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mRNA Expression levels of genes involved in antitumor immunity: Identification of a 3-gene signature associated with prognosis of muscle-invasive bladder cancer

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ABSTRACT

Immunotherapy for bladder cancer has given promising results. Here we aimed to evaluate the possible involvement and prognostic value of 33 genes involved in the immune response during bladder carcinogenesis. Expression levels were assessed by quantitative real-time RT-PCR in normal and tumor human bladder samples. Immunohistochemistry was performed to evaluate the protein expression of 2 genes and relation of the mRNA and protein levels was analyzed. Tumors were obtained from 154 patients (83 with muscle-invasive bladder cancer [MIBC] and 71 non-MIBC [NMIBC]) who underwent transurethral bladder resection or radical cystectomy between 2002 and 2006. All patients signed an informed consent. Results of molecular analyses were coupled with survival analyses. Overall, 25 genes (75.8%) were significantly overexpressed in MIBC and 15 (45.5%) were deregulated in NMIBC as compared with normal tissue. On multivariate analysis, risk of NMIBC recurrence was increased with high *FOXP3/CD8* ratio and overexpression of *OX40L* (p = 0.016 and p = 0.0039, respectively). In MIBC, a molecular signature of 3 genes (*OX40L, CD8 and TIGIT*) was significantly associated with prognosis in terms of recurrence-free and overall survival (p = 0.0007 and p = 0.007). RT-PCR findings were confirmed by immunohistochemistry for CD8 and FOXP3, with high association between mRNA and protein levels. Finally, risk of recurrence of non–muscle-invasive bladder cancer was increased with high *FOXP3/CD8* ratio and *OX40L* overexpression. We identified a 3 gene molecular signature associated with prognosis of muscle-invasive bladder cancer. These results confirm the useful role of immune checkpoints in bladder carcinogenesis and suggest targets for therapy.

Abbreviations: BCG, bacillus Calmette–Guerin; Cdna, cDNA; MIBC, muscle-invasive bladder cancer; mRNA, messenger ribonucleic acid; NMIBC, non–muscle-invasive bladder cancer; OS, overall survival; PFS, progression-free survival; RFS, recurrence-free survival; RT-PCR, reverse-transcriptase polymerase chain reaction; TNM, tumor-node metastasis

Introduction

Bladder cancer is the sixth most common cause of cancer mortality, and its incidence has increased markedly in recent decades. Urothelial carcinoma is the predominant histologic type in the United States and Europe, accounting for 90% of all cases. About 2-thirds of newly diagnosed cases are non–muscle-invasive bladder cancer (NMIBC). These have a 60% recurrence rate and, in 10% of cases, evolve to muscle-invasive tumors. Muscle invasive bladder cancer (MIBC) occurs in one-third of cases at diagnosis. Survival greatly differs between early and advanced bladder cancer.

Currently, the prognosis of metastatic MIBC remains poor because treatment options are limited. The lack of individual prognostic factors in urothelial neoplasms calls for new molecular markers that may also serve as therapeutic targets.

However, the treatment landscape and outcomes for bladder cancer may be transformed by recently developed novel therapies, the most promising of which is immunotherapy.[1,2]

The immune system is present in almost all solid tumors, and its role in controlling growth and metastasis has been demonstrated in a large array of tumors. Indeed, the adaptive immune environment, mainly CD8+ T cells and T helper 1 cells, has been reported to have prognostic value.[3] However, the presence of tumor underlines the capacity of cancer cells to escape immune control, through physiologic mechanisms, as an immune-checkpoint inhibition of T-lymphocyte function.[4]

The immune checkpoint genes programmed death 1 (PD-1) and cytotoxic T-lymphocyte-associated protein 4 (CTLA4) have been described for bladder cancer,[5] but to our knowledge,

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🔓 Supplemental data for this article can be accessed on the publisher's website.

previous studies have included mostly MIBC samples and almost exclusively assessed the PD-1 pathway. However, the complexity and changes of the immune environment over time require additional studies of other genes involved in antitumor immunity.

To identify new molecular markers of clinical interest, we analyzed the mRNA and protein expression of a large panel of genes (n = 33) involved in the immune process in normal and tumor bladder samples, including 71 NMIBC and 83 MIBC samples, and determined any association with recurrence and survival.

Results

Patients characteristics

Patients included 25 women and 129 men (median age 70 y [range 31–9]). Pathological staging showed NMIBC in 71 patients (25 low-grade pTa, 17 high-grade pTa, 29 high-grade pT1) and high-grade MIBC in 83. Clinical and histological features and survival of NMIBC and MIBC patients are in Table 1. Characteristics for both groups were consistent with urothelial carcinoma presentation and evolution.

