Expression balance of ectoenzymes CD39 and CD73 predicts invasiveness in human bladder cancer

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Abstract

Background

An accurate prediction of progression is critical to define the management of bladder cancer (BC). The ectonucleotidases CD39 and CD73 play strategic roles in calibrating purinergic signals via an extracellular balance between ATP and adenosine. The altered expression of these enzymes plays a potential role in tumor invasion and metastasis, therefore, it has been proposed in the prognosis of solid tumors. In BC this is not yet clear. This study aimed to evaluate CD39 and CD73 expression in a cohort of patients with non-muscle-invasive (NMI) and muscle-invasive (MI) BC regard to its association with clinicopathological features.

Methods

Retrospective clinical follow-up data and primary urothelial BC specimens of 162 patients were used. Tissue microarrays were constructed, and immunohistochemistry for CD39 and CD73 was performed to make associations with clinicopathological data.

Results

Overall, 96 were NMI (59.3%) and 66 MI (40.7%). CD39 immunoreactivity in BC cells was found in 72% of the cases, while CD73 was found in 97%. High CD39 expression alone was more frequent in NMI BC ($p < 0.001$), while high expression of CD73 was more frequent in MI BC. The association of both markers potentiated the prediction, in that high CD39 expression and low CD73 expression was strongly associated with NMI BC and low CD39 with high CD73 was associated with MI BC.

Conclusion

The invasiveness stage of BC may be predicted by the altered expression of CD39 and CD73. Therefore CD39 serves as an additional tool for the selection of patients with favorable prognosis.

Background

Bladder cancer (BC) is the most common malignancy of the urinary tract, being the fourth most common cancer in men and the ninth in women [1]. Approximately 70% to 80% of BC cases are non-muscle-invasive (NMI), but a significant portion of those patients recurs and progresses to muscle-invasive (MI) form (10–25%) despite transurethral resection and adjuvant intravesical therapies [2]. MI BC is a more aggressive disease, being associated with a 5-years survival rate of 60% and 10% for patients with localized disease and metastases, respectively[2]. In these cases, radical cystectomy is often necessary. In this context, the management of BC and its complications results in a major economic burden.

Nucleotides and nucleosides constitute important groups of molecules known to modulate many pathophysiological functions in the extracellular space through activation of the purinergic receptors. The equilibrium of the purinergic signals is acquired by the conversion of ATP/ADP to AMP and AMP to adenosine, which occurs by hydrolytic enzymes [3]. One of these enzymes is the CD39 (ecto-nucleoside triphosphate diphosphohydrolase-1, NTPDase-1), an integral membrane protein that hydrolyzes ATP and ADP in order to yield AMP in the extracellular space [4]. The CD39 enzyme usually works with another enzyme named CD73 (ecto-5'-nucleotidase, Ecto5'NTase), which dephosphorylates AMP into adenosine [5]. While ATP drives pro-inflammatory immune cell activity, adenosine promotes a depressive action on the immune cell

activity and has anti-inflammatory effects [3]. Therefore, the combination of CD39 and CD73 may determine an immunossuppressive environment to support neoplastic growth, survival, and progression [6]. Expression of CD39 and CD73 has been identified in various human tumors, including in kidney cancer, ovarian cancer, testicular cancer, pancreatic cancer, lung cancer, thyroid cancer, lymphoma, sarcoma, and leukemia [7–10]. Regarding BC, the role of CD39/CD73 is not clear, although purinergic signaling has been described in bladder tumors [11].

Stella et al. have shown that the profile of ectonucleotidase activity correlates to malignancy in BC. There is a distinct pattern in metabolizing nucleotides between NMI and MI bladder tumors. Working with human BC cell lines they demonstrated that BC cell with low malignancy phenotype exhibited a high level of hydrolysis of tri- and diphosphonucleosides due to high expression of NTPDase 3. In contrast, cells with a high malignancy phenotype exhibited a robust reduction in the NTPDase 3 expression [12]. A few years later the same group worked with a chemical-induced bladder tumor model demonstrating that the immunostaining for NTPDase is progressively lost during the disease progression, in contrast to CD73 expression that increased over time [13].

