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Clinical significance of changes of expression of the Wnt/β-catenin signaling pathway in renal clear cell carcinoma

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Abstract. – OBJECTIVE: To explore the changes and clinical significance of expression of β-catenin in renal carcinoma.

PATIENTS AND METHODS: We selected 46 patients with renal clear cell carcinoma who were hospitalized from May 2013 to March 2016 and healthy adults (controls) matched for age and body weight, who were hospitalized in the physical examination center of our hospital during the same period. Peripheral blood of patients and controls was drawn for ELISA. After surgery, renal carcinoma and normal peritumoral tissue samples were harvested for immunohistochemical staining, Western blot analysis and qRT-PCR was used to observe changes of β-catenin expression in renal carcinoma tissues.

RESULTS: Compared with controls, ELISA showed that there were significant differences in β-catenin levels in peripheral blood of patients with renal carcinoma ($p<0.05$). Western blot and qRT-PCR showed that the expression levels of β-catenin in renal carcinoma tissues were higher than in normal peritumoral tissues and the differences were statistically significant ($p<0.05$). Immunohistochemical staining showed that β-catenin was increased significantly in renal carcinoma tissue compared with normal peritumoral tissues ($p<0.05$).

CONCLUSIONS: β-catenin expression was significantly increased in the tumor tissues of patients with renal carcinoma. The measurement of β-catenin expression levels in peripheral blood from patients could be used for early diagnosis of renal carcinoma, which is of great clinical significance.

Key Words:
Renal clear cell carcinoma; β-catenin.

Introduction

Renal cell carcinoma (RCC) is the most common primary malignant tumor of the kidney and the most deadly malignant tumor of the urinary and reproductive systems. Renal carcinoma originates from the parenchyma of uriniferous tubular epithelium, including various subtypes of RCC stemming from different parts of the uriniferous tubule, but excludes renal pelvic tumors and renal interstitium tumors[1-3]. Epidemiologic studies showed that renal carcinoma accounted for 2-3% of adult malignant tumors and 80-90% of adult renal malignant tumors. The rates of morbidity vary according to the region. In general, the rates of morbidity in developed countries were higher than in developing countries, the rates of morbidity of urban areas were higher than in rural areas, and the rates of morbidity of males were higher than in females, with a ratio of male to female patients of 2:1. The age of onset was seen across all age groups and the highest age range was from 50-70 years old. The pathogenesis of renal carcinoma remains unknown. Factors firmly established as being related to renal carcinoma include heredity, smoking, obesity, hypertension and antihypertensive treatment[3].

Among various signaling molecules and proteins related to the growth and metastasis of tumors, various studies showed that there was abnormal activation of the Wnt/β-catenin signaling pathway in breast cancer[4,5], prostate cancer[6], hepatocellular carcinoma[7] and other tumors. However, the expression level and clinical significance of the Wnt/β-catenin signaling pathway in RCC is not clear. Therefore, in the present study, histological and serological samples from patients with renal carcinoma were collected to evaluate the expression levels of β-catenin, thus providing a theoretical basis for the targeted therapy of renal carcinoma.

Patients and Methods

Patients

Forty-six patients with renal clear cell carcinoma treated in our hospital from May 2013 to March 2016 and healthy adults (controls) matched for

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age and weight treated in the physical examination center of our hospital during the same period were selected. Data collected included general condition, disease history, and the physical signs of patients. The study was approved by the Ethical Commettee (No. 2013-009) of our University. Enrolled patients conformed to the following inclusion and exclusion criteria.

Inclusion Criteria

1) Confirmed as having renal clear cell carcinoma through pathological diagnosis; 2) Enrolled patients were without primary or secondary benign or malignant tumors in other tissues/organs; 3) Enrolled patients were diagnosed with RCC for the first time; 4) Patients participated in the study voluntarily and signed the informed consent.

Exclusion Criteria

1) Patients and their families were unable to cooperate with doctors; 2) Patients refused treatment; 3) Within 3-4 weeks before resection of pathological tissue, patients underwent chemotherapy, radiotherapy, targeted therapy or immune regulation therapy; 4) Patients with mental illness or consciousness disorders.