Table 1. Clinical and pathological characteristics of patients.

a) 71 patients with non–muscle-invasive bladder cancer (NMIBC)

	No recurrence(n = 25)	Recurrence(n = 36)		Muscle-invasive progression(n = 10)	
	No. (%)	No. (%)	p-value*	No. (%)	p-value**
Age (years)					
≥ 60	Nineteen (76.0)	Twenty-seven (75.0)	0.93	Ten (100.0)	0.08
<60	Six (24.0)	Nine (25.0)		0 (0.0)	
Sex					
Male	22 (88.0)	32 (88.9)	0.91	9 (90.0)	0.89
Female	3 (12.0)	4 (11.1)		1 (10.0)	
Smoking status					
Non-smoker	13 (52.0)	15 (41.7)	0.43	5 (50.0)	0.81
Smoker	12 (48.0)	21 (58.3)		5 (50.0)	
History of NMIBC					
No	22 (88.0)	13 (36.1)	<0.0001	4 (40.0)	0.31
Yes	3 (12.0)	23 (63.9)		6 (60.0)	
Associated pTis					
No	25 (100.0)	36 (100.0)	0.99	8 (80.0)	0.0004
Yes	0.0 (0.0)	0 (0.0)		2 (20.0)	
Grade					
Low grade	10 (40.0)	14 (38.9)	0.93	1 (10.0)	0.07
High grade	15 (60.0)	22 (61.1)		9 (90.0)	
Tumor stage					
Ta	15 (60.0)	24 (66.7)	0.59	3 (30.0)	0.043
T1	10 (40.0)	12 (33.3)		7 (70.0)	

*Chi^2 test (recurrence vs no recurrence)
**Chi^2 test (muscle-invasive progression vs others)

b) 83 patients with muscle-invasive bladder cancer (MIBC)

	No. of patients (%)	Recurrence-free survival(n = 48)		Overall survival(n = 46)	
		No. of events (%)[a]	p-value*	No. of events (%)[b]	p-value*
Age (years)					
≥ 60	61 (73.5)	40 (65.5)	0.017	39 (63.9)	0.009
<60	Twenty-two (26.5)	Eight (36.4)		Seven (31.8)	
Sex					
Male	66 (79.5)	36 (54.5)	0.23	38 (57.6)	0.44
Female	17 (20.5)	12 (70.6)		8 (47.1)	
Smoking status					
Non-smoker	34 (41.0)	18 (52.9)	0.45	12 (35.3)	0.002
Smoker	49 (59.0)	30 (61.2)		34 (69.4)	
History of NMIBC					
No	59 (71.1)	30 (50.8)	0.043	31 (52.5)	0.41
Yes	24 (28.9)	18 (75.0)		15 (62.5)	
Associated pTis					
No	73 (88.0)	43 (58.9)	0.59	40 (54.8)	0.76
Yes	10 (12.0)	5 (50.0)		6 (60.0)	
Tumor stage					
T2	34 (41.0)	17 (50.0)	0.10	13 (38.2)	0.009
≥T3	49 (59.0)	31 (63.3)		33 (67.3)	
Lymph node status					
N-	58 (69.9)	27 (46.6)	0.002	25 (43.1)	0.0006
N+	25 (30.1)	21 (84.0)		21 (84.0)	

[a]first recurrence (local or metastatic);
[b]death
*Chi^2 test

For the 71 NMIBC cases, the median follow-up was 57.4 months (range 1–158, mean 61 months); 36 (50.7%) recurred as NMIBC during follow-up. The progression rate to muscle-invasive tumor was 14.1% (n = 10).

For the 83 MIBC cases, the median follow-up was 12.3 months (range 1–152, mean 28 months). During follow-up, 41 patients (48.8%) died of bladder cancer and 5 (5.9%) of unrelated causes.

Gene expression

For all genes except *LGALS9*, the expression profile differed between MIBC and NMIBC samples (Table 2). All genes except CD96 were overexpressed in MIBC as compared with NMIBC samples. In MIBC samples, 25/33 genes (75.8%) were significantly deregulated as compared with normal bladder tissue ($p < 0.05$), all showing overexpression, except for *TGFB3* which was significantly under-expressed. In NMIBC, 15/33 genes (45.5%) were significantly deregulated as compared with normal bladder tissue ($p < 0.05$), with 8 downregulated (*PD-L2, CD4, CD8, CD226, TIM3, IFNG, IL10*, and *TGFB3*) and 7 upregulated (*CD80, LGALS9, CD96, TNFRSF9, TDO2, OX40* and *FOXP3*). Regarding NMIBC samples, 15 genes showed significant differences in expression by stage, most overexpressed in pT1 tumors as compared with pTa tumors. In contrast, only 2 genes (i.e., *TNFSF9* and *CD86*), showed different expression profiles by grade (low versus high) (Supplemental data 1).

