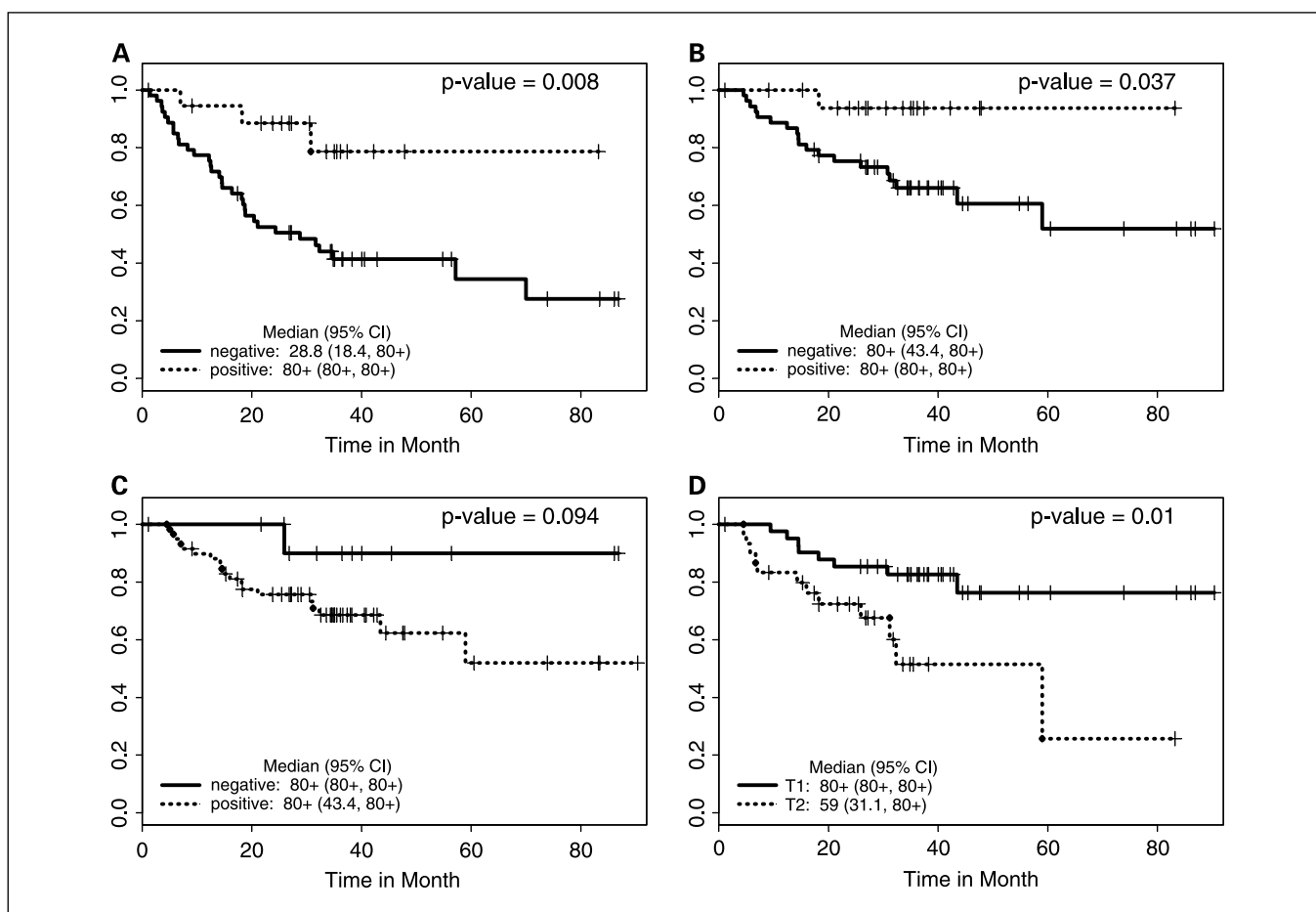


Fig. 4. Kaplan-Meier plots of disease-free survival (A) and overall survival (B-D) according to immunohistochemical expression of CT10.5 or disease stage. P values shown are from log-rank tests. A, disease-free survival according to positive versus negative staining for CT10.5 (CT10). B, overall survival according to positive versus negative staining for CT10.5. C, overall survival according to positive versus negative staining for 57B (MAGE-A4). D, overall survival according to disease stage (pT₁ or T_a versus pT₂-T₄).