Experimental Instruments

GeneAmp®9700 PCR System (ABI Biosystems, Carlsbad, CA, USA); Centrifuge 5415D (Eppendorf, Hamburg, Germany); PB3002-S electronic balance (Mettler Company, Columbia, MD, USA); 96-Well plates (AB gene); TL-5.0 table centrifuge (Shanghai Lixin Machinery Research Institute, China); micropipette (Eppendorf, Hamburg, Germany); NanoDrop1000 spectrophotometer (Thermo Fisher, Waltham, MA, USA); SpeedVac vacuum centrifugal concentration system (Thermo Fisher, Waltham, MA, USA); NimbleGen HX1Mixer (Roche NimbleGen, Madison, WI, USA); NimbleGen Elution System (Roche NimbleGen, Madison, WI, USA); pH meter (Φ71) (Bio-Rad, Hercules, CA, USA); electric thermostatic water bath (WS2-261-79) (Beijing Medical Equipment Factory, China); Whirlpool oscillator (Beijing Medical Equipment Factory, China), pathological slicer (Leica, Wetzlar, Germany).

Experimental Reagents

Taq Master Mix (SinoBio, Walpole, MA, USA), agarose (Biowest, Rosenberg, TX, USA), sterile double distilled water, anti-phospho-β-catenin (p-β-catenin) (1:1,000; Cell Signaling, Danvers, MA, USA), β-actin antibody (1:5,000; Invitrogen, Carlsbad, CA, USA), 0.9% sterile saline (Otsuka Pharmaceutical, Tokyo, Japan), Trizol (Invitrogen, Carlsbad, CA, USA).

Double-Antibody and One-step Sandwich method

Tumor samples, standards and horseradish peroxidase (HRP) labeled antibodies were added accordingly to peridium micropores for incubation. After washing, TMB substrate was used for color development. Since peroxidase has catalytic action, TMB turned blue. The solutions then turned yellow when treated with acid. Shades of color were positively correlated with the amount of target protein in the samples. Optical densities (OD) of samples at a wavelength of 450 nm were read in a microplate reader to calculate the specific concentration levels of samples.

Sample Processing

(1) Serum: At room temperature, whole blood samples were left to stand for 2 h. Next, samples were placed at 4°C overnight and centrifuged at 2000 rpm for 20 min. Appropriate amounts of supernatant were used for experiments, or stored at -20°C or -80°C. Repeated cycles of freeze-thaw were avoided.

(2) Plasma: EDTA or heparin was used as an anticoagulant. Samples were centrifuged (2-8°C, 2000 rpm, 30 min) within 30 min after being collected, or stored at -20°C or -80°C. Repeated cycles of freeze-thaw were avoided.

Determination of β-catenin Levels in Serum by ELISA

Fasting venous blood (3 ml) was collected from patients in the early morning. After 30 min incubation at room temperature, venous blood was centrifuged at a speed of 400×g for 10 min. An appropriate amount of serum was collected for storage at -40°C until use.

1. Plates required for the experiment were placed at room temperature for 20 min. Unused plates were sealed with a valve bag and stored at 4°C.
2. The wells for standards and samples were determined. A volume of 50 μl of standards at different concentrations was added to the appropriate wells.
3. A volume of 50 μl of samples was added to the appropriate wells.
4. A volume of 100 μl HRP-labeled detection antibody was added to all wells of the standards. The reaction wells were sealed and allowed to react in an incubator or water bath set to 37°C for 60 min.

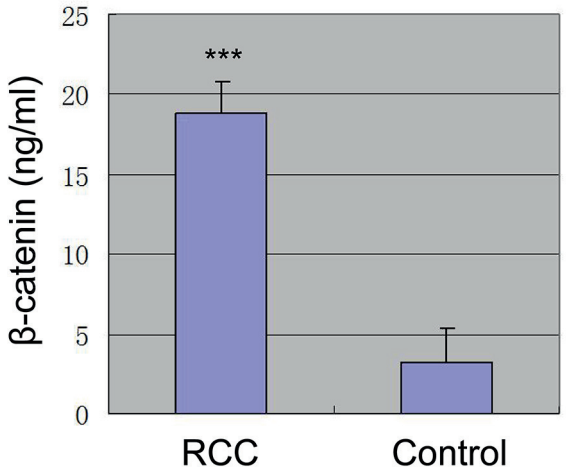

Figure 1. β-catenin expression detected by ELISA: Serum β-catenin levels were increased in patients with renal carcinoma compared with the control group, and the difference was statistically significant ($^{***}p<0.001$).