Supplemental Data 2 shows for the 4 main drug-targeted genes, the number with co-altered mRNA expression (Venn diagram).

Association between gene expression and prognosis of NMIBC

On univariate analysis, only the expression of *FOXP3* and *OX40L* was associated with RFS, and only *FOXP3* expression was associated with PFS. Risk of recurrence was increased with *OX40L* overexpression (5 year-recurrence rate = 90.9% vs. 71.8%, log rank p = 0.0023). Risk of both recurrence and progression to muscle-invasive tumors was increased with *FOXP3* overexpression (5 year-recurrence rate = 81.4% vs. 69.4%, p = 0.0062 and 5 year-progression rate = 34.4% vs. 9.2%, p = 0.018, respectively).

As described in previous studies, we analyzed FOXP3 and CD8 expression together by defining a *FOXP3/CD8* ratio determined by using *FOXP3* and *CD8* mRNA values in each NMIBC sample to obtain a quantitative value (median value [range] = 6.25 [0.94–45.01]). Prognosis in terms of RFS was worse with *FOXP3/CD8* ratio ≥ 6.25 than with a lower ratio (p = 0.010) (Fig. 1).

Multivariate analyses included covariates associated with RFS or PFS showing significance at p<0.05 on univariate analysis, i.e., history of NMIBC, *FOXP3/CD8* ratio and *OX40L* status

Table 2. mRNA expression of the 33 studied genes in normal and tumor bladder samples.

