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ORIGINAL ARTICLE

NTPDase3 and ecto-5′-nucleotidase/CD73 are differentially expressed during mouse bladder cancer progression

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Abstract According to the World Health Organization, bladder cancer is the seventh most common cancer among men in the world. The current treatments for this malignancy are not efficient to prevent the recurrence and progression of tumors. Then, researches continue looking for better therapeutic targets which can end up in new and more efficient treatments. One of the recent findings was the identification that the purinergic system was involved in bladder tumorigenesis. The ectonucleotidases, mainly ecto-5′-nucleotidase/CD73 have been revealed as new players in cancer progression and malignity. In this work, we investigated the NTPDase3 and ecto-5′-nucleotidase/CD73 expression in cancer progression in vivo. Bladder tumor was induced in mice by the addition of 0.05 % of N-butyl-N-(hydroxybutyl)-nitrosamine (BBN) in the drinking water for 4, 8, 12, 18, and 24 weeks. After this period, mice bladders were removed for histopathology analysis and immunofluorescence assays. The bladder of animals which has received BBN had alterations, mainly inflammation, in initial times of tumor induction. After 18 weeks, mice's bladder has developed histological alterations similar to human transitional cell carcinoma. The cancerous urothelium, from mice that received BBN for 18 and 24 weeks, presented a weak immunostaining to NTPDase3, in contrast to an increased expression of ecto-5′-nucleotidase/CD73. The altered expression of NTPDase3 and ecto-5′-nucleotidase/CD73 presented herein adds further evidence to support the idea that alterations in ectonucleotidases are involved in bladder tumorigenesis and reinforce the ecto-5′-nucleotidase/CD73 as a future biomarker and/or a target for pharmacological therapy of bladder cancer.

Keywords Bladder cancer · BBN · Purinergic signaling · NTPDase3 · Ecto-5′-nucleotidase/CD73

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Introduction

Bladder cancer is the second most prevalent tumor in the genitourinary tract [1, 2]. It is the seventh most common cancer worldwide with about 336,000 new cases per year [3]. The main risk factors are smoking, which increase the risk up to six times, and occupational and environmental exposure to carcinogens [4, 5]. About 90 % of bladder cancer corresponds to transitional cell carcinoma (TCC) [1, 6] due to the fact that urothelium is constantly exposed to potential carcinogens [7]. The tumor invasiveness of TCC defines the patient's prognosis. For example, 70–80 % of patients present superficial non-muscle-invasive TCCs which are generally not life threatening; while 20–30 % of individuals have muscle-invasive TCCs with increased risk of metastasis and death [8, 9]. Superficial cancers are treated by transurethral resection (TUR), followed by chemo/immunotherapy. However, nearly 70 % of patients present tumor recurrence, where 30 % of the recurrent tumors progress to muscle-invasive disease within 5 years of TUR [8, 10]. Given the high recurrence rates and the need for frequent monitoring, this disease is one of the most expensive cancers to treat on a per patient basis from diagnosis until death [11], and hence, poses a tremendous burden on health systems worldwide [4]. Therefore, new therapeutic targets, which end up in more efficient treatments, are necessary to prevent bladder cancer recurrence and progression.

Accordingly, recent researches have focused in the potential involvement of purinergic system in bladder tumors [1, 12–14]. Nucleosides and nucleotides mediate a variety of biological functions in both short- and long-term signaling functions (development, regeneration, differentiation, proliferation, and cell death) [1, 12]. These events are mediated by the activation of P1 (for adenosine) or P2 (for ATP, ADP, UDP, and UTP) receptors and are controlled by the action of ectonucleotidases [15]. The ecto-nucleoside triphosphate diphosphohydrolases refer to a family of cell-surface enzymes that hydrolyze extracellular ATP and ADP to AMP. Studies have demonstrated the involvement of these enzymes in cancer progression [16–18]. The final dephosphorylation of nucleotides, conversion of nucleoside monophosphates (e.g., AMP) to their respective nucleosides (e.g., adenosine), is catalyzed by ecto-5′-nucleotidase/CD73 (ecto-5′-NT/CD73). This enzyme is highly expressed in a variety of solid tumors [19–22] and has both its enzymatic activity and its adhesion protein function associated with cancer progression [23, 24]. Besides, ecto-5′-NT/CD73 was found to be involved in cancer cell growth, maturation, differentiation, adhesion, migration, invasiveness, metastasis, immune escape, and drug resistance [19, 21–27].

