Original Article
Yes-associated protein 1 promotes bladder cancer invasion by regulating epithelial-mesenchymal transition

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Abstract: Purpose: To investigate the expression of Yes-associated protein 1 (YAP1) in bladder cancer, and to study its role in regulating epithelial-mesenchymal transition in bladder cancer cells. Material and methods: The expression of YAP1, vimentin, and E-cadherin was detected by immunohistochemistry in bladder cancer and para-carcinoma tissues. The relation between expression levels and overall survival of patients was evaluated by Kaplan-Meier estimates. Furthermore, YAP1 expression was knocked down in T24 and UMUC3 bladder cancer through transfection with YAP1-targeted small interfering RNA (siRNA), and the impact on invasiveness and epithelial-mesenchymal transition was detected. Results: Expression levels of YAP1 were higher in bladder cancer tissues, and increased YAP1 expression significantly correlated with poor patient outcomes and poor overall survival in bladder cancer patients. Furthermore, YAP1 siRNA significantly attenuated the invasion of bladder cancer cells and could reverse their epithelial-mesenchymal transition. Conclusion: YAP1 appears to play an important role in bladder cancer progression and is highlighted as a novel potential therapeutic target.

Keywords: Bladder cancer, yes-associated protein 1, e-cadherin, epithelial-mesenchymal transformation, invasion

Introduction

Bladder cancer (BC) is the second most common malignant neoplasm in the urological organs and is considered to be the fourth most prevalent neoplasm in men [1]. BC is associated with high rates of tumor recurrence and progression, which represent major challenges in treatment. The epithelial-mesenchymal transition (EMT) is an important event during tumor progression involving acquisition of a plastic, motile, and mesenchymal phenotype by tumor cells originating from epithelial tissue. During this process, expression of the mesenchymal markers vimentin and N-cadherin are upregulated while expression of the epithelial marker E-cadherin is downregulated [2]. Recent studies indicate that EMT plays a vital role in the initiation, invasion, and metastasis of several types of cancers, including BC, contributing to tumor progression [3-5].

The Hippo signaling pathway restricts cell proliferation and promotes apoptosis in many human malignancies. The core components of the pathway include LATS1/2, WW45, MST1/2, and MOB1, which constitute a protein kinase cascade that regulates Yes-associated protein 1 (YAP1) [6], a proline-rich phosphoprotein [7]. YAP1 is overexpressed in many types of cancers, including liver [8], colon [9], ovarian [10], lung [11], breast [6] and prostate [12] cancer, which usually leads to a poor prognosis [13]. Accordingly, YAP1 has been suggested as a potent oncogene and a novel therapeutic target [14-16]. We also recently reported that YAP1 overexpression promoted the proliferation and migration of BC cells [9]. However, the specific mechanism underlying these effects was not explored. We hypothesized that YAP1 might influence the EMT of BC. Thus, the aim of this study was to examine the role of YAP1 in the process of EMT in BC cells. We first compared
YAP1 promotes invasion by regulating EMT

The positive expression rate of YAP1, vimentin and E-cadherin in case of BC and in para-carcinoma tissues

<table>
<thead>
<tr>
<th>Number of patients</th>
<th>Yap1</th>
<th>Vimentin</th>
<th>E-Cad</th>
</tr>
</thead>
<tbody>
<tr>
<td>BC tissues</td>
<td>55</td>
<td>27</td>
<td>33</td>
</tr>
<tr>
<td>Para-cancer tissues</td>
<td>10</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

Figure 1. The representatives of immunohistochemical staining (A-L): YAP1 was negative expressed in para-cancerous tissues (A, B) and positive expressed in BC tissues (C, D). Vimentin was negative expressed in para-cancerous tissues (E, F) and positive expressed in BC tissues (G, H). E-cadherin was positive expressed in para-cancerous tissues (I, J) and negative expressed in BC tissues (K, L).

