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Expression of *MAGE-A3, NY-ESO-1, LAGE-1* and *PRAME* in urothelial carcinoma

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BACKGROUND: The potential for cancer-testis (CT) antigens as targets for immunotherapy in cancer patients has been heavily investigated, and currently cancer vaccine trials based on the CT antigens, MAGE-A3 and NY-ESO-1, are being carried out.
METHODS: We used specific q-RT-PCR assays to analyse the expression of the CT genes *MAGE-A3*, *NY-ESO-1* (*CTAG1B*), *LAGE-1* (*CTAG2*) and *PRAME* in a panel of bladder tumours from 350 patients with long-term follow-up and detailed treatment information.
RESULTS: Overall, 43% of the tumours expressed *MAGE-A3*, 35% expressed *NY-ESO-1*, 27% expressed *LAGE-1* and 20% expressed *PRAME*. In all, 56% of the tumours expressed at least one of the CT genes analysed. Univariate Cox regression analysis of CT gene expression in non-muscle-invasive tumours showed that expression of *MAGE-A3* ($P = 0.026$), *LAGE-1* ($P = 0.001$) and *NY-ESO-1* ($P = 0.040$) was significantly associated with a shorter progression-free survival. In addition, we found that patients with tumours expressing *PRAME* responded poorly to chemotherapy ($P = 0.02$, χ^2-test).
CONCLUSION: Cancer-testis genes are frequently expressed in bladder cancer and especially in tumours of high stage and grade. In addition, the CT gene expression may have both prognostic and predictive value. Development of specific immunotherapy against the CT antigens in bladder cancer may ultimately increase patient survival.
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Cancer-testis (CT) antigens are normally only expressed in the testis, apart from some expressed in the early developing embryo and the placenta. However, CT antigens are also found expressed in various tumour types including melanoma, lung cancer, bladder cancer, liver cancer and breast cancer (Simpson *et al*, 2005). More than 100 CT antigens have been reported so far and ~30 belong to multigene families on the X chromosome (Caballero and Chen, 2009). As the testis is an immunoprivileged site owing to low expression of HLA molecules, CT antigens are the promising targets of cancer immunotherapy. The development of cancer-specific immunotherapy has been ongoing for years, with some candidates reaching early-phase clinical trials (Nishiyama *et al*, 2001; Odunsi *et al*, 2007; Uenaka *et al*, 2007; Old, 2008; Francois *et al*, 2009). Supportive of this anticancer immunotherapy, T cells and antibodies reactive against tumour CT antigens have been detected in patients with improved disease outcome (Knuth *et al*, 1989; Kurashige *et al*, 2001).

Urothelial carcinoma of the urinary bladder is a common malignant disease. A total of 70 530 new cases and 14 680 deaths were estimated in United States alone in 2010 (Jemal *et al*, 2010). Patients follow two distinct disease courses with different prognosis. Approximately 75% of patients are initially diagnosed with non-muscle-invasive bladder tumours (stage Ta–T1). These patients experience a high recurrence rate but progression to a muscle-invasive stage is relatively low, depending on the stage and grade of the disease (Millan-Rodriguez *et al*, 2000). Risk factors for disease progression to a muscle-invasive cancer include lamina propria invasion, tumours of high grade of dysplasia and large size, concurrent carcinoma *in situ* (CIS), tumour multiplicity and recurrence of high-risk non-muscle-invasive tumours (Hermann *et al*, 1998). The recurrent nature of bladder cancer is a major medical problem, and it also makes bladder cancer one of the most expensive cancers to treat (Avritscher *et al*, 2006). The remaining 25% of patients presents with muscle-invasive bladder cancer at initial diagnosis. The tumours are aggressive, poorly differentiated and show a poor treatment response and frequent development of metastases despite radical cystectomy.

Patients with non-muscle-invasive tumours are often treated with a local, organ-sparing transurethral resection. This surgical procedure is often accompanied by immunotherapy consisting of intravesical administration of the Bacillus Calmette–Guérin vaccine against tuberculosis, which by an unknown mechanism reduces the risk of recurring tumours, suggesting that anticancer vaccines could be especially useful for the treatment of bladder cancer (Simons *et al*, 2008). Accordingly, the expression of several CT antigens in urothelial carcinoma has been investigated for potential immunotherapy purposes (Kurashige *et al*, 2001; Sharma *et al*, 2003, 2006; Picard *et al*, 2007; Bergeron *et al*, 2009).

We used specific q-RT-PCR assays to analyse the expression of the CT genes *MAGE-A3*, *NY-ESO-1* (*CTAG1B*), *LAGE-1* (*CTAG2*) and *PRAME* in a panel of bladder tumours from 350 patients with long-term follow-up and detailed treatment information. This is the first report to document *PRAME* expression in bladder cancer and to show a relation between *PRAME* expression and chemotherapy response in the bladder cancer. Furthermore,

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we used Illumina methylation microarrays to address the possible epigenetic regulation of these genes in normal bladder mucosa and in bladder cancer.