GENES	Normal(n = 15)	NMIBC(n = 71)	MIBC(n = 83)	MIBC/NMIBC p-value[**]	AUC[£]	NMIBC/N p-value[**]	AUC[£]	MIBC/N p-value[**]	AUC[£]
CD8	1.00 [0.28–3.89][*]	0.31 [0.04–6.13][*]	1.00 [0.09–45.55][*]	$<10^{-7}$	0.766	0.00026	0.199	1	0.500
CD4	1.00 [0.35–2.38]	0.63 [0.13–3.95]	1.41 [0.15–14.20]	0.00000028	0.746	0.014	0.3	0.15	0.616
FOXP3	1.00 [0.00–2.51]	2.42 [0.34–20.44]	6.16 [0.71–56.57]	$<10^{-7}$	0.742	0.00011	0.815	$<10^{-7}$	0.956
PRF1	1.00 [0.27–2.47]	0.37 [0.00–5.59]	1.50 [0.11–36.45]	$<10^{-7}$	0.820	0.067	0.235	0.051	0.659
GZMA	1.00 [0.34–5.23]	2.03 [0.04–38.43]	7.93 [0.56–168.88]	$<10^{-7}$	0.791	0.11	0.695	0.00000031	0.916
INFG	1.00 [0.00–9.24]	0.28 [0.00–13.74]	1.18 [0.00–93.38]	0.00000043	0.742	0.0080	0.285	0.35	0.576
IL10	1.00 [0.35–2.88]	0.23 [0.00–1.50]	1.59 [0.14–12.89]	0.00000044	0.902	0.00000092	0.402	0.11	0.629
TGFB3	1.00 [0.29–1.79]	0.03 [0.00–0.38]	0.19 [0.01–1.80]	$<10^{-7}$	0.880	$<10^{-7}$	0.002	0.00015	0.072
TIGIT	1.00 [0.14–1.87]	0.82 [0.08–14.73]	3.55 [0.26–68.06]	$<10^{-7}$	0.796	0.19	0.477	0.0000028	0.881
PVR	1.00 [0.47–1.77]	0.84 [0.00–1.80]	1.35 [0.35–5.26]	$<10^{-7}$	0.815	0.11	0.366	0.0025	0.746
CD96	1.00 [0.21–4.28]	2.84 [0.00–15.59]	2.00 [0.02–15.44]	0.013	0.384	0.0043	0.766	0.066	0.650
CD226	1.00 [0.35–1.63]	0.19 [0.00–3.28]	0.80 [0.05–7.32]	$<10^{-7}$	0.810	0.000041	0.100	0.29	0.414
OX40	1.00 [0.00–2.04]	1.45 [0.25–12.54]	4.20 [0.36–30.09]	$<10^{-7}$	0.806	0.043	0.684	$<10^{-7}$	0.937
OX40L	1.00 [0.00–6.08]	0.72 [0.00–12.23]	10.56 [0.00–110.27]	$<10^{-7}$	0.892	0.99	0.536	0.0000021	0.886
TNFSF9	1.00 [0.00–4.07]	0.54 [0.00–10.45]	2.63 [0.05–25.00]	$<10^{-7}$	0.845	0.9	0.396	0.00021	0.802
TNFRSF9	1.00 [0.00–4.81]	1.92 [0.00–23.68]	12.69 [0.52–254.52]	$<10^{-7}$	0.840	0.047	0.667	$<10^{-7}$	0.948
TNFSF18	1.00 [0.00–3.77]	0.53 [0.00–14.71]	2.67 [0.00–213.81]	$<10^{-7}$	0.787	0.72	0.513	0.00043	0.787
TNFRSF18[££]	2.19 [0.00–14.21]	5.08 [0.44–165.03]	20.35 [1.89–185.72]	$<10^{-7}$	0.804	0.14	0.687	$<10^{-7}$	0.941
IDO1	1.00 [0.14–2.18]	1.58 [0.00–54.40]	10.25 [0.35–2896.87]	$<10^{-7}$	0.811	0.15	0.699	$<10^{-7}$	0.948
IDO2[££]	0.00 [0.00–3.57]	0.50 [0.00–8.70]	0.95 [0.00–174.89]	0.027	0.604	0.34	0.674	0.0038	0.735
TDO2	1.00 [0.00–6.66]	19.61 [0.84–277.11]	77.17 [3.61–596.90]	$<10^{-7}$	0.793	0.0055	0.954	$<10^{-7}$	0.997
LGALS9	1.00 [0.21–1.61]	2.56 [0.22–8.96]	2.72 [0.20–70.56]	0.33	0.546	0.00062	0.834	0.0000067	0.867
TIM3	1.00 [0.43–3.26]	0.42 [0.02–3.58]	2.37 [0.19–20.16]	$<10^{-7}$	0.899	0.0079	0.200	0.00084	0.772
LAG3	1.00 [0.44–2.57]	0.47 [0.00–24.07]	2.96 [0.05–159.10]	$<10^{-7}$	0.840	0.9	0.291	0.00075	0.774
ICOSLG	1.00 [0.41–2.12]	0.88 [0.19–7.01]	1.74 [0.22–10.75]	0.00000038	0.738	0.51	0.462	0.00079	0.773
ICOS	1.00 [0.00–2.32]	1.09 [0.00–19.86]	3.88 [0.16–56.58]	$<10^{-7}$	0.775	0.14	0.561	0.0000016	0.891
PD-L1	1.00 [0.31–2.68]	0.60 [0.08–7.96]	3.94 [0.09–45.68]	$<10^{-7}$	0.863	0.13	0.376	0.000027	0.841
PD-L2	1.00 [0.43–1.92]	0.17 [0.01–3.54]	1.37 [0.06–26.77]	$<10^{-7}$	0.884	0.00000025	0.074	0.25	0.593
PD-1	1.00 [0.00–4.67]	0.79 [0.00–11.27]	4.31 [0.28–106.99]	$<10^{-7}$	0.815	0.26	0.526	0.0000040	0.876
CTLA4	1.00 [0.00–5.35]	1.63 [0.00–25.08]	9.12 [0.39–306.94]	$<10^{-7}$	0.809	0.085	0.613	0.00000023	0.921
CD28	1.00 [0.18–3.86]	0.64 [0.02–8.28]	1.58 [0.07–14.06]	$<10^{-7}$	0.767	0.61	0.343	0.037	0.669
CD80	1.00 [0.00–5.39]	2.46 [0.00–24.17]	12.46 [1.00–185.95]	$<10^{-7}$	0.855	0.024	0.700	$<10^{-7}$	0.984
CD86	1.00 [0.29–2.23]	0.45 [0.07–5.98]	2.11 [0.12–24.28]	$<10^{-7}$	0.832	0.38	0.257	0.0035	0.738

[*]Median value [range]
[**]Kruskal Wallis H test
[£]Area under the Receiver Operating Characteristic (ROC) curve
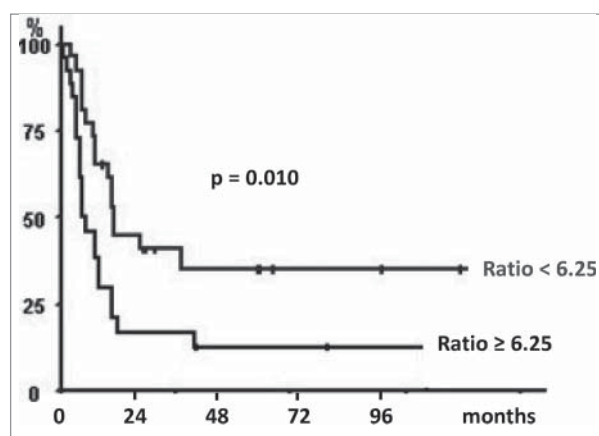[££]The mRNA values of the samples were normalized so that a Ct value of 35 was set to 1.