Considering the exposed above we hypothesized that information on the expression of CD39 and CD73 genes in human specimens of bladder tumor could be useful in the prognosis of BC. In the present study, we investigated the expression of CD39 and CD73 in patients treated for NMI and MI urothelial bladder carcinoma.

Methods

Retrospective clinical follow-up data and tumor specimens of 165 patients were used in this study. Our cohort is represented by a series of 165 consecutive primary urothelial bladder tumors consisting of pTa, pT1, pT2, pT3, and pT4 collected between 2011 and 2017. The samples were made available by the Department of Pathology of the Hospital de Transplante Euryclides de Jesus Zerbini, the hospital where the patients underwent transurethral resection (TUR) or radical cystectomy. Tumor stage and grade were assigned according to AJCC/TNM 2010 and WHO 2004 classifications. CB recurrence or metastasis was determined via imaging and/or histological analysis. The descriptive characteristics of the cohort are depicted in Table 1. The exclusion criteria were: (i) patients with the previous history of other cancers; (ii) patients had received preoperative chemotherapy, BCG-adjuvant therapy or radiation therapy; and (iii) unavailability of clinicopathological data or paraffin blocks. Approval for the study was given by the Institutional Board of Ethics of the Hospital Brigadeiro UGA V-SP endorsed by the Institutional Board of Ethics of the Nove de Julho University (UNINOVE) (CAAE: 49446515.0.0000.5511).

Table 1
Correlation of CD39 expression with clinicopathological parameters of 162 BC patients.

Parameters	No. of Patients	CD39			P Value
		Negative	Low expression	High expression	
Gender					
Male	129	39 (30%)	34 (26%)	56 (44%)	0.538
Female	33	07 (21%)	11 (33%)	15 (46%)	
Age					
≤ 65	48	13 (27%)	12 (25%)	23 (48%)	0.782
> 65	114	33 (29%)	33 (29%)	48 (42%)	
Smoking					
No	31	06 (19%)	06 (19%)	19 (61%)	0.128
Yes	120	35 (29%)	35 (29%)	50 (42%)	
Unknown	11	05 (46%)	04 (36%)	02 (18%)	
Grade					
Low	42	8 (19%)	16 (38%)	18 (43%)	0.140
High	120	38 (32%)	29 (24%)	53 (44%)	
Tumor stage					
NMI	96	11 (11%)	35 (36%)	50 (52%)	< 0.001
MI	66	35 (53%)	10 (15%)	21 (32%)	
Tumor stage					
pTa	71	09 (13%)	24 (34%)	38 (54%)	< 0.001
pT1	25	02 (08%)	11 (44%)	12 (48%)	
pT2	21	14 (67%)	02 (10%)	05 (24%)	
pT3	26	11 (42%)	05 (19%)	10 (39%)	
pT4	19	10 (53%)	03 (16%)	06 (32%)	
Treatment					
TUR	87	09 (10%)	32 (37%)	46 (53%)	< 0.001
Cystectomy	75	37 (49%)	13 (17%)	25 (33%)	
Recurrence*					
No	37	04 (11%)	14 (38%)	19 (51%)	0.970
Yes	50	05 (10%)	18 (36%)	27 (54%)	
Progression*					

* Only patients who underwent transurethral resection (TUR).

Parameters	No. of Patients	CD39			P Value
No	73	09 (12%)	28 (38%)	36 (49%)	*0.210*
Yes	14	02 (14%)	02 (14%)	10 (71%)	
* Only patients who underwent transurethral resection (TUR).					

Tissue microarrays (TMA) were constructed from 165 formalin-fixed, paraffin-embedded surgical specimens (from TUR or cystectomy), represented in triplicate tissue cores of 1-millimeter diameter. A single pathologist reevaluated the hematoxylin-and-eosin-stained slides of all specimens and considered the areas that best represented the whole tumor.

