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ORIGINAL ARTICLE

Transitional cell carcinoma matrix stiffness regulates the osteopontin and *YAP* expression in recurrent patients

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Abstract

Cells translate the mechanosensing of extracellular matrix component dysregulation and stiffness into the signal transduction including Osteopontin (OPN) through the Hippo pathway. But how extracellular matrix (ECM) component dysregulation and stiffness are ultimately linked to transitional cell carcinoma (TCC) development remains poorly understood. This study was aimed to evaluate the possible links between ECM component alteration after cancer surgery and OPN and Yes-associated protein (YAP) expression in TCC and adjacent tissues. In this study, we used 50 TCC (25 newly diagnosed and 25 recurrent) and 50 adjacent tissues to determine the tissue stiffness using atomic force microscopy. The mRNA expression of *SPP1*, Indian hedgehog (*IHH*), and *YAP* was also determined using qRT-PCR. Western blotting and ELISA were performed to assess the tissue and serum levels of OPN, respectively. To assess the glycoproteins and elastic fibers content, Periodic Acid Schiff, and Verhoeff-Van Gieson Staining were performed, respectively. Matrix stiffness was markedly higher in TCCs than adjacent tissues ($p < 0.05$). Gene expression analysis showed that *YAP, SPP1,* and *IHH* genes were upregulated in TCC tissues ($p < 0.05$). Additionally, the OPN protein overexpression was observed in the tissue and the serum of TCC patients ($p < 0.05$). We also found that glycoproteins, elastic fibers content of recurrent TCC tissues was remarkably higher as compared to adjacent tissues ($p < 0.05$). Our results suggest that glycoproteins and elastic fibers content modulation and ECM stiffness may upregulates the expression of *YAP*, *SPP1* and *IHH* genes, and possibly contribute to the TCC development and relapse.

Keywords Transitional cell carcinoma (TCC) · Yes-associated protein (YAP) · Tissue stiffness · Osteopontin (OPN) · Indian hedgehog (*IHH*) · Cancer

Introduction

Transitional cell carcinoma (TCC) is the commonly distributed neoplasm of the urinary bladder which causes ~ 200 thousands of deaths annually worldwide [1, 2]. In total, TCC is classified into two categories including non-muscle invasive bladder cancer (NMIBC) which accounts for 70 percent of TCC cases, and muscle invasive bladder cancer (MIBC) which comprises the 30% remaining TCC cases. According to the WHO grading system, TCC is divided into three following grades: papillary neoplasm, low-grade, and high-grade papillary carcinoma [3, 4]. Patients with TCC that treated with transurethral resection are at high risk of cancer recurrence, progression and cancer related death [5].

Extracellular matrix (ECM) modifications are mandatory for tumor relapse and progression [5]. Reported data have suggested that ECM may have a principal as a substitute for supporting role in the onset of cancer [6]. Indeed, ECM component dysregulation and stiffness are related with a lack of asymmetric division and differentiation of cancer cells [7, 8]. The ECM as a complex structure provide not only physical microenvironment in which cells embedded but also play essential roles in many cellular processes including migration, growth and differentiation [9, 10]. Furthermore, ECM remodeling and stiffness has been shown to have pivotal role in cancer development and progression. The major constituents of the ECM are collagens, proteoglycans, elastin

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and fibronectin which forms fibrous proteins [10]. Change in ECM composition and aberrant collagen crosslinking is the main cause of ECM stiffness, which is mechanically sensed via cancer cells. In addition to collagen, main ECM components including elastin, proteoglycans and fibronectin bind to collagen and play an important role in ECM stiffening, and thereby have effects on mechanosignaling [11]. Recently published data have suggested a critical role for integrin mediated focal adhesion kinase (FAK) activation in ECM stiffness to changes in genes expression involved in cancer proliferation and progression [12, 13]. Osteopontin (OPN), as another member of ECM protein, is a secreted phosphorylated glycoprotein which interacts with cell-surface receptors i.e. integrin. Moreover, *SPP1* overexpression has been found to promote cancer development, progression and metastasis [14]. Prior studies have suggested that ECM stiffness and rigidity might play a major role in *SPP1* expression and activation [15], however the precise molecular mechanism remain unclear.

The transcriptional coactivator Yes-associated protein (YAP) as a converging effector of the Hippo pathway is activated via dephosphorylation and involved in cancer development [16]. It is postulated that YAP as a central mediator of hippo signaling is closely implicated in cancer cell proliferation in in response to ECM rigidity and stiffness and acts as mechanotransducer in cancer cells. YAP acts as an upstream regulator of several genes including Indian hedgehog (*IHH*) and *SPP1* [17]. Notably, YAP as a potential mechanotransducer, translocate to the nucleus and upregulates the expression of *IHH* and *OPN* in cancer cell [18]. On the other hands, it has been demonstrated that *IHH* regulates the *SPP1* gene expression [19], indeed, *OPN* is the downstream for *IHH* signaling in cancer and play a complex role in ECM associated cancer development. Therefore, present study was planned to assess the hypothesis that alteration in ECM components including elastin and proteoglycans and subsequent matrix stiffness may correlate with *SPP1* expression in patients with recurrent and newly-diagnosed TCC.

