Original Article

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

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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 years old, with an 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
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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.

(H & E) stained to confirm the diagnosis. Methods and reagents used in Immunohistochemical staining of HA, the evaluation criterion for positive staining were similar to previous report[6].

Data Processing and Statistical Methods

Obtained values of relative copy number of each aim gene's mRNA were normalized by that of GAPDH mRNA. These normalized values were used to compare the expression level of each gene's mRNA. The relative quantity of every aimed gene's protein expression were also standardized by the house- keeping gene's (β-actin), and one positive control was used to eliminate the error among different experiments.

SPSS 11.5 analysis software (SPSS Inc) was used, the differential expression of HASs mRNA were analyzed with rank-sum test, the expression of each tissue sample's protein manifested as '$x\pm s$', Independent-Sample T Test and One-Way ANOVA were used to analyze the different proteins expression. Chi-square test was selected to discover the differences in HA Immunohistochemical staining.

RESULTS

HAS1, HAS2 and HAS3 mRNA expression were discovered in all samples of RCCC tissue. HAS3 mRNA had the maximum expression, while the mRNA expression levels of HAS1 and HAS2 were similar (mean rank: HAS1:114.06; HAS2: 122.87, HAS3: 183.06). The differences among the mRNA expression of the three isoforms were statistically significant (χ^2=40.299, P<0.001), there was statistical significance for HAS1/HAS3, HAS2/HAS3 (Z=-5.308, -5.602, P values all <0.001), while the differences between HAS2 and HAS3 were not significant (Z=-1.263, P=0.207). HAS1, HAS2 and HAS3 mRNA expression were also discovered in adjacent non-neoplastic kidney tissues, with HAS3 of the maximum expression and HAS2 of the minimum expression (mean rank: HAS1: 42.67, HAS2: 25.67, HAS3: 54.67), the differences among the mRNA expression of the three were statistically significant (χ^2=20.741, P<0.001). There was statistical significance for HAS1/HAS3, HAS2/HAS3 and HAS1/HAS2 (Z=-3.196, -2.417, -3.976; P=0.001, 0.016, <0.001, respectively). Protein expression of HAS1-3 were also discovered in all tumor samples, with gray values shown as follows: HAS1: 1.1266±0.6474; HAS2: 1.6103±0.9019; HAS3: 1.2245±0.6021. There was no statistical significance among them (F=1.845, P=0.171), the differences for HAS1/HAS2, HAS1/HAS3 and HAS2/HAS3 were of no statistical significance (P values all >0.05). While in nephridial tissues, the protein expressions were: HAS1: 1.0235±0.3562; HAS2: 1.4043±1.007; HAS3: 0.5700±0.2007, the differences among them or for HAS1/HAS2, HAS1/HAS3 and HAS2/HAS3 also had no statistical significance (P values all >0.05).

In comparison with adjacent non-neoplastic kidney tissues, the RCCC tissues had a higher mRNA expression of HAS1-3. The differences were of great statistical significance (HAS1: mean rank 67.71/35.67, Z=-4.215, P<0.0001; HAS2: mean rank 70.71/25.33, Z=-5.969, P<0.0001; HAS3: mean rank 68.97/31.33, Z=-4.950, P<0.0001). As for protein expression, there was statistical significance between the tumor tissues and the adjacent kidney tissues in HAS3 protein expression (P=0.022), On the other hand, there were non-significance in the protein expression of HAS1 or HAS2 between the tumor tissues and the adjacent kidney tissues (P=0.373, 0.701).

Renal tubular epithelial cells showed strong positive staining for HA in the sections of 27 adjacent kidney tissue samples. The percentage of positive stained cells were all more than 80%, and these cells all showed cytoplasma staining (Figure1A, B). Weak cytoplasma positive staining were detected in only 16 samples of RCCC tumor tissues, with 5%-30% percentage of positive staining tumor cells, and the tumor mesenchyme showed little positive staining (Figure 1C, D). No positive staining for HA was detected in the remaining 77 RCCC samples (Figure 1E, F). Chi-square test showed the difference of HA staining between adjacent kidney tissues and RCCC tissues were of great statistical significance (P<0.0001).

Patients with renal clear cell carcinoma were regrouped according to their clinic staging (T1/T2/T3), pathological grading (well differentiation/moderate differentiation/poor differentiation), lymphatic metastasis or not (N0/N1), distant metastasis or not (M0/M1). Detailed information of statistical comparison between different subgroups on the HAS1-3 mRNA expression was shown in Table 3 (P values all >0.05), their differences all had no statistical significance; HAS1-3 protein expression levels of different subgroups were compiled in Table 4, their differences all were not statistically significant (P values all >0.05).

