The expression of apolipoproteinA1 and its correlation with infiltration of urologic neoplasm

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Background: To explore the differential expression of apolipoproteinA1 (APOA1) in urologic neoplasm patient compared with controls, as well as investigates whether APOA1 correlated with infiltration of urologic neoplasm.

Methods: A total of 59 tissue sections of surgically-resected urologic neoplasm and 6 cases of normal tissue sections were collected. Fourteen cases of urine samples from transitional cell carcinoma patients and 6 cases urine samples from controls were also applied in this experiment. We also selected 6 cases of fresh bladder transitional cell carcinoma tissues. The urologic neoplasm tissue sections were classified into infiltration and non-infiltration urologic neoplasm groups. The expressions of APOA1 between urologic neoplasm and normal control were detected by Western blot, Immunohistochemistry and qRT-PCR. The method of Immunohistochemistry was applied to examine the differences of APOA1 expression between infiltration and non-infiltration urologic neoplasm tissue section groups.

Results: Compared with none expression in normal controls, APOA1 was exhibited higher level in urologic neoplasm patient’s urine and fresh bladder transitional cell carcinoma tissues (P<0.05). There were statistical differences of APOA1 between infiltration and non-infiltration urologic neoplasm tissue section groups. APOA1 expressions were found to be up-regulated in the infiltration neoplasm tissue sections compared to non-infiltration group (P<0.001).

Conclusions: APOA1 could act as a valuable biomarker for predicting the occurrence and development of urologic neoplasm.

Keywords: ApolipoproteinA1 (APOA1); urologic neoplasm; infiltration; tissue sample; urine; immunohistochemistry

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Introduction

Urologic neoplasm is ranked the second most common tumor that occurs in the urogenital system, including kidney, bladder, prostate, and testicle (1). Carcinogen exposure and the habit of smoking are considered the main etiological factors of urologic neoplasm (2). According to the previous reports, the most common clinical urologic neoplasms are prostate cancer (PC), renal cell carcinoma
(RCC), bladder cancer (BC) (3). The 5-year survival rate of RCC ranges from 15% to 50%, according to the different of tumor stage, treatment, and selection. For BC, the 80% of the BC is non-invasive (4). Invasive BC accounts for about 20% of the annual incidence of BC, amounting to approximately 15,000 deaths per year in the China (3). The patients at early stage (I or II) are more likely to survive with a 5-year survival rate of 97%, while the patients at advanced stage (III or IV) the 5-year survival rate is only 6%, as to the cancer has already invaded to other organs, which makes it quite difficult to therapy (4). The previous studies have reported, almost all of the PCs is non-cutaneous cancer in males (5). The most risk of PC is environment factors, such as diet, smoking etc. Because of the gradual progression and nonspecific symptoms PC is usually diagnosed at advanced stage (6). So finding a valuable biomarker is becoming vital important for the survival rate of urologic neoplasm.

ApolipoproteinA1 (APOA1) which is a major component of the high-density lipoprotein complex has higher expression in the human serum. It also has the function to activate lecithin-cholesterol-acyltransferase (LCAT) in vivo (7). APOA1 is synthetic mainly in the liver and small intestine. The gene methylation of APOA1 determines the expression of the protein (8,9). APOA1 only expresses a little amount in the liver, small intestine, and pancreas in the human body, so the abnormal expression of APOA1 in human is of great significance for the early diagnosis of tumor (10). According to the reports, APOA1 plays a significant role in the process of fat metabolism in the human body. The ration of APOA1/APOB is now used to judge the lipid metabolism in the diseases of coronary atherosclerosis and stroke (11). A large number of studies have reported APOA1 participates in the PPAR-RXR pathways, which involved in the metabolism of tumor cell. The relationship between APOA1 and human tumor is a hot topic, such as APOA1 was expressed lower in the pancreatic cancer and ovarian cancer tissues compared with normal tissues (12,13). Lei et al. has reported APOA1 expressed higher in the BC urine than the controls (14).

