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ORIGINAL ARTICLE

Peptidyl Arginine Deiminase, Type II (PADI2) Is Involved in Urothelial Bladder Cancer

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Abstract

Peptidyl arginine deiminase, type II (PADI2) expression has been shown to potentiate multiple different carcinogenesis pathway including breast carcinoma and spontaneous skin neoplasia. The objective of this study was to examine the role of PADI2 in urothelial bladder cancer which has not been evaluated previously. Analysis of mutation and genome amplification of bladder cancer within The Cancer Genome Atlas (TCGA) showed that *PADI2* is both mutated and amplified in a cohort of bladder cancer patients, with the largest number of mutations detected in urothelial bladder cancer. Even though *PADI2* expression was not significantly correlated to survival in bladder cancer patients, it was significantly overexpressed at the mRNA and protein levels, as revealed by TCGA data and immunohistochemistry analysis, respectively. PADI2 showed wide expression pattern in bladder cancer tissues but was hardly detected in tumor adjacent normal tissue. RNAi mediated silencing of *PADI2* in the bladder cancer cell line T24 did not result in a change of proliferation. Interestingly knockdown of *PADI2* expression did not affect Snail1 protein, which is associated with metastatic progression, in these cells. However, *PADI2* silencing remarkably attenuated both in vitro migration and invasion- in T24 cells indicating a Snail1-independent effect of PADI2 on invasive potential of urothelial bladder cancer. This was further corroborated by in vivo xenograft assays where *PADI2* shRNA harboring T24 cells did not have detectable tumors by week 4 as compared to robust tumors in the control *Luciferase* shRNA harboring cells. *PADI2* silencing did not affect proliferation rates and hence this would suggest that *PADI2* knockdown is perhaps causing increased apoptosis as well as transition through the cell cycle, which needs to be confirmed in future studies. Our results reveal a yet undefined role of *PADI2* as an oncogene in urothelial bladder cancer.

Keywords PADI2 · Urothelial bladder cancer · Overexpressed · Tissue microarray analysis

Introduction

Bladder cancer is the 17th most common malignancy in women and the 7th most common malignancy in men and is one of the major reasons for cancer related mortality [1]. Even though environmental risk factors, occupational hazards, schistosomiasis, and genetic variants have been indicated as the cause of bladder cancer [1–9], the exact etiology of bladder cancer is not known and thus presents as a challenge to the field of oncology.

The peptidyl arginine deiminase (PADI), commonly known as (PAD) family of posttranslational modification enzymes play a role by converting positively charged arginine residues on a substrate protein into neutrally charged citrulline, which is, known as the citrullination or deimination process [10]. Aberrant PAD activity has been linked to different diseases [10], which in most cases is linked to inflammation [11]. However, in case of cancer the precise role of PAD dysfunction is under intense investigation [10, 12–15]. It has been shown to be involved in the pathogenesis of skin neoplasia and breast cancer [10, 12–15].

However, it is not known if PADI2 is involved in the pathogenesis of bladder cancer, which was the aim of this study. Our results cumulatively suggest that PADI2 gene product

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functions as a proto-oncogene in the context of urothelial bladder cancer.

Materials and Methods

The Cancer Genome Atlas (TCGA) Data Analysis

TCGA data analysis was performed using cBioPortal for Cancer Genomics (http://cbioportal.org) [16, 17]. This included 2019 patients over 2066 samples. Analysis was done to determine genome amplification, somatic mutations, correlation to survival, and mRNA expression.

Tissue Samples and Processing

The current study was approved by the Institutional Review Committee of the First Hospital of Jilin University, China. Both fresh-frozen and paraffin-embedded urothelial bladder carcinoma tissue specimens along with any corresponding adjacent non-tumorous ovary tissue samples were obtained from 35 Chinese patients (9 T1 cases, 21 T2/3 cases, and 5 T4 cases) at the First Hospital of Jilin University between 2016 and 2017. All cases were included in this study after review by a pathologist. None of the 35 patients received preoperative local or systemic therapy. All of the freshly harvested samples were immersed in RNAlater (Life Technologies, Shanghai, China). They were then quickly frozen within 30 min after surgery. All of the tissue samples were then stored in liquid nitrogen for any further use.