Table 3. The statistical analysis result of the different HASs' mRNA expression in variant subgroups (rank-sum test)

	Mean rank			Z/χ value			*P* value		
	HAS1	HAS2	HAS3	HAS1	HAS2	HAS3	HAS1	HAS2	HAS3
Clinic staging									
T1/T2/T3	52.57/40.33/44.75	50.21/41.67/47.38	53/37.67/47	3.607	1.656	5.309	0.165	0.437	0.070
Lymphatic metastasis									
N0/N1	45.83/50.38	45.17/52.25	45.70/50.75	-0.712	-1.107	-0.791	0.477	0.268	0.429
Distant metastasis									
M0/M1	49.14/42.50	48.15/43.60	48.43/44	-1.110	-0.980	-0.740	0.267	0.337	0.459
Pathological grading (differentiation)									
W/M/P	52.13/41.25/45	51.18/46.50/59.50	53.06/47.25/59	5.258	4.753	3.458	0.072	0.093	0.177

Note: W=well differentiation; M=moderate differentiation; *P*=poor differentiation.

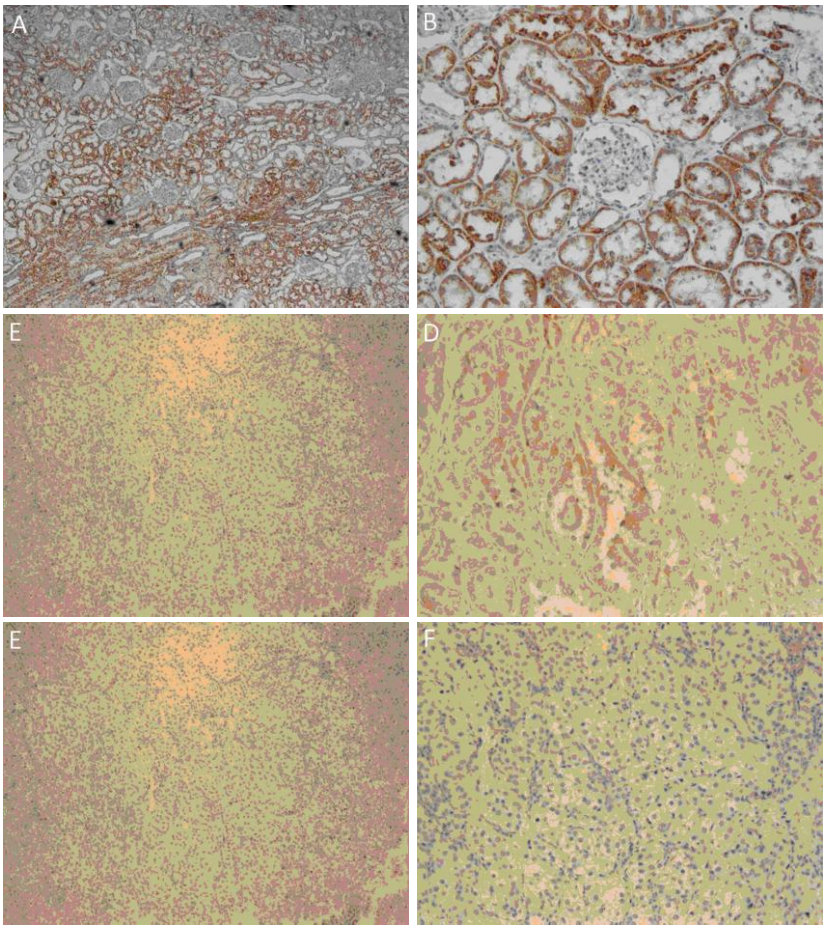

Figure 1. The HA immuno-histochemical staining result in variant tissues. In the adjacent kidney tissue sections, renal tubular epithelial cells showed strong HA positive staining and the stain located in cytoplasma (A, B). In human renal clear cell carcinoma (RCCC) tissue, weak cytoplasma positive HA staining were detected in a few tumor cells in some cases (16/93), but the tumor mesenchyme showed little positive staining (C, D); no HA positive staining was detected in the remaining 77 RCCC samples (E, F). Magnification: A, C, E=10×10; B, D, F=10×20.