This study aimed to explore the expression of APOA1 between urologic neoplasm tissues and normal urologic tissues. Furthermore, we wanted to verify APOA1 whether correlated with infiltration of urologic neoplasm. The differential expression of APOA1 was investigated between the infiltrated urologic neoplasm tissue sections and non-infiltrated urologic neoplasm tissue sections by the method of immunohistochemistry. Fifty-nine cases of surgically resected urologic neoplasm tissue sections and 6 cases of normal tissue sections were analyzed. The methods of PCR and Western-blot were applied to explore the differential expressions of APOA1 between 6 cases of fresh urologic neoplasm tissues and normal human bladder tissues. The differential expressions of APOA1 between urologic neoplasm patients’ urine and normal control urine were investigated by the method of Western-blot. We expected APOA1 could be considered as the biomarker of the occurrence and development of urologic neoplasm, especially the differential expression of APOA1 was found in the urine samples.

Methods

Sample characteristics

This study contained 59 cases of surgically resected urologic neoplasm tissue samples and 6 cases of normal tissues. We also collected 14 cases of urine sample respectively from transitional cell carcinoma patients and 6 cases of urine sample from controls. Furthermore, 6 cases of fresh bladder transitional cell carcinoma tissues were also selected. The 6 cases of normal tissues and 6 cases of normal human urine sample acted as the control groups in this experiment. The tissue samples were selected from Beijing Shijitan Hospital and Hebei Xingtai Hospital from 2010.10 to 2013.10. All the patients underwent initial surgery and without any radiotherapy and chemotherapy before surgery. And the inspection found no history of other malignant tumors, eliminated the abnormal metabolism related diseases. The cancer tissue samples were divided into two groups depending on infiltration (not infiltration, infiltration). The details were listed in Table 1. We extracted the protein from the urine and tissues by the methods of centrifugation (12,000 rpm, 4 °C, 30 min); The urine needed ethanol extraction, ethanol was 4 times more than urine (100% ethanol, 4 °C, 30 min). Before centrifugation, the tissues must be cleavage by protease, 37 °C, 30 min. The extraction was ultrasound on the ice (ultrasonic 3S, stop 6S), 30 min on the ice, concussion every 10 min. After centrifugation, the protein was collected. The concentration of the protein was quantitative by the Bradford assay, then was preserved at −70 °C. The method of Western-blot was used to explore the differential expression of APOA1 protein expression in the urine and fresh urologic neoplasm tissues. Loading quantity of protein sample was 30 µg. Each patient signed the informed consent and this study received approval from the Institutional Review Board for the
Protection of Human Subject.

**Immunohistochemistry**

The paraffin-embedded tissue blocks were sectioned in 5 mm slices. The tissues dewaxed 20 min by xylene, dehydrated with 100%, 100%, 95% and 75% serial ethyl alcohol. After three rinses using PBS 15 min, endogenous peroxidase (Dingguo, Biotechnology, China) blocked 20 min at room temperature to deprive endogenous peroxidase activity. After three rinses using PBS 15 min, the slides were preincubated with normal goat serum for 30 min to reduce nonspecific antibody binding. After antigen retrieval, the samples were incubated with antibody against APOA1 (Thermo Fisher MAI-83002, USA) at a dilution of 1:500 (diluted in PBS buffer); after three rinses using PBS, the samples were incubated in HRP-conjugated secondary antibody (DAKO, USA), 30 min at 37 ℃. Then chromogen DAB solution (DAKO, USA) colored 5 min. Then the sections were counterstained with hematoxylin for 2 min, washed in running water, dehydrated with 75%, 95%, 100%, and 100% serial ethyl alcohol. The slides were cleared with xylene.

**Assessment of APOA1 in Immunohistochemistry**

Semi-quantitative immunohistochemical detection was used to determine the APOA1 protein level of tissue samples. Cytoplasm immunoreactivity for the APOA1 was scored by evaluating the percentage of positive BC cells and the intensity of cancer cells. Both the scores were decided under double-blind conditions by three independent pathologists. The percent of positive cells was scored as “0” (0%), “1” (1–10%), “2” (11–50%), “3” (51–80%), “4” (81–100%). Intensity was scored as “0” (no staining), “1” (weakly stained), “2” (moderately stained), and “3” (strongly stained). The scores were then classified as follow: “−” (score 0), “+” (score 1–4), “++” (score 5–8), and “+++” (score 9–12). In order to do the statistical analysis, the immunohistochemical scores were grouped into low expression group (“−”, “+”) and high expression group (“++”, “+++”).