Cell Culture

T24 cell lines were cultured in DMEM medium supplemented with 10% FBS (Gibco, Thermo Fisher Scientific, Shanghai, China), as well as 100 U/mL penicillin and 0.1 mg/mL streptomycin, and were maintained in a 37 °C, 5% carbon dioxide humidified atmosphere.

Plasmid Constructs

Renilla Luciferase and *PADI2* shRNAs were obtained from OriGene (Rockville, MD, USA). Firefly Luciferase lentiviral particles were obtained from AMS Biotechnology.

Transient Transfection, Recombinant Lentivirus Production, and Stable Cell Lines Generation

T24 cells (4×10^4 cells) were transiently transfected using Lipofectamine LTX (Life Technologies, Shanghai, China). Transiently transfected cells were harvested and analyzed at 72 h after transfection. T24 cells were transduced with the lentiviral soup containing Firefly Luciferase (FF+), and stable transductants were selected using blastocidin (5 μg/ml). The pGFP-V-RS (containing the shRNAs) lentiviral particles were generated by transfection of 293Ts using Mirus Transit-293 T. T24 FF+ cells transduced with pGFP-V-RS-shRNA-*Renilla Luciferase* or *PADI2* were selected with Puromycin (2 μg/mL) to generate stable pools. In all cases, gene silencing was verified both by visualization of GFP expression and by immunoblotting.

Immunohistochemistry

Tissue specimens from all 35 patients with urothelial bladder carcinoma were stained for PADI2 expression (#ab-16,478, Abcam, Waltham, MA, USA). The stained slides were scored by a pathologist blinded to the identity of the tissue cores as percent staining (low, ≤25%; medium, 25–50%, and high, ≥50%).

Cell Lysis and Western Blot

Cells were lysed in buffer containing 25 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% NP-40 and 5% glycerol containing complete, Mini protease inhibitor cocktail (Roche Diagnostics, Indianapolis, USA). Twenty micrograms of whole cell lysate was resolved on a NuPAGE 4–12% gel (Life Technologies, Shanghai, China), transferred to an Immobilon PVDF membrane (Millipore, Billerica, USA), and probed with anti-PADI2 antibody (#ab-16,478, Abcam) anti-Snail1 antibody (#ab-53,519, Abcam). The blots were subsequently stripped, and re-probed with antibody against GAPDH (#ab-9485, Abcam) each time to confirm equal loading. The blots were imaged using ECL Plus western blotting substrate.

Cell Proliferation Assay

Cell proliferation was quantitated using a mitochondrial colorimetric assay (MTT assay, Sigma-Aldrich, St. Louis, MO) as per the manufacturer's recommendations. Results were expressed in terms of relative optical density (OD), as mean ± standard deviation.

In Vitro transwell migration and invasion assays

Migration and invasion analyses were performed using Culturex 96 well cell migration and Culturex 96 well BME cell invasion assay kits following the manufacturer's recommendations (R&D Systems, Shanghai, China), respectively. Images were obtained at 10X magnification. Data obtained from both sets of experiments were used to analyze percent migration and invasion and were expressed as mean ± standard deviation.

Xenograft Assay

Experiments received approval from the Institutional Animal Care and Use Committee of The First Hospital of Jilin University and were performed in accordance with the "Guidelines for the Welfare of Animals in Experimental Neoplasia" published by The United Kingdom Coordinating Committee on Cancer Research. For xenograft assays, 10^6 FF-T24/sh-PADI2 or sh-Renilla Luciferase cells were injected (sub-cutaneous) in the hind-flank of six-week-old female nude mice ($n = 5$ per group). Mice were assessed weekly for tumor formation up to 35 days using in vivo bioluminescence imaging using an IVIS Imaging System (IVIS imaging system 200, Xenogen Corporation, PerkinElmer, Waltham, USA) fitted with an ultrasensitive CCD camera. Images on day 35 were obtained along with X-ray to confirm presence or absence of tumor formation.

Results

We initially performed in silico to determine *PADI2* expression in bladder cancer subtypes, including urothelial bladder cancer. As shown in Fig. 1a, somatic mutations and genomic amplification of PADI2 is observed in about 1.5–4.5% (median, 2.2%) of bladder cancer cases. Whereas genomic amplification of *PADI2* is most pronounced in bladder/urinary tract cancer, somatic mutation rate of *PADI2* is highest among urothelial bladder cancer patients (Fig. 1b).