A previous study with mouse bladders showed that mouse healthy urothelium expresses only NTPDase3, not expressing the ecto-5′-NT/CD73 [28]. This is in agreement with a previous work from our group, where we showed a differential pattern of ectonucleotidases expression in two malignant bladder cancer cells. We showed that a less malignant lineage from a TCC grade 1 of malignancy (RT4) expresses NTPDase3 and ecto-5′-NT/CD73, while a more malignant lineage, from a TCC grade 4 of malignancy (T24) only expresses ecto-5′-NT/CD73 [14]. Although little is known about the role of NTPDase3 in cancer, these findings prompted us to suspect that the loss of NTPDase3 expression and the parallel ecto-5′-NT/CD73 expression might be involved in the bladder cancer progression.

Therefore, herein we investigate the NTPDase3 and ecto-5′-NT/CD73 expression in a model of mouse bladder cancer induced by N-butyl-N-(hydroxybutyl)-nitrosamine (BBN).

Materials and methods

Reagents

BBN was purchased from Sigma (Sigma Chemical Company, St. Louis, MO, USA). Rabbit antibodies anti-rat ecto-5′-nucleotidase (rNu-9_L) and anti-rat NTPDase3 (rN3-1_L) were obtained from http://ectonucleotidases-ab.com. Alexa fluor 568 goat anti-rabbit IgG and Alexa fluor 488 phalloidin were purchased from Invitrogen (Invitrogen Co., Carlsbad, CA, USA). Optimum cutting temperature (OCT) freezing medium (Tissue-Tek; Sakura Finetek, Torrance, CA, USA). All other chemicals and solvents used were of analytical grade.

Animals

Male mice from the Balb-c lineage were used at the age of 10 weeks. Animals were obtained and maintained in the Unidade de Experimentação Animal do Hospital de Clínicas de Porto Alegre (HCPA) under a standard dark–light cycle (lights on between 7:00 a.m. and 7:00 p.m.) at room-controlled temperature (22±2 °C). The mice had free access to standard laboratory chow and water. Mice were euthanatized by isoflurane inhalation. After euthanasia, the bladders were rapidly excised and processed as described below. All animal studies were carried out in strict accordance with the recommendations in the Brazilian national law number 11.794, from October 8, 2008, which determines the procedures to scientific use of animals. The protocol (protocol 10-0104) was approved by the Research Ethics Committee at group of research and graduation of HCPA. All efforts were made to minimize animal suffering.

Bladder cancer induction

The animal model of bladder cancer induction by BBN have been used in many studies [29–33] mainly because BBN

induced alterations in the bladder of rodents that are correspondent to histopathological and molecular features of human transitional cell carcinoma [34–36]. In this work, bladder cancer was induced by the addition of 0.05 % of BBN in the drinking water for 4, 8, 12, 18, and 24 weeks with a respective control group for each induction time. The number of mice per group was 8 for control groups of 4, 8, and 12 weeks and 9, 10, and 10 for BBN groups of 4, 8, and 12 weeks, respectively; and for the cancer induction times of 18 and 24 weeks, it was 3 for control groups and 5 and 4 for groups that received BBN, respectively. The animals were weighed in an analytical balance, as well as the excised bladders. Then, the bladder wet weight was expressed as milligrams of bladder per 100 g of animal as an additional measure of edema [37].

Bladder processing

A Y-shaped cut was made in the excised bladders for further tissue processing. Thereby, bladders were disposed as a monolayer and were divided in two parts: apex and base. The tissues were embedded in OCT freezing medium and snap frozen in isopentane in dry ice and stored at −80 °C until use. In this study, we analyzed only the base of bladders due to its major and constant contact with urine, which ends up in the major probability of cancer. For this, the frozen base of bladders was sliced in cryostat to achieve histological slides (5 μm) that were used for histopathological analysis or for immunofluorescence.

Histopathological analysis

Histological slides were stained with hematoxylin and eosin (HE) for histopathological analysis. The lesions induced by BBN were classified in three groups: degeneration and inflammation, pre-neoplastic lesion, and cancer in accordance with the histological characteristics of each one. The analyses were done by a pathologist, blinded for the experimental data.

Immunofluorescence analysis of NTPDase3 and ecto-5′-NT/CD73 expression

Frozen cryostat sections (5 μm) from mouse bladder tissues were fixed in 10 % phosphate-buffered formalin mixed with cold acetone and washed three times for 5 min in Tris-buffered saline (TBS). Tissue sections were then incubated in 5 % fetal bovine serum prepared in TBS containing 0.25 % Triton X-100 for 30 min at room temperature. These sections were incubated 120 min at room temperature with the following primary antibodies: rabbit rN3-1$_L$ [38]; rabbit rNu-9$_L$ [39, 40]; each diluted in 5 % fetal bovine serum prepared in TBS containing 0.25 % Triton X-100. They were then incubated with Alexa 568-conjugated goat anti-rabbit IgG secondary antibody and Alexa 488 phalloindin (1:40) for 120 min at room temperature. Sections were counterstained with 4′, 6-diamidino-2-phenylindole, dihydrochloride (DAPI) blue (1:10,000) for 5 min at room temperature. All immunofluorescent localization data shown are representative images of staining performed on at least three individual bladders.