Material and methods

Experimental design

The study was reviewed and approved by the Medical Ethics Committee of Hamadan University of medical sciences. Tissue and blood samples were obtained from 50 patients with TCC of the bladder undergoing transurethral resection of bladder at the urology center of Shahid Beheshti Hospital, Hamadan University of Medical Sciences (Hamadan, Iran). 25 Patients had newly diagnosed TCC and 25 had recurrent TCC. All tissue samples were re-viewed for TCC by the skillful pathologist. Exclusion criteria were: patients who had a coexisting of another and a history of urolithiasis within the past six months. A total 30, age-matched control venous blood samples were obtained from patients referred to the hospital. Clinic-pathological characteristics of TCC patients were presented in table 1.

Gene expression determination by qRT-PCR

Total RNA was extracted from frozen tissue samples using AccuZol reagent. The cDNA synthesized using 1000 ng total RNA and cDNA synthesis commercial kit (Thermo Fisher Scientific, USA) according to the manufacturer's protocol. Quantitative RT-PCR was performed in triplicate with SYBR Green master mix (Amplicon, Denmark) on a Light Cycler (Roche Life Science Deutschland GmbH, Germany). Specific primer sets were as follows: *SPP1* (forward: 5′- CAGACCCTTCCAAGTAAGTC-3′ and reverse: 5′- TCATCAGTGTCATCTACATCATC-3′), *YAP* (forward: 5′-CTTTCCTTAACAGTGGCACCT-3′ and reverse: 5′-TCACCTGTATCCATCTCATCC-3′), *IHH* (forward: 5′-AAGGACGAGGAGAACACAGG-3′ and reverse: 5′- AGA TAGCCAGCGAGTTCAGG-3′), *ACTB* (forward: 5′-GAG CCTCGCCTTTGCCGATCC-3′ and reverse: 5′-ACATGC CGGAGCCGTTGTCG-3′), Relative expression of target genes was normalized using Ct values obtained for the house keeping β-actin gene. Fold change expression was calculated using $2^{-\Delta\Delta CT}$ formula[32].

Table 1 Clinic-pathological characteristics of newly-diagnosed (n = 25) and recurrent (n = 25) bladder transitional cell carcinoma (TCC) patients

Characteristics	Newly-diagnosed (%)	Recurrent (%)	p-value[a]
Number	25(50.00)	25(50.00)	
Age (years)			
≤ 68.5[b]	9 (36.00)	10 (40.00)	0.771
> 68.5	16 (64.00)	15 (60.00)	
Smoking			
Yes	14 (56.00)	21 (84.00)	**0.031**
No	11 (44.00)	4 (16.00)	
Tumor size (cm)			
≤ 2.5[b]	16 (64.00)	11 (44.00)	0.078
> 2.5	9 (36.00)	14 (56.00)	
Histologic grade			
Grade I	10 (40.00)	4 (16.00)	**0.036**
Grade II	14 (56.00)	15 (60.00)	
Grade III	1 (4.00)	6 (24.00)	

[a]Chi-square test, [b]median

Western blot analysis

To evaluate OPN expression at the protein level, we performed western blot technique. In this technique, 20 mg of tissue specimens were powdered using liquid nitrogen, then added 600 µl of protease inhibitor (Melford) supplemented radioimmunoprecipitation assay (RIPA) buffer. Prepared tissues lysate centrifuged and then supernatants were collected. To determine the total protein contents of collected supernatants, we used bicinchoninic acid (BCA) method. After separating on SDS-PAGE electrophoresis, proteins transferred to nitrocellulose membrane using electrical current. Then blocking of membrane was done using 5% nonfat milk dissolved in tris buffered saline with tween 20 (TBST) in duplicate for 40 min. primary antibodies: OPN (1:1000; STJ94832) and β-Actin (1:2000; Abcam; ab8227) were added to the membrane and incubated overnight at 4 °C. To visualize the proteins, we used secondary horseradish peroxidase-conjugated antibody (horseradish peroxidase-conjugated anti-rabbit IgG). To normalize target protein content, the band density of each sample is evaluated by image J (https://imagej.nih.gov/ij/). In the next step, the density of the target protein band is normalized using the density of the β-Actin band.

Serum OPN enzyme-linked immunosorbent assay (ELISA)

To investigate the serum level of OPN protein, serum samples of TCC patients and control subjects were applied for a determination of serum OPN level by ELISA method (Eastbiopharm; CK-E10857), following manufacturer's instructions.

Extracellular matrix glycoproteins assay

To investigate the glycoprotein content in tissue sections, we performed the periodic acid schiff (PAS) staining method. In this technique, 4-µm paraffin embedded sections of the bladder cancer tissue arrays were used. Briefly, the slides were subsequently deparaffinized, rehydrated to distilled water, following oxidizing in 0.5% periodic acid solution for 5 min, then after rinsing in water, sections were incubated with Schiff's reagent for 15 min, followed by washing with lukewarm tap water for 5 min. After all, sections were counterstained using Mayer's hematoxylin solution for 2 min. At last, slides were dehydrated and coversliped using mounting medium.

