Original Article
Expression of Fas/FasL and c-myc in bladder cancer and their correlation with tumor immune function

Jian Li¹, Yibin Zhou², Qingwen Li¹, Jiajun Zhang¹, Wenyan Sun¹, Changyuan Dai¹, Yuxi Shan²

¹Department of Urology, The First Affiliated Hospital of Bengbu Medical College, Bengbu, Anhui, China; ²Department of Urology, The Second Hospital Affiliated to Soochow University, Suzhou, Jiangsu, China

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Abstract: Fas/FasL can induce cell apoptosis and maintain body homeostasis. As one transmembrane glycoprotein, it exerts important biological functions. C-myc is one important oncogene involving in progression of multiple tumors and is closely correlated with tumor proliferation, differentiation and apoptosis. This study thus investigated the expression level of Fas/FasL and c-myc in bladder cancer, to analyze its correlation with tumor immunity. A total of 40 bladder cancer patients were recruited. Enzyme linked immunosorbent assay (ELISA) and immunohistochemistry (IHC) were employed to detect the expression of Fas, FasL and c-myc in both serum and bladder cancer tissues. In situ end labeling approach was used to determine the apoptotic rate of cancer and adjacent tissues along with infiltrated lymphocytes (TIL) in bladder mucus. The correlation between Fas/FasL and c-myc expression and TIL apoptotic rate were analyzed. Serum Fas in experimental group was lower than control group, with elevated FasL and c-myc (P<0.05). The expression of Fas in tumor tissues was lower than adjacent or control tissues, while FasL and c-myc levels were elevated (P<0.05). TIL apoptotic rate in bladder cancer tissues was higher than adjacent tissues (P<0.05). The expression of FasL and c-myc along with TIC apoptotic rate was higher in higher grade, multiple lesion, recurrent patients (P<0.05). FasL and TIL were positively correlated with TIL apoptotic rates, while Fas was negatively correlated (P<0.05). FasL was over-expressed while Fas was down-regulated in bladder cancer, facilitating apoptosis of lymphocytes. C-myc expression can up-regulate the immune escape latency of tumor cells.

Keywords: Bladder cancer, Fas/FasL, C-myc, lymphocytes, apoptosis

Introduction

Previous study regarding immune surveillance indicated the body’s potency to recognize tumor cells via body’s immune system and exerts timely eradication via immune cells [1]. Both basic and clinical studies in immunology revealed that, although T cells could recognize tumor-specific antigens at the early stage, those recognized tumor cells are impossible for elimination, thus can proliferate and differentiate for tumor infiltration and migration [2]. Fas/FasL is an important signal pathway for clearing aged cells, as it can induce apoptosis at all body sites and help the normal cellular metabolism, thus accelerating immune process for clearing abnormal cells to inhibit unfavorable consequences [3, 4]. As one important member of apoptotic factors, Fas/FasL plays a critical role in immune escape mechanism of tumors. Fas is one transmembrane glycoprotein on cell surface mainly in activated lymphocytes, fibroblasts and endothelial cells. It shares homology with tumor necrosis factor (TNF) receptor, and is one family of TNF receptor superfamily [5]. FasL is the ligand of Fas, and is one type II transmembrane glycoprotein [6]. Previous knowledge has shown the binding of FasL and Fas inside human body to induce cell apoptosis. Tumor cells, however, have immune escape to get rid of normal cytotoxicity by immune cells. During this process Fas/FasL pathway is one important component [7, 8]. C-myc is one nuclear regulation protein, and is abnormally expressed in various tumors. It participates in cell cycle alternation, cell metabolism, differentiation and apoptosis, and is closely related with Fas/FasL. This study thus recruited bladder cancer patients, whose Fas/FasL, and c-myc levels were determined to analyze its correlation with tumor immune functions.
Molecular marker of bladder cancer

Materials and methods

General information

A total of 40 bladder cancer patients in our hospital from January 2014 to January 2015 in the First Affiliated Hospital of Bengbu Medical College were recruited in this study. The confirmed diagnosis was made by lab indexes, imaging and post-operative pathological methods. There were 20 males and 20 females in patient group, with aging between 25 and 75 years (average = 46.1±9.4 years old). The average period of disease onset was 22.5±9.1 months. All patients had hematuria and pollakiuria, precipitant urea and urodynia. Under UIC standard, there were 14 cases of T0-T1 phase for superficial bladder cancer, 26 cases with infiltrated bladder cancer (stage T2-T4). In WHO criteria, there were 10, 19 and 11 patients for G1, G2 and G3, respectively. In all patients, there were 21 with primary diagnosis and 19 recurrent cases. Single lesion occupied 23 cases while 17 cases belong to multiple lesions. All patients had not received chemo-/radio-/immune therapy before endoscopy. Another 20 individuals from out hospital were recruited as the control group (12 males and 8 females, aging between 20 and 70 years, average age = 45.1±3.4 years). No significant difference regarding sex or age existed between two groups (P>0.05), which were thus comparable.