MATERIALS AND METHODS

Patients and biological specimens

Informed written consent was obtained from all patients, and research protocols were approved by the local ethical committees in Aarhus. Biological materials were obtained directly from surgery after removal of the necessary amount of tissue for routine pathology examination. Samples were submerged in guanidinium thiocyanate and stored at $-80\,^{\circ}C$. Recurrence-free survival was recorded from sampling visit and censored at the time of the last control cystoscopy. Disease recurrence was defined as detection of a new tumour. Progression-free survival time was recorded from sampling visit and censored at the time of the last control cystoscopy or at cystectomy. Progression of the disease was defined as invasion into the bladder muscle – verified by microscopy. Cancer-specific survival was recorded from sampling visit and until the last annotation of the patient being alive. The cause of death was obtained by a review of the hospital files. Chemotherapy response was evaluated by metric evaluation of an appropriate marker lesion (primary tumour or metastases), using sequential computer tomography scans. Positive response was assumed if the marker lesion vanished or regressed in size. Stable conditions, increasing size of the marker lesion or appearance of further metastases were considered as no response. If a salvage cystectomy was performed after primary or neo-adjuvant chemotherapy, results of the pathological evaluation were taken into consideration. In the adjuvant setting (stage pN2 at cystectomy), no later appearance of metastases was considered complete response, whereas the occurrence of any further metastases was considered no response.

Total RNA extraction and cDNA generation

Total RNA was extracted from the tumour biopsies by the Trizol RNA extraction method (Invitrogen, Carlsbad, CA, USA). Genomic DNA was removed by DNase I Amplification Grade (Invitrogen) using an RNeasy kit (Qiagen, Valencia, CA, USA) according to the instructions of the manufacturer. Total RNA was eluted in RNase-free water and RNA quality was measured using a 2100 Agilent Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). One μg DNAse treated total RNA was converted to cDNA using oligo (dT) priming. Synthesis was performed in 20-μl volumes containing $1\times$ first strand buffer, 0.5 mM of each dNTP, 10 mM of dithiothreitol, 20 U of RNase inhibitor (Promega, Madison, WI, USA), 5 μM of oligo(dT)24 and 200 U of SuperScript II Reverse Transcriptase (Invitrogen) for 60 min at 42 °C and for 15 min at 70 °C thereafter.

q-RT-PCR assays

MAGE-A3, *PRAME*, *NY-ESO-1*, *LAGE-1* and *beta-actin* transcripts were amplified by q-RT-PCR, using TaqMan chemistry and 7900 ABI system (Life Technologies, Carlsbad, CA, USA) in 96-well plates. Duplicates were performed for all PCR amplifications. To verify successful genomic DNA removal, we performed PCR amplification of an intron of the *MAGE-3* gene. All primer sequences are listed in Table 1. The cDNA corresponding to 50 ng of total RNA was amplified by PCR in a 25-μl mixture containing $1\times$ TaqMan buffer, 5 mM of $MgCl_2$, 0.4 mM of dUTP, 0.2 mM of dATP, 0.2 mM of dGTP, 0.2 mM of dCTP, 0.625 U of Ampli Taq Gold DNA polymerase, 0.05 U of UNG, 0.2 μM of each oligonucleotide primers and 0.2 μM of TaqMan MGB probe. The amplification profile was 1 cycle of 2 min at 50 °C, 1 cycle of 12 min at 95 °C and 40 cycles of 15 s at 95 °C and 1 min at 60 °C. The fluorescent signal generated by the degradation of the TaqMan probe was detected in real time during all elongation steps at 60 °C.

We included the following positive controls: (1) cDNA corresponding to 50 ng (100%) and to 0.5 ng (1%) of total RNA from Gerl melanoma cell culture (positive for MAGE-A3 and PRAME); (2) cDNA corresponding to 50 ng (100%) of total RNA from LNCap cell culture (positive for NY-ESO-1 and LAGE-1). Cell lines were obtained from LICR, USA. As negative controls, we included: (1) RNA extracted from Staq cell culture, known to be MAGE-A3-negative; (2) water submitted to reverse transcriptase and PCR steps and (3) only water submitted to PCR steps.

Calculation of CT gene expression level

Gene expression cut-off values were calculated on the basis of positive Gerl and LNCap cell RNA. The *MAGE-A3* and *PRAME* expression level of the 1% Gerl (equivalent to 0.5 ng of RNA), normalised by the beta-actin expression level of the 100% Gerl (equivalent to 50 ng of RNA), was arbitrarily taken as cut-off value. For *LAGE-1* and *NY-ESO-1*, the expression level of the 1% LNCap (equivalent to 0.5 ng of RNA), normalised by the beta-actin expression level of the 100% LNCap (equivalent to 50 ng of RNA),

Table 1 Primer sequences

Target gene	Sequence	Specificity	Primer	Dye
MAGE-A3	5′-TAAGCCTTTGTTAGAGCCTCCAA-3′	MAGE-A3 INTRON3	Forward	
	5′-GGAGAGAGGGAGCATGTGAGA-3′	MAGE-A3 INTRON3	Reverse	
	5-′TTCCATTCAGTACTCAG-3′	MAGE-A3 INTRON3	Probe	FAM, NFQ-MGB
MAGE-A3	5′-GTCGTCGGAAATTGGCAGTAT-3′	MAGE-A3 EXON 3	Forward	
	5′-TGGGGTCCACTTCCATCAG-3′	MAGE-A3 EXON 3	Reverse	
	5′-AAAGCTTCCAGTTCCTT-3′	MAGE-A3 EXON 3	Probe	FAM, NFQ-MGB
LAGE-1	5′-CCAGGAGGCCGGACAGC-3′	LAGE-1	Forward	
	5′-GGACCAGCTCCGCTTCCAT-3′	LAGE-1	Reverse	
	6-FAM-5′-CATCACGATGCCTTTCT-3′-MGB	LAGE-1	Probe	FAM, NFQ-MGB
NY-ESO-1	AGTTCACTGTGTCCGGCAACAT	NY-ESO-1	Forward	
	GACCTGATGGAGAGCTGCAGTT	NY-ESO-1	Reverse	
	6-FAM-5′-CTGACTATCCGACTGACT-3′-MBG	NY-ESO-1	Probe	FAM, NFQ-MGB
β-actin	5′-CTGGAACGGTGAAGGTGACA-3′	β-actin	Forward	
	5′-CGGCCACATTGTGAACTTTG-3′	β-actin	Reverse	
	5′-TGCTCGCTCCAACC-3′	β-actin	Probe	
PRAME	ABI assay on demand (Hs00196132_m1)			