5. The solutions were discarded and the liquid was patted dry. Each well was treated with 350 µl cleaning solution. After incubation for 1 min, the cleaning solution was removed and the liquid was patted dry. The operation was repeated 4-5 times or the plate was washed by a plate washer.
6. Each well was treated with 50 µl solution A and 50 µl solution B (from the ELISA kit) and incubated in the dark for 15 min at 37°C.
7. Each well was treated with 50 µl stop buffer and absorbance was measured within 15 min at 450 nm.
8. OD value of the blank well was subtracted from the OD values of all standards and samples. With concentration as the dependent variable and light absorption as the independent variable, a relevant software was used to construct a standard curve and calculate the levels of β-catenin.

Detection of β-catenin by Immunohistochemistry

Paraffin sections of breast cancer tissues were dewaxed with water and 3% H_2O_2, incubated at 20°C for 10 min, washed with distilled water and soaked twice in phosphate buffered saline (PBS) for 5 min. Goat serum (Life Technologies, Carlsbad, CA, USA) was diluted in 5% PBS for blocking. After the tissues had been incubated at 20°C for 10 min, the goat serum was drained off and primary antibody (β-catenin, ab60709, Abcam, Cambridge, UK) was added. The tissues were incubated at 37°C for 1-2 h or stored at 4°C overnight. Biotin-labeled working solution (Perkin-Elmer, Norwalk, CT, USA) was added to samples in appropriate amounts and incubated at 37°C for 10 min. HRP (Sigma-Aldrich, S7571, St. Louis, MO, USA) was added and the tissues were incubated at 37°C for 10 min. Next, 1-2 drops of DAB Plus Chromogen (TL-125-HD, Thermo Scientific, Waltham, MA, USA) was added to 1 ml DAB Plus Substrate (TA-125-HDX, Thermo Scientific, Waltham, MA, USA). After mixing, the solution was added dropwise onto sections. Slides were mounted and washed after incubation at 37°C for 10 min.

Statistical Analysis

Statistical analysis was conducted with SPSS 20.0 software (SPSS Inc., Chicago, IL, USA). Normally distributed continuous variables are presented as mean ± standard deviation. The absolute percentage was applied for categorical variables and Student's t-test for continuous variables. p-values < 0.05 were considered statistically significant.

Results

Comparison of Serum β-Catenin Levels Between Patients and Controls

By ELISA, serum β-catenin levels were increased in patients with renal carcinoma compared with healthy controls, and the difference was statistically significant ($p<0.05$) (Figure 1).

Immunohistochemical Analysis of β-catenin in Renal Carcinoma Tissue

Through immunohistochemical staining of renal clear cell carcinoma tissue from patients after surgery, the expression levels of β-catenin in renal clear cell carcinoma tissue were increased compared with normal peritumoral tissues, and the difference was statistically significant ($p<0.05$) (Figure 2A and B).

Western blot analysis of β-catenin in Renal carcinoma tissue

According to Western blot analysis, the levels of β-catenin increased in renal carcinoma tissue compared with normal peritumoral tissue, and the difference was statistically significant ($p<0.05$). This indicates that in patients with RCC, the Wnt/β-catenin signaling pathway is activated (Figure 3).

Table I. Comparison of serum β-catenin levels between patients and healthy controls.

Group	Number of cases	β-catenin (ng/ml)
RCC	46	18.77±4.28
Control group	46	3.27±1.09
t-value	-	22.67
p-value	-	0.001

Correlation of expression level of β-catenin in renal carcinoma tissue and Clinical Prognosis

After surgery, adjuvant treatment for patients was given and follow-up of 3 years was carried out. Higher serum β-catenin levels were correlated with poorer prognosis (Figure 4).