Extracellular matrix elastic fibers assay

For determination of elastic fibers content of bladder cancer tissue, sections were stained using Verhoeff-Van Gieson (VVG) staining method. In this method, formalin fixed, paraffin embedded tissues were sectioned and stained. Briefly, tissue sections were dewaxed and rehydrated with ethanol to distilled water, next, the sections were stained with Verhoeff's solution for 1 h (Tissue stained in black). After rinsing in tap water with 2–3 change, tissues were differentiated using ferric chloride (2%) for 2 min. Then differentiation of tissues was stopped with several changes of tap water (elastic fiber was appeared in black and background was gray in microscopically check). In the next step, the sections were treated with 5% sodium thiosulfate (for 1 min), then after washing in running water (for 5 min), sections were Counterstained using Van Gieson's solution for 5 min. At least, sections were dehydrated quickly with 96% ethanol (2 changes). Slides were mounted and coversliped to visualize the elastic fibers using light microscopy.

Atomic force microscopy (AFM)

Mechanical properties of tissue samples were determined using atomic force microscopy (JPK Instruments, Germany). To evaluate the mechanical properties, we used fresh tissue specimen with 5×5 mm dimension and 400 µm thickness under liquid nitrogen conditions. Prepared tissue samples were placed and kept in phosphate-buffered saline (PBS) containing protease inhibitor (Proteinase k, Melford). After calibration of AFM microscopy, Specimens were subjected to the AFM scanner and process performed for 15 min at room temperature. The force curves were recorded and analyzed using AFM software (JPK Instruments).

Statistical analysis

Statistical analysis was performed using SPSS software version 16 with analysis of variance (ANOVA) followed by tukey post-doc test. All graphs were depicted using GraphPad prism software version 8. Spearman's and Pearson's correlation coefficient tests were performed to investigate the correlation between various variables. All descriptive analysis on categorical variables were carried out using Fischer's exact test. Analyzed data were presented as mean ± SD and $p < 0.05$ considered as significant difference.

Results

Of the 50 patients diagnosed with bladder TCC, 25/50 (50%) was newly-diagnosed and 25/50 (50%) was recurrent. Our investigations showed that there was no significant difference in age ($p = 0.771$) and tumor size ($p = 0.078$) between newly-diagnosed and recurrent TCC patients. While the smoking history showed a considerable effect on recurrent rate in TCC patients ($p = 0.031$). We also found that there

was a significant relationship between histological grading and recurrent rate in TCC patients (p = 0.036) (Table 1).

ECM glycoprotein and elastic fibers were aberrantly higher in Recurrent TCC

To evaluate the ECM content including glycoproteins and elastic fibers, the tissues from newly-diagnosed and recurrent TCCs and their adjacent normal-appearing tissues as well as normal urinary bladder tissues as control were stained with PAS and VVG, respectively. VVG stained slides of TCCs tumors showed blue-black elastic fibers that were not uniform and had variation in size, thickness and density, while normal and adjacent normal-appearing tissues groups revealed minimal or no demonstrable elastic fibers around tumor cells (Fig. 1). When TCCs cases were compared to normal and adjacent normal-appearing tissues, for blue-black color intensity, 'p' value was not statistically significant, indicating that fibrillar elastic levels are similar in normal urinary bladder tissues and TCCs (Fig. 2A).

The percentage of PAS positive area was significantly higher in newly-diagnosed and recurrent TCCs groups in compared to normal and adjacent normal-appearing tissues groups. However, staining intensity of glycoproteins was higher in recurrent tumors in compared to newly-diagnosed tumors, but this difference was not statistically significant (Fig. 2B).