From the available patient data, we further saw that PADI2 expression was not correlated to survival in bladder cancer patients (Fig. 2a; $P = 0.394$). However, analysis of RNAseq data showed uniform upregulation of *PADI2* in bladder cancer tissue compared to tumor adjacent normal tissue (Fig. 2b). This was further corroborated by immunohistochemistry analysis. (Fig. 3). Robust PADI2 expression was detected in tumor specimens (Fig. 3a, c) compared to tumor adjacent normal bladder tissue, where hardly any PADI2 expression was detected (Fig. 3b, d). PADI2 expression was very low in benign papilloma lesions (Fig. 3e) and barely detected in normal urothelial transitional epithelium (Fig. 3f). This suggested that PADI2 over-expression might be associated with bladder cancer and can either be as a result of carcinoma formation or potentiate carcinoma formation.

To evaluate if PADI2 expression was reliant on the EMT inducer Snail1 expression [18, 19], the expression of Snail1 in T24 bladder cancer cell lines were evaluated following transfection with shRNA targeting Firefly luciferase or *PADI2*. Even though we obtained robust silencing of PADI2 expression (Fig. 4a, *top panel*), there was no change in Snail1 protein expression following silencing of *PADI2* (Fig. 4a, *middle panel*).

We next evaluated if PADI2 expression dictated cell proliferation. However, as shown in Fig. 4b, silencing of *PADI2* expression did not alter cell proliferation in T24 cells compared to those that harbored the shRNA targeting *Luciferase* shRNA.

We next scored each of the individual T24 transfectants for migration (Fig. 4a, 5b, e) and invasion (Fig. 5c, d, f) in standard transwell assays. Using these criteria, phase contrast imaging showed that silencing *PADI2* expression completely attenuated in vitro migration (Fig. 5a, b, e) and invasion (Fig. 5c, d, f) in T24 cells. Given that silencing of *PADI2* expression did not affect expression of Snail1, our results indicate that the effect of PADI2 on in vitro migration and invasion was a Snail1-independent pathway.

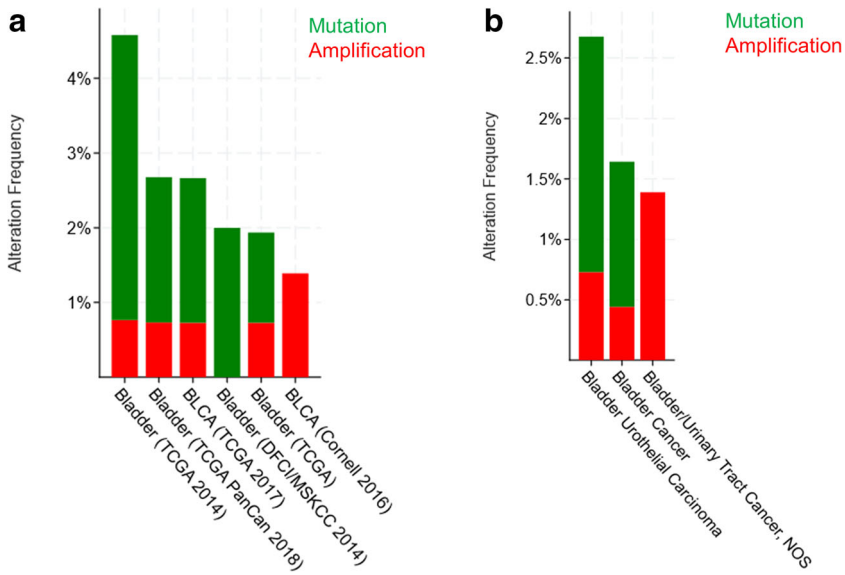

Fig. 1 ***PADI2* amplification and somatic mutations in bladder cancer patients.** Data analyzed was from TCGA that has 2019 bladder cancer patients from 13 studies. **a** Analysis based on cancer studies, and **b** bladder cancer sub-types