Scanning laser confocal analysis of fluorescently labeled cells

Imaging was performed on a Olympus FluoView™ 1,000 confocal microscope equipped with solid state lasers of 405, 473, 559, 635 nm (Centro de Microscopia Eletrônica da Universidade Federal do Rio Grande do Sul). Images were acquired by sequential scanning with Olympus UPLSAPO ×40 N.A 0.9 objective and the appropriate filter combinations. All images were acquired with the same power of lasers that resulted in images with the same size (512×512 pixels). The images quantification was made in MacBiophotonics ImageJ software. Only the red channel of the enzyme (ecto-5′-NT/CD73 or NTPDase3) immunostaining was chosen, and then background subtraction was done. Following, the whole area of urothelium (with or without cancer) was selected with a region of interest (ROI) and the mean of fluorescence from ROI was acquired in number. With the mean of fluorescences, statistical analysis comparing the different times of bladder cancer induction with control was performed. The images were quantified and saved as TIFF files in MacBiophotonics ImageJ software, and finally imported in CorelDRAW X6 software.

Statistical analysis

All results are presented as mean ± SD. Bladder weight data were analyzed by two-way ANOVA (between-group factor: treatments; within-group factor: weeks of treatment), followed by Bonferroni post test. Quantified immunofluorescence data were analyzed by one-way ANOVA, followed by Tukey post hoc test. Differences between mean values were considered significant at $p<0.05$.

Results

Mouse bladder cancer induction by BBN exposition

Over the time of bladder cancer induction, the animals did not exhibit unusual or altered behavior. Importantly, BBN toxicity was limited to bladder tissue and the visual analysis of other organs, including liver, kidney, lung, and heart showed no morphological alterations between BBN-treated and control mice. Macroscopically, the bladders of animals that received BBN had thicker walls than that of control animals. Figure 1 shows the bladder wet weight in different times of bladder cancer induction where animals that received BBN for 4 and

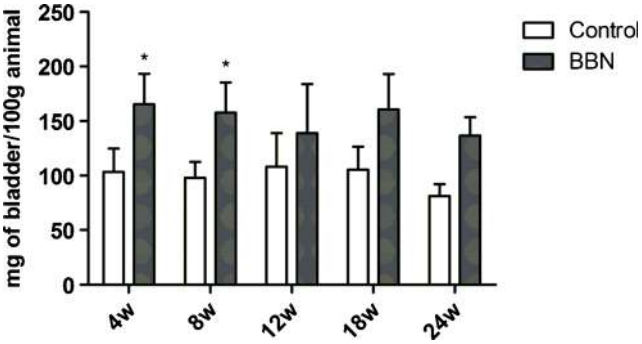

Fig. 1 Bladder wet weight expressed as mg of bladder/100 g animal to groups which received BBN in different times of cancer induction: 4, 8, 12, 18, and 24 weeks and their respective control groups. Values represent mean ± standard deviation and were analyzed through two-way ANOVA. *Differences between mean values of control and BBN, with $p<0.0001$

8 weeks had a significant increase in bladder weight in comparison with their respective control groups [$F_{(1,57)}=44.96$, $p<0.0001$] without difference between different times of bladder cancer induction [$F_{(4,57)}=1.361$, $p=0.26$]. For example, the bladder weight of control (healthy) animals killed at 4 weeks, was 103±22 mg of bladder/100 g animal, while the bladder weight of animals which received BBN for 4 weeks was 166±28 mg of bladder/100 g of animal.