Methods

Patients

A paraffin-embedded tissue microarray (55 BC samples, and 10 para-carcinoma samples) was used for immunohistochemical analysis. The clinicopathological parameters of the included
YAP1 promotes invasion by regulating EMT

Table 2. Distribution of Yap1/E-cadherin status in bladder cancer according to clinicopathological characteristics

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Number of patients</th>
<th>E-Cadherin n (%)</th>
<th>p&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Yap1 n (%)</th>
<th>p&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;69&lt;sup&gt;b&lt;/sup&gt;</td>
<td>25</td>
<td>5 (9.09)</td>
<td>20 (36.36)</td>
<td>16 (29.09)</td>
<td>9 (16.36)</td>
</tr>
<tr>
<td>≥69</td>
<td>30</td>
<td>12 (21.52)</td>
<td>18 (32.72)</td>
<td>0.15</td>
<td></td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>46</td>
<td>14 (25.45)</td>
<td>32 (58.18)</td>
<td>22 (40)</td>
<td>24 (43.64)</td>
</tr>
<tr>
<td>Female</td>
<td>9</td>
<td>3 (5.45)</td>
<td>6 (10.91)</td>
<td>5 (9.09)</td>
<td>4 (7.27)</td>
</tr>
<tr>
<td>pTNM stage</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pTis-T1</td>
<td>18</td>
<td>10 (18.18)</td>
<td>8 (14.55)</td>
<td>5 (9.09)</td>
<td>13 (23.64)</td>
</tr>
<tr>
<td>pT2-4</td>
<td>37</td>
<td>7 (12.73)</td>
<td>30 (54.55)</td>
<td>0.011</td>
<td>22 (40)</td>
</tr>
<tr>
<td>Histological grade</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G1-G2</td>
<td>23</td>
<td>11 (20)</td>
<td>12 (21.82)</td>
<td>7 (12.73)</td>
<td>16 (29.09)</td>
</tr>
<tr>
<td>G3</td>
<td>32</td>
<td>6 (10.91)</td>
<td>26 (47.27)</td>
<td>0.037</td>
<td>20 (36.36)</td>
</tr>
<tr>
<td>Tumor size(cm)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;4.1&lt;sup&gt;c&lt;/sup&gt;</td>
<td>32</td>
<td>12 (21.82)</td>
<td>20 (36.36)</td>
<td>16 (29.09)</td>
<td>16 (29.09)</td>
</tr>
<tr>
<td>≥4.1</td>
<td>23</td>
<td>5 (9.09)</td>
<td>18 (32.73)</td>
<td>0.25</td>
<td>11 (20)</td>
</tr>
<tr>
<td>Vimentin</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>22</td>
<td>14 (25.45)</td>
<td>8 (14.55)</td>
<td>8 (14.55)</td>
<td>14 (25.45)</td>
</tr>
<tr>
<td>Positive</td>
<td>33</td>
<td>3 (5.45)</td>
<td>30 (54.55)</td>
<td>&lt;0.001</td>
<td>19 (34.55)</td>
</tr>
</tbody>
</table>

a: One-Way ANOVA analysis, b: mean age, c: mean size.

Figure 2. High YAP1 expression is related with prognosis of bladder cancer patients. Kaplan-Meier survival analysis of the overall survival time (n=55), P=0.0075.

Patients are summarized in Table 1. The tissue microarrays were obtained from the National Engineering Center for Bio Chips in Shanghai, China. All cases were evaluated by two experienced pathologists. Grading and staging were performed according to the WHO classification of 2009. This study was approved by the Ethics Committee of Nanfang Hospital. Follow-up was conducted by telephone and/or clinical examination, and the mean follow-up period was 36 months.

Cell culture

Human urothelial carcinoma cell lines (UMUC3 and T24) were obtained from Southern Medical University. Both T24 and UMUC3 cells were cultured in RPMI1640 with 10% fetal bovine serum and 100 units/mL of penicillin.

Quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

To quantify the YAP1, E-cadherin, and vimentin mRNA expression levels in the urothelial carcinoma cell lines (T24 and UMUC3), total RNA was extracted using TRIzol reagent (Invitrogen). The extracted RNA was reverse-transcribed into cDNA that was used for a template in qRT-PCR on SYBR Master Mix (Takara) with an Applied Biosystems 7500 machine. All mRNA expression levels were normalized to that of the...
YAP1 promotes invasion by regulating EMT

For immunohistochemical staining, formalin-fixed, paraffin-embedded tissue sections were de-paraffinized in xylene, and then hydrated in alcohol. Antigen retrieval was performed with sodium citrate buffer (10 mmol/L citrate buffer, pH 6.0). The samples were blocked to check for peroxidase activity and non-specific protein binding. The sections were washed with phosphate-buffered saline (PBS) and incubated with primary antibodies (YAP1, 1:1500 ABclonal, MA, USA; E-cadherin, 1:1000, CST, MA, USA; vimentin, 1:1000, CST, MA, USA) for 12 h at 4°C. The Dako En Vision staining technique