Abbreviations: FAM = 6-carboxyfluorescein; MGB=Minor Groove Binder; NFQ = Non fluorescent quencher.

was arbitrarily taken as cut-off value. The cut-off value was calculated by the formula: Cut-off value $= 2^{(a-b)}$, a = beta-actin Ct obtained from the equivalent of 50 ng (100%) of Gerl or LNCap RNA, b = CT gene Ct obtained from the equivalent of 0.5 ng (1%) of Gerl or LNCap RNA.

A tumour sample was considered CT gene-positive when the expression level after beta-actin normalisation was equal or greater than the cut-off value described above. The expression levels for the tumour samples were calculated by the formula: CT gene expression level $= 2^{(c-d)}$, c = beta-actin Ct obtained from the equivalent of 50 ng of tumour RNA, d = CT gene Ct obtained from the equivalent of 50 ng of tumour RNA.

In addition, the following conditions had to be fulfilled before a sample was considered positive for the expression of *MAGE-A3*, *PRAME*, *NY-ESO-1* or *LAGE-1*: (1) absence of PCR contamination using the three negative controls, (2) the two positive controls had to be in an appropriate range. (3) The beta-actin Ct obtained for this sample had to be <23. If this value was >23, we consider that the quality of the RNA was too degraded to perform the assay. (4) The absence of genomic DNA contamination by requiring a difference of at least five Ct between the *MAGE-A3* intron PCR and the *MAGE-A3* exon PCR.

Promoter methylation analysis

Infinium methylation microarray (Illumina, San Diego, CA, USA) data was generated as described previously (Reinert *et al*, 2011). In brief, we used 1 μg of DNA from each sample for whole-genome amplification and hybridised this to the Infinium arrays. Slides were scanned by a BeadXpress Reader instrument (Illumina) and data analysed by the Bead Studio Methylation Module Software (Illumina). For each probe, a beta value was calculated, which approximately corresponded to the average methylation percentage in the sample analysed.

Table 2 Clinical and histopathological characteristics

Total number of patients	350
Median age (range)	70 (31–93)
Male–female ratio	3.5
Median follow-up time for all patients (range)	34 (0–141) months
Median follow-up time for stage Ta–T1tumour patients	59 (0–141) months
Median follow-up time for stage T2–4 tumour patients	9 (0–124) months
Stage	
Ta	87
T1	49
T2–4	214
Grading (Bergkvist)[a]	
1	23
2	56
3	213
4	41
Unknown	17
Number of progression events to stage T2–4 bladder cancer (no prior muscle-invasive cancer)	
Ta	5
T1	9
Chemotherapy	
Primary chemotherapy	28
Secondary chemotherapy	23
Adjuvant chemotherapy	5
Neoadjuvant chemotherapy	1

[a]The Bergkvist grading system may be translated into the WHO 2004 grading system using the following grouping: G1 + G2 = LOW grade, G3 + G4 = HIGH grade.

Statistical analysis

We used STATA 10.0 statistical analysis software (Stata Corporation, College Station, TX, USA) for calculation of log-rank tests for equality of survival function, Kaplan–Meier survival plots, Wilcoxon rank-sum tests and univariate and multivariate Cox regression analysis. Variables with a *P*-value <0.01 in univariate analysis were included in multivariate analysis to identify variables with independent significance. The assumptions of proportional hazards were verified.

RESULTS

Expression of CT genes

We analysed expression of *MAGE-A3*, *PRAME*, *LAGE-1* and *NY-ESO-1* in a panel of tumours from 350 patients with bladder cancer by q-RT-PCR. Clinical and histopathological characteristics are listed in Table 2. First, we analysed the expression levels of the CT genes. We found that *MAGE-A3* was expressed significantly higher than *PRAME* ($P<0.001$), *LAGE-1* ($P<0.001$) and *NY-ESO-1* ($P<0.001$). Furthermore, *LAGE-1* and *NY-ESO-1* were expressed significantly higher than *PRAME* ($P<0.001$) (Figure 1A). High expression was also significantly correlated with high stage and grade (Figure 1B and C and Table 3). Following, we dichotomised the CT gene expression data into positive and negative CT gene expression (see Materials and Methods for detailed description). We found that overall 43% of the tumours expressed *MAGE-A3*, 20% expressed *PRAME*, 27% expressed *LAGE-1* and 35% expressed *NY-ESO-1*. In the group of non-muscle-invasive tumours, we found that 24% expressed *MAGE-A3*, 8% expressed *PRAME*, 17% expressed *LAGE-1* and 24% expressed *NY-ESO-1*. In total, 56% of the tumours expressed at least one of the CT genes analysed. Overall, *MAGE-A3*, *LAGE-1* and *NY-ESO-1* expression showed the highest correlation (Table 4). The dichotomised expression of all CT genes analysed was significantly correlated with age, tumour size, concomitant CIS, stage and grade. The detailed results of the PCR screening are listed in Table 5.