Pathological analysis

To characterize the tumor induced by BBN, pathological analysis was performed. As shown in Table 1, after the initial weeks of tumor induction (4, 8, and 12 weeks) BBN-treated

Table 1 Incidence (%) of pathological features which comprises the different pathological conditions observed in HE staining of bladders from animals, which received BBN 0.05 % for 4 (n=9), 8 (n=10), 12 (n=10), 18 (n=5), and 24 (n=4) weeks

Pathological condition	Pathological features		Positive animals (%) of each cancer induction time				
			4 weeks	8 weeks	12 weeks	18 weeks	24 weeks
Inflammation	Cytoplasmic eosinophilia		100	100	90	–	–
	Bulkier nuclei		56	90	90	–	–
	Atypical apoptosis		33	80	70	–	–
	Degenerative changes		56	100	90	20	–
	Surface erosion		89	50	60	20	–
	Endothelial proliferation		100	90	70	20	–
	Submucosal edema		100	100	70	40	–
	Submucosal Inflammatory infiltrate	Mild	22	50	40	100	–
		Moderate	11	40	30	–	–
		Strong	56	10	30	–	–
	Muscle inflammatory infiltrate	Mild	78	70	70	100	–
		Strong	–	30	30	–	–
Pre-neoplastic lesion	Chromatin deposition		33	70	80	100	100
	Loss of umbrella cells		11	–	40	80	100
	Dysplasia		22	–	70	–	–
	Loss of intercellular cohesion		22	40	90	80	100
	Hyperplasia	Focal	44	20	10	–	–
		Diffuse	–	–	70	–	–
Cancer	Papillary		–	–	–	20	25
	In situ		–	–	–	–	–
	Invasive	Until lamina propria	–	–	–	–	–
		Until muscle	–	–	–	80	100
	Lesion degree	Low	–	–	–	40	50
		High	–	–	–	60	50
	Higher number of cells and cell crowding		–	–	–	80	100
	Pleomorphism		–	–	–	80	100
	Nuclear hyperchromia		–	–	–	80	100
	Giant cells		–	–	–	–	25
	Increased mitotic index		–	–	–	80	100
	Inflammatory infiltrate	Peritumoral	–	–	–	–	75
		Intratumoral	–	–	–	–	50

mice developed bladder inflammation, which was eventually followed by cell degenerative alterations and pre-neoplastic transformation. In addition to promote inflammatory features, BBN exposition for 18 weeks also induced pathological alterations related to bladder cancer in 80 % of treated mice (Table 1). The BBN exposition for 24 weeks was effective to induce bladder cancer in 100 % of treated mice, as observed by the presence of pathological bladder alterations that resemble the human bladder tumors, including higher cell number and cell crowding, increased mitotic index, presence of giant cells, loss of umbrella cells, chromatin deposition, and other features as shown in Table 1. Figure 2 illustrate the features described on Table 1, showing HE staining from bladder of control mouse and from mouse that received BBN for 12, 18, and 24 weeks.

Immunofluorescence analysis of NTPDase3 and ecto-5′-NT/CD73 in bladder after induction of cancer

Taking in account that the results presented in Table 1 demonstrate that the bladders from animals receiving BBN after 4, 8, and 12 weeks showed pathological features similar to each other typical of inflammation, and after 18 and 24 weeks typical features of cancer, although we have been done the immunofluorescence analysis of all times of treatment, here, we are showing only the results of control, and 12 and 24 weeks, which are representative of all groups.

Previously, we have reported that the expression of NTPDase3 is absent in the T24 cell line, a representative in vitro model of invasive and metastatic bladder cancer [14]. To better investigate the involvement of NTPDases in an in vivo bladder cancer model, cryosections of bladder tissues from control or BBN-treated mice were labeled with antibody against NTPDase3. Accordingly, to the results previously published by Yu et al. [28], NTPDase3 was expressed only in the urothelium of bladder tissue from control mice while a decrease of NTPDase3 expression was observed over the time of BBN exposition (Fig. 3a). The decrease of NTPDase3 expression along the development of malignant alterations of bladder tissue was confirmed by quantitative analysis showed in the Fig. 3b. Importantly, the weaker NTPDase3 expression during the induction of bladder cancer suggests the participation of this enzyme in in vivo bladder cancer progression, which is in agreement to our previously published data [14].

Next, we evaluate whether the ecto-5′-NT/CD73 could also be associated to bladder cancer progression in our model. As shown in Fig. 4a, the ecto-5′-NT/CD73 expression in bladder of control mice was absent in the urothelium cell layer, being restricted to detrusor smooth muscle cells as also previously shown by Yu et al. [28]. Notably, and in contrast to