Immunohistochemistry

TMA was cut and used on 3-μm-thick paraffin sections. After rehydration, antigen retrieval (microwave irradiation in citrate buffer), and blocking procedures, the sections were incubated overnight with mouse monoclonal anti-human CD39 antibody (1:500; catalog ab178572; Abcam, Cambridge, MA, USA) or rabbit polyclonal anti-human CD73 antibody (1:50; catalog ab115289; Abcam, Cambridge, MA, USA). The negative control was performed omitting the primary antibodies. To complete the sandwich, sections were incubated with LSAB + System-HRP reagents (K0690; Dako Co., Glostrup, Denmark). Finally, DAB substrate-chromogen was used to complete the reaction (K346811; Dako Co., Glostrup, Denmark), and the slides were counterstained with Harris hematoxylin. Slides were then dehydrated, coverslipped, and observed under a light microscope. The expression of CD39 and CD73 was evaluated blindly by a single pathologist (KRML) who has considered the cases as categorically negative or positive. Thus the proportion score reflects the percentage of tumor cells with CD39 or CD73 staining: score 0, negative expression; score 1, positive tumor area < 50% (low expression); and score 2, positive area > 50% (high expression). For statistical analysis, it was considered the higher score of the three TMA's cores or the sum of the three scores corresponding to the three cores for each patient. In this case, the median was considered as the cut-off to discriminate between high expression and low expression.

Statistical analysis

Pearson's Chi-square test, Fisher's exact test, and logistic regression were used to assess the association between clinical-pathological features and CD39 and CD73 expression. The odds ratio was also evaluated, in which a confidence interval of 95% was adopted. Receiver Operator Characteristic curve was generated using SPSS (version 22; SPSS, Inc., Chicago, IL), the same program for graphing. Differences with $p < 0.05$ were considered significant.

Results

Of all 165 BC patients, 162 offered viable samples for CD39 and CD73 immunostaining analysis. The samples of three patients were deteriorated during the immunohistochemistry process. Of the 162 patients, 96 were NMI (59.3%) and 66 MI (40.7%). Overall, there were 129 males (79.6%) and 33 females (20.4%) in this cohort. Most were elderly (70.4% > 65 years old), and smokers (79.5%) (Table 1). Regarding histological grade, 74% of patients were high grade and 26% were low grade. The tumor stage between pT0 to pT4 was found in our cohort, with pT0 being more prevalent (46.8%). Of all 87 patients undergoing TUR for a primary pTa or pT1, 50 had tumor recurrence (57.5%) and 14 had disease progression (16.1%) (Table 1).

[Table 1]

All tumor samples could be evaluated for CD39 and CD73 immunostaining (three TMA cores per patient). The immunoreactivity for both CD39 and CD73 was predominantly cytoplasmatic in neoplastic cells. Of the total number of

patients, 46 (28.4%) were negative for CD39 immunostaining, 46 (28.4%) presented weak CD39 immunostaining (low expression), and 71 (43.8%) presented strong CD39 immunostaining (high expression). As demonstrated in Table 1, high CD39 expression was associated with NMI (P < 0.001), and lower tumor stage (p < 0.001). High CD39 expression was also associated with the modality of treatment (TUR), probably due to the association between the high CD39 expression and NMI BC. CD39 expression was not associated with grade, recurrence, or tumor progression (Table 1).

Regarding CD73 immunostaining, five patients were negative, 24 presented low expression of CD73, while 133 presented high expression (Table 2). CD73 immunostaining was not associated with clinicopathological features (Table 2). However, when combined to the CD39 expression, a significant association with tumor stage was found (Table 3). Low expression of both CD39 and CD73 was associated with advanced BC, similarly to the condition of low expression of CD39 and high expression of CD73 (Tables 3 and 4). In contrast, high expression of CD39 and low expression of CD73 as well as the high expression for both proteins was associated with the NMI form of BC (Tables 3 and 4).

Table 2
Correlation of CD73 expression with clinicopathological parameters of 162 BC patients.