Prognostic value of CT gene expression in patients with non-muscle-invasive tumours

When analysing CT gene expression in non-muscle-invasive tumours from patients without any prior muscle-invasive tumour, we found that the expression of *LAGE-1* ($P=0.0004$; log-rank test) and *MAGE-A3* ($P=0.0599$) was significantly and in trend associated with a shorter progression-free survival (Figure 1). Analysis of Ta and T1 tumours separately and analysis of combinations of all, or subgroups of the CT genes, did not result in better prediction of outcome (results not shown). Univariate Cox regression analysis showed that expression of *MAGE-A3* ($P=0.026$), *LAGE-1* ($P=0.001$) and *NY-ESO-1* ($P=0.040$) was significantly associated with progression-free survival (Table 6). Multivariate Cox regression analysis including disease stage and grade showed that *LAGE-1* expression was an independent prognostic variable of muscle invasion in non-muscle-invasive bladder cancer (HR = 3.4 (95% CI: 1.26–9.25), $P=0.016$).

Cancer-testis gene expression in non-muscle-invasive tumours was not associated with recurrence-free survival (results not shown).

Prognostic and predictive value of CT gene expression in patients with muscle-invasive tumours

Cox regression analysis and Kaplan–Meyer survival estimates of cancer-specific- and metastasis-free survival as the function of CT gene expression showed no significant differences in muscle-invasive tumours (results not shown). A total of 57 patients

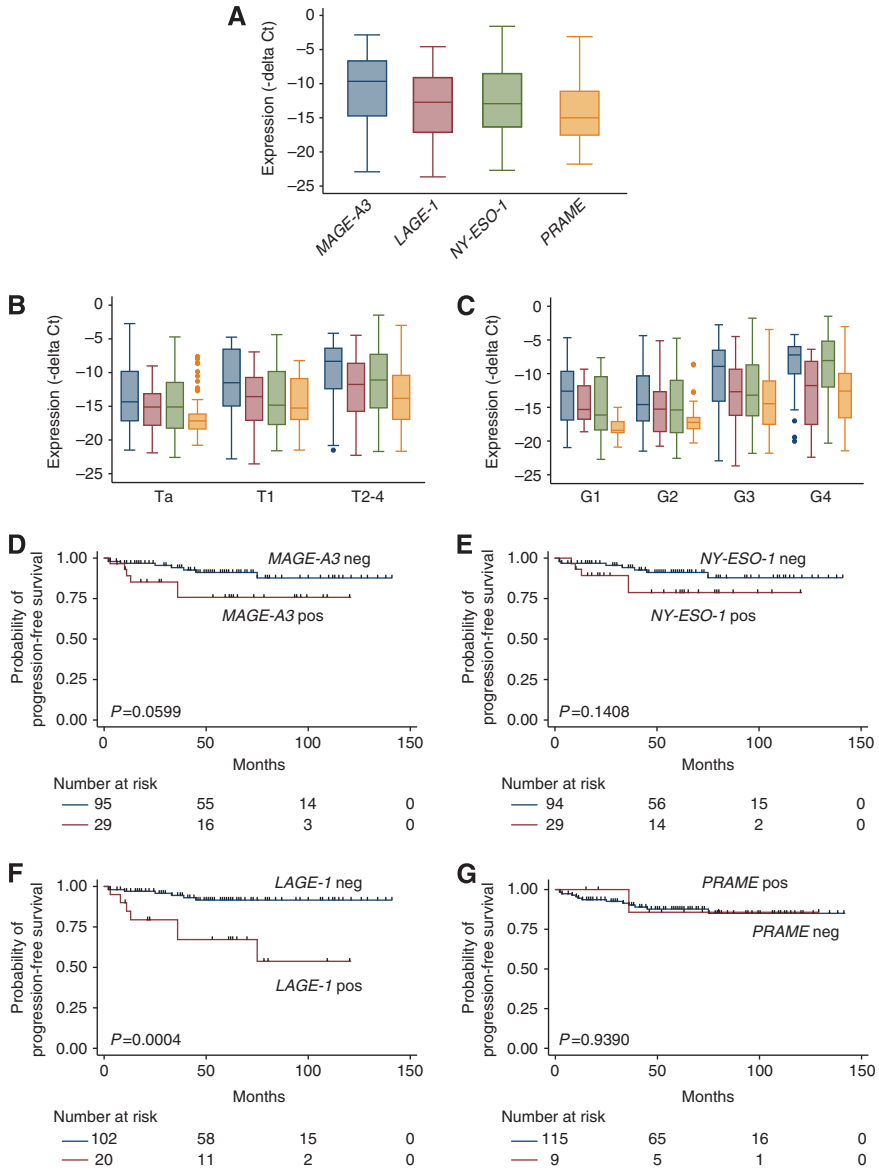

Figure 1 Expression of CT genes and correlation to outcome. Comparison of CT gene expression for all tumours combined (**A**) and stratified for stage (**B**) and grade (**C**). Expression is plotted as: − 1×(cross threshold (Ct) CT gene − Ct beta actin). High Ct values indicate high gene expression and *vice versa*. The colour coding in (**B** and **C**) is the same as in **A**. Kaplan–Meier survival estimates of progression-free survival as a function of *MAGE-A3* expression (**D**), *NY-ESO-1* expression (**E**), *LAGE-1* expression (**F**) and *PRAME* expression (**G**). Only patients with non-muscle-invasive tumours and no prior muscle-invasive tumours were included in the analysis ($n = 124$).