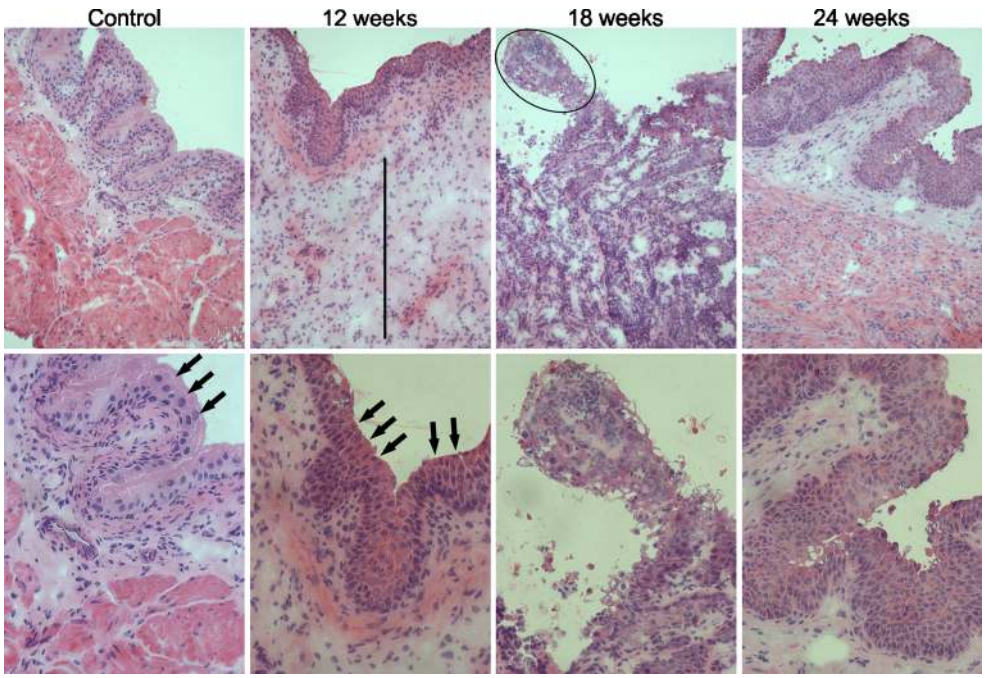

Fig. 2 The images correspond to hematoxylin and eosin staining of bladders from control animal and bladders from animals which received BBN for 12, 18, and 24 weeks, correspondent to each column respectively. Images of the *top row* were taken with ×200 of increase and the images of the *second row* with ×400. *Black arrows* indicate examples of umbrella cells, specific from urothelium. It could be observed that the bladder from animals that received BBN for 12 weeks present features of inflammation, mainly edema (*black line* indicates the length of edema area). Cancer features as a papillary carcinoma (indicated by *black circle*) and loss of umbrella cells, increase of number of cells and cell crowding, could be observed in bladders from 18 to 24 weeks