Parameters	No. of Patients	CD73			P Value
		Negative	Low expression	High expression	
Gender					
Male	129	04 (03%)	22 (17%)	103 (80%)	0.281
Female	33	01 (03%)	02 (06%)	30 (91%)	
Age					
≤ 65	48	00 (00%)	06 (13%)	42 (87%)	0.273
> 65	114	05 (04%)	18 (16%)	91 (80%)	
Smoking					
No	31	01 (03%)	06 (19%)	24 (78%)	0.619
Yes	120	04 (03%)	15 (13%)	101 (84%)	
Unknown	11	00 (00%)	03 (27%)	08 (73%)	
Grade					
Low	42	01 (02%)	06 (14%)	35 (34%)	0.945
High	120	04 (03%)	18 (15%)	98 (82%)	
Tumor stage					
NMI	96	03 (03%)	13 (14%)	80 (83%)	0.860
MI	66	02 (03%)	11 (17%)	53 (80%)	
Tumor stage					
pTa	71	03 (04%)	09 (13%)	59 (83%)	0.612
pT1	25	00 (00%)	04 (16%)	21 (84%)	
pT2	21	01 (05%)	04 (19%)	16 (76%)	
pT3	26	00 (00%)	02 (19%)	24 (92%)	
pT4	19	01 (05%)	05 (26%)	13 (69%)	
Treatment					
TUR	87	02 (02%)	12 (14%)	73 (84%)	0.746
Cystectomy	75	03 (04%)	12 (16%)	60 (80%)	
Recurrence*					
No	37	01 (03%)	05 (14%)	31 (84%)	0.976
Yes	50	01 (02%)	07 (14%)	42 (84%)	
Progression*					

* Only patients who underwent transurethral resection (TUR).

Parameters	No. of Patients	CD73			P Value
No	73	02 (03%)	09 (12%)	62 (85%)	*0.564*
Yes	14	00 (00%)	03 (21%)	11 (79%)	

* Only patients who underwent transurethral resection (TUR).

Table 3
Correlation of different profiles of CD39 and CD73 expression with clinicopathological parameters of 162 BC patients.

Parameters	No. of Patients	CD39-CD73				P Value
		$CD39_{Low}CD73_{Low}$	$CD39_{High}CD73_{Low}$	$CD39_{Low}CD73_{High}$	$CD39_{High}CD73_{High}$	
Gender						
Male	129	30 (23%)	28 (22%)	24 (19%)	47 (36%)	*0.457*
Female	33	06 (18%)	04 (12%)	07 (21%)	16 (49%)	
Age						
≤ 65	48	09 (19%)	11 (23%)	10 (21%)	18 (38%)	*0.837*
> 65	114	27 (24%)	21 (18%)	21 (18%)	45 (40%)	
Smoking						
No	31	06 (19%)	12 (39%)	02 (07%)	11 (36%)	0.007
Yes	120	25 (21%)	19 (16%)	25 (21%)	51 (43%)	
Unknown	11	05 (46%)	01 (09%)	04 (36%)	01 (09%)	
Grade						
Low	42	08 (19%)	12 (29%)	05 (12%)	17 (41%)	*0.253*
High	120	28 (23%)	20 (17%)	26 (22%)	46 (38%)	
Tumor stage						
NMI	96	15 (16%)	26 (27%)	08 (08%)	47 (49%)	< 0.001
MI	66	21 (32%)	06 (09%)	23 (35%)	16 (24%)	
Tumor stage						
pTa	71	12 (17%)	22 (31%)	05 (07%)	32 (45%)	< 0.001
pT1	25	03 (12%)	04 (16%)	03 (12%)	15 (60%)	
pT2	21	09 (43%)	02 (09%)	05 (24%)	05 (24%)	
pT3	26	07 (27%)	01 (04%)	10 (39%)	08 (31%)	
pT4	19	05 (26%)	03 (16%)	08 (42%)	03 (16%)	
Treatment						
TUR	87	12 (14%)	26 (30%)	06 (07%)	43 (49%)	< 0.001
Cystectomy	75	24 (32%)	06 (08%)	25 (33%)	20 (27%)	
Recurrence*						
No	37	08 (22%)	09 (24%)	02 (05%)	18 (49%)	*0.291*

* Only patients who underwent transurethral resection (TUR).