Table 3 Statistical differences in CT gene expression levels between stages and grades

	MAGE-A3	*PRAME*	*LAGE-1*	*NY-ESO-1*
Ta *vs* T1	0.002	<0.001	0.237	0.609
Ta *vs* T2–4	<0.001	<0.001	<0.001	<0.001
T1 *vs* T2–4	0.03	0.209	0.115	<0.001
Grade 1+2 *vs* Grade 3+4	<0.001	<0.001	0.024	<0.001

Table 4 Pairwise correlation coefficients for dichotomized CT gene expression

	MAGE-A3	*PRAME*	*LAGE-1*	*NY-ESO-1*
MAGE-A3	1			
PRAME	0.2826	1		
LAGE-1	0.4691	0.2831	1	
NY-ESO-1	0.5777	0.2761	0.5732	1

received chemotherapy and 65 patients received radiation therapy during the disease courses, and we were therefore able to investigate if any of the analysed CT genes were predictive of treatment response. Interestingly, we found that patients with tumours expressing *PRAME* responded poorly to chemotherapy ($P = 0.02$, χ^2-test) when comparing tumours from patients with complete and partial response with tumours from patients with no response. *LAGE-1*, *NY-ESO-1* and *MAGE-A3* expression was not correlated to chemotherapy response. None of the markers showed any correlation to radiation response (results not shown).

Table 5 Cancer-testis (CT) gene expression according to clinical and histopathological characteristics

	MAGE-A3			*NY-ESO-1*			*LAGE-1*			*PRAME*			Combined		
	N	Positive *n* (%)	*P*	*N*	Positive *n* (%)	*P*	*N*	Positive n (%)	*P*	*N*	Positive *n* (%)	*P*	*N*	Positive *n* (%)	*P*
Age															
⩾70	175	86 (49%)		175	68 (39%)		174	61 (35%)		178	43 (24%)		178	113 (63%)	
<70	171	62 (36%)	**0.017**	171	51 (30%)	0.09	172	34 (20%)	**0.002**	171	28 (16%)	0.084	172	82 (48%)	**0.004**
Sex															
Male	270	120 (44%)		270	99 (37%)		269	78 (29%)		272	56 (21%)		273	158 (58%)	
Female	76	28 (37%)	0.294	76	20 (26%)	0.102	77	17 (22%)	0.250	77	15 (19%)	0.486	77	37 (48%)	0.153
Tumour size															
<3 cm	166	55 (33%)		164	43 (26%)		164	36 (22%)		166	22 (13%)		166	82 (49%)	
⩾3 cm	168	86 (51%)	**0.001**	170	71 (42%)	**0.004**	171	57 (33%)	**0.021**	171	45 (26%)	**0.003**	172	106 (62%)	**0.028**
Stage															
Ta	86	16 (19%)		86	18 (21%)		85	8 (9%)		86	3 (3%)		87	27 (31%)	
T1	49	17 (35%)		48	14 (29%)		48	14 (29%)		49	8 (16%)		49	27 (55%)	
T2–4	211	115 (55%)	<**0.0001**	212	87 (41%)	**0.003**	213	73 (34%)	<**0.0001**	214	60 (28%)	<**0.0001**	214	141 (66%)	<**0.0001**
Grade[a]															
1	23	3 (13%)		23	4 (17%)		23	2 (9%)		23	0 (0%)		23	6 (26%)	
2	55	9 (16%)		55	8 (15%)		56	5 (9%)		56	2 (4%)		56	14 (25%)	
3	211	102 (48%)		210	78 (37%)		209	63 (30%)		212	48 (23%)		213	135 (63%)	
4	40	24 (60%)		41	19 (46%)		41	16 (39%)		41	13 (32%)		41	29 (71%)	
ND[b]	17	10 (59%)	<**0.0001**	17	10 (59%)	<**0.0001**	17	9 (52%)	<**0.0001**	17	8 (47%)	<**0.0001**	17	11 (65%)	<**0.0001**
CIS[c]															
No CIS	83	16 (19%)		83	15 (18%)		82	10 (12%)		83	2 (2%)		83	24 (29%)	
CIS	19	9 (47%)	**0.017**	18	7 (39%)	0.064	18	8 (44%)	**0.004**	18	4 (22%)	**0.009**	19	14 (78%)	<**0.0001**
BCG[d]															
CR	11	3 (27%)		11	2 (18%)		11	2 (18%)		11	1 (9%)		11	4 (36%)	
PR	61	28 (46%)		63	24 (38%)		63	20 (32%)		63	19 (30%)		63	38 (60%)	
NR	49	28 (57%)	0.164	49	23 (47%)	0.208	49	22 (45%)	0.183	50	14 (28%)	0.432	50	34 (70%)	0.153

Abbreviations: BCG = Bacille Calmette-Guérin; CIS = carcinoma *in situ*; CR = complete response; ND = not determined; NR = no response; PR = partial response. Fisher's exact test statistics was used for measuring differences in categorical variables. In the combined analysis, tumours should be positive for at least one of the CT genes. [a]Bergkvist grading system. [b]Not determined – only associated with stage T2–4 tumours. [c]CIS in selected site biopsies. Analysis only performed for patients with non-muscle-invasive tumours from whom selected site biopsies were taken ($n = 102$). [d]Response to BCG installations. Bold indicates significant *P*-values ($P < 0.05$).

Table 6 Univariate Cox regression analysis of progression-free survival

	Hazard ratio (95% CI)	*P*-value
Age (per 5 year increment)	1.28 (0.99–1.67)	0.063
Sex (men *vs* women)	1.51 (0.53–4.27)	0.442
Stage (T1 *vs* Ta)	4.41 (1.55–12.52)	**0.005**
Grade (G3 + G4 *vs* G1 + G2)[a]	3.69 (1.20–11.35)	**0.023**
Concomitant CIS (presence *vs* absence)	2.23 (0.69–7.26)	0.182
Tumour size (>3 cm *vs* ⩽3 cm)	0.48 (0.16–1.48)	0.204
MAGE-A3 expression (pos vs neg)	2.96 (1.14–7.68)	**0.026**
PRAME expression (pos vs neg)	0.61 (0.08–4.61)	0.633
LAGE-1 expression (pos vs neg)	4.88 (1.88–12.65)	**0.001**
NY-EOS-1 expression (pos vs neg)	2.82 (1.04–7.61)	**0.040**

Abbreviations: CI = confidence interval; CIS = carcinoma *in situ*. Bold indicates significant *P*-values ($P < 0.05$). [a]Bergkvist grading system.