The experimental protocol has been pre-approved by the ethical committee of the First Affiliated Hospital of Bengbu Medical College and written consents have been obtained from all patients and healthy volunteers.

Inclusive criteria: All patients have been diagnosed by pathology. There was no connective tissue disease, immune disorder, or severe major heart failure including heart, liver and kidney. Patients had not received any anti-tumor treatment such as radio-, chemo- or immune therapy before surgery.

Table 1. Serum Fas, FasL and c-myc contents

<table>
<thead>
<tr>
<th>Group</th>
<th>N</th>
<th>Fas (ng/ml)</th>
<th>FasL (ng/ml)</th>
<th>c-myc (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experiment</td>
<td>40</td>
<td>0.16±0.04*,#</td>
<td>1.47±0.07*,#</td>
<td>1.58±0.04*,#</td>
</tr>
<tr>
<td>Control</td>
<td>20</td>
<td>1.03±0.01</td>
<td>0.02±0.01</td>
<td>0.02±0.02</td>
</tr>
</tbody>
</table>

Note: *, P<0.05 compared to control group.

Reagents and instruments

DAB kits; Hydrogen peroxide; Polylysine; 0.01% citric acid buffer; TdT enzyme buffer and reaction solution; Anti-DIG antibody labeled by peroxidase; Xylene; Absolute ethanol; Paraffin; Hematoxylin; Resin. Ultra-clean workstation (Formal, US); Centrifuge (Feige, China); Inverted microscope (Olympus, Japan); Microplate reader (TECNA, UK); Tissue embedding workstation (SAKURA, Japan); Tissue processor (TIYODA, Japan); Ultra-think microtome (Leica, Germany); Temperature-controlled vibrator (Jinghong, China); Image analyzing system (HP, US).

ELISA

Fasted venous blood samples were collected from all patients. After centrifugation, supernatants were saved for further use. ELISA was applied to quantify serum level of Fas, FasL and c-myc. EDTA-Na anticoagulant; ELISA kits for Fas, FasL and c-myc; PBS buffer; Blocking solution plus primary antibody for Fas, FasL and c-myc were purchased from Abcam, USA. In brief, the test kit was incubated at room temperature for 30 min. Standard samples were diluted along with test samples (N = 5 each). Samples were loaded into the plate for washing, adding reaction buffer, development and quenching. A microplate reader was used to measure absorbance value at 450 nm by Microplate reader (TECNA, UK). Linear regression formula was employed to calculate sample concentration.

Expression of Fas, FasL and c-myc

Tissues were fixed in formalin, dehydrated, immersed in paraffin and embedded with tissue embedding workstation (SAKURA, Japan) and ultra-think microtome (Leica, Germany). Tissues sections were de-waxed, re-hydrated and were heated for antigen retrieval with tissue processor (TIYODA, Japan). Hydrogen peroxide (Sigma, USA) was used to quench slides, which were then blocked in normal goat serum. Primary antibody (Abcam, USA) was then added for 1 h incubation at room temperature, followed by secondary antibody conjugated with horseradish peroxidase (Rabbit antimouse IHC secondary antibody, Abcam, USA) for 10 min incubation. Streptomycin-peroxidase
complex (Abnova, USA) was then added for 10 min incubation. DAB (Sigma, USA) was used to develop slides. After stopping, hematoxylin (Sigma, USA) was used to counter-stain slides, which were differentiated by HCl-ethanol. Slides were dehydrated and mounted. Imaging system was used to capture 5 randomly selected fields for recording by inverted microscope (Olympus, Japan) and image analyzing system (HP, US).

IHC results judgment

Using positive controlled slides and negative (PBS) controlled slides, IHC staining results were analyzed with HE staining as the reference.

The positive staining for Fas, FasL and c-myc was identified as brown or dark yellow granules in cytoplasm. Negative (-): ≤10% positive cells; Weak positive (+): 11%~25% positive cells; Positive (++): 26%~50% positive cells; Strong positive (+++): >50% positive cells. 5 fields were randomly selected from each slide for image capture and recording in softwares.

TUNEL assay

Tissue samples were prepared for paraffin-based slides, which were incubated in 2% hydrogen peroxide in PBS. After rinsing, TdT enzyme buffer and reaction buffer were added for reaction (Thermofisher, USA). Anti-DIG antibody conjugated with peroxidase (Abcam, USA) was then added to develop slides in DAB substrates. Positive signal was identified as brown granules in nucleus. 5 randomly selected fields were selected to calculate cell apoptotic rate (AR), which was equal to positive cell number/total cell number ×100%.

Statistical analysis

SPSS 17.0 software (IBM, USA) was used to process all collected data, of which enumeration data were processed by chi-square analysis, while measurement data were compared by analysis of variance (ANOVA) and were presented as mean ± standard deviation. Logistic regression model was used in multi-factorial analysis. A statistical analysis was defined when P<0.05.

Results

Serum Fas, FasL and c-myc contents

ELISA approach was used to determine serum levels of Fas, FasL and c-myc from peripheral venous blood samples. Results showed that Fas level in experimental group were (0.16±0.04) ng/ml, which was lower than control group. FasL and c-myc levels were (1.47±0.07) ng/ml and (1.58±0.04) ng/ml, respectively, which were significantly higher than control group (P<0.05, Table 1).

Fas and FasL in bladder tissues

IHC staining was performed to test the expression level of Fas and FasL in bladder tissues. Results found strong positive expression of Fas (1 case) and 14 cases of positive expression, comprising positive rate at only 37.5%, which was significantly lower than adjacent and control tissues (P<0.05). There were 11 strong positive and 19 positive cases of FasL in bladder tissues, comprising 75% positive rate, which was elevated than adjacent and control tissues (P<0.05, Table 2; Figure 1).

Expression of c-myc in patient bladder tissues by IHC staining

The expression of c-myc was tested in bladder tissues in all patients. Result showed 10 cases of strong positive and 23 positive cases, comprising positive rate as high as 82.5%, which was significantly higher than adjacent tissues or control ones. See Table 3 and Figure 2 for details.

TUNEL assay of lymphocytes

Lymphocytes were distributed in the lamina propria of bladder mucus and has infiltrated into tumor mesenchymal and tumor solid. Positive signals were shown as yellow-brown granules in nucleus of lymphocytes. The apo-

Table 2. Fas and FasL expression in bladder tissues

<table>
<thead>
<tr>
<th>Group</th>
<th>N</th>
<th>Fas expression strength</th>
<th>FasL expression strength</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Bladder cancer</td>
<td>40</td>
<td>25</td>
<td>14</td>
</tr>
<tr>
<td>Adjacent tissue</td>
<td>40</td>
<td>5</td>
<td>25</td>
</tr>
<tr>
<td>Control</td>
<td>20</td>
<td>1</td>
<td>9</td>
</tr>
</tbody>
</table>

Note: *, P<0.05 compared to adjacent tissues; #, P<0.05 compared to control group.
Molecular marker of bladder cancer

Totic rates of lymphocytes infiltrated in bladder cancer tissue, adjacent tissue and normal bladder mucous were (31.47±17.99)%, (6.59±7.46)%, and (3.37±1.50)%, respectively, with statistical significance (P<0.05). See Figure 3 for details.

Correlation between Fas, FasL, c-myc expression and TIL apoptotic rate

The analysis between expression of Fas, FasL and c-myc in bladder tissues showed elevated expression of FasL and c-myc in bladder cancer tissues compared to adjacent or control groups, while Fas expression was lower than adjacent or control tissues (P<0.05). In bladder cancer tissues, the expression of FasL and c-myc, along with TILAR were elevated while Fas was down-regulated in tumors with higher UIC or G1-G3 grade (P<0.05). In tumors with advanced WHO grade (T0-T4), FasL, c-myc expression and TILAR were also elevated while Fas expression was decreased (P<0.05). Regarding number of lesions, FasL, c-myc and TILAR were all elevated in multiple lesions while Fas was down-regulated (P<0.05). Compared to primary cases, recurrent patients had higher expression of FasL and c-myc, along with higher TILAR but lower Fas expression (P<0.05). See Table 4 for details.

In analyzing the correlation between all parameters, we found a positive correlation between FasL and TILAR (r = 0.84, P<0.05), between c-myc and TILAR (r = 0.51, P<0.05) and a negative correlation between Fas and TILAR (r = -0.65, P<0.05). C-myc was positively correlated with FasL (r = 0.38, P<0.05), and was negatively correlated with Fas (r = -0.39, P<0.05). FasL was negatively correlated with Fas (r = -0.27, P<0.05).

