Original Article

Detection of RECK and MT1-MMP expression in bladder urothelial carcinoma using quantum-dot immunofluorescence and its clinical significance

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Received March 14, 2016; Accepted September 6, 2016; Epub October 15, 2016; Published October 30, 2016

Abstract: Objectives: To explore the role of Kazal motifs (RECK) and Membrane type matrix metalloproteinase-1 (MT1-MMP) in the occurrence and development of bladder urothelial carcinoma (BUC). Methods: A new labeling reagent-quantum-dots (QDs) immunofluorescence histochemistry technique was adopted to detect RECK and MT1-MMP expression in a 150-tissue microarray of human BUC (including 70 cases of BUC tissues and 5 cases of cystitis tissues), and its correlation with the clinicopathological features of BUC was analyzed. Results: (1) RECK Expression: The RECK expression in cystitis tissues was high. Image analysis results showed that the average optical density of RECK was 0.4571±0.0312 and the positive area ratio was 0.4219±0.0304. Otherwise, RECK expression in BUC tissues was low. The average optical density of RECK was 0.1254±0.0187, and the positive area ratio was 0.1253±0.0174. According to QD staining and statistical analyses, RECK expression in cystitis tissues was significantly higher than that in BUC tissues (P<0.05); (2) MT1-MMP Expression: MT1-MMP expression in BUC tissues was high. Image analysis results showed that the average optical density of MT1-MMP was 0.4520±0.0316 and the positive area ratio was 0.4982±0.0326. MT1-MMP expression in cystitis tissues was low. Image analysis results showed the average optical density was 0.1651±0.0179 and the positive area ratio was 0.1482±0.0133. According to QDs staining and statistical analyses, MT1-MMP expression in BUC tissues was significantly higher than that in cystitis tissues (P<0.05); (3) RECK expression was not obviously correlated with patients’ age and gender (P>0.05), but there was a significant difference between RECK expression and the tumor invasion depth, differentiation degrees or clinical UICC staging (P<0.05). The over-expression of MT1-MMP was not obviously correlated with patients’ age and gender (P>0.05), but there was a significant difference between the expression of MT1-MMP and the tumor invasion depth, differentiation degree or clinical UICC staging (P<0.05). Conclusions: In BUC tissues, RECK expression was low while MT1-MMP expression was high. They both may involve in the occurrence and play an important role in the invasion and metastasis of BUC. And there is a synergistic effect between them.

Keywords: Bladder urothelial carcinoma, RECK, MT1-MMP, tissue microarrays, quantum-dots

Introduction

Bladder urothelial carcinoma (BUC) is the most common urinary malignant tumor in China, accounting for more than 90% of the bladder cancer [1, 2]. The clinical characteristics include multiple tumor occurrence and high recurrence rate. The malignancy of about 16%~25% of the recurrent tumors worsens. About 75%~85% of the BUC is caused by non-muscular-invasion bladder tumors. 10% of the superficial BUC is predicted to develop into invasive cancer or have metastasis; however, the pathogenesis has not been completely clarified.

The occurrence and development of tumor are characterized by serious disorders resulting from the interaction between tumor cells and their surrounding extracellular matrix (ECM). Membrane type matrix metalloproteinase-1 (MT1-MMP) can cause the release of various bioactive molecules by degrading EMC components, and can regulate the cell migration, growth, differentiation, and survival by regulating cell adhesion as well as the structure and development of cytoskeletons. Studies show that MT1-MMP is one of the key factors influencing the tumor biological characteristics, as MT1-MMP expression level is closely related to tumor occurrence and development [3, 4].

The reversion-inducing-cysteine-rich protein with Kazal motifs (RECK) genes can inhibit the
secretion and activity of various MMPs through a variety of mechanisms, thereby inhibiting tumor invasion and metastasis [5].

In the present study, a new method for QDs immunofluorescence was adopted to explore RECK and MT1-MMP expression in human BUC tissue microarrays and their relationships with the occurrence, development and clinical pathology of BUC, for providing objective indicators for making judgments about the invasion, metastasis and prognosis of BUC.