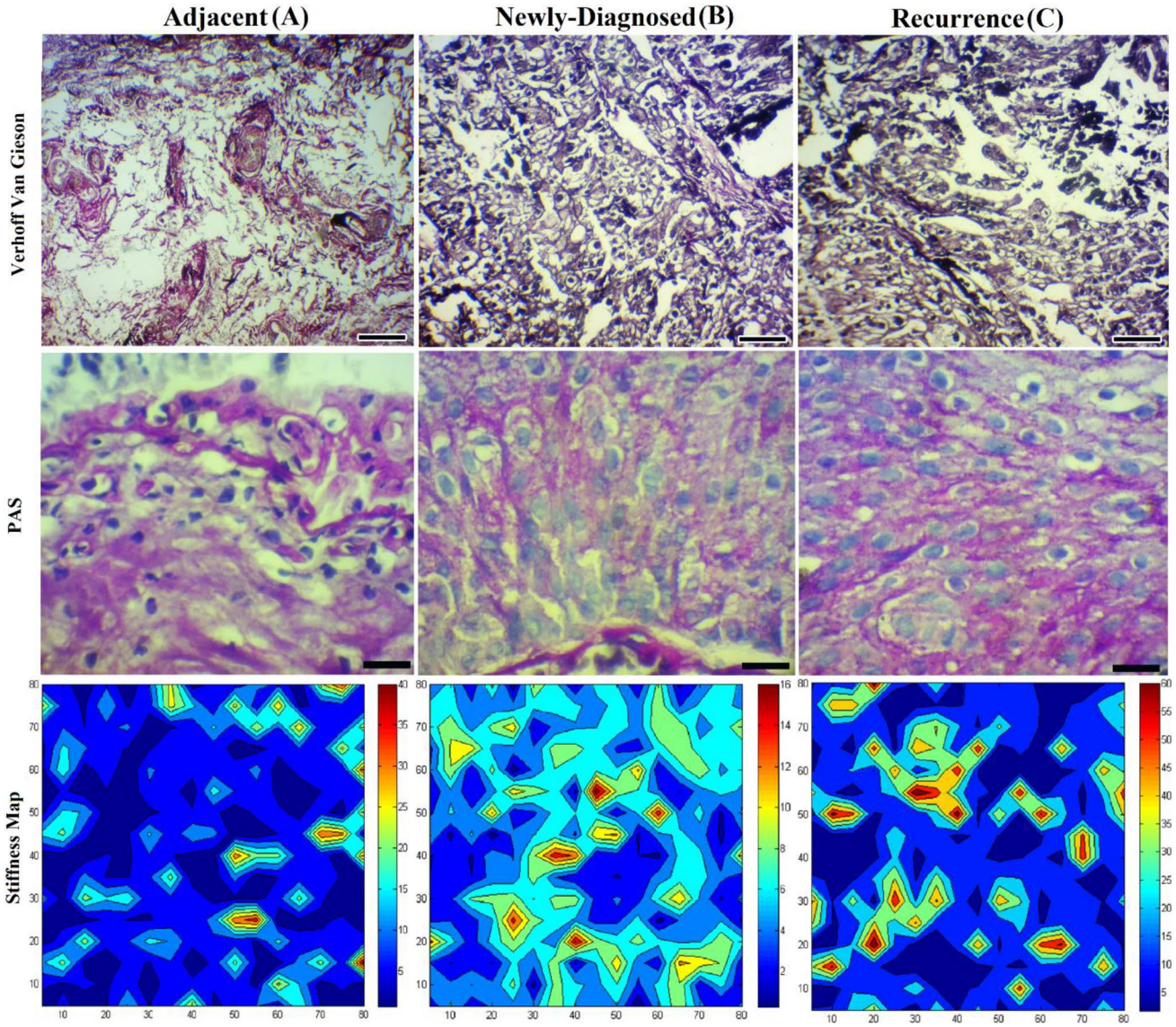

Fig. 1 Verhoff-Van Gieson (Bar = 60 µm), PAS (Bar = 25 µm) staining and stiffness map of bladder TCCs. **A** Adjacent normal-appearing tissues. **B** Newly-diagnosed TCC. **C** Recurrent TCC

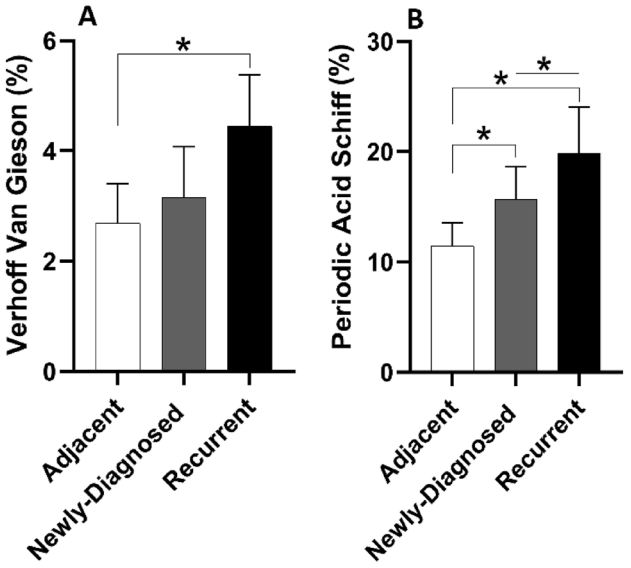

Fig. 2 Verhoff-Van Gieson and periodic acid schiff (PAS) staining of TCCs (25 newly diagnosed and 25 recurrent) and normal appearing adjacent (n = 50) tissue. **A** Mean percentage area of VVG staining in different group. **B** Mean percentage area of PAS staining in different group. *p < 0.05 for all comparisons

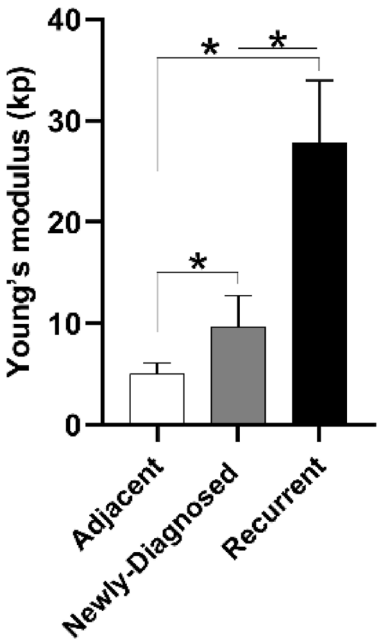

Fig. 3 Young's modulus (kp) values of the newly-diagnosed bladder TCC (n = 25), recurrent bladder TCC (n = 25) and 50 normal appearing adjacent tissue. *p < 0.05 for all comparisons

Matrix stiffness was considerably higher in TCCs subjects

The heterogeneity of matrix structure plays a crucial role, usually leading to the broadening stiffness and Young's modulus distribution. As presented in Fig. 3, the calculated Young's modulus values from recorded force curves were demonstrated that cancerous tissues had considerably stiffened matrix as compared to the normal adjacent tissues. Moreover, there was a significant difference between Young's modulus values of recurrent and newly diagnosed TCCs. Representative image for stiffness map was presented in Fig. 1.