Discussion

Fas/FasL belongs to the tumor necrosis factor/neural growth factor receptor family. Fas/FasL had interaction for body signal transduction and can participate in the induction of cell apoptosis to maintain the homeostasis of internal environment, thus exerting important biological functions [9]. Study has indicated that FasL could induce apoptosis of infiltrated T lymphocytes, thus facilitating immune escape in eyes or testicles [10]. Fas is expressed in various tumor tissues. FasL is mainly existed in the surface of immune activated spleen cells or thyroid cells, including activated T cells and natural killer cells [11]. In multiple tumors, Fas and FasL were co-expressed. The positive

Table 3. C-myc expression in bladder tissues by IHC staining

<table>
<thead>
<tr>
<th>Group</th>
<th>N</th>
<th>Expression strength</th>
<th>Positive rate (%)</th>
</tr>
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<tr>
<td>Bladder cancer</td>
<td>40</td>
<td>7</td>
<td>23</td>
</tr>
<tr>
<td>Adjacent tissue</td>
<td>40</td>
<td>32</td>
<td>2</td>
</tr>
<tr>
<td>Control</td>
<td>20</td>
<td>18</td>
<td>2</td>
</tr>
</tbody>
</table>

Note: *, P<0.05 compared to adjacent tissues; #, P<0.05 compared to control group.

Figure 1. The expressions of Fas and FasL in bladder tissues detected by Immunohistochemistry (×200). A. Fas expression in tumor adjacent tissues; B. Fas expression in bladder cancer tissues; C. FasL expression in adjacent tissues; D. FasL expression in bladder cancer tissues.

Figure 2. The expression of c-myc in bladder tissues detected by Immunohistochemistry (×200). A. The expression of c-myc in tumor adjacent tissues; B. The expression of c-myc in bladder cancer tissues.

Figure 3. The expressions of Fas and FasL in bladder tissues detected by Immunohistochemistry (×200). A. Fas expression in tumor adjacent tissues; B. Fas expression in bladder cancer tissues; C. FasL expression in adjacent tissues; D. FasL expression in bladder cancer tissues.
expression of FasL had anti-attack potency to resist against death signal, endowing tumor cells the ability of immune escape, thus facilitating tumor proliferation and differentiation [12, 13]. c-myc is one transcriptional regulatory protein, and exerts its function via facilitating/inhibiting target gene transcription. The abnormal expression of c-myc can induce the development of tumors. When c-myc was inactivated, cell growth suppressor genes p15, p21 and p27 were all up-regulated. In cells with over-expression of c-myc, these genes were down-regulated.

In this study, bladder cancer patients were recruited to collect peripheral blood samples, in which serum Fas, FasL and c-myc contents were determined by ELISA. Results showed lower Fas and higher FasL or c-myc in experimental groups compared to control group. Further IHC staining found elevated FasL and c-myc along with lower Fas expressions in bladder cancer tissues. These results suggested depressed Fas expression level and elevated FasL/c-myc level in bladder cancer patients. Previous study has shown the down-regulation of Fas in various malignant tumor cells. Under such circumstances, tumor cell can escape from body immune killing, thus avoiding cell apoptosis. Meanwhile, in tumor cells with co-expression with those two factors, the local increase of FasL can facilitate the killing of body immune cells by tumors, further aggravating local environment for immune escape, helping tumor cells to survive under immune surveillance for continuous infiltration and migration [14-16].

Further analysis of Fas, FasL and c-myc in bladder cancer tissues found higher FasL and c-myc expression along with TICAR and lower Fas expression in those tumors with higher G1-G3 grade or WHO T0-T4 grade, multiple lesions or recurrence. These results suggested that bladder cancer with higher grade and lower differentiation grade may manifest with elevated FasL expression, which may be correlated with the differentiation grade of tumors. For those tumor tissues with good differentiation, FasL is

Figure 3. TUNEL assay for apoptosis of lymphocytes (TILAR %). *, P<0.05 compared to adjacent tissue; #, P<0.05 compared to control tissue.