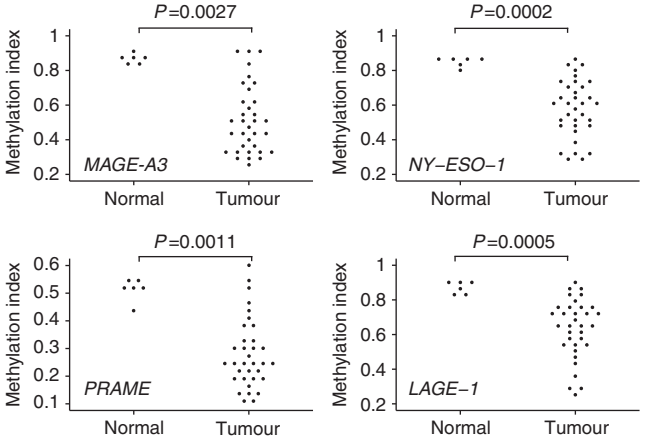

Figure 2 Methylation status of *MAGE-A3*, *PRAME*, *LAGE-1* and *NY-ESO-1* promoter regions. Distribution plots of methylation index (beta values) differences between normal samples and tumours. Beta values range from 0 to 1, where 0 indicates no methylation and 1 indicates full methylation. Statistical differences were calculated using the Mann–Whitney test.

Methylation status of *MAGE-A3*, *PRAME*, *LAGE-1* and *NY-ESO-1* promoter regions

Cancer-testis genes are normally silenced because of the promoter hypermethylation (Sigalotti *et al*, 2004). To address this further for the CT genes studied here in bladder tumours, we analysed the previously generated Illumina methylation array data from six normal urothelial samples and 36 bladder tumours (19 Ta tumours, 9 T1 tumours and 8 T2-4 tumours) (Reinert *et al*, 2011). We found that all four CT genes showed a significant hypomethylation in bladder tumours compared with normal tissue (Figure 2). No significant differences between the different tumour stages were observed in this relatively small sample set. No RNA was available from this tumour series to make direct correlations between the methylation levels and the mRNA levels for the CT genes studied.

DISCUSSION

The potential for CT antigens as targets for immunotherapy in cancer patients has been heavily investigated in recent years, and currently cancer vaccine trials based on the CT antigens MAGE-A3 and NY-ESO-1 are being carried out. Here, we describe the expression of the CT genes *MAGE-A3*, *PRAME*, *NY-ESO-1* and *LAGE-1* in tumours from a large cohort of patients with bladder cancer. We found that the expression of the CT genes was highly associated with high stage and grade of the disease. In addition, we found that the expression of *LAGE-1* was significantly associated with poor outcome for patients with non-muscle-invasive bladder cancer – also when stratifying for disease stage and grade in multivariate analysis. Interestingly, we found that patients with tumours expressing *PRAME* responded poorly to chemotherapy.

To our knowledge, this is the largest expression study of CT genes in bladder cancer. Our results are in agreement with earlier studies of CT gene expression in bladder cancer. *MAGE-A3* was reported to be expressed in 25%–58% of tumours (Patard *et al*, 1995; Nishiyama *et al*, 2001; Sharma *et al*, 2006; Picard *et al*, 2007), whereas *NY-ESO-1* and *LAGE-1* were reported to be expressed in ~30% and 40%–47% in a maximum of 104 bladder tumours, respectively (Kurashige *et al*, 2001; Sharma *et al*, 2003; Sharma *et al*, 2006). *PRAME* is a CT gene expressed in various solid tumours as well as different haematological malignancies (Epping *et al*, 2008; Santamaria *et al*, 2008). Until now, this CT gene has remained uncharacterised in urothelial carcinoma.

Several previous reports have shown that increased level of CT gene expression is associated with a poor outcome. In lung cancer, it was shown that *NY-ESO-1* and *MAGE-A3* expression was significantly associated with a poor outcome in 523 patients (Gure *et al*, 2005). In another study of gastrointestinal cancer it was shown that patients with MAGE-A1, MAGE-A3, MAGE-A4, MAGE-C1 and NY-ESO-1 antigen-positive tumours had a significantly shorter recurrence-free survival (Perez *et al*, 2011). Expression of several CT genes has been reported to be associated with castrate-resistant prostate cancer (Suyama *et al*, 2010), and in ovarian cancer *MAGE-A4* expression was reported to be associated with poor survival (Yakirevich *et al*, 2003). Similar observations have also been observed previously in bladder cancer for MAGE-*A4* and *MAGE-A9* (Bergeron *et al*, 2009). Using immunohistochemistry, it was shown that expression of the CT antigens MAGE-A4 and MAGE-A9 was significantly associated with a shorter progression-free survival. Furthermore, the expression of MAGE-A9 was also significantly associated with a shorter recurrence-free survival. In this present study, we showed that the expression of the CT genes *MAGE-A3*, *LAGE-1* and *NY-ESO-1* was significantly associated with a poor progression-free survival, whereas no association was observed regarding recurrence-free survival for any of the CT genes studied.