Figure 3. The EMT relative mRNA and protein expression after transfection of YAP1siRNA in UMUC3 cells. *P<0.05

Figure 4. The EMT relative mRNA and protein expression after transfection of YAP1siRNA in T4 cells. *P<0.05.
YAP1 promotes invasion by regulating EMT

was used for visualization using the DAB substrate. Sections were counter-stained in hematoxylin and then dehydrated with xylene. The staining results of BC tissue sections treated with PBS instead of primary antibodies were regarded as the negative control. To assess the immunoreactivity scores, slides were scored and evaluated by two blinded independent, investigators. Staining intensity was graded from 0 to 3 as follows: 0 for negative, 1 for weak, 2 for moderate, or 3 for strong staining. The percentage of positive cells was graded from 0 to 4: 0 for 0-5% positive cells, 1 for 6-25% positive cells, 2 for 26-50% positive cells, 3 for 51-75% positive cells, and 4 for 76-100% positive cells. The final score was calculated by multiplying the staining intensity score by the fraction of positive cells score: a final score of 6 or above was defined as high expression.

Gene suppression

The small interfering RNA (siRNA) used in this study (YAP1 siRNA) to suppress YAP1 expression was synthesized by RIBOBIO (Guangzhou, China): 5'-CAGUGGCACCUAUCACUCU dTdT-3'. Cells were transfected with siYAP1 using Lipofectamine 2000, and triplicate experiments were performed.

Western blot analysis

Western blotting was performed as described previously [2]. The following antibodies were used: rabbit monoclonal anti-YAP1 (1:1000, CST, USA), anti-E-cadherin, (1:1000, CST, USA) anti-vimentin (1:1000, CST, USA), and mouse monoclonal anti-GAPDH (1:2000, Sigma, USA). Secondary antibodies were purchased from Cell Signaling Technology (1:2000, CST, USA).

Transwell invasion assay

Cell invasion was investigated using a matrigel invasion assay (8 µm pore size; BD Falcon). 1×10⁵ cells were suspended in 100 µl serum-free DMEM and then placed in a transwell plate (3422 Corning, NY) insert precoated with 1 µg/µl Matrigel (BD Biosciences). Medium containing 20% FBS was added to the well. After 48 h incubation at 37°C, the cells were fixed in 4% paraformaldehyde and stained with 0.1% crystal violet (Sigma-Aldrich). Cells in four random fields were counted under a microscope (magnification, ×400).

Statistical analyses

Each experiment was repeated at least three times. Stata 14.0 (Stata Corp, College Station, TX, USA) was used for data analyses. A Student’s t test, and Pearson’s chi-square (χ²) test were used. Kaplan-Meier survival analysis was used to estimate the probability of patients’ survival. Data were presented as mean ± SD. P<0.05 was considered significant.

Results

Expression of YAP1, E-cadherin, and vimentin in BC tissues

YAP1 protein was primarily localized in the cytoplasm and nucleus of BC tissues compared to para-carcinoma tissues. Moreover, the positive expression rates of YAP1 and vimentin were higher in the BC than para-carcinoma tissues, whereas the E-cadherin-positive expression rate was lower in the BC than in para-carcinoma tissues (Figure 1 and Table 1).

Relationship between YAP1 expression and clinicopathological variables

To analyze the correlation between the expression of YAP1/E-cadherin and the clinicopathological variables, we compared the positive
YAP1 promotes invasion by regulating EMT

expression rate of YAP1 between samples from patients with muscle-invasive BC (MIBC) and non-muscle-invasive BC (NMIBC). YAP1 expression was higher in MIBC than in NMIBC cases, indicating an association of YAP1 with more aggressive forms of BC (Table 2). In addition, YAP1-positive expression also correlated with a higher pathological grade (P=0.009), but not with tumor size, age, or sex (P > 0.05).

Relationship between YAP1 expression and survival

Based on the immunohistochemistry results, all 55 patients were divided into positive and negative YAP1 groups. Kaplan-Meier analysis indicated that the group of patients with positive YAP1 expression had significantly shorter overall survival rates (Figure 2).