OPN gene and protein overexpression was detected in recurrent TCCs

Gene expression evaluation showed that *SPP1* was over expressed in TCCs tumor tissue as compared to the healthy adjacent bladder tissue. Additionally, our findings revealed that *SPP1* mRNA was markedly overexpressed in TCCs patients which diagnosed as recurrent as compared to the newly-diagnosed TCCs patients (Fig. 4A). Further investigations demonstrated that OPN gene expression was not correlated with histologically grading in bladder TCCs (Fig. 4F). To validate these findings, the gene expression levels were confirmed via western blot technique. Western blot analysis was in line with *SPP1* gene expression and showed that OPN protein expression was higher in TCCs tissue samples when compared to the healthy adjacent tissue samples. However, recurrent TCCs cases showed a slightly higher OPN protein expression than newly-diagnosed TCCs and the difference was not statistically significant (Fig. 4B). Most importantly, our observations were confirmed the positive relationship between OPN protein and gene expression in TCC cases (Fig. 4D).

Serum OPN level might be a relatively good tumor marker for TCCs cases

As shown in Fig. 5, serum OPN level was significantly higher in TCCs cases as compared to the control group. However, there was no significant deference between newly-diagnosed and recurrent TCC cases. Correlation analysis was performed to investigate whether OPN serum level might be positively related to the TCCs histological grading. Our findings showed that serum OPN was positively associated with grading and also, we have showed that grade III TCCs cased had truly higher serum OPN level. To investigate whether OPN expression might be considered as tumor marker for TCCs, we have carried out the receiver operating characteristic (ROC) curve analysis. We have found that the area under curve (AUC) for serum OPN between TCCs subjects and control group was [0.771

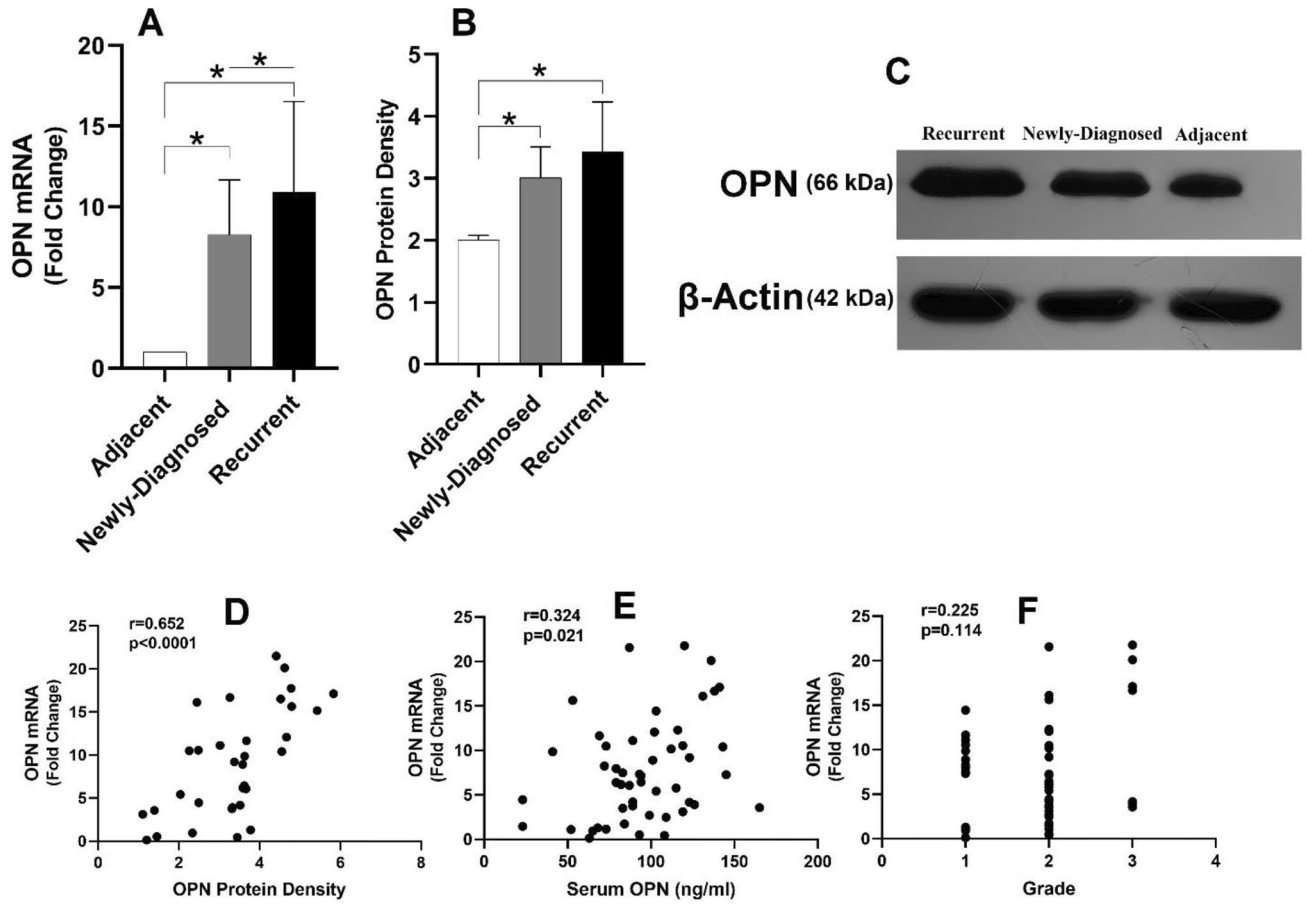

Fig. 4 **A** Gene expression of *SPP1* in TCC (25 newly diagnosed and 25 recurrent) and adjacent tissue samples (n = 50). **B** Representative image for western blot analysis of OPN in bladder cancer (recurrent and newly-diagnosed) and adjacent tissue samples. **C** OPN and β-Actin protein levels. **D** Correlation between *SPP1* gene and protein expression. **E** Correlation between *SPP1* gene expression and serum protein level. **F** Correlation between *SPP1* gene expression and various histological grades. *p < 0.05 in all comparisons