Discussion

RCC occurs primarily in renal parenchyma[8]. About 90% of renal carcinoma is RCC, among which clear cell RCC is the most common histological subtype[1,9-11]. In recent years, renal carcinoma has had the highest steadily rising rates of morbidity and mortality of cancers of the urinary and reproductive systems. There are 200,000 new cases and over 100,000 patients dying from renal carcinoma in the world annually[12]. For the treatment of local RCC, nephrectomy is usually considered to be effective. However, the rate of mortality of RCC at the progressive stage is still high and the annual 5-year survival rate was reported to be only 53%[13-15]. Until now, the recognition of changes of molecular mechanisms and signaling pathways during the development and progression of renal clear cell carcinoma remained relatively limited. Therefore, identification of biomarkers will be useful for identification and judgment of the prognosis of RCC.

Previous studies[16-21] showed that Wnt family genes play important roles in the growth and development of normal tissues and organs during human embryogenesis, as well as in tumor formation. Moreover, Wnt signaling was shown to be involved in kidney development and occurrence of malignant tumors of the kidney. The Wnt signaling pathway, regulated by β-catenin is also known as the classical Wnt signaling pathway. After binding the active frizzled receptor, Wnt ligand can activate the classical Wnt signaling pathway to inhibit β-catenin. This is mediated by intracellular glycogen synthase kinase 3β which phosphorylates β-catenin, thus causing accumulation of intracellular β-catenin. Studies showed that only the classical pathway could promote the degradation of β-catenin, the key regulatory protein[10], by its phosphorylation[12].

Stable β-catenin can also enter the cell nucleus and initiate transcription of Wnt target genes through interaction with proteins such as c-myc, cyclin D1, and *T cell factor*/*lymphoid enhanced factor*[11-13]. These genes can increase cell proliferation and differentiation, promote intercellular adhesion and increase cell migra-

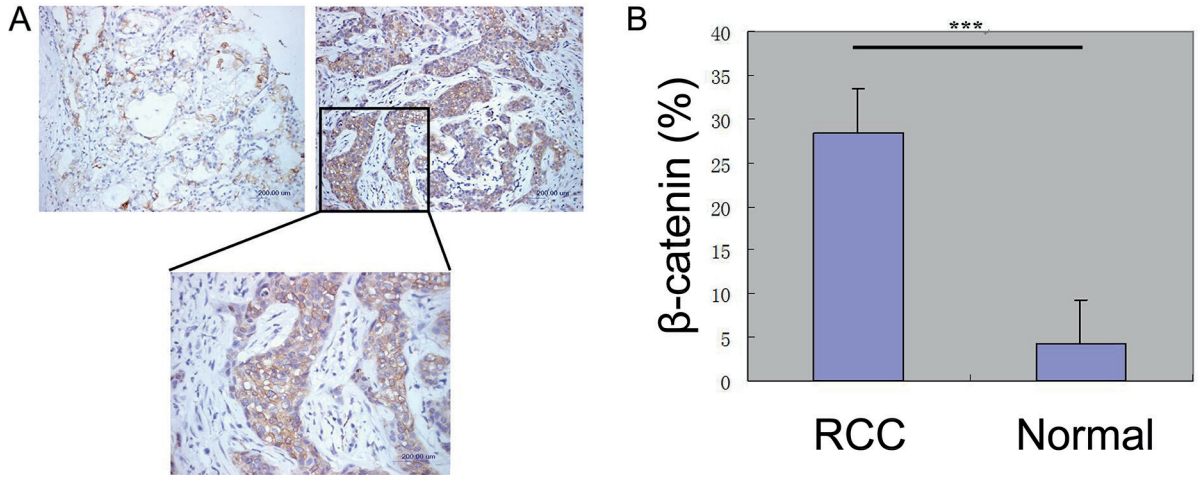

Figure 2. β-catenin expression detected by immunohistochemistry: ***A,*** (×200), Left: Normal peritumoral tissues; Right: In renal carcinoma tissue, the positive area of β-catenin expression increased significantly. Analysis by inverted microscopy (×400) shows that β-catenin is mainly expressed in the renal interstitium. ***B,*** Compared with normal peritumoral tissues, β-catenin expression levels in renal carcinoma tissue increased significantly, and the differences were statistically significant ($p<0.05$).