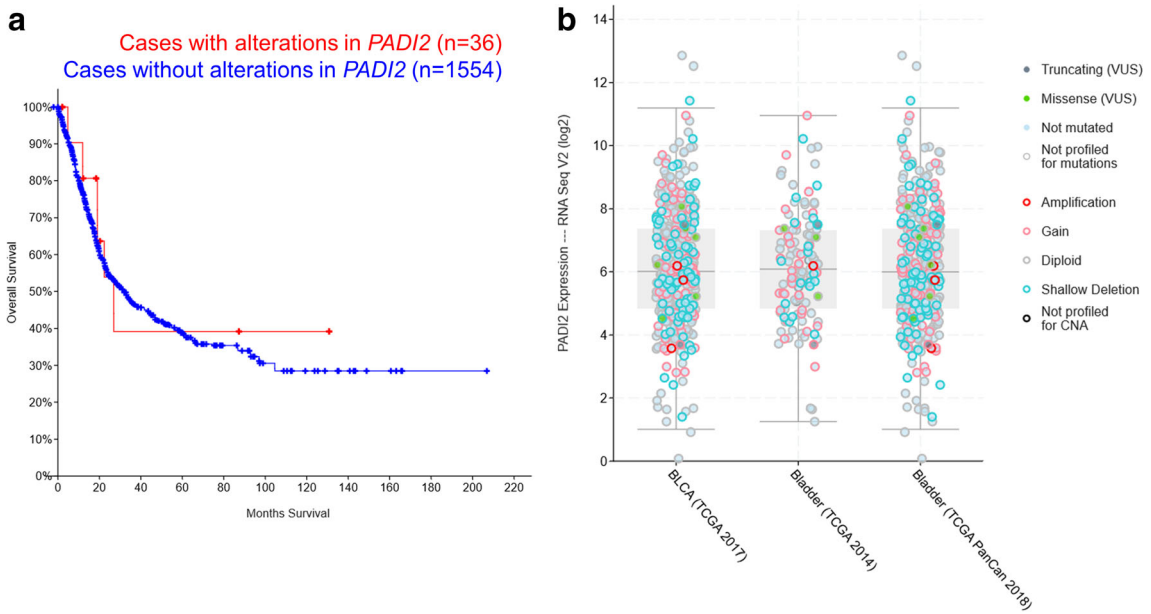

Fig. 2 ***PADI2*** **is overexpressed in patients with bladder cancer. a** Kaplan-Meyer survival analysis curve did not show any difference between bladder cancer patients with and without genomic amplification or somatic mutation in *PADI2*. $P = 0.394$. **b** Normalized expression of *PADI2* with overlaid genomic changes in bladder cancer patients

In order to understand the in vivo role of *PADI2* in bladder cancer formation, we finally did xenograft assays with stably expressing firefly luciferase/ T24 cells, stably expressing shRNA targeting either *Luciferase* or *PADI2*. The T24/ shRNA-Luciferase cells formed steadily growing tumors by Day 35 in all 5 mice injected, the T24/shRNA-*PADI2* cells formed small tumor that regressed by end of Week 3 ($p < 0.05$) (Fig. 6a).

To confirm the role of PADI2 in bladder cancer progression, IHC scoring of PADI2 expression in the 35 patients were