Figure 1. Recurrence-free survival according to *FOXP3/CD8* ratio in non–muscle-invasive bladder cancer (NMIBC).

for recurrence, and stage, grade and *FOXP3* status for progression to muscle-invasive tumor. *FOXP3/CD8* ratio and *OX40L* status remained independent prognostic factors significantly associated with RFS (p = 0.016 and p = 0.0039, respectively) (Table 3a), with none associated with PFS.

Response to bacillus Calmette–Guerin (BCG) therapy

In total, 21/28 (75%) patients with BCG therapy showed recurrent NMIBC or progression to invasive tumor during follow-up, including 15 (53.6%) over the first 2 y. None of the 33 investigated genes was associated with response to BCG-therapy, although FOXP3 expression was 3-fold increased but not significantly associated with response (Supplemental data 3).

Association between mRNA expression and survival of MIBC

On univariate analysis for RFS and OS, prognosis was poor with *OX40L* overexpression (p = 0.027 and p = 0.014, respectively) but was improved with *CD8* overexpression (p = 0.024 and p = 0.029, respectively). The expression of *TIGIT* was also associated with OS (p = 0.042).

Identification of a molecular signature predictive of MIBC prognosis

We performed unsupervised hierarchical clustering analyses of 83 MIBC samples with the 3 genes previously found associated with survival in MIBC (*OX40L, CD8,* and *TIGIT*) and found 3 major clusters composed of 29 (Group A), 27 (Group B), and 27 (Group C) samples, respectively (Fig. 2A). Group A was characterized by normal expression of the 3 selected genes compared with normal tissue samples, group B by marked overexpression of *OX40L* only, and group C by simultaneous overexpression of the 3 genes (Fig. 2B).

The groups did not differ in clinicopathological factors (Supplemental Data 4) but did differ in RFS and OS (log rank p = 0.0002 and p = 0.0005, respectively): Kaplan-Meier survival curves showed poorer outcome associated with group B (Fig. 2C). Five-year RFS and OS rates were 11.2% and 8.3% for group B vs. 47.9% and 44.2% for group A, and 47.1% and 52.8% for group C, respectively.

Multivariate analyses included covariates associated with RFS or OS showing significance at p < 0.05 on univariate analysis, i.e., tumor stage, lymph node status and the 3 gene signature. It retained lymph node status and the 3 gene signature as independent prognostic factors in both RFS (p = 0.002 and
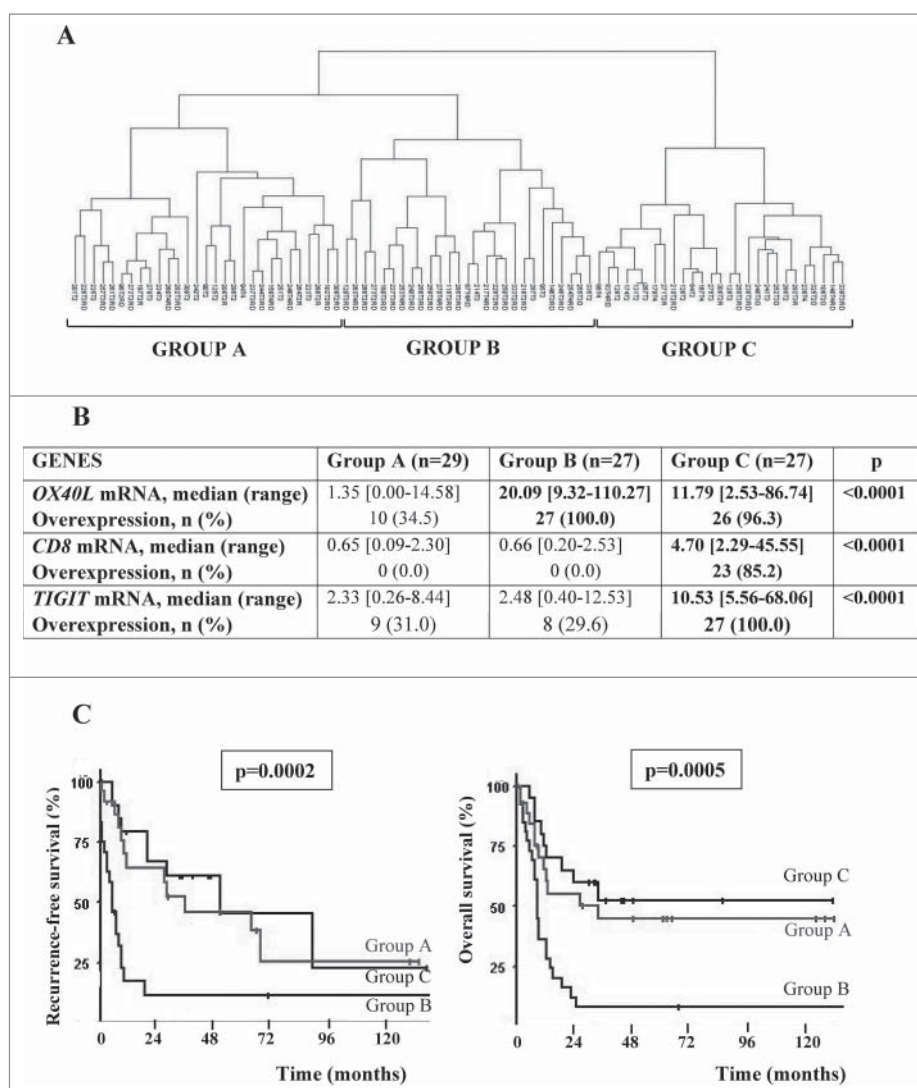
Table 3. Cox proportional-hazards regression analysis.