**Western-blot**

We extracted the protein from the fresh tissues and urine by cracking. The Bradford assay was performed for measuring protein concentration. The protein of 30 µg was separated by 10% SDS-PAGE. Separated proteins in SDS-PAGE were electrotransferred to a PVDF membrane (Bio-Rad, USA). The membrane was blocked in 5% nonfat dry milk in PBST (1× PBS, 0.05% Tween-20) at room temperature and incubated with mAb against APOA1 (Thermo Fisher MAI-83002, USA) at a dilution of 1:1,000, mAb against β-actin (Santa Cruz, CA) at a dilution of 1:200 overnight at 4 ℃. Then the PVDF membrane was incubated in horseradish-peroxidase-conjugated goat anti-mouse IgG antibody (diluted 1:2,000 in PBST buffer) 90 min at room temperature. The membrane was washed three times in PBST buffer. Electrophoresis strip was detected by the enhanced HRP-DAB chromomeric kit. The intensities of Electrophoresis strip was tested by Image J software. The value of APOA1 was normalized with respect to the

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**Table 1 Tissue characteristics of urologic neoplasm patients**

<table>
<thead>
<tr>
<th>Group</th>
<th>Number (n)</th>
<th>Age (y)</th>
<th>Gender (F/M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Infiltration</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-infiltration</td>
<td>14</td>
<td>62.7±9.3</td>
<td>2/12</td>
</tr>
<tr>
<td>Infiltration</td>
<td>45</td>
<td>64.4±6.0</td>
<td>7/38</td>
</tr>
<tr>
<td>Classification</td>
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<td></td>
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<tr>
<td>Bladder cancer</td>
<td>38</td>
<td>64.5±8.1</td>
<td>8/30</td>
</tr>
<tr>
<td>Urethral carcinoma</td>
<td>7</td>
<td>60.6±7.5</td>
<td>1/6</td>
</tr>
<tr>
<td>Mixed tumor*</td>
<td>1</td>
<td>62</td>
<td>0/1</td>
</tr>
<tr>
<td>Epithelioid angiosarcoma</td>
<td>1</td>
<td>58</td>
<td>0/1</td>
</tr>
<tr>
<td>Inverted papilloma</td>
<td>1</td>
<td>65</td>
<td>0/1</td>
</tr>
</tbody>
</table>

*, epithelial and mesenchymal mixed tumor. Age is presented as mean ± SD.
intensities of β-actin. All of the western blotting analyses were repeated three times.

qRT-PCR

The RNA from fresh bladder transitional cell carcinoma tissues was extracted using TRIzol reagent (Ambition, life technologies, USA) according to manufacturer’s instructions. The RNA weighted 2 µg from each sample was obtained to be reverse transcribed and synthesized cDNA. The reaction was performed with oligo d(T) (Dingguo Biotech, China), dNTP (Genview, USA) under the action of M-MLV reverse transcriptase (Promega, USA). qRT-PCR was conducted with Roche Lightcycler 480 Real Time PCR System with TransStart Top Green qPCR SuperMix (TransGen Biotech, China). Primers of APOA1, GAPDH were as follows: APOA1 forward 5’-CGGCAGACTATGTGTCCCA-3’, and reverse 5’-CTGAAGGTGGAGGTCACGCT-3’; GAPDH forward 5’-TTTGGTATCGTGGAAGGACT-3’ and reverse 5’-AGTAGAGGCAGGGATGATGT-3’. The program consisted of a pre-incubation step (5 min at 95 °C, 10 s at 55 °C, and 10 s at 72 °C). Then we performed the melting curve step at the condition of 5 s at 95 °C, 1 min at 65 °C, then melting at 0.11 °C/s with continuous acquisition mode until 97 °C. At last, the sample was cooled at 40 °C for 30 s. 5GAPDH was used as endogenous reference. 2−ΔΔCt method was applied for evaluated mRNA expression of the target gene. All of the qRT-PCR experiment was repeated three times.

Statistical analysis

The software of SPSS18.0 was used to do statistical analysis. The differential expressions of APOA1 among each groups were verified by analysis of the Wilcoxon rank and test, Kruskal Wallis rank and Fisher’s exact probability method. Two-tailed P<0.05 was accepted as statistically significant difference.