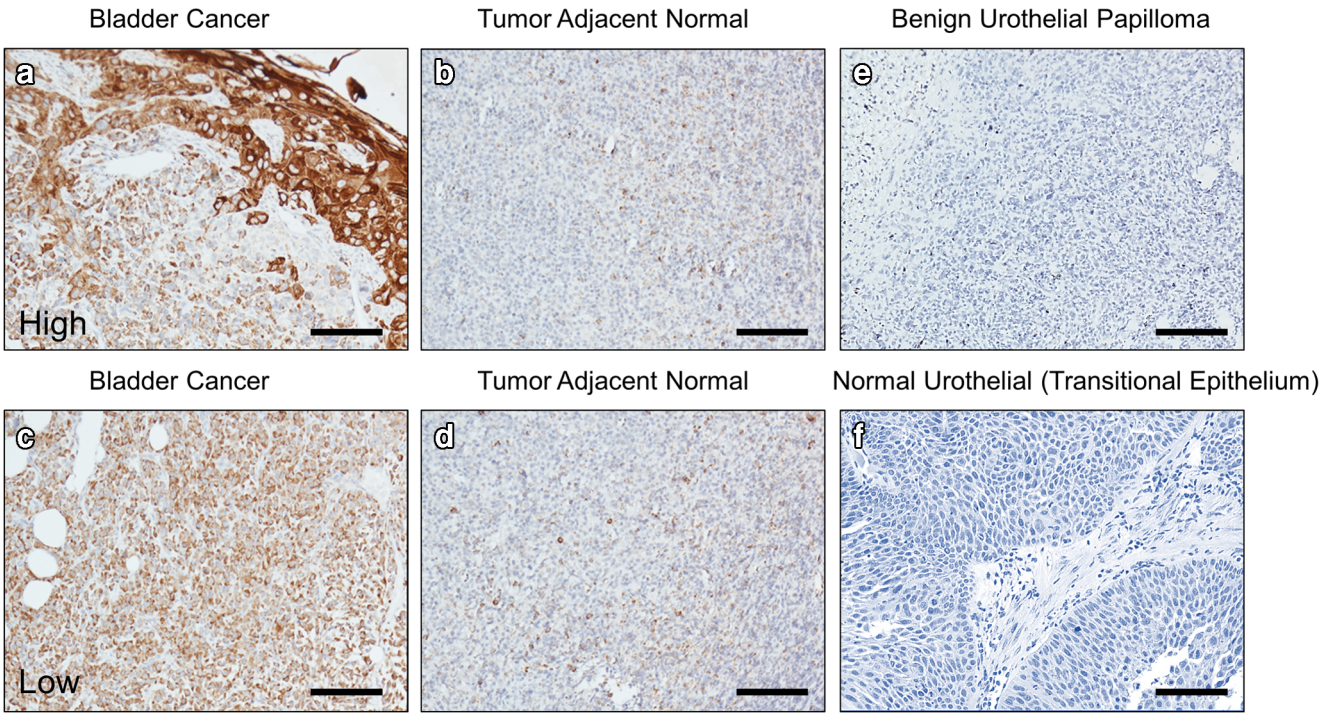

Fig. 3 PADI2 expression is increased in urothelial bladder cancer. Representative immunohistochemistry images showing high (**a**) and low (**c**) PADI2 expression in bladder cancer tissue. No significant staining was observed in the tumor adjacent normal tissues (**b**, **d**). PADI2 expression was minimal in benign lesions (papilloma) (**e**) and normal urothelial or transitional epithelium (**f**). Scale bar, 30 μm

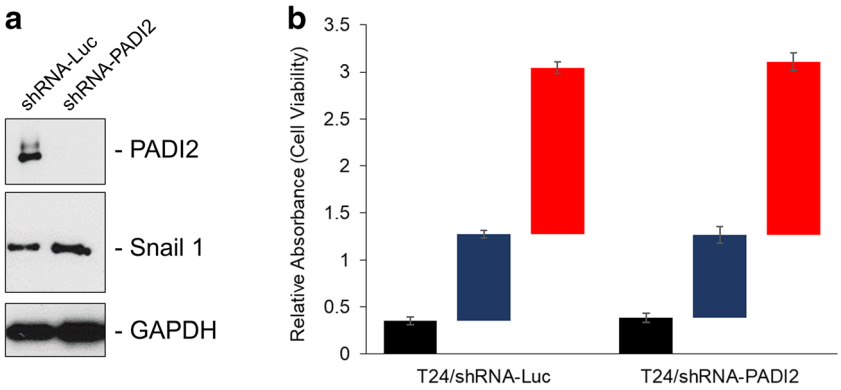

Fig. 4 PADI2 silencing does not affect Snail 1 expression or cell proliferation. a Western blot analysis of PADI2 and Snail 1 in T24 cells stably transfected with shRNA targeting either Luciferase or PADI2. The blot was stripped and probed with GAPDH as a loading control. **b** Cell viability was measured in the indicated PADI2 cells at 24, 48, and 72 h after transfection by the MTT assay

correlated to disease stage. Patients in T1 stage had significantly lower PADI2 expression compared to patients in both T2/T3 and T4 stages (Fig. 6b; $P < 0.05$ in each case), confirming that PADI2 expression is positively correlated to muscular invasion in patients with bladder cancer.

Discussion

Our data in the current manuscript describes a role of PADI2 in bladder cancer. Given that (a) PADI2 expression is not correlated to survival in bladder cancer patients (Fig. 2a), and (b) knockdown of *PADI2* did not affect proliferation of the T24 cells in vitro (Fig. 4b) or in vivo (Fig. 6a) indicate that PADI2 is not a driver oncogene or protooncogene in urothelial bladder cancer pathogenesis but associated with invasion of urothelial bladder cancer. Indeed, PADI2 expression was significantly higher in Stage T4 and T2/3 bladder cancer patients with muscular invasion compared to patients in T1 stage, confirming a role of PADI2 in bladder cancer progression. It remains to be determined if this is causal or correlative of disease progression in bladder cancer; of note though our results indicate that might be causal.