a: Factors affecting recurrence-free survival with NMIBC.

Prognostic factor	Recurrence-free survival		
	Adjusted HR	95% CI	p value[*]
History of NMIBC	2.66	[1.43–4.94]	0.0019
FOXP3/CD8 *ratio*	2.11	[1.15–3.88]	0.016
OX40L *overexpression*	2.27	[1.04–4.97]	0.0039

b: Factors affecting recurrence-free and overall survival with MIBC.

Prognostic factor	Recurrence-free survival			Overall survival		
	Adjusted HR	95% CI	p value[*]	Adjusted HR	95% CI	p value[*]
T Stage			0.19			0.23
≤T2	1			1		
T3–4	1.62	[0.79–3.34]		1.57	[0.75–3.28]	
N+ status	3.37	[1.56–7.30]	0.002	3.26	[1.61–6.60]	0.001
3 gene signature			0.0007			0.007
Group C	1			1		
Group A	2.08	[1.36–3.19]		1.74	[1.16–2.61]	
Group B	4.34	[1.85–10.19]		3.04	[1.36–6.80]	

[*]Cox model
HR: hazard ratio; CI: confidence interval

Figure 2. Supervised classification analysis of 83 muscle-invasive bladder cancer (MIBC) samples with the 3 gene signature of *OX40L, CD8* and *TIGIT*.

p = 0.0007, respectively) and OS (p = 0.001 and p = 0.007, respectively) (Table 3b).

Immunohistochemistry analyses

CD8 protein expression ($\geq 1+$) in immune cells was significantly more common in MIBC than NMIBC samples (p = 0.0084) (Table 4). Importantly, mRNA and protein data for both CD8 and FOXP3 were strongly associated (p = 0.00000016 and p = 0.00000093 respectively) (Supplemental data 5). The prognostic significance of FOXP3/CD8 ratio was analyzed by protein scoring (0, 1+ or 2+) and we found no significant difference in RFS or PFS for NMIBC (data not shown). For OX40L and TIGIT, identified as genes of interest in MIBC, immunohistochemistry analysis was not possible because of lack of available reliable antibodies.

Discussion

The lack of individual prognostic factors in urothelial neoplasms calls for new molecular markers that might also serve as

therapeutic targets. Because of the potential implication of anti-tumor immunity in bladder cancer, we assessed the mRNA levels of 33 genes related to the immune environment and identified prognostic markers in both NMIBC and MIBC. In our study population, half of the tumors were superficial and half invasive. In these 2 populations, the classical prognostic factors were confirmed, and recurrence and progression rates agree with those usually reported. As expected, expression profiles for most of the genes differed significantly between NMIBC and MIBC. Specific cell markers and cytokines involved in the immune response, such as *CD8, FOXP3, TGFB3, IFNG, IL10*, were deregulated in the early stage of bladder carcinogenesis, with downregulation in NMIBC samples. However, genes involved in immune checkpoint pathways were predominantly overexpressed at the muscle-invasive stage.

In NMIBC, risk of disease recurrence was increased with *OX40L* and *FOXP3* overexpression and high *FOXP3/CD8* ratio. Regarding the literature, the role of *FOXP3* in immune control or dysregulation is still unclear. Indeed, FoxP3 has been found a key regulator in the development and function of regulatory T cells and seems to play a major role in preventing

Table 4. Immunohistochemistry scores for CD8 and FOXP3 expression in immune cells.