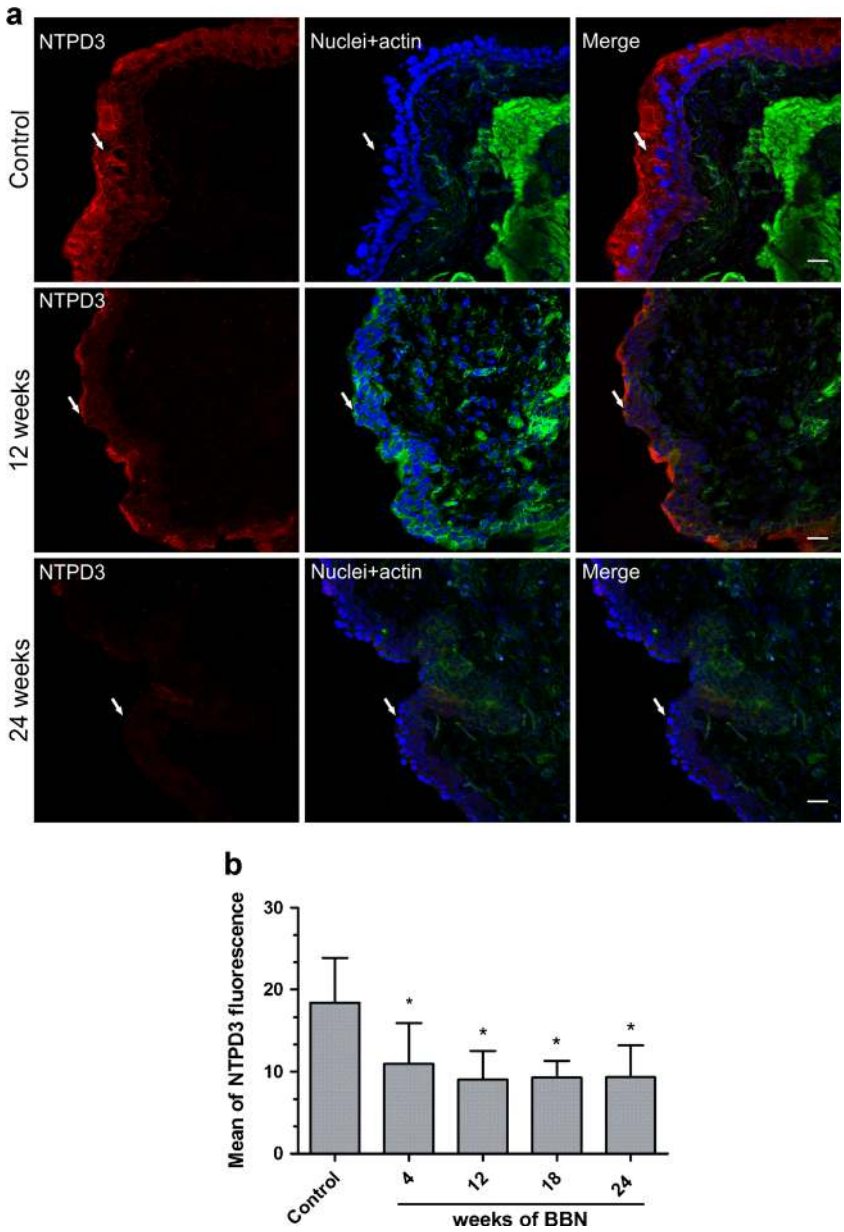

Fig. 3 **a** The images correspond to immunofluorescence of NTPDase3 in bladder urothelium at different times of bladder cancer induction. Cryosections of mouse bladders were labeled with antibody to NTPDase3 (*red*), Alexa 488 phalloidin to label actin cytoskeleton (*green*), and DAPI to label nuclei (*blue*). Color-merged panels are shown on the *right*. Pictures correspond to increase of ×400, whose focus was in urothelium (*white arrows*). *White scale bars*=25 μm. **b** Quantification of NTPDase3 staining of bladder urothelium from mice which received BBN for 4, 8, 12, 18, and 24 weeks. The immunofluorescent images of NTPDase3 were quantified in MacBiophotonics ImageJ software as described in Materials and methods. *Bars* represent mean ± standard deviation of at least 30 mean of fluorescence of Z project acquired from three different animals. The data were analyzed for statistical significance by one-way ANOVA, followed by Tukey post hoc. Differences between mean values were significant at $p<0.001$. * Significantly different compared to control

NTPDase3, ecto-5′-NT/CD73 expression increased dramatically in the urothelium of bladder of BBN-treated mice (Fig. 4a). This result was confirmed by quantitative analysis of the ecto-5′-NT/CD73 immunofluorescence, which increased in a time-response profile of BBN exposition (4, 8, 12, 18, and 24 weeks). Moreover, it was observed that an increase in the areas of increased cell proliferation and decreased presence of umbrella cells are important pathological characteristics of a malignant bladder process. These features can be better observed in the cancer urothelium from mice following 24 weeks of BBN exposition (Fig. 4a), which had an increase of ecto-5′-NT/CD73 significantly different of all other groups (Fig. 4b). Taken together, these results suggest that the increasing in the ecto-5′-NT/CD73 expression correlates to bladder cancer progression.

Discussion

Bladder cancer remains a tremendous burden for health systems worldwide [4] mainly due to the high rate of tumor recurrence and progression. Wherefore, the molecular biology of this tumor needs to be better understood in an attempt to find new therapeutic targets, which end up in more efficient treatments.

The alterations induced by BBN exposition for 4 and 8 weeks have shown histopathological features typical of bladder inflammation, with hard edema in submucosal and few pre-neoplastic lesions. After 12 weeks and more clearly after 18 weeks, progressive decrease of inflammation and increase of malignant features transformations could be observed up to 24 weeks, when all mouse bladder presented features of bladder transitional cell carcinoma (Table 1 and