Table 4. The HASs' protein expression in variant subgroups

	HAS1	HAS2	HAS3
Clinic staging			
T1	1.6085±1.1178	1.6390±0.4561	1.4388±0.6634
T2	0.9843±0.3482	1.7118±1.2877	1.2827±0.5206
T3	0.9118±0.2409	1.4656±0.7589	0.9834±0.6901
Lymphatic metastasis			
N0	1.2155±0.8364	1.5346±0.6568	1.3806±0.6977
N1	1.0250±0.3731	1.6969±1.1741	1.0461±0.4563
Distant metastasis			
M0	1.1656±0.8458	1.2893±0.5962	1.2195±0.6327
M1	1.1036±0.3479	1.9773±1.0903	1.2303±0.6154
Pathological grading (differentiation)			
Well	0.9645±0.3200	1.4245±0.3217	1.4263±0.6501
Moderate	1.1930±0.8056	1.8788±1.0542	1.1948±0.6481
Poor	1.1520±0.4724	0.7740±0.0396	0.9550±0.3620

DISCUSSION

HA has broad participation in the embryogenesis of mammalian kidney[7], has important role in the process of urinary concentration in normal kidney[8]. Furthermore, HA has taken part in the process of occurrence, development and fibrosis in different kinds of nephritis and diabetic nephropathy; HA also makes a difference to the pathologic process of renal ischemia-reperfusion injury and kidney tubular crystallization[9-12]. Many studies have confirmed that HA plays very important roles in the biological process of many kinds of malignancy such as mammary cancer, gastric cancer, colorectal cancer, ovarian cancer, melanoma, glioma, lung cancer, prostatic cancer and head & neck cancer, etc.[1]. But the report of relationship between HA and the biological process of renal clear cell carcinoma is infrequent.

HAS is the key enzyme of the enzymatic synthesis of

HA. Human HAS family has three subtypes: HAS1, HAS2, HAS3; different HAS isoforms has different character, catalyzes HA of different molecular weight. The HA catalyzed by HAS3 is the shortest (molecular weight: 1×10^5–1×10^6 Da); the molecular weight of HA catalyzed by HAS1 is 2×10^5–2×10^6 Da; and the mean molecular weight of HA catalyzed by HAS2 is more than 2×10^6 Da[2]. On the other hand, HA with different length has different biological function, for example, the HA bigger than 10^6 Da has important function in keeping the structure and glutinousness of tissues, regulating the adhesion between cell and intercellular matrix, reducing contact inhibition, accelerating cellular immigration or transfer; the HA with the mean molecular weight less than 10^6 Da takes part in the process such as new vessels generation, cell multiplication and immigration, inflammatory reaction, etc[13]. Over-expression of one or more subtypes of HAS and overcompound of HA of different molecular weight in many kinds malignant cells have important function in the malignant biological behaviour process such as malignant transformation, multiplication, immune escape, invasion, adhesion, transfer, generation of tumor vessels and lymphatic, multidrug resistance, etc[1, 14-16].

Our study found that HAS3 mRNA had highest expression in human RCCC tissues and adjacent non-neoplastic kidney tissues and had significant statistical difference when compared with HAS1 and HAS2; but the expression of different subtype HAS protein had no significant statistical difference. It clewed that in RCCC tissue and adjacent non- neoplastic kidney tissue, because of being degraded or other reasons, these HAS mRNA (especially HAS3) may not thoroughly being translated into corresponding protein to catalyze different length HA, play respective role in the biological process of RCCC or physiological process of adjacent non-neoplastic kidney. However, the function of the differential expression of every HAS subtype mRNA in RCCC tissues or adjacent non-neoplastic kidney tissues are still unknown, need advanced study.

The expression of different subtype HAS mRNA in human RCCC tissues are higher than that of adjacent non-neoplastic kidney tissues and HAS3 mRNA had the maximum expression. But on protein level, only the HAS3 expression in RCCC tissues are higher than adjacent non-neoplastic kidney tissues, and had significant statistical difference. It showed that HAS3 and its catalyzed HA might have important function in the biological process of RCCC. But immunohistochemical staining showed that HA staining in human RCCC tissues are very weak while in adjacent non-neoplastic kidney tissues are strong. These results are inexplicable! The reason, we presume, the functions of every HAS isoforms (include HAS3) mRNA & protein in human RCCC tissues are not to catalyzed HA synthesis, might be others we still unknown. On the other hand, the HA catalyzed by HAS3 is very short, maybe not being tested by the linking protein used in our experiment. If true so-called, it needs more sensitive and specific means to measure different length HA to take advanced study.

When being redivided according to clinical stages, pathological grading, lymphatic metastasis or not and distant metastasis or not, the expression of all subtypes of HAS mRNA or protein had no significant statistical difference in different subgroup RCCC tissues. It showed that every HAS isoforms may have no associativity with the malignancy and clinical course of human RCