Original Article

Correlation Between Hyaluronic Acid，Hyaluronic Acid Synthase And Human Renal Clear Cell Carcinoma

Jian-liang Cai[1], Ming Li[2], Yan-qun Na[1]

[1]*Peking University Wu Jieping Urological Center, Peking University Shougang Hospital, Beijing 100144, China*
[2]*Key Laboratory of Carcinogenesis and Translational Research (Ministry of Education), Department of Urology, Peking University School of Oncology, Beijing Cancer Hospital & Institute, Beijing 100142, China*

DOI: 10.1007/s11670-011-0059-6

ABSTRACT

Objective: To study the correlation between hyaluronic acid (HA), hyaluronic acid synthase (HAS) and human renal clear cell carcinoma (RCCC).

Methods: The expression of three HAS isoforms' gene and HA in 93 RCCC tissues, 27 nephridial tissues by the side of RCCC from two hospitals were measured with Real-Time RT-PCR、Western Blot and immunohistochemical methods and analyzed.

Results: All RCCC and adjacent normal tissues expressed three HASs' mRNA & protein; at the mRNA level, both RCCC and adjacent normal tissues, expressed more HAS3 than HAS1 or HAS2, their differences were statistically significant (all *P* values <0.05); but, at the protein level, all HAS isoforms presented the equivalent expression. Compared with the adjacent non-neoplastic kidney tissues, the expression of all HAS isoforms' mRNA in RCCC tissues were increased evidently and their differences were significant (all *P* values <0.0001); but at the protein level, only the expression of HAS3 increased evidently (*P*=0.022). In all adjacent normal tissues, more than 80% renal tubular cells strongly expressed HA, however, only the minority RCCC cases (16/93) presented weakly positive HA staining in few cancer nests (5%-30%), the difference were significant (*P*<0.0001). In RCCC tissues subgrouped according to clinical stage, pathological grade, lymphatic metastasis or not and distant metastasis or not, the HASs' mRNA & protein differential expression all had no statistical significance (all *P* values >0.05).

Conclusion: Different from other malignancy, HA and HASs (except for HAS3) may not play important roles in the biological progress of human RCCC.

Key words: Renal clear cell carcinoma; Hyaluronic acid; Hyaluronic acid synthase; Gene

INTRODUCTION

Hyaluronic acid (HA), a kind of high molecular weight glycosaminoglycan with negative charge, is the impotent composition of extracellular matrix. Recent years, many studies have confirmed that HA has important effect on the progress of malignant transformation, multiplication, transfer, invasion and metastasis in malignancy[1]. But the report of relationship between HA and renal clear cell carcinoma (RCCC) is infrequent. Hyaluronic acid synthase (HAS) is the key enzyme for the synthesis of HA. Human HAS family has three subtypes: HAS1, HAS2, HAS3, enzymatic synthesis HA with different molecular weight & function[2]. To study the correlation between HA, HAS and the biological behavior of RCCC, we tested the expression of all HAS subtypes' mRNA , protein and HA in 93 cases of human RCCC tissues and 27 cases of nephridial tissue by the side of RCCC (abbreviate as nephridial tissue) with technique of Real-Time RT-PCR, Western Blot and immunohistochemical method, and analyzed the results according to the corresponding clinical data.

MATERIALS AND METHODS

Clinical Data

The 93 renal carcinoma patients were hospitalized for operations from September 2008 to March 2010, 65 male and 28 female, 30 to 81 yeas old, with a average of 56.6. Thirty-eight tumors located in left kidney and 55 right. The size of tumors was 2.0 cm×1.5 cm×1.0 cm to 18 cm×12 cm×9.5 cm, average 6.5 cm×5.6 cm×4.8 cm. Thirty-five cases had clinical symptoms such as lumbago, gross (macroscopic) hematuria, etc. Clinical stages (according to 2010 revised edition of AJCC guideline of Diagnosis and treatment of RCC): 41 T1, 28 T2, 16 T3a, 7 T3b, 1 T3c, no T4; 22 N1; 29 M1 with metastasis in lung, bone, pleura, brain, parenchyma, etc. Seventy-three patients given radical nephrectomy, 20 had partial nephrectomy, 71 open and 22 Laparoscopic surgery.

Received 2010–08–25; **Accepted** 2010–11–17
*Corresponding author.
E-mail: nayanqun@163.com

Cases with lymphatic metastasis had lymph node dissection and those with lymphatic or distant metastasis had biological treatment or targeted therapy after operation. Pathologic diagnosis was made after operation, tumor cell differentiation were 50 well, 36 moderate and 7 poor. The 27 nephridial tissue were obtained from the cases whose tumour were single, size <4.0 cm, without lymphatic and distant metastasis.