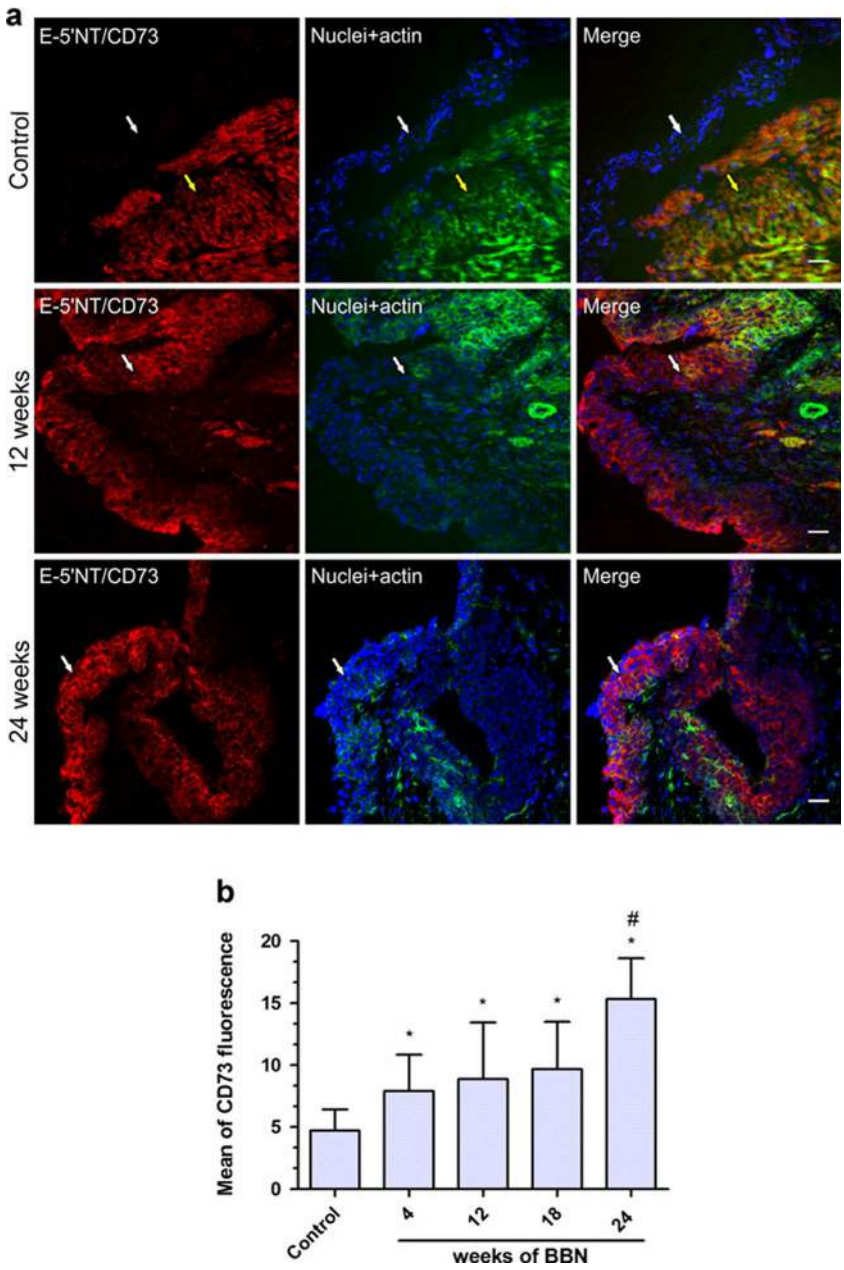

Fig. 4 **a** The images correspond to immunofluorescent staining of ecto-5′-NT/CD73 in different times of bladder cancer induction. Cryosections of mouse bladders were labeled with antibody to ecto-5′-NT/CD73 (*red*), Alexa 488 phalloidin to label actin cytoskeleton (*green*), and DAPI to label nuclei (*blue*). Color-merged panels are shown on the right. Pictures correspond to increase of ×400 whose focus was in urothelium (*white arrows*), but in the control lamina propria and muscle (*yellow arrows*) can also be observed. *White scale bars*= 25 μm. **b** Quantification of ecto-5′-NT/CD73 staining of bladder urothelium from mice which received BBN for 4, 8, 12, 18, and 24 weeks. The immunofluorescent images of ecto-5′-NT/CD73 were quantified in MacBiophotonics ImageJ software as described in Materials and methods. *Bars* represent mean ± standard deviation of at least 30 mean of fluorescence of Z project acquired from three different animals. The data were analyzed for statistical significance by one-way ANOVA, followed by Tukey post hoc. Differences between mean values were significant at $p<0.001$. * Significantly different compared to control. # Significantly different to all other groups

Fig. 2). This sequence of alterations are in agreement with data from literature, where long-time administration of BBN and other carcinogenic compounds results in a series of proliferative changes in bladder mucosa before the invasive bladder cancer [29–31]. Initially, hyperplasia and papillary or nodular hyperplasia occurs, followed by invasive carcinoma [31]. In addition, the bladder wet weight was significantly increased in animals which received BBN for 4 and 8 weeks (Fig. 1), according to the strong edema observed in their bladders by HE staining (Table 1 and Fig. 2).

As described, for healthy urothelium [28] and bladder cancer cell lines (T24 and RT4) [14], our results showed that, in fact, there is a decrease in NTPDase 3 expression (Fig. 3) and the outset followed to increase of ecto-5′-NT/CD73 expression (Fig. 4) during bladder cancer progression in vivo.