Interestingly, this was independent of Snail1 protein expression. In case of skin neoplasia, it was shown that *PADI2*

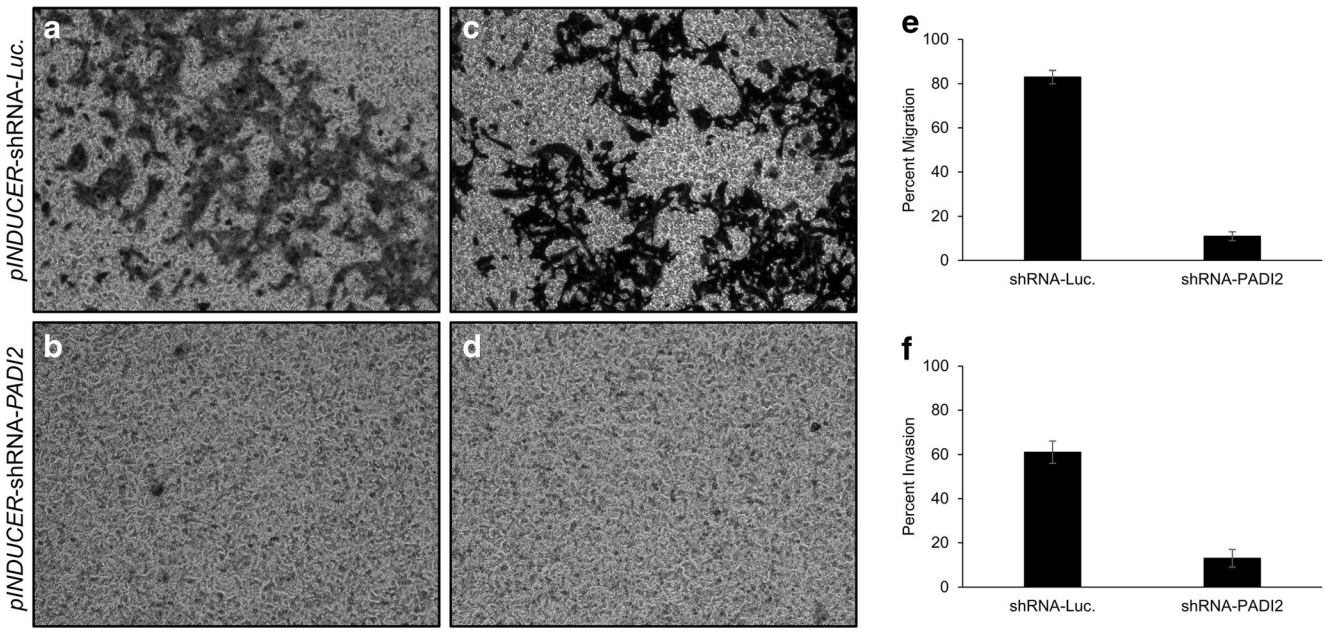

Fig. 5 PADI2 silencing significantly downregulates both in vitro migration and invasion in bladder cancer cells. RNAi-mediated silencing of PADI2, but not Luciferase, in T24 cells inhibited in vitro migration (**a**, **b**) and invasion (**c**, **d**). The migrated and invasive cells were photographed using a microscope, and the number of the migrated and invasive cells in every field was counted and represented as percent of total cells at the beginning of the assay (**e**, **f**). Error bars, S.D.