	CD8				FOXP3			
	0 + (%)	1+ (%)	2 + (%)	P*	0 + (%)	1 + (%)	2 + (%)	P*
All samples (n = 108)	19 (17.6)	53 (49.1)	36 (33.3)		26 (24.1)	46 (42.6)	36 (33.3)	
NMIBC (n = 50)	14 (28.0)	27 (54.0)	9 (18.0)	0.0084	16 (32.0)	21 (42.0)	13 (26.0)	0.074
MIBC (n = 58)	5 (8.6)	26 (44.8)	27 (46.0)		10 (17.2)	25 (43.1)	23 (39.7)	

*Chi-2 test (0 + versus 1+/2+)

autoimmune disease or maintaining self-tolerance.[6] In cancer, FOXP3 upregulation has been found to have protective or pro-tumorigenic effects in various solid tumors.[7] Some authors suggest that tumor cells could have FOXP3-dependent suppressive effects on T cells and that the mimicking of the regulatory T-cell function by tumor cells may represent a possible mechanism of tumor resistance to immune destruction in the microenvironment and thus facilitate tumor progression.[8,9] Increased number of intra-tumoral infiltrating FOXP3+ cells and increased FOXP3/CD8 ratio were described as prognostic factors associated with worse survival in several cancers.[10-12]

Our results agree with previously published studies, finding the *FOXP3/CD8* mRNA ratio significantly associated with RFS in NMIBC, with worse prognosis when this ratio was high. Blocking the suppressive T-cell function by stimulating CD8+ T-effector cells should be an effective immunotherapy to improve outcomes in this cancer. Finally, although *FOXP3* mRNA level seemed to be higher in patients with than without response to BCG therapy, FOXP3 did not significantly predict response to BCG therapy, but the small number of patients who received the treatment may explain the lack of statistical power in our study.

In MIBC, we identified a 3 gene signature, *CD8, OX40L* and *TIGIT*, associated with both RFS and OS that allowed us to distinguish 3 groups of tumors with differential expression of these genes. Group C was characterized by a relative high expression level of *CD8* compared with the 2 other groups, which seemed to be associated with improved prognosis. In contrast, group B was characterized by a marked overexpression of *OX40L* but downregulation of *CD8*, and this molecular profile was significantly associated with poor prognosis. The relative reduction in *CD8* expression may reflect reduced tumor immunogenicity, thereby allowing for immune tolerance toward tumor cells or a tumor ignorance by the immune system.[13] However, when neither *OX40L* nor *TIGIT* were overexpressed, the prognosis remained good, as if the tumor's ability to stimulate the immune response was too low, perhaps related to a weak mutation load (group A). The 3 groups did not differ in clinicopathological criteria, which suggests that our 3 gene signature was associated with MIBC prognosis, regardless of conventional tumor-aggressive features. These results were confirmed on multivariate analyses, so the prognostic ability of the 3 gene signature to identify patients with poor outcomes may be even more powerful than stage, which probably reflects the biologic behavior of tumor cells. This molecular profiling may help identify patients who would be most likely to benefit from adjuvant treatments and closer follow-up.

In other cancers, the therapeutic efficacy of immune checkpoint inhibitors such as TIGIT and OX40L are still under investigation. TIGIT has recently been identified as a co-inhibitory receptor that critically limits CD8+ T-cell–dependent immune responses.[14,15] A recent publication underlined that OX40L upregulation, like PD-1 and CTLA-4 pathway activation, is associated with the epithelial-to-mesenchymal transition and drug response,[16] but its role in bladder cancer was still unknown. Accumulating preclinical evidence supports the clinical development of anti-OX40 monoclonal antibodies in several solid tumors and thus in bladder cancer according to our results.[17]

In recent years, many trials testing immune checkpoint inhibition in bladder cancer were launched, and most of them are still ongoing. Assessing the association of gene expression with response to systemic therapies is of interest. The results of our study suggest that the expression of specific cell markers, particularly *CD8*, may be good biologic markers reflecting the immunogenicity of the tumor and is easily assessable by RT-PCR or immunohistochemistry, because we found a good association of mRNA and protein levels.

In conclusion, regulation of several immune genes is associated with urothelial carcinogenesis, which suggests an interplay between both the tumor and immune compartment. Some of these molecular alterations could appear very early during carcinogenesis, involving mostly specific cell genes encoding for adaptive immune markers or cytokines. RFS was worse with *OX40L* and *FOXP3* overexpression, and high *FOXP3/CD8* ratio in NMIBC. As well, immune checkpoint alterations might emerge at invasive steps of carcinogenesis, which suggests their role in tumor escape from the immune response. We identified a 3 gene signature associated with both RFS and OS in MIBC; patients with poor prognosis because of this gene profile may benefit from new adjuvant strategies and/or intense follow-up.

Modification of the tumor microenvironment by combined administration of new immunotherapies represents a promising pharmacological approach, as suggested by our results. However, considering the cost of the drugs, their accurate prescription is warranted and response to treatment should be correctly assessed. CD8 may be the best prognostic marker, well reflecting the immunogenicity of the tumor and easily assessable. Large prospective clinical studies with molecular evaluation of tumors are needed.

Materials and methods

Patients and Samples

We analyzed 154 urothelial carcinoma samples from patients who had undergone transurethral bladder resection or radical cystectomy in our hospital between January 2002 and January

2006. Specimens of normal bladder tissue from 15 patients undergoing surgery unrelated to bladder tumors (transurethral resection of the prostate or prostatic adenomectomy) were used as normal bladder tissue. All patients signed an informed consent. This study received approval from an institutional review board and was conducted according to the principles outlined in the Declaration of Helsinki.