**Materials and methods**

**Materials**

Four tissue microarrays of human BUC were provided by Fanpu Biotech, Inc. The microarrays had a dot matrix 15×10, a dot diameter of 1.1 mm and a thickness of 4 μm, including 70 cases of BUC tissues and 5 cases of cystitis tissues, using twin-core arraying. BUC tissues were obtained from resected specimens in clinical surgeries of the received patients from Tongji Hospital of Huazhong University of Science & Technology (15 cases), Renmin Hospital of Wuhan University (45 cases), and Zhongnan Hospital of Wuhan University (10 cases) between Aug. 10, 1999 and Dec. 30, 2011. The tissues have been fixed for 24 hours with 10% neutral-buffered formalin.

Among the patients with BUC, there were 42 male and 28 female cases, with age ranging from 26 to 80 years (average age, 56.8 years; median age, 65 years). According to WHO’s histological classification of urinary tract tumors, there were 42 cases of invasive urothelium carcinoma (35 high-grade cases; 7 low-grade cases) and 28 cases of non-invasive papillary urothelium carcinoma (22 high-grade cases; 6 low-grade cases). According to the clinical stages of UICC in 2002, there were 38 cases of T1, 22 cases of T2, and 10 cases of T3~4. The 5 samples of cystitis tissues were obtained from the patients received by Renmin Hospital of Wuhan University in the corresponding period, involving 3 males and 2 females, with age ranging from 58 to 76 years (average age, 56 years; median age, 68 years).

Rabbit anti-human MT1-MMP polyclonal antibody, and mouse anti-human RECK polyclonal antibody were obtained from Beijing Zhongshan Biotechnology Co., Ltd. at a dilution of 1:100. Goat anti-rabbit/mouse biotinylated secondary antibodies, and QDs high sensitive fluorescent assay kits containing 605 nm quantum-dot labelled streptavidin complex (605 nm QDs-SA) were all purchased from Wuhan Jiayuan Quantum Dots Co., Ltd.

**Methods**

MT1-MMP and RECK expression in BUC tissue microarrays were detected by using the QDs double staining method. The experimental procedures were conducted in strict accordance with the manufacturer’s instructions. The methods were as follows: BUC tissue sections (thickness, 4 μm) were dewaxed, hydrated, microwave antigen retrieved and swashed with TBS buffer. The tissue sections were blocked by incubation with blocking buffer solution (Beyotime Institute of Biotechnology, Shanghai, China) in a wet chamber for 30 min at 37°C. Then the sections were added MT1-MMP and RECK antibodies dropwise, and incubated for 2 h at 37°C, swashed with TBS-Tween® for three times, each time for 5 minutes. Then they were blocked by incubation with blocking buffer solution in the wet chamber for 10 min at 37°C. Next, biotinylated sheep anti-mouse or sheep anti-rabbit IgG antibody was added to the wet chamber and incubated for 30 min at 37°C, swashed repeatedly with TBS-Tween for three times, each time for 5 minutes. The tissue sections were then again blocked by incubation with blocking buffer solution in the wet chamber for 20 min at 37°C. QDs-SA diluted with blocking buffer solution was dripped onto the tissue sections and incubated in a wet chamber for 30 min at 37°C. They were then swashed with TBS-Tween for three times, each time for 5 minutes, and finally treated with 90% glycerin buffer.

The tissue microarrays were observed with a fluorescence microscope after the addition of 900 mL/L glycerin. The QDs were excited by the blue light. The positive expression of MT1-MMP and RECK were indicated by red fluorescence under fluorescence microscope. When the positive area ratio was ≥25%, it was considered as positive protein expression. In the negative control group, the primary antibody was substituted with TBS, and the available known positive section was used as the posi-
RECK and MT1-MMP expression in BUC

RECK and MT1-MMP expression was quantitatively analyzed by using NuanceFx™ Multispectral Imaging System (Cooperative Resources International). Five complete and non-overlapping visual fields under a high power lens (original magnification ×400) were selected randomly from every section. The average optical density, positive reaction area, and total area of all cells in each visual field were determined to calculate the positive area ratio. The measurements of each section were determined by the average value of the average optical density or positive area ratio of the 5 visual fields in that section (positive area ratio = total area of positive reaction in unit area/total cell area in unit area ×100%).