Table 4. Fas, FasL, c-myc expression and apoptotic rates

<table>
<thead>
<tr>
<th>Item</th>
<th>N</th>
<th>Fas</th>
<th>FasL</th>
<th>c-myc</th>
<th>TILAR %</th>
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<tbody>
<tr>
<td>UIC grade</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G1</td>
<td>10</td>
<td>7 (70)</td>
<td>7 (70)</td>
<td>7 (70)</td>
<td>21.12±9.23</td>
</tr>
<tr>
<td>G2</td>
<td>19</td>
<td>6 (91.7)</td>
<td>13 (83.3)</td>
<td>16 (75)</td>
<td>28.35±11.46</td>
</tr>
<tr>
<td>G3</td>
<td>11</td>
<td>2 (100)</td>
<td>10 (89.5)</td>
<td>10 (100)</td>
<td>31.67±17.89</td>
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<tr>
<td>P value</td>
<td></td>
<td>0.016</td>
<td>0.023</td>
<td>0.042</td>
<td>0.028</td>
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<tr>
<td>WHO grade</td>
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<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>T0-T1</td>
<td>14</td>
<td>8 (36.4)</td>
<td>10 (36.4)</td>
<td>9 (36.4)</td>
<td>20.12±8.65</td>
</tr>
<tr>
<td>T2-T3</td>
<td>20</td>
<td>6 (70.6)</td>
<td>15 (76.5)</td>
<td>17 (82.4)</td>
<td>26.53±12.22</td>
</tr>
<tr>
<td>T4</td>
<td>6</td>
<td>1 (88.9)</td>
<td>5 (94.4)</td>
<td>5 (88.9)</td>
<td>32.03±17.97</td>
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<tr>
<td>P value</td>
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<td>Single</td>
<td>23</td>
<td>10 (89.3)</td>
<td>14 (85.7)</td>
<td>17 (85.7)</td>
<td>21.24±11.56</td>
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<td>Multiple</td>
<td>17</td>
<td>5 (87.5)</td>
<td>16 (84.3)</td>
<td>16 (87.5)</td>
<td>32.01±17.54</td>
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<tr>
<td>P value</td>
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<td>0.028</td>
<td>0.017</td>
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<td>13 (88.4)</td>
<td>15 (88.4)</td>
<td>22.43±12.01</td>
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<td>19</td>
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<td>17 (78.9)</td>
<td>18 (84.2)</td>
<td>31.89±17.02</td>
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<tr>
<td>P value</td>
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<td>0.015</td>
<td>0.044</td>
<td>0.013</td>
</tr>
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</table>
Molecular marker of bladder cancer

highly expressed in peripheral tumor cells around the lesion, thus benefiting tumor cell infiltration and migration. C-myc protein expression is also related with the stage and differentiation grade of bladder cancer patients, thus may working as one important factor evaluating disease condition and prognosis of bladder cancer patients. C-myc was found to be positively correlated with FasL while negatively correlated with Fas, which was negatively related with Fas. The combined assay of those three factors in disease diagnosis and prognosis prediction may better analyze the biological activity of bladder cancer, thus having clinical implications [17].

This study assayed TIL by TUNEL method and found more infiltration of TIL in mesenchymal and mucosal layer of bladder cancer than solid. TILAR in cancer infiltrated tissues was also higher than adjacent tissue or normal bladder mucosal tissues. These results suggested the facilitating of TILAR by bladder cancer cells. Further analysis of related indexes found the negative correlation between Fas and TILAR, and positive correlation among FasL, c-myc and TILAR. Inside body TIL can can received antigen stimulus for rapid activation. The abundantly positive expression of Fas increased FasL sensitivity. For TIL which kill tumor cells, they were susceptible for strong attack for FasL-positive tumor cells, leading to abundant apoptosis. Those apoptotic TIL exist in cancer lesion or the peripheral region [18]. Basic study has found 4-times decrease of TIL in FasL-positive tumor cells. For those tumor cells with FasL-negative, TIL was increased by almost 2 folds [19]. These data suggested the close correlation between TIL loss and FasL expression by tumor cells. Inside the body, activation of TIL rapidly down-regulate Fas-resistant TIL to highly express FasL by tumor cells, which strongly attack TIL to induce their apoptosis, thus escaping immune surveillance and toxicity, making tumor growth and progression [20].

In summary, in bladder cancer, tumor cells highly expressed FasL while expressed Fas at low levels. They can help to clear locally specific lymphocytes for facilitating lymphocyte apoptosis, thus making the focal growth, proliferation, infiltration and migration of malignant tumor cells. Moreover, c-myc was up-regulated in bladder cancer and facilitated the interaction between Fas and FasL, thus potentiating immune escape ability of tumors. There might be one coordinated function between Fas/FasL and c-myc to modulate the occurrence and progression of lymphocyte apoptosis. The detailed mechanism of Fas/Fasl and c-myc still require substantiation. Further exploration can be made via inhibiting its transduction pathway and may help in treating bladder cancer.

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Yuxi Shan, Department of Urology, The Second Hospital Affiliated to Soochow University, 1055, San Xiang Road, Suzhou 215004, Jiangsu, China. Tel: +86-512-67784135; Fax: +86-512-67784135; E-mail: shanyuxiv@163.com

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