Parameters	No. of Patients	CD39-CD73				P Value
Yes	50	04 (08%)	17 (34%)	04 (08%)	25 (50%)	
Progression*						
No	73	11 (15%)	21 (29%)	05 (07%)	36 (49%)	*0.867*
Yes	14	01 (07%)	05 (36%)	01 (07%)	07 (50%)	
* Only patients who underwent transurethral resection (TUR).						

Table 4
Expression of CD39 and CD73 in the prediction of BC stage.

	Odds Ratios	95% CI	P-value	Prediction of
$CD39_{High}$	0.158	0.079−0.315	< 0.001	NMI
$CD73_{High}$	1.077	0.570−2.034	*0.872*	None
$CD39_{Low}CD73_{Low}$	2.520	1.183−5.368	0.013	MI
$CD39_{High}CD73_{Low}$	0.269	0.104−0.698	0.005	NMI
$CD39_{Low}CD73_{High}$	5.884	2.432−14.232	< 0.001	MI
$CD39_{High}CD73_{High}$	0.334	0.167−0.666	0.002	NMI
NMI, non-muscle-invasive; MI, muscle-invasive.				

[Table 2−4]

Discussion

The ectonucleotidase enzymes play distinct roles in mammalian organisms by regulating the extracellular concentration of ATP/ADP/AMP/adenosine. The balance of nucleotides/adenosine determines the activation of distinct purinergic receptors triggering specific effects. Like other pleiotropic molecules, the expression of these enzymes may be found in several normal tissues. CD39 and CD73 are constitutively expressed in a variety of tissues and leukocytes and may be upregulated by pro-inflammatory cytokines, hypoxia, and oxidative stress [3]. Regarding normal bladder, the ectonucleotidases play role in nerve-mediated detrusor contractions and in exocytosis of the umbrella cell layer by controlling the ATP/adenosine concentrations in the extracellular space [14−15]. It is possible that CD39 and CD73 are involved in other physiological processes of the lower urinary tract, but this remains uncertain.

In the pathophysiological context, CD39 and CD73 have been found in neoplasias raising the hypothesis of their involvement in tumorigenesis [7, 16]. In the present study, we have shown that the upregulation of CD39 is associated with the non-muscle-invasive form of BC, while upregulation of CD73 with low expression of CD39 is associated with the muscle-invasive form, raising the hypothesis that the nucleotides/adenosine balance may be effective in determining invasive phenotype in BC cells.

With regard to CD39, its expression has not been studied in BC until now. On the other hand, there is some information about CD73 in urothelial tumors. Wettstein and coworkers showed that CD73 predicted favorable prognosis in patients with NMI urothelial BC [17]. In their study, cases of high expression of CD73 were more frequent in pT1 and pTa stages, although no association with CD39 expression was made [17]. More recently, Koivisto and coworkers demonstrated similar

results, expanding the analysis to MI BC. They demonstrated that high expression of CD73 by neoplastic cells was more frequent in NMI than in MI BC [18], a phenomenon not found in our study. In our study, isolated CD73 expression was not sufficient to predict the BC stage (Table 4), suggesting the use of isolated CD39 expression or CD39/CD73 expression balance as a useful strategy to access invasive status of the BC. In other tumors, CD73 has been associated with the worst prognosis. CD73 overexpression promotes invasion, migration, adhesion, and metastasis of human breast cancer cells and in melanomas [19, 20].

Although there is little information about CD39 in BC, it has been widely described in other tumor types. CD39 is overexpressed in pancreatic cancer correlating positively with long-term survival after surgery treatment [21]. In rectal adenocarcinoma, although CD39 was strongly expressed in malignant cells being associated with early tumor stage, its association with CD73 offered a better strategy to predict the prognosis [22]. The combined analysis displaying low CD39 and high CD73 expression in both protein and mRNA levels was associated with invasion and metastasis leading to an unfavorable clinical outcome [22], similarly as we have proposed herein for BC.