Statistical methods

The result data of QDs staining was represented as the average value ± standard deviation. One-way ANOVA and SNK(q) tests were carried out with Software SPSS13.0 (SPSS, Inc., Chicago, IL, USA) to analyze the average optical density and positive area ratio of the positive reaction cells in each group (α = 0.05). The count data were analyzed using χ² test. A P value of less than 0.05 was considered to indicate a significant difference.

Results

RECK expression

The positive expression of RECK was mainly discovered in the cell cytoplasm. Strong red fluorescence was observed in the cytoplasm of urocystitis cells, indicating a high expression of RECK (Figure 1A). According to the image analysis results, the average optical density of RECK was 0.4571±0.0312, and the positive area ratio was 0.4219±0.0304. RECK expression in BUC tissues was low (Figure 1B), with an average optical density of 0.1254±0.0187 and a positive area ratio of 0.1253±0.0174. One-way ANOVA results showed that there were significant differences between RECK expression in BUC and urocystitis tissues (P<0.05, Table 1). RECK expression was not obviously correlated with patients' age and gender (P>0.05), but there was a significant difference between RECK expression, and the tumor invasion depth, differentiation degree or clinical UICC staging (P<0.05, Table 1).

MT1-MMP expression

The positive expression of MT1-MMP was mainly discovered in the cell cytoplasm. The cytoplasm of BUC cells exhibited strong red fluorescence, indicating a high expression of MT1-MMP (Figure 1C). According to the image analysis results, the average optical density of MT1-MMP was 0.4520±0.0316, and the positive area ratio was 0.4982±0.0326. MT1-MMP expression in urocystitis tissues was low (Figure 1D), with an average optical density of 0.1651±0.0179 and a positive area ratio of 0.1482±0.0133. One-way ANOVA results showed that there were significant differences between MT1-MMP expression in BUC and urocystitis tissues (P<0.05).
of MT1-MMP was not statistically correlated with patients' age and gender (P>0.05), but there were statistically significant differences between the over-expression of MT1-MMP and the tumor invasion depth, differentiation degree or clinical UICC staging (P<0.05, Table 1).

**Discussion**

Bladder tumor is the most common urogenital infinite tumor. 95% of the bladder cancers are bladder urothelial carcinoma (BUC). The postoperative recurrence rate of BUC is up to 70%, with 30% of the recurrent tumors developed towards the later stages of the disease at a faster pace. In addition, 15-30% of the primary tumors are invasive at the early stage, tend to early metastasize even after treatment [6, 7]. The prognosis of BUC patients depends on the invasive depth, metastasis, and recurrence of the carcinoma. Therefore, studying the factors associated with the invasion and metastasis of BUC is of important clinical significance.

Tissue “chips” (or tissue microarrays) refer to tissue sections consisting of paraffin blocks, in which up to 1000 separate tissue cores are assembled in array fashion. It is another important biochip technique besides DNA microarrays and protein microarrays. A tissue microarray is a high throughput tissue information carrier [8, 9], which is characterized by its small volume and large quantity of information. One-time experiment on tissue microarrays produces a large number of results. It enables us to obtain all the data about RECK and MT1-MMP expression in BUC tissues within the shortest possible time. When used for immunohistochemical studies, tissue microarrays help improve the sample comparability and eliminate deviation caused by man-made factors as many tissues are tested on the same section and the consistency of experiment conditions is thus maintained. The experiment results are consistent, reliable, and highly comparable. Such experiments are time saving, economic, and informative [10].

This research has used QDs immunofluorescence and tissue microarrays to explore RECK and MT1-MMP expression in human BUC tissues and their relation to the occurrence, development, clinic, and pathology of bladder cancer. QDs have the characteristics of high sensitivity, strong fluorescence intensity and non-quenchability. The fluorescence exhibited by QDs is completely distinguishable from the tissues’ autofluorescence. Due to QDs’ many advantages and in order to raise people’s awareness of the molecular mechanism of the occurrence and development of BUC and its biological behavior, we adopted QDs immunofluorescence technique to detect RECK and MT1-MMP expression in BUC tissues and to explore the relation between their expression and the BUC.