Several studies have documented a link between the expression of CT antigens and chemotherapy resistance. It was shown by Monte *et al* (2006) that MAGE-A2 inhibited p53 function through histone deacetylase recruitment, and thereby conferred resistance to etoposide. Perez *et al* (2011) showed a correlation between CT antigen expression and poor tumour response to imatinib in gastrointestinal tumours. In a study of prostate cancer, it was shown that MAGE-A2 siRNA knockdown in LNCaP cells treated with docetaxel resulted in a significant reduction in cell survival (Suyama *et al*, 2010). In CML patients it was shown by De Carvalho *et al* (2011) that PRAME regulated TRAIL, and upon PRAME knock down it was possible to induce TRAIL expression, and thereby increase imatinib sensitivity. These results are in line with our observations that *PRAME* expression was associated with poor response to chemotherapy in the bladder cancer patients. Our results were based on only 57 patients receiving different chemotherapy regimens, and the specific effect of CT gene expression on drug response needs to be further characterised in larger patient cohorts and through *in vitro* studies. This finding might indicate that patients with high *PRAME* expression should have adjuvant immunotherapy treatment either alone or together with cisplatin-based treatment.

We found that the CpG islands in the DNA promoter regions of the CT genes analysed were hypermethylated in normal bladder tissue and that significant hypomethylation occurred in the tumour samples. Consequently, the general global hypomethylation observed in cancer may be the reason for reexpression of selected genes in the bladder tumours. However, it is not known whether other transcriptional regulatory mechanisms might also be involved in CT gene expression, and further work is needed in order to establish a direct link between promoter methylation and CT gene expression.

Cancer-testis antigens for immunotherapy must be highly expressed in order to maximise immunotherapy efficiency. In addition, multifunctional immunotherapy targeting several antigens from several different tumour types and metastases may be beneficial to increase efficiency. Alternatively, the components of the immunotherapy could be tailored to the specific cancer or even for the individual patient. Such a personalised approach would be interesting to include in future clinical trials.

In our research, we found that the *MAGE-A3*, *NY-ESO-1* and *LAGE-1* were expressed in a large fraction of bladder tumours of high stage and grade, and consequently, this may pave the way for successful immunotherapy with high efficiency.

In conclusion, CT genes are frequently expressed in bladder cancer and especially in tumours of high stage and grade. In addition, the antigens may have both prognostic and predictive value. Development of specific immunotherapy against the CT antigens in bladder cancer may ultimately increase patient survival.

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REFERENCES

Avritscher EB, Cooksley CD, Grossman HB, Sabichi AL, Hamblin L, Dinney CP, Elting LS (2006) Clinical model of lifetime cost of treating bladder cancer and associated complications. *Urology* **68**(3): 549–553

Bergeron A, Picard V, LaRue H, Harel F, Hovington H, Lacombe L, Fradet Y (2009) High frequency of MAGE-A4 and MAGE-A9 expression in high-risk bladder cancer. *Int J Cancer* **125**(6): 1365–1371

Caballero OL, Chen YT (2009) Cancer/testis (CT) antigens: potential targets for immunotherapy. *Cancer Sci* **100**(11): 2014–2021

De Carvalho DD, Binato R, Pereira WO, Leroy JM, Colassanti MD, Proto-Siqueira R, Bueno-Da-Silva AE, Zago MA, Zanichelli MA, Abdelhay E, Castro FA, Jacysyn JF, Amarante-Mendes GP (2011) BCR-ABL-mediated upregulation of PRAME is responsible for knocking down TRAIL in CML patients. *Oncogene* **30**(2): 223–233

Epping MT, Hart AA, Glas AM, Krijgsman O, Bernards R (2008) PRAME expression and clinical outcome of breast cancer. *Br J Cancer* **99**(3): 398–403

Francois V, Ottaviani S, Renkvist N, Stockis J, Schuler G, Thielemans K Colau D, Marchand M, Boon T, Lucas S, van der Bruggen P (2009) The CD4(+) T-cell response of melanoma patients to a MAGE-A3 peptide vaccine involves potential regulatory T cells. *Cancer Res* **69**(10): 4335–4345

Gure AO, Chua R, Williamson B, Gonen M, Ferrera CA, Gnjatic S, Ritter G, Simpson AJ, Chen YT, Old LJ, Altorki NK (2005) Cancer-testis genes are coordinately expressed and are markers of poor outcome in non-small cell lung cancer. *Clin Cancer Res* **11**(22): 8055–8062

Hermann GG, Horn T, Steven K (1998) The influence of the level of lamina propria invasion and the prevalence of p53 nuclear accumulation on survival in stage T1 transitional cell bladder cancer. *J Urol* **159**(1): 91–94

Jemal A, Siegel R, Xu J, Ward E. Cancer statistics (2010) *CA Cancer J Clin* **60**(5): 277–300

Knuth A, Wolfel T, Klehmann E, Boon T, Meyer zum Buschenfelde KH (1989) Cytolytic T-cell clones against an autologous human melanoma: specificity study and definition of three antigens by immunoselection. *Proc Natl Acad Sci USA* **86**(8): 2804–2808

Kurashige T, Noguchi Y, Saika T, Ono T, Nagata Y, Jungbluth A, Ritter G, Chen YT, Stockert E, Tsushima T, Kumon H, Old LJ, Nakayama E (2001) Ny-ESO-1 expression and immunogenicity associated with transitional cell carcinoma: correlation with tumor grade. *Cancer Res* **61**(12): 4671–4674

Millan-Rodriguez F, Chechile-Toniolo G, Salvador-Bayarri J, Palou J, Algaba F, Vicente-Rodriguez J (2000) Primary superficial bladder cancer risk groups according to progression, mortality and recurrence. *J Urol* **164**(3 Pt 1): 680–684