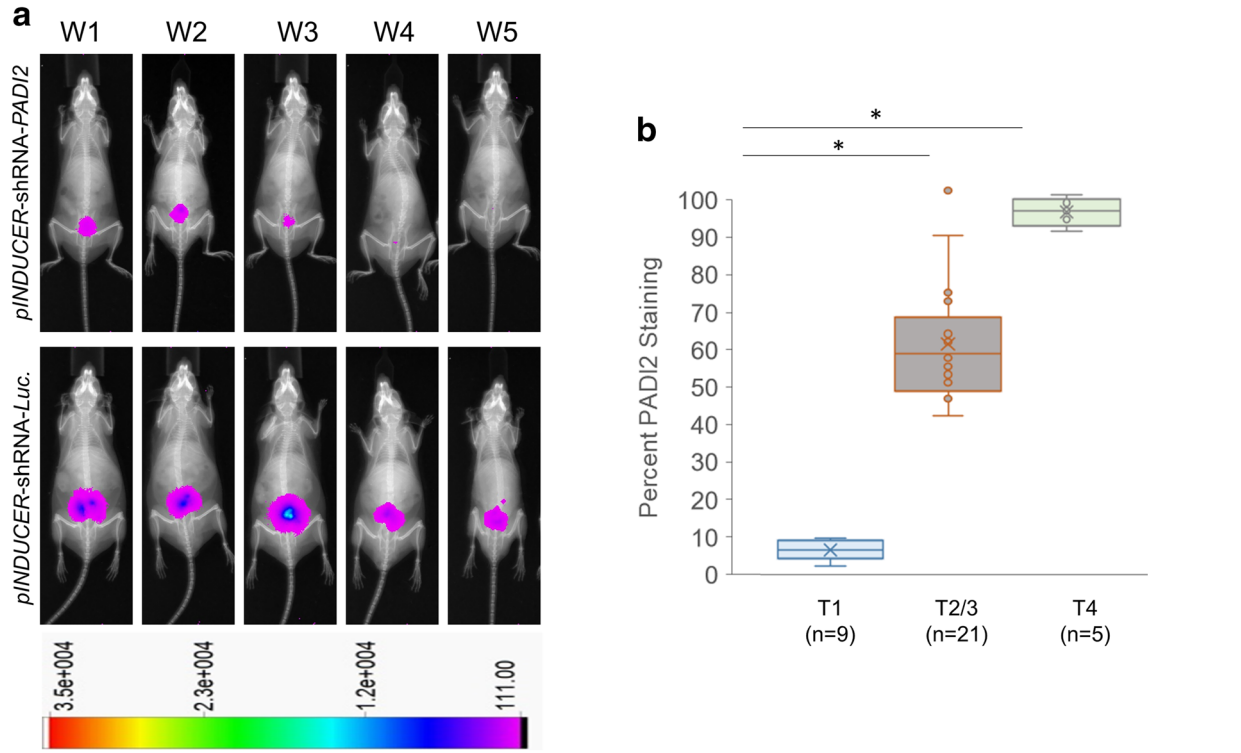

Fig. 6 PADI2 silencing significantly inhibits in vivo tumor growth in bladder cancer cells. a Luciferase expressing T24 cells expressing either a shRNA targeting *PADI2* (*top*) or Luciferase (*bottom*), were injected (sub-cutaneous) into nude mice. Tumor formation in the T24/shRNA-*PADI2* cells-injected mice was largely attenuated as observed by overlay of X-ray and in vivo luciferase imaging in representative animals in the two experimental groups. **b** Quantification of PADI2 expression, as determined by percent IHC staining in 35 bladder cancer patients in T1 ($n = 9$), T2/3 ($n = 21$), and T4 ($n = 5$). Data was analyzed by Kruskal-Wallis test. *$P < 0.05$

overexpression lead to increased markers of EMT and invasiveness. In fact, in the case of skin neoplasia, the epithelial cell marker, E-cadherin was down regulated and both Snail 1 and Vimentin, another mesenchymal cell marker was overexpressed following overexpression of PADI2 [10, 20]. Our findings in bladder cancer are in apparent contradiction to these findings in skin neoplasia.

It might be that PADI2's role on tumorigenesis is context dependent. It might also be possible that PADI2 might affect EMT in bladder cancer by impacting effectors downstream of Snail 1. A counter argument to the aforementioned assertion is that following EMT, Snail 1 is upregulated, irrespective of as a cause or as an effect. That we did not observe Snail1 protein going down following silencing of PADI2 would thus point to a role in tumorigenesis per se.

In fact, our xenograft assays provide further evidence for this. The T24 cells harboring the shRNA targeting *PADI2* did form small tumors which regressed quickly. This is indicative of a role of PADI2 in directly affecting bladder cancer formation. However, knockdown of PADI2 did not cause any change in cell proliferation. Hence, it will be important to determine in future if PADI2 potentiates bladder cancer formation by inhibiting cell death instead. Overall, our study results establish PADI2 as a putative biomarker in bladder cancer that needs to be further validated in patients' samples.

Authors' Contributions BSG and CSR participated in the design of this study. HMX and TS performed the analysis. JH and YX drafted the manuscript. All authors read and approved the manuscript.

Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no competing interests.

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