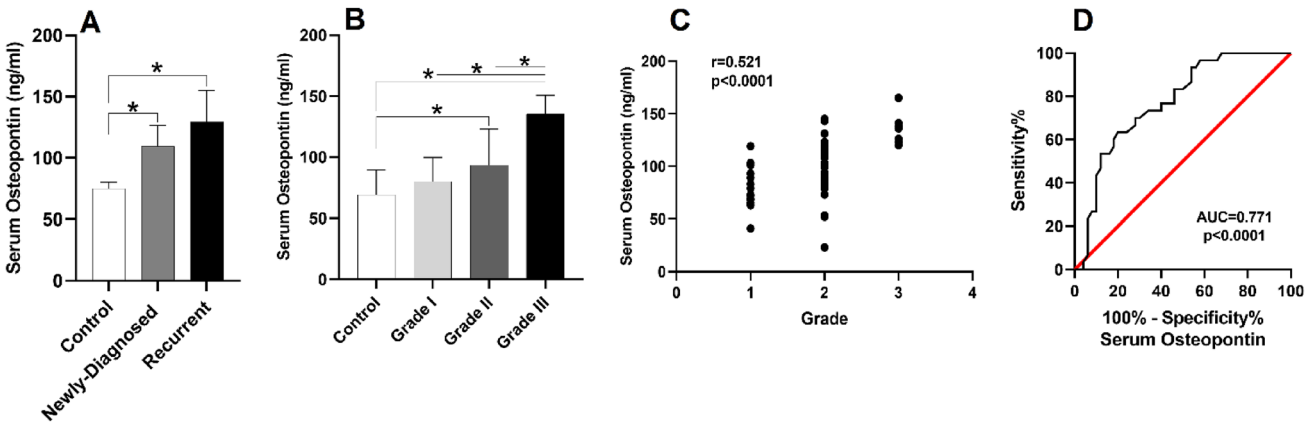

Fig. 5 **A** Serum level of Osteopontin (OPN) in TCC (n = 50) and control subjects (n = 30). **B** Serum level of OPN in control and various grades of TCCs. **C** Correlation between serum OPN level and various histological grades. **D** ROC curve analysis of serum OPN. *p < 0.05 in all comparisons

(95% CI, 0.668–0.873)] on a sample set of 40 TCC cases and 30 controls (Fig. 5D).

Overexpression of YAP gene detected in TCCs and positively correlated with IHH and SPP1 gene expression

We conducted *YAP* and *IHH* gene expression on human bladder TCCs and healthy adjacent tissue samples to investigate the possible correlation between *YAP* and *IHH* as well as OPN expression. In the present study, TCCs cases showed a strikingly higher *YAP* expression as compared to healthy adjacent. Of note, *YAP* expression was significantly differing between newly-diagnosed and recurrent TCCs, and recurrent TCCs showed a markedly higher *YAP* expression when compared to the newly-diagnosed cases (Fig. 6A). Further, *IHH* expression analysis revealed that TCCs tissue samples had significantly higher *IHH* gene expression as compared to the healthy adjacent tissues. Next, we hypothesized that the *YAP* gene expression as upstream regulator of *IHH* and *SPP1*, may be correlated with *IHH* and *SPP1* gene expression. To test this hypothesis, we conducted correlation between *YAP* mRNA expression and *IHH* and *SPP1* mRNA expression. Our results revealed a striking correlation between *YAP* and *IHH* mRNA expression levels ($r = 0.588$, $p < 0.0001$) (Fig. 6). We also showed that there was a slightly positive correlation between *YAP* and *SPP1* mRNA expression ($r = 0.426$, $p = 0.002$) (Fig. 6C). Our investigations also revealed that *YAP* gene expression was strikingly associated with TCCs histological grading ($r = 0.525$, $p < 0.0001$) (Fig. 6E).