In terms of mechanism, acting sequentially, CD39 and CD73 efficiently hydrolyze extracellular ATP to adenosine, the latter having an effect in down-modulating the antitumor immunity via binding to purinergic receptors. Adenosine-activated A2A receptor protects tumors from antitumor T cells [23]. The blockage of CD73 using a monoclonal antibody enhances antitumor response [24]. Different from adenosine, ATP is a potent pro-inflammatory mediator limiting tumor growth. To promote antitumor immunity, ATP activates inflammasome [25], and pyroptosis [26], promotes chemotaxis of monocytes, macrophages, and neutrophils[27], and controls tumor-infiltrating CD8 + T cells [28] and NK cells [29].

Considering the above, we would propose that the overexpression of CD39 and/or CD73 could increase the adenosine concentration in the extracellular space, which could induce local immunosuppression and consequently tumor progression. However, cases of $CD39_{Low}CD73_{Low}$ and $CD39_{High}CD73_{High}$ were more frequent in MI and NMI BC, respectively (Table 4). In fact, access to ATP/adenosine status would be critical in understanding this controversial phenomenon. An important limitation of our study is the lack of technical approaches to determine ATP/adenosine in patient tumor specimens. Immunostaining analysis for CD39 and CD73 is a simplistic form to access the complex nucleotides/adenosine system. The final effects will be determined by multiple factors. First, the amount of ATP that moves into the extracellular space, either through cell injury or through channel transport. The tumor microenvironment can present a 1000-fold higher ATP concentration into interstitial space compared to normal tissues [30]. Second, in addition to CD39 and CD73, both nucleotides and nucleosides can be removed from extracellular space by several other ectoenzyme families[5]. Third, ATP and adenosine exert effects by binding purinergic receptors - P1 receptors, with adenosine as the main ligand, and P2 receptors, with ATP and ADT as the main ligands; P2 receptors are subdivided into seven ionotropic P2X and eight metabotropic P2Y subtypes [31, 32]. Therefore, the roles of ATP/adenosine balance in BC will depend on various expression levels and coexpression patterns.

Finally, our study complements the findings of Stella and coworkers, in which the profile characterized by downregulation of NTPDase 3 and upregulation of CD73 was associated with the late stage of BC [12], a phenomenon also demonstrated in an animal model [13]. In their hypothesis, that profile establishes an extracellular microenvironment with high levels of ATP and adenosine [12] favorable to tumor progression [12].

In conclusion, the altered expression of CD39 and CD73 presented herein supports the idea that these ectonucleotidases are involved in bladder tumorigenesis. Our results suggest that malignant urothelial cells of human BC strongly express CD39 and CD73; however, high expression of CD39 in tumor cells is correlated with the early stage of BC. Further studies are necessary to clarify the effective role of the CD39 and CD73 in the establishment of invasive phenotype in BC cells.

Abbreviations

BC

bladder cancer

NMI

non-muscle-invasive

MI

muscle-invasive

TMA

Tissue microarray

Declarations

Ethics approval and consent to participate

This study received Institutional Review Board approval by the Ethical Board under the protocol CAAE: 49446515.0.0000.5511.

Consent for publication

Not applicable

Availability of data and materials

The dataset supporting the conclusions of this article is included within the article.

Competing interests

The authors have no conflicts of interest to declare.

Authors' contributions

JMF participated in the design of the study, carried out the immunohistochemistry assays, performed data analysis and interpretation, statistical analysis, and drafted the manuscript.

LHGM participated in the design of the study, data analysis and interpretation, statistical analysis, and drafted the manuscript.

KRML participated in the immunohistochemistry analysis, data analysis and interpretation, and drafted the manuscript.

CBM participated in the design of the study, in the sample collection, and drafted the manuscript.

JFAC participated in the design of the study, in the sample collection, and drafted the manuscript.

CPC participated in the design of the study, performed data analysis and interpretation, statistical analysis, and drafted the manuscript.

JPJ participated in the design of the study, performed data analysis and interpretation, statistical analysis, and drafted the manuscript.

HD participated in the design of the study, performed data analysis and interpretation, statistical analysis, and drafted the manuscript.

All authors read and approved the final manuscript.

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