Immediately after surgery, tumor samples were frozen in liquid nitrogen and stored at $-80°C$ (for RNA extraction) and fixed in formaldehyde. Each tumor was reviewed by 2 pathologists (DD and MS) who were blinded to clinical outcomes. Tumors were re-staged according to the 2009 American Joint Committee on TNM classification of bladder tumors and graded according to the 2004 World Health Organization grading scheme.[18,19]

Data were obtained from the patients' medical records. Patients were followed up according to current guidelines.

Gene selection

From the literature on antitumor immunity, we selected 33 genes involved in the immune process, including PD-1, CTLA4, the pathways indoleamine 2,3-dioxygenase (IDO) and T-cell immunoreceptor with immunoglobulin and immunoreceptor tyrosine-based inhibition motif (ITIM) domains (TIGIT), tumor necrosis factor (TNF) receptor superfamily members (including TNF superfamily, member 4 [OX40] and its ligand OX40L), and other specific cellular markers of immune response such as CD4, CD8, interferon γ (INFγ), transforming growth factor-β3 (TGFB3) and interleukin 10 (IL-10) (Supplemental data 6). We chose one endogenous RNA control gene, namely TBP (GenBank Accession No. NM_003194) which encodes the TATA box-binding protein.

Real-time quantitative RT-PCR

The theoretical basis, primers and PCR consumables, RNA extraction, cDNA synthesis, and PCR reaction conditions have been described previously in detail.[20]

Quantitative values were obtained from the cycle threshold (Ct) number at which the increase in signal associated with exponential growth of PCR products was first detected. Each sample was normalized to TBP level. Results, expressed as N-fold differences in target gene expression relative to the TBP gene, and termed "Ntarget," were determined as $Ntarget = 2^{\Delta Ctsample}$, where the ΔCt value of the sample was determined by subtracting the Ct value of the target gene from the Ct value of the TBP gene. Ntarget values for samples were normalized such that the median of the 15 normal bladder Ntarget values was 1. For 2 genes, namely TNFRSF18 and IDO2, because of low expression, the mRNA values were normalized such that a Ct value of 35 was set to 1.

For each investigated gene, mRNA values ≥ 3 were considered to represent overexpression and ≤ 0.33 under-expression. We previously used the same cut-off value for altered tumor gene expression.[20]

Analysis of protein expression

Representative blocks of paraffin-embedded tumor were available for 108 patients (50 NMIBC and 58 MIBC). For each tumor, 2 observers, including at least 1 expert pathologist, selected the tumor block containing the highest density of immune cells on hematoxylin and eosin-safranin–stained slides. Briefly, serial 5-μm tissue sections were deparaffinized, rehydrated and pretreated in appropriate buffer for antigen retrieval by using a Leica automat. Tissue slides were then incubated at 48°C with a primary antibody, anti-CD8 (SP16, Spring Biosciences) or anti-FOXP3 (263A/E7, Abcam) (both 1:100), then appropriate secondary antibodies.

We used a semi-quantitative analysis of protein expression with the following scores: 0 (no positive cells), 1+ (few positive cells) and 2+ (numerous positive immune cells). The same score was used for analysis of CD8 and FOXP3 on tumor immune infiltrating cells. All quantification was performed with blinding to patient status by an expert pathologist (D.D.).

Statistical analysis

The clinicopathological features of NMIBC and MIBC were tested for association with tumor recurrence and survival by using Student's t test for continuous variables or chi-square test for categorical variables. Data are presented as median (range). The associations between clinical and histological variables and mRNA levels were tested by the non-parametric Mann-Whitney U and Kruskal-Wallis H tests (a link between 1 categorical and 1 quantitative variable).

Unsupervised hierarchical cluster analyses were performed using WARD algorithm to identify homogenous tumor groups regarding molecular data.

Overall survival (OS) was calculated from the date of surgery until death or the last follow-up. Recurrence-free survival (RFS) was defined as the time from the date of surgery until the first local relapse or first metastasis. For NMIBC, progression-free survival (PFS) was defined as the time from the date of surgery until progression to muscle-invasive disease. Patients were censored if they had not experienced the end-point of interest at the time of last follow-up. Survival curves were derived by the Kaplan-Meier method, with the log-rank test used to compare survival between groups.

Cox proportional-hazards regression was used to estimate hazard ratios (HRs) and their 95% confidence intervals (95% CIs) for covariates associated with RFS, PFS or OS showing significance at p < 0.05 on univariate analysis.

Differences between 2 populations were judged significant at confidence levels greater than 95% (p < 0.05).

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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