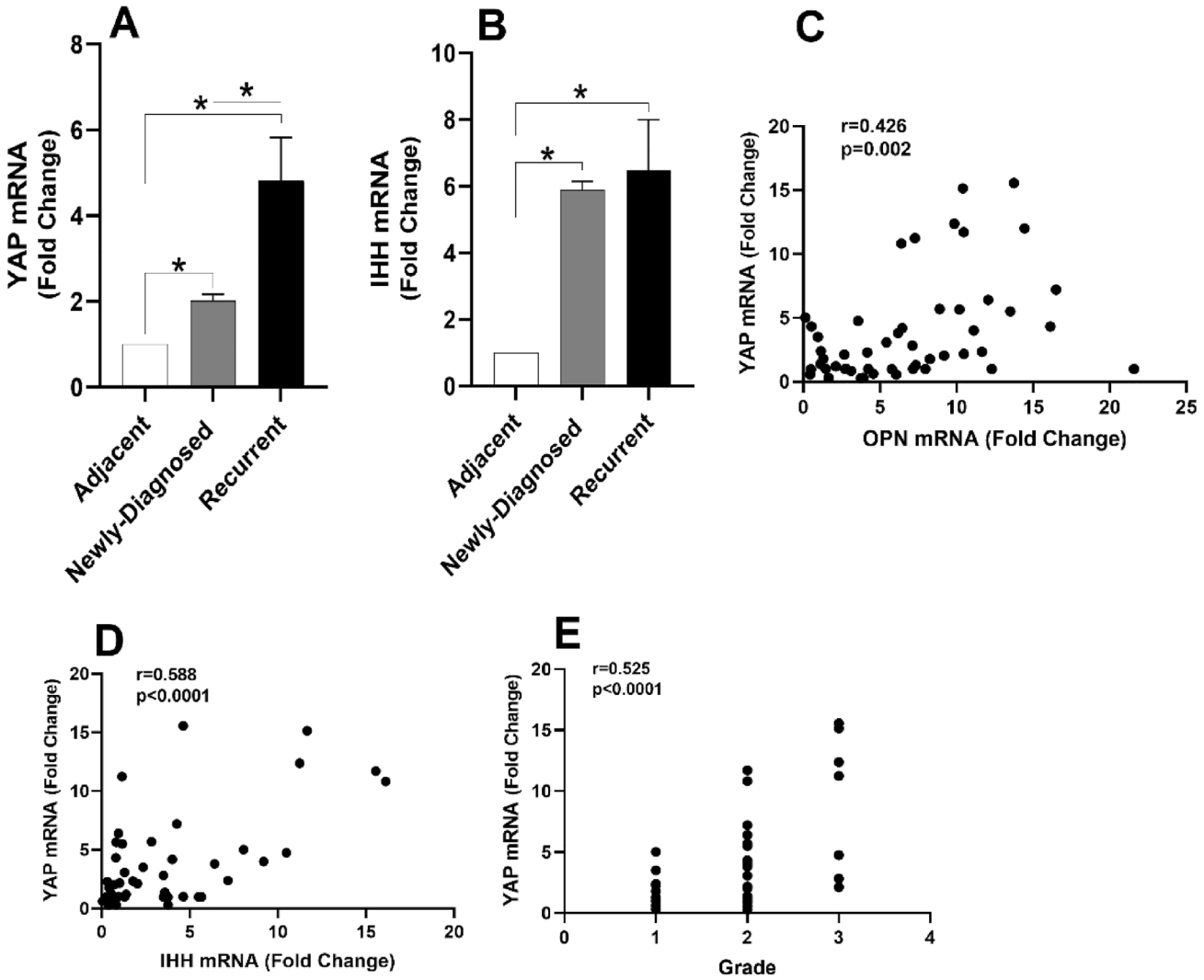

Fig. 6 **A** Gene expression of *YAP* (25 newly diagnosed and 25 recurrent) and adjacent tissue (n = 50). **B** Gene expression of *IHH* (25 newly diagnosed and 25 recurrent) and adjacent tissue. **C** Correlation between *YAP* and *SPP1* gene expression. **D** Correlation between *YAP* and *IHH* gene expression. **E** Correlation between *YAP* gene expression and various histological grades. *p < 0.05 in all comparisons

Discussion

Elevated ECM component and stiffness, commonly found in solid tumor, can drive mechanotransduction which might be the potential trigger of tumorigenesis [20]. While it is well known that various cell types, including immune cells, vasculatures and stromal cells constitute the cancer tissue microenvironment [21], present study here focused on evaluation the role of PAS-stained glycoproteins and VVG stained elastic fibers content with bladder cancer. Glycoproteins and elastic fibers were previously thought to be inactive scaffolds that gives the tissue its shape and prevents the cells from surroundings. While emerging studies have demonstrated that extracellular glycoproteins and elastic fibers act as functional ligands that crosstalk with cell surface receptors such as integrins to mediates the intracellular mechanosensing in cancer cells. However, this crosstalk between elevated ECM contents and intracellular signaling awaits further investigation in bladder TCCs. Our present study reports ECM contents including glycoproteins and elastic fibers increased in recurrent TCCs as compared to newly-diagnosed and healthy adjacent samples. These findings are in line with the results of the studies from other cancer researches, demonstrating that elevated ECM composition including collagens, glycoproteins and elastic fibers and then ECM stiffening contributes to the cancer development and progression. In accordance with ECM composition assay, matrix stiffness evaluation with AFM demonstrated that TCCs subjects had remarkably higher Young's modulus values as compared to normal adjacent tissues, as well as our investigations revealed that TCCs belonging to the recurrent subjects showed stiffened matrix in comparison to the newly diagnosed TCCs. Remodeling and dysregulation in matrix synthesis during cancer and fibrotic diseases is the main cause of ECM stiffness mediated mechanotransduction which can trigger a wide range of signaling cascade such as FAK/CDC42/YAP and FAK/PI3K/Akt inside the cancer cells [22].