Table 1. Multivariate analyses of disease-free and overall survival

	Coefficient	Hazard ratio	Z	P
Disease-free survival				
57B (positive vs negative)	1.088	2.97	2.03	0.042
CT10.5 (positive vs negative)	-1.897	0.15	-3.08	0.0021
p stage (T ₂ + vs T ₁ /T _a)	0.857	2.36	2.45	0.014
Overall survival				
57B (positive vs negative)	1.9	6.676	1.85	0.065
CT10.5 (positive vs negative)	-2.46	0.0852	-2.38	0.017
p stage (T ₂ + vs T ₁ /T _a)	1.48	4.4049	3.18	0.0015

NOTE: 57B, mAb used to detect MAGE-A4; CT10.5, mAb used to detect CT10; p stage, disease stage at pathologic examination.

protein expression can be absent or rather heterogeneous in a high percentage of tumors (16, 21–25).

The present study, instead of analyzing selected antigens, evaluated a broad panel of CTAs by immunohistochemistry (22–24, 26). The immunohistochemical data in this analysis were complemented by mRNA analyses for the same antigens as those detected with the mAbs. Interestingly, the parallel RT-PCR/immunohistochemical analyses revealed congruent results in the vast majority of cases. Because CTAs have high homology, the possibility of cross-reactivity of the mAbs to related antigens cannot be excluded. This point is exemplified by discussions about the specificity of mAb 57B, which was generated to MAGE-A3 but is now considered to be reactive to MAGE-A4 (25, 27, 28). The high percentage of congruent mRNA expression and mAb reactivity suggest that the present antibodies did in fact detect their target antigen. We further broadened the panel of test antigens by including RT-PCR analyses for LAGE-1 and MAGE-A10, both of which are highly expressed in various tumors and for which no mAbs are available.

Our findings show that the CTA distribution pattern in urothelial carcinoma follows that in other tumors such as lung carcinoma and melanoma, with MAGE-A4 and MAGE-A3 present most often and the others to a lesser extent. Urothelial carcinoma of the urinary bladder was previously analyzed by RT-PCR for several MAGE antigens and their incidence in advanced-stage tumors compares well with the findings of the present study (29). NY-ESO-1 has previously been analyzed by our group and some of the data in the present study were derived from this prior analysis (14). GAGE mRNA expression was originally studied in superficial and invasive urinary bladder carcinoma, but no further distinction was made on

disease stage (30). Prior findings evaluating the presence of CTA expression in infiltrating tumors are similar to the present analysis (31). Our results of 20% expression for CT10 differs slightly from the limited previous data in unstaged tumors in which CT10 mRNA was expressed in almost half of the samples (32). However, only nine specimens were analyzed in that prior study and the 95% confidence for staining for both studies is overlapping. Previous studies have addressed the expression of SSX antigens in various tumors or expression of MAGE and other antigens in breast carcinoma, melanoma, and non-small-cell lung carcinoma (7, 33–35); however, none of these studies addressed the expression pattern of a combination of CTAs as was done in the present analysis. With the present panel of mAbs, 77% of all tumors showed expression of at least one antigen. We anticipate that as more CTAs become available, more tumors will express one or more antigens that can be used as an immunotherapy target.

A very novel finding in the present study was the association of CT10 expression with better disease-free and overall survival. Several previous studies have found an *inverse* correlation of CTA expression and survival in ovarian cancer, non-small-cell lung cancer, and gastric cancer; however, reports of CTA expression being associated with better survival have been rare (36–39). Potential reasons for the discordance between our results and those from other studies include the different tumor types, the small number of patients in all studies, heterogeneity in the stages of evaluable tumors, and different immunohistochemical methods across studies. This new correlation between CT10 expression and disease outcome will need an additional prospective study in an independent cohort of urothelial cancer patients to confirm our preliminary findings.

Table 2. Fisher’s exact test P values for assessing relationships between antigen expression and disease stage

	E978 (NY-ESO-1)	MA454 (MAGE-A1)	M3H67 (MAGE-A3)	57B (MAGE-A4)	CT7.33 (CT7)	CT10.5 (CT10)	GAGE
p stage	0.07	1.00	1.00	1.00	0.03	0.10	0.47
E978		0.21	0.78	0.52	0.04	0.00	0.00
MA454			0.12	1.00	0.23	0.10	0.22
M3H67				0.01	0.04	0.24	1.00
57B					0.03	0.16	1.00
CT7.33						0.00	0.34
CT10.5							0.00

In conclusion, the present study showed that one or more CTAs were expressed in advanced transitional-cell carcinoma of the urinary bladder and established an association between better prognosis and CT10, a recently identified CTA. Our

findings of substantial heterogeneity of antigen expression among urothelial tumors imply that successful strategies for the immunotherapy of urinary bladder carcinoma may require vaccines targeting several antigens.

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