Results

The location of APOA1 expression in different type of urologic neoplasm tissues by the method of Immunobistochemistry

APOA1 was located in the membrane and cell plasma of BC and ureteral carcinoma cells. In the epithelial and mesenchymal mixed tumor and epithelioid angiosarcoma, APOA1 was expressed in the cytoplasm. The expression of APOA1 mainly was positioned in the cell membrane of the inverted papilloma. The result is shown in Figure 1.
APOA1 expressed higher in urologic neoplasm patient compared to normal human

We compared the differential expression of APOA1 between normal human urologic tissues and urologic neoplasm by Immunohistochemistry, Western-blot, and qRT-PCR, APOA1 was expressed higher in the urologic neoplasm than normal human tissues by Immunohistochemistry (P<0.001). In 53 cases of urologic neoplasm tissues, the positive expression of APOA1 is 81.1%; while in the normal control group, the rate is 28.6% (Figure 2). While in the fresh urologic neoplasm tissues, APOA1 exhibited higher level compared with normal tissues by the method of Western-blot and qRT-PCR (Figure 3). Compared with none expression of APOA1 in normal human urine, higher expression of APOA1 is found in urologic neoplasm patient’s urine (Figure 4).

APOA1 expression in urologic neoplasm tissues is associated with the invasion by Immunohistochemistry

Results showed, higher expression of APOA1 presents in 81.8% of non-invasive urologic neoplasm tissues (14
cases), while in the 45 cases of invasive neoplasm tissues; the positive rate is 92.3%. APOA1 was noticeably higher in invasive urologic neoplasm tissues than non-invasive, the differences had statistical significant (P<0.001; Figure 5).

Discussion
Reports show APOA1 is the main component of plasma high density lipoprotein (HDL) which is constituted by phospholipids, cholesterol, and other lipoprotein and plasma factors. APOA1 accounts for 70% of HDL protein content (7). APOA1 mediates the important physiological function in human body, including resistance to atherosclerosis, anti-inflammatory, anti-oxidation, anti-endotoxin, combined with vascular walls ATP transporter A-I (ABCA-I), and so on. APOA1 is mainly synthesized in the liver and small intestine. The status of APOA1 gene methylation determines the high tissue specificity of protein expression. The methylation of APOA1 is merely found in the liver, small intestine, pancreas and vascular intima (15). All above indicate APOA1 plays a diverse role in the human body.

In the recent studies, APOA1 correlated with the occurrence and development of tumors in human body had become a hot topic. Li et al. found the APOA1 had higher expression in BC patients’ urine by the method of 2-DE and MS, which was confirmed by Western blot and ELISA results. They also showed the expression of Apo-A1 in the urine could be used as a biomarker to diagnosis BC with a sensitivity and specificity of 89.2% and 84.6% (16). Steel et al. had reported, in the proteomics study, the expression of APOA1 significantly decreased in the hepatic cancer patient’s serum (17). Gillard et al. verified the APOA1 was expressed little in the HpG2 cell, and the secretion of APOA1 also correlated with the lipid status of the HpG2 cell (18). The expression of APOA1 was also found to have differences between breast cancer patient and controls. Huang compared the differential expressions of APOA1 in plasma protein between breast cancer patient and controls. However, the relationship between APOA1 and urologic neoplasm had been rarely reported.

In the present study, APOA1 was expressed higher in urologic neoplasm tissues and urine compared with normal control group. The results demonstrated that APOA1 was up-regulated in urologic neoplasm patient compared with

Figure 4 APOA1 expressed higher in urologic neoplasm patient’s urine compared with normal control urine. In the Figure 4A, C represents the control electrophoresis strip. (A) N1-N6 represents six cases of normal control urine. 1–14 represent 14 cases of urologic neoplasm patients’ urine samples. (B) The result of APOA1 protein expression in urologic neoplasm urine by Western-blot. APOA1, ApolipoproteinA1.
normal control people. Especially in the urine sample, APOA1 was found to have expression in urologic neoplasm patient, while had no expression in controls. Considering the urine had the advantage of noninvasive and enrichment, APOA1 might function as a potential molecular marker in the occurrence and development of urologic neoplasm. In conclusion, APOA1 could be considered as a valuable biomarker for the clinicians and patients. APOA1 could afford clinical aid for the diagnosis and therapy of urologic neoplasm. The mechanism between APOA1 and the occurrence and development of urologic neoplasm needed further investigation.

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Footnote

Conflicts of Interest: The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. This study had gained approval from the Institutional Review Board for the Protection of Human Subjects. The study protocol was approved by the Institutional Review Board of Beijing Shijitan Hospital (No. 2017-q17), and all of the participants signed an informed consent form. All the participants were given informed consent.

References