Effect of YAP1 on EMT and invasion in BC cells in vitro

YAP1 expression was significantly reduced at both the protein and mRNA levels following transfection of YAP1 siRNA. RT-PCR analysis showed that inhibition of YAP1 caused downregulation of vimentin, Snail1, and ZEB1 expression (Figures 3-5). Western blotting also showed that YAP1 downregulation resulted in a significant decrease in the expression level of vimentin, but increased the expression level of E-cadherin in both T24 and UMUC3 cells. Silencing YAP1 expression also clearly inhibited the cell invasion capacity (P<0.05) in the transwell invasion assays (Figure 6).

Discussion

EMT is associated with many processes, including embryonic development, wound healing and tissue repair, and cell migration [17]. Recent evidence also points to a clear role of EMT in cancer progression by promoting tumor invasion and metastasis [18-22]. Given that the Hippo pathway contributes to EMT, 23, 24 and that YAP is a key effector in this pathway to induce proliferation and EMT in mammary epithelial cells [25], we explored the role of YAP in BC progression via promoting EMT. Indeed, overexpression of YAP1 has been associated with tumor progression in many human cancers [13]. Nuclear YAP1 can bind to TEAD and induce cell proliferation and EMT; it also has anti-apoptotic effects, which contributes to a poor prognosis [26, 27]. However, this is the first study to suggest a role of YAP1 in BC via upregulating EMT.

YAP1 expression was significantly increased in BC tissues, and was correlated with pathological grading, muscle invasiveness, and lower...
YAP1 promotes invasion by regulating EMT

overall survival. These findings support previous studies on the prognostic relevance of YAP1 in other tissues [13]. Moreover, siRNA-mediated suppression of YAP1 expression in UMUC3 and T24 cells resulted in changes in the protein and mRNA levels of EMT markers, and inhibited the cell invasion capacity.

There might be multiple mechanisms through which YAP1 regulates EMT. YAP1 was shown to potentiate TGFβ-Smad2/3/4 signaling by regulating the expression of Slug, Snail, and Twist1 [28]. Moreover, the Hippo pathway was shown to be activated in high-density Eph4 cells, and the increased cytoplasmic YAP1 sequestered the Smad2/3/4 complexes to inhibit TGFβ signaling [29]. Further, YAP1 and KRAS may promote EMT together through upregulating the transcription factor FOS, leading to the increased expression of vimentin and Slug in colorectal cancer cells [30]. In line with these previous mechanisms, we found that the mRNA and protein levels of Snail1 and ZEB1 were reduced in the YAP1 siRNA-transfected BC cells.

Overall, our results suggest that expression of YAP1 is essential for the process of EMT, and high expression of YAP1 was associated with a more aggressive tumor phenotype and poor clinical outcomes. Thus, inhibition of YAP1 could impair the progression of EMT in BC and drugs targeting YAP1 may be a potentially effective approach for BC treatment and prognosis.

Disclosure of conflict of interest

None.

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YAP1 promotes invasion by regulating EMT

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**Supplementary Table 1.** Primer sequences for PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence</th>
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<tbody>
<tr>
<td>GAPDH F</td>
<td>5'-ACAACTTTGGTATCGTGGAAGG-3'</td>
</tr>
<tr>
<td>GAPDH R</td>
<td>5'-GCCATCACGCCACAGTTTC-3'</td>
</tr>
<tr>
<td>YAP1 F</td>
<td>5'-CGCTCTTCAACGCGTCGCA-3'</td>
</tr>
<tr>
<td>YAP1 R</td>
<td>5'-AGTACTGGCCTGTCGGGAGT-3'</td>
</tr>
<tr>
<td>Vimentin F</td>
<td>5'-GTTTCCAAGCCTGACCTCAC-3'</td>
</tr>
<tr>
<td>Vimentin R</td>
<td>5'-GCTTCAACCGCAAAGTTCTC-3'</td>
</tr>
<tr>
<td>E-cadherin F</td>
<td>5'-GCCGAGCAGCTACGTGCAC-3'</td>
</tr>
<tr>
<td>E-cadherin R</td>
<td>5'-ACTTTGAATCAG GTGTCGAG-3'</td>
</tr>
<tr>
<td>Snail1 F</td>
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</tr>
<tr>
<td>Snail1 R</td>
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<td>ZEB1 F</td>
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<td>ZEB1 R</td>
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</table>

F: Forward primer. R: Reversed primer.