Monte M, Simonatto M, Peche LY, Bublik DR, Gobessi S, Pierotti MA, Rodolfo M, Schneider C (2006) MAGE-A tumor antigens target p53 transactivation function through histone deacetylase recruitment and confer resistance to chemotherapeutic agents. *Proc Natl Acad Sci USA* **103**(30): 11160–11165

Nishiyama T, Tachibana M, Horiguchi Y, Nakamura K, Ikeda Y, Takesako K, Murai M (2001) Immunotherapy of bladder cancer using autologous dendritic cells pulsed with human lymphocyte antigen-A24-specific MAGE-3 peptide. *Clin Cancer Res* **7**(1): 23–31

Odunsi K, Qian F, Matsuzaki J, Mhawech-Fauceglia P, Andrews C, Hoffman EW, Pan L, Ritter G, Villella J, Thomas B, Rodabaugh K, Lele S, Shrikant P, Old LJ, Gnjatic S (2007) Vaccination with an NY-ESO-1 peptide of HLA class I/II specificities induces integrated humoral and T cell responses in ovarian cancer. *Proc Natl Acad Sci USA* **104**(31): 12837–12842

Old LJ (2008) Cancer vaccines: an overview. *Cancer Immun* **8**(Suppl 1): 1

Patard JJ, Brasseur F, Gil-Diez S, Radvanyi F, Marchand M, Francois P, Abi-Aad A, Van Cangh P, Abbou CC, Chopin D, Boon T (1995) Expression of MAGE genes in transitional-cell carcinomas of the urinary bladder. *Int J Cancer* **64**(1): 60–64

Perez D, Hauswirth F, Jager D, Metzger U, Samartzis EP, Went P, Jungbluth A (2011) Protein expression of cancer testis antigens predicts tumor recurrence and treatment response to imatinib in gastrointestinal stromal tumors. *Int J Cancer* **128**(12): 2947–2952

Picard V, Bergeron A, Larue H, Fradet Y (2007) MAGE-A9 mRNA and protein expression in bladder cancer. *Int J Cancer* **120**(10): 2170–2177

Reinert T, Modin C, Castano FM, Lamy P, Wojdacz TK, Hansen LL, Wiuf C, Borre M, Dyrskjot L, Orntoft TF (2011) Comprehensive genome methylation analysis in bladder cancer: identification and validation of novel methylated genes and application of these as urinary tumor markers. *Clin Cancer Res* **17**(17): 5582–5592

Santamaria C, Chillon MC, Garcia-Sanz R, Balanzategui A, Sarasquete ME, Alcoceba M, Ramos F, Bernal T, Queizan JA, Penarrubia MJ, Giraldo P, San Miguel JF, Gonzalez M (2008) The relevance of preferentially expressed antigen of melanoma (PRAME) as a marker of disease activity and prognosis in acute promyelocytic leukemia. *Haematologica* **93**(12): 1797–1805

Sharma P, Gnjatic S, Jungbluth AA, Williamson B, Herr H, Stockert E, Dalbagni G, Donat SM, Reuter VE, Santiago D, Chen YT, Bajorin DF, Old LJ (2003) Frequency of NY-ESO-1 and LAGE-1 expression in bladder cancer and evidence of a new NY-ESO-1 T-cell epitope in a patient with bladder cancer. *Cancer Immun* **3**: 19

Sharma P, Shen Y, Wen S, Bajorin DF, Reuter VE, Old LJ, Jungbluth AA (2006) Cancer-testis antigens: expression and correlation with survival in human urothelial carcinoma. *Clin Cancer Res* **12**(18): 5442–5447

Sigalotti L, Fratta E, Coral S, Tanzarella S, Danielli R, Colizzi F, Fonsatti E, Traversari C, Altomonte M, Maio M (2004) Intratumor heterogeneity of cancer/testis antigens expression in human cutaneous melanoma is methylation-regulated and functionally reverted by 5-aza-2'-deoxycytidine. *Cancer Res* **64**(24): 9167–9171

Simons MP, O'Donnell MA, Griffith TS (2008) Role of neutrophils in BCG immunotherapy for bladder cancer. *Urol Oncol* **26**(4): 341–345

Simpson AJ, Caballero OL, Jungbluth A, Chen YT, Old LJ (2005) Cancer/testis antigens, gametogenesis and cancer. *Nat Rev Cancer* **5**(8): 615–625

Suyama T, Shiraishi T, Zeng Y, Yu W, Parekh N, Vessella RL, Luo J, Getzenberg RH, Kulkarni P (2010) Expression of cancer/testis antigens in prostate cancer is associated with disease progression. *Prostate* **70**(16): 1778–1787

Uenaka A, Wada H, Isobe M, Saika T, Tsuji K, Sato E, Sato S, Noguchi Y, Kawabata R, Yasuda T, Doki Y, Kumon H, Iwatsuki K, Shiku H, Monden M, Jungbluth AA, Ritter G, Murphy R, Hoffman E, Old LJ, Nakayama E (2007) T cell immunomonitoring and tumor responses in patients immunized with a complex of cholesterol-bearing hydrophobized pullulan (CHP) and NY-ESO-1 protein. *Cancer Immun* **7**: 9

Yakirevich E, Sabo E, Lavie O, Mazareb S, Spagnoli GC, Resnick MB (2003) Expression of the MAGE-A4 and NY-ESO-1 cancer-testis antigens in serous ovarian neoplasms. *Clin Cancer Res* **9**(17): 6453–6460