Membrane type matrixmetalloproteinase-1 (MT1-MMP) is an important extracellular matrix degrading enzyme. MT1-MMP plays an important role in the tumor invasion and metastasis by degrading different components of extracellular matrix (ECM). The increasing activity or over-expression of MT1-MMP is closely related to tumor growth, invasion, and metastasis. MMPs are Zn$^{2+}$-dependent endogenous proteolytic enzymes. At least 23 types of MMPs have been identified. According to their degradation substrates and sequence particularity, the MMPs can be divided into 5 broad categories:

**Table 1.** RECK and MT1-MMP expression and the correlation with the pathological type, and clinical stage of bladder urothelial carcinoma

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>n</th>
<th>RECK</th>
<th>P value</th>
<th>MT1-MMP</th>
<th>P value</th>
</tr>
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<tbody>
<tr>
<td>Gender</td>
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<td></td>
<td></td>
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<tr>
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<td>42</td>
<td>14</td>
<td>0.05</td>
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<td>28</td>
<td>8</td>
<td></td>
<td>9</td>
<td></td>
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<tr>
<td>Age, years</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;60</td>
<td>36</td>
<td>9</td>
<td>0.05</td>
<td>11</td>
<td>0.05</td>
</tr>
<tr>
<td>≥60</td>
<td>34</td>
<td>13</td>
<td></td>
<td>10</td>
<td></td>
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<tr>
<td>Depth of tumor invasion</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Invasive</td>
<td>42</td>
<td>9</td>
<td>0.05</td>
<td>8</td>
<td>0.05</td>
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<tr>
<td>Non Invasive</td>
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<td></td>
<td></td>
<td></td>
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<tr>
<td>High</td>
<td>22</td>
<td>12</td>
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<td>12</td>
<td></td>
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<td>Low</td>
<td>6</td>
<td>1</td>
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<td>1</td>
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<td>UICC stage</td>
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<td>T1</td>
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<td>0.05</td>
<td>7</td>
<td>0.05</td>
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<tr>
<td>T2</td>
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<td>9</td>
<td></td>
<td>13</td>
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<td>T3-4</td>
<td>10</td>
<td>7</td>
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<td>3</td>
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UICC, Union for International Cancer Control.
collagenase, gelatinase,stromelysin, matrilysin, and membrane type MMPs (MT-MMPs) [11, 12]. Research shows that MT1-MMP can degrade many ECM components, and catalyze the activation of pro-MMP-2 at cell surface as a “membrane-dependent” zymogen activator [13]. MT1-MMP can also activate pro-MMP-9 with some factors. MMP-2 and MMP-9 play important roles in the oncogenesis and progression of human malignancies [14-16] as they can effectively degrade IV type collagen, BM’s main component, and they are related to the cell differentiation and apoptosis, vasculogenesis, immune responses, and tumor cell growth. The results of this study showed that the MT1-MMP expression level in BUC tissues was significantly higher than that in urocystitis tissues. These results suggest that MT1-MMP participates in the occurrence of BUC and promotes its invasion and metastasis by degrading ECM. MT1-MMP also plays an important role in the tumor angiogenesis process. The detection of MT1-MMP can be used as a clinical and biological indicator for research on the occurrence and development of BUC. The results of this study also showed that the over-expression of MT1-MMP in BUC tissues was not obviously correlated with patients’ age and gender (P > 0.05), but it was correlated with the stage and grade of bladder cancer (P < 0.05). These results suggest that the increased MT1-MMP expression level is related to the invasion, metastasis, and clinically pathological process of BUC. One of the possible mechanisms could be as follows: MT1-MMP promotes the activation of MMP-2 and MMP-9, and degrades the physiological barrier of ECM and BM independently or collaboratively. So cancer cells can invade the surrounding tissues and enter the circulatory system, thus promoting the invasion and metastasis of tumors. This could be one of the reasons why BUC has an early metastasis. It indicates that the high expression of MT1-MMP can be related to the stage and grade of bladder cancer. It also provides some thoughts to the further search of restraining methods for the early invasion and metastasis of BUC.

Reversion-inducing-cysteine-rich protein with Kazal motifs (RECK) gene is an important tumor suppressor gene. It plays a crucial role in regulating the expression and activity of various matrix metalloproteinases (MMPs) [17]. RECK genes are highly expressed in normal tissues, but they are not expressed or poorly expressed in cell lines derived from various tumor tissues or cells transfected by oncogene, such as ras. Many oncogenes, such as ras, fos, and myc, can down-regulate RECK gene expression, indicating that RECK genes might be common negative regulation target sites of different oncogenes [18-20].

The results of this research showed RECK gene high expressed in urocytis tissues and low expressed in BUC tissues. These results suggest that RECK can inhibit the activity of at least 3 MMP family members, including MMP-2, MMP-9, and MMP1-MMP. In addition, proper RECK expression can inhibit angiogenesis, indicating a close relationship between RECK genes and angiogenesis. Low RECK expression in BUC tissues shows that RECK affects the activation of proMMP-2 by inhibiting the activity of MT1-MMP. The inhibition of MMP-9 by RECK is at post-translation level. That is, RECK restrains the total amount of MMP-9 and its activity by inhibiting its release from cells and by directly inhibiting its proteolytic activity, so as to exert an anti-neoplastic effect. The results of this research also showed that low RECK expression in BUC tissues was not obviously correlated to the patients’ age and gender (P > 0.05), but there was a significant difference between low RECK expression and the tumor invasion depth, differentiation degrees, and clinical UICC staging (P < 0.05), indicating MT1-MMP expression is related to tumor invasion. Since MMP can degrade various ECM components and it is closely related to tumor invasion and metastasis, inhibition of MMP may effectively suppress the invasion and metastasis of tumors. RECK can inhibit the expression and activity of matrix metalloproteinase. It is closely related to oncogene signals and extracellular matrix rebuilding.

Among the research on BUC, there is little on the interrelation between RECK and MT1-MMP. RECK mainly plays a role by directly inhibiting MMP-2, MMP-9, and MT1-MMP, or suppresses the activation of proMMP-2 and proMMP-9 by inhibiting MT1-MMP. However, the mechanism by which RECK inhibits MT1-MMP functions remains unclear. The research of Takao Miki and others [5] showed that RECK could form a complex with MT1-MMP in order to inhibit its
RECK and MT1-MMP expression in BUC

activity; RECK also increased the amount of MT1-MMP which binds the detergent-resistant membranes (DRM) during the glucose gradient centrifugation process. Moreover, the interference with membrane cholesterol significantly affected the inhibition of MT1-MMP by RECK. These results show that, other than by inhibiting enzyme activity, RECK also regulates MT1-MMP by directly affecting its biological behaviors on the cell membrane. Takao Miki and others also found that, in case of RECK expression loss, the internalization of MT1-MMP was achieved through cage protein or caveolae; and when RECK was expressed, the internalization of MT1-MMP was achieved in other ways. These results show that RECK regulates the approach of MT1-MMP internalization. Changes in the approach accelerate the internalization and degradation of MMPs. In this research, MT1-MMP and RECK expression levels in BUC tissues were negatively correlated, indicating that MT1-MMP and RECK play an important role in the invasion and metastasis of BUC and that there is a synergistic effect between them.

In conclusion, MT1-MMP and RECK expression is related to the invasion and metastasis of BUC. High MT1-MMP expression not only accelerates the degradation of BM and ECM components, but it also leads to the activation of a series of MMPs, thereby accelerating the tumor invasion and metastasis. The inhibition of MMPs by RECK is not without discrimination, so using medicine to simulate RECK expression or directly activate endogenous RECK expression in order to implement its activities as a tumor suppressor gene can be a new idea for tumor therapy. Joint detection of RECK and MT1-MMP expression in BUC tissues has provided a significant theoretical basis for the clinical prediction of the invasion, metastasis, and prognostic evaluation of BUC and its comprehensive treatment.

Acknowledgements

This study was partially supported by the National Natural Science Foundation of China (Grant No. 81402242).

Disclosure of conflict of interest

None.

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