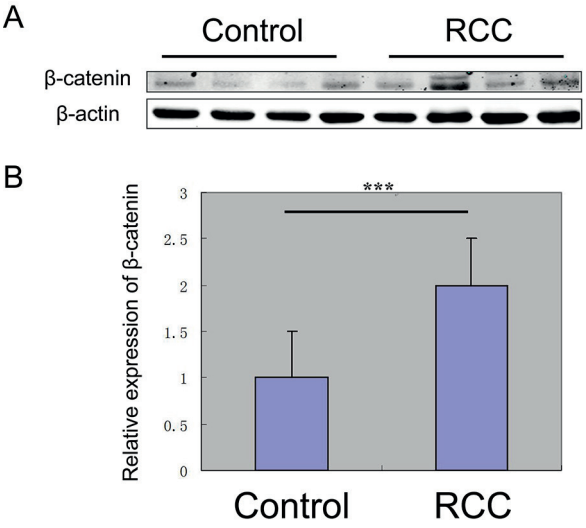

Figure 3. β-catenin expression detected by Western blot: A, B. The expression levels of β-catenin were increased in renal carcinoma tissues compared with normal peritumoral tissues, and the difference was statistically significant ($^{***}p<0.01$).

tion and tumor formation[14-16]. Recent studies showed that overexpression of β-catenin was related to high rates of morbidity and poor prognosis in patients with renal carcinoma[17]. There were similar findings in our study. In serum of patients with renal carcinoma, the expression levels of β-catenin were significantly higher than those of healthy controls and the expression levels in cancer tissue were also significantly higher than in normal peritumoral tissues. This suggested there was overexpression of the Wnt/β-catenin signaling pathway and was limited to renal carcinoma tissue. This observation provided insight into the targeted therapy of renal carcinoma. Since β-catenin was specifically increased in renal carcinoma tissue, targeted therapy could significantly reduce the damage to normal tissues and organs normally caused by adjuvant chemotherapy drugs. Moreover, through the comparison of β-catenin levels of patient serum after surgery, it was determined that higher serum β-catenin levels correlated with lower survival rate. This might be related to the ability of the activated Wnt signaling pathway to promote growth and metastasis of cancer tissues. It is generally recognized that β-catenin positively regulates this pathway and it can regulate expression of target genes that take part in the occurrence and development of tumors. For example, c-myc and cyclin D1 can modulate the proliferation of cancer cells and promote matrix metalloproteinase-7 to modulate the invasiveness of tumors[22,23]. However, the effects of β-catenin on the prognosis of renal clear cell carcinoma have not yet been determined.

Conclusions

We believe that early detection of β-catenin levels has important clinical significance regarding the occurrence and development of renal carcinoma. β-catenin can be used as a biomarker for diagnosis of renal carcinoma, and its increased expression levels in renal clear cell carcinoma suggest that it may be a candidate for targeted therapy.

Conflict of Interests

The Authors declare that they have no conflict of interests

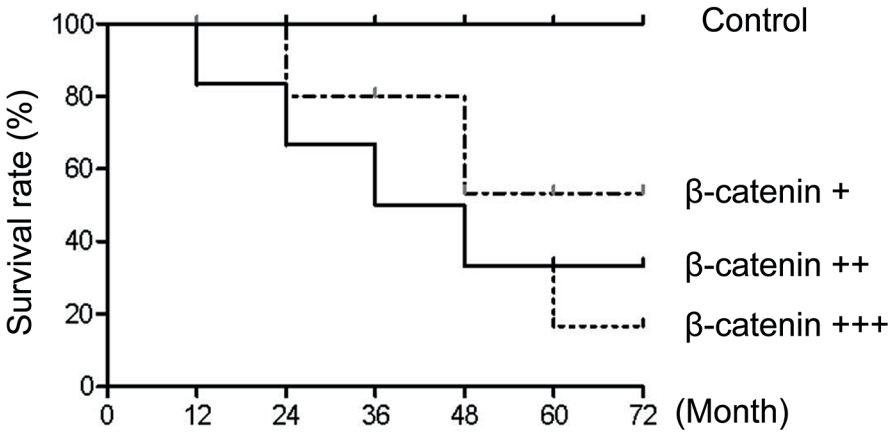

Figure 4. Correlation between survival rates and serum β-catenin levels.

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