YAP as the central mediator for Hippo pathway, may activated through translocating into the cell nucleus as a results of matrix stiffness. Recent studies revealed that integrin/FAK/CDC42 is the main cascade for microenvironment niches sensing to activates the YAP in hippo-independent manner [23, 24]. Of note, we found that was markedly overexpressed in recurrent TCC patients as compared to the newly-diagnosed subjects and healthy adjacent tissue samples. Importantly, *YAP* expression was positively correlated with histological grading of TCCs. These findings were supported with the other previous studies on the other cancers. In addition to hippo cascade kinases including thousand and one amino acid (TAO) and Macrophage-stimulating 1/2 (MST1/2), Protein phosphatase 1 (PP1A) as a functional phosphatase plays a critical role in the dephosphorylation and transcriptional activity of YAP [25]. Owing to the ability of YAP in regulating of the expression of genes involved in cancer development, it is possible that YAP dephosphorylation in hippo-independent manner might be contributed in cancer cell proliferation.

Activated YAP as a mechanoregulator of biochemical signals such as OPN linked to tumor progression and development [26]. OPN as a functional matrix protein had commonly enhanced expression during several cancer types, and was progressively overexpressed with clinical stage in various cancer including gastric, renal, breast, pancreatic, lung, and colorectal cancers [27]. In the present study, both western blot and gene expression data indicate that bladder cancer studied highly express OPN as compared to healthy adjacent tissue. Moreover, our findings showed a higher *SPP1* gene expression in subjects with recurrent TCCs in comparison with newly-diagnosed TCC subjects. We also found a positive correlation among *SPP1* gene and protein expression which confirm the gene expression data. Our findings are in consistence with studies that have showed *SPP1* expression significantly up-regulated in gastric, lung and colon cancers [27–29]. Recently, it has been reported that the OPN play an essential role in cancer biology and development, OPN has enhanced the tumor stem-cell mediated cancer development through involvement in Wnt-β-catenin pathway in cancer patients [30]. Most importantly, it has been suggested that OPN as a secreted matrix protein in body fluid, as well as involvement in several steps of tumor progression (cell proliferation, survival, chemoresistance, angiogenesis, stem-like properties, tumor invasion, and metastasis) found to be an appropriate tumor marker in cancers [31]. Several lines of research demonstrate that serum OPN may contribute to be a unique tumor marker in predicting the cancer prognosis and invasion [32–35]. Notably, we found that serum OPN was significantly elevated in bladder TCC subjects as compared to control. However, there was no significant difference between recurrent and newly-diagnosed TCCs subjects in serum OPN level. Interestingly, serum OPN was positively associated with histological grading of TCCs. Further, we found that serum OPN was markedly elevated in higher TCC grade, investigations revealed that patients with grade III TCCs had significantly higher serum OPN level as compared to control, grade I and II TCC. ROC curve analysis showed that OPN might be considered as a good tumor marker in TCC patients.

The relevant study on the role of abnormal increased ECM composition and stiffness has been well performed. Owing to the ability of YAP in perceiving the cell microenvironment and ECM stiffness, which causes mechanotransduction, YAP could upregulate its downstream elements

including *SPP1* and *IHH*. It has been suggested that there is a feedback loop between stiffened matrix and OPN upregulation, OPN as a matricellular protein overexpressed through YAP activation and induce collagen type I expression which results in increased matrix stiffness [36]. YAP mRNA expression was found to be highly correlated with *SPP1* gene expression, but is YAP direct relevant mediator for *SPP1* expression in response to the mechanical cues? Most recently, it has been postulated that hedgehog pathway plays a fundamental role in *SPP1* regulation, *IHH* as the main mediator for hedgehog pathway have shown to act as the upstream for *SPP1* [19, 37]. Accordingly, we found that *IHH* mRNA expression was strikingly higher in TCCs as compared to adjacent samples. However, there was no significant difference among newly diagnosed and recurrent TCCs. Further, we observed that *YAP* expression was positively associated with *IHH* expression. and *SPP1* were found to be upregulated in Hippo pathway dependent manner in fibrotic diseases and cancer [17, 38–40].

Although, the *SPP1* expression regulated through various signaling cascades including *GSK-3β/β-catenin* and *TGF-β* [15, 41–43], crosstalk between β-catenin and YAP and also links among YAP and TGF-β have postulated that various signaling cascades might be involved to link the YAP to OPN. However, OPN may contribute to be directly upregulated through YAP activation. Beside the transcriptional activity of IHH in OPN regulation, it can also play fundamental role cancer development. Functionally, we observed that *IHH* gene expression was strikingly elevated in both newly diagnosed and recurrent TCCs as compared to the healthy adjacent tissue samples. In line with this observation, several cancer studies have shown that hedgehog pathway play a pivotal role cancer growth and progression [44–46]. This data importantly implies the complexity of links between overexpressed ECM composition and then stiffened matrix and TCC malignancy.

Conclusion

Given the relationship between alteration in ECM composition including glycoproteins and elastic fibers and followed matrix stiffness, mechanical cue can upregulate the *YAP* gene expression as a central mediator in Hippo pathway. Overexpressed *YAP* may contribute to be a mechanotransducer and correlates with the expression of *SPP1* and *IHH* genes which possibly involved in TCC development and growth.

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Declarations

Conflict of interest All the authors have declared that no conflict interest exists.

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or National Research Committee of National Institute for Medical Research Development.

Informed consent Informed consent was obtained from all participant.

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