Specimen Collection and Preservation

Informed consents were obtained before surgery. Fresh tumor specimens were taken immediately after nephrectomy, tumor bulks resected from the solid area of the tumors, the nephridial tissue obtained far from the tumor mass (distance >2.0 cm). Every specimen was cut into two portion, one was fixed in neutral formaldehyde solution, the other was collected in cryotubes, snap frozen in liquid nitrogen for 24-48 h, then stored at -70℃. The study was approved by the Ethical Committee for Clinical Research of Peking University Shougang Hospital.

RNA Extraction

Small tissue bulks were weighted, homogenized with trizol reagent (Invitrogen, USA; 1 ml/50-100 mg tissue). Total RNA was extracted according to the manufacturer's instructions. RNA pellets were resuspended in 20 μl DEPC water, their concentration and purity were measured by a UV-spectrophotometer.

Primers

Using Array Designer 2.03 software, PCR primers were designed (Table 1) according to the sequence of HAS1 (NM_001523), HAS2 (NM_005328), the consensus sequence of HAS3v1 (spliceosome1 NM_005329) and HAS3v2 (spliceosome2, NM_138612), and GAPDH (NM_002046).

Reverse Transcription

Reverse transcriptions were performed according to previous report[3], A reverse transcriptase assay kits was used (Promega, USA). The cDNA collected stored at -70℃.

Real-Time PCR

Preparation of plasmid standard: PCR reaction system was shown in Table 2 (without SYBR Green I), and amplification conditions were: 95℃ for 2 min, then 95℃ for 10 sec, 57℃ (HAS1)/59℃ (HAS2)/58℃ (HAS3)/61℃ (GAPDH) for 10 sec, 72℃ for 45 sec, 35 cycles in total. Methods for plasmid standard preparation were demonstrated by Tingmin Yu[4]. Plasmid standards were serial 10-fold diluted, stored at -20℃.

Real-time PCR reaction system is compiled in Table 2 (The Taq DNA polymerase is TaqBeadTM, Promega, USA), an Applied Biosystems 7000 Real Time PCR System (Applied Biosystems, USA) was used. Real time PCR protocol for each gene: 95℃ for 2.0 min, followed by 40 cycles of 95℃ for 10 s, 57℃ (HAS1)/59℃ (HAS2)/58℃ (HAS3)/61℃ (GAPDH) for 10s, 72℃ for 45 s. The plasmid standards of each gene and GAPDH were used as template to generate a standard curve. Melting curve was generated to confirm the specificity of the reaction products afterwards. Relative copy number of each target gene and the house-keeping gene is quantitated by a fluorescent quantitative analysis software. The entire experiment was repeated twice. Mean values were used for statistical analysis.

Western Blotting

Total protein extraction and SDS-PAG gel were prepared with conventional method. For detailed procedures of Western Blotting, see Nakata S[5]. Primary antibodies (Santa Cruz, USA) were HAS1, HAS2 (1:400 dilution), HAS3, β-actin (1:200 dilution). Straps pictures were scanned and saved as hypertexts. The density and area of each strap were digitalized with the help of GIS1000 software.

Immunohistochemical Staining

Fixed tissue was embedded in paraffin and sliced. A random section for each tissue was Haematoxylin and eosin

Table 1. The information about primers used

Gene	Up steam primers	Down steam primers	Product length
HAS1	5′-TGTGACTCGGACACAAGGTTG-3′;	5′- GCCT CAAGAAACTGCTGCAA -3′	262 bp
HAS2	5′- ATCCCATGGTTGGAGGTGTT -3′;	5′- TGCCTGTCATCACCAAAGCT -3′;	252 bp
HAS3	5′- AGCCTTTTTGCCTTCCTGGA -3′;	5′- AAGTTGCTGCGCCACACAA -3′;	286 bp
GAPDH	5′-ACC ACA GTC CAT GCC ATC AC-3′;	5′-TCC ACC ACC CTG TTG CTG TA-3′	140 bp

Table 2. The reaction system of Real time PCR

Reagent	Working concentration	Final concentration	Volume used
H_2oO			15.1 μl
U-primer	10 μmol/L	0.12 μmol/L	0.3 μl
D-primer	10 μmol/L	0.12 μmol/L	0.3 μl
SYBR Green Ⅰ	20 ×	0.4 ×	0.5 μl
dNTP	2.5 mmol/L	200 μmol/L	2.0 μl
Mg++	2.5 mmol/L	2 mmol/L	2.0 μl
10 × buffer		1 × buffer	2.5 μl
Taq enzyme	5 u/μl	1.5 u/reac	0.3 μl
cDNA		2 μl	2.0 μl

Note: bulk volume=25 μl; U-primer=Upsteam prime; D-primer=Downsteam prime; reac=reaction.