The enzymatic action of NTPDase3 and ecto-5′-NT/CD73 results to ATP hydrolysis and generation of immunosuppressive adenosine, respectively. Inflammation acts in all stages of tumorigenesis, at early stages, creates a favorable microenvironment which favors mutations, genomic instability, and epigenetic modifications and in cancer progression stimulating angiogenesis, immune escape, and tumor growth [41, 42]. In addition, ATP and adenosine participates in inflammatory signaling [43]. ATP has been described as a pro-inflammatory molecule acting as a chemotactic signal to immune phagocytes [44, 45] while adenosine has an immunosuppressive role

suppressing innate and adaptative immune responses [44]. The participation of altered ectonucleotidases expression in the modulation of immune cells to contribute to cancer progress have been described to gliomas [46], that have a similar ATP/ADP/AMPase activity pattern of bladder cancer. This profile of nucleotide metabolism may favor extracellular ATP and adenosine accumulation within the tumor interstice, so while ATP could induce the dead of healthy cells, tumor proliferation, and recruitment of immune cells, adenosine is responsible for angiogenesis and immunosuppression [46]. Although, the BCG antimutor activity is due to stimulation of the local and acute immune response and the recruitment of polymorphonuclear neutrophil granulocytes [33], the presence of ATP and adenosine may be important to create chronic inflammatory conditions observed in tumor microenvironment, which suppress the immune response. This hypothesis is reinforced by the increasing density of immune cells in the bladder tissues during the course of BBN treatment. Moreover, the massive presence of tumor-associated macrophages in late clinical staging of patients with bladder cancer [47] and the association between tumor infiltrating lymphocytes and the recurrence of non-muscle-invasive bladder cancer [48] further suggest the chronic inflammatory process and cancer progression association.

Moreover, both, ATP and adenosine have been extensively described to participate in bladder signaling [28, 49–53]. Urothelium is a source of ATP release [28, 50, 51] and it is also an important site of adenosine biosynthesis. Importantly, both ATP and adenosine have functions in exocytosis of umbrella cell layer [52, 53], which is the mechanism to increase the luminal surface area when the bladder fills (cytoplasmatic discoidal/fusiform vesicles fuse with the apical plasma membrane) [54]. The ATP released by urothelium is responsible for micturition reflex, through P2X3 from subepithelial nerve fibers [55]. Then, although it needs to be better elucidated, the changes in NTPDase3 and ecto-5′-NT/CD73 expression with malignant transformation of urothelial cells described in these work, would be expected to perturb nucleotide signaling in the bladder and thus affect some bladder functions.

Although little is known about the role of NTPDase3 in cancer, an important finding of the present study was the absence of ecto-5′-NT/CD73 in healthy urothelium [28] followed by increase in ecto-5′-NT/CD73 expression with the increase of features of malignancy in mice that received BBN (Fig. 4). These findings indicate that this enzyme is involved in bladder cancer progression, and makes it a promissory therapeutic target to local treatments by instillation of inhibitors of this enzyme. This result is in agreement with the literature that shows the increase of ecto-5′-NT/CD73 expression in many other cancers such as breast cancer, glioma, and melanoma [19, 23, 56]. Furthermore, ecto-5′-NT/CD73 overexpression promotes invasion, migration, adhesion, and metastasis of human breast cancer cells [20, 57], indicating higher invasiveness and metastatic capability to melanomas [56, 58] and poor prognosis in human colorectal cancer [22]. Ecto-5′-NT/CD73 expression in cancer cells has been also linked with drug resistance [26, 59] and immune escape [44, 60]. Moreover, researchers have already targeted ecto-5′-NT/CD73 in an attempt to develop promising therapies, for instance, ecto-5′-NT/CD73 inhibitors have shown antiproliferative effects in bladder cancer cell lines and gliomas [61, 62], and decrease in ovarian cancer progression [63]. The treatment of mice with anti-CD73 antibody inhibited breast tumor growth and metastasis [20], and the use of RNA interference of ecto-5′-NT/CD73 inhibited cell growth and invasion in human breast cancer cells [25].

In conclusion, the altered expression of NTPDase3 and ecto-5′-NT/CD73 presented herein add further evidence to support the idea that alterations in the activity and expression of ectonucleotidases are involved in bladder tumorigenesis. Although additional studies are needed to determine whether the alterations in the ectonucleotidases expression are the cause or consequence of malignant transformation of urothelium, we bring the ecto-5′-NT/CD73 as a future biomarker and/or a target for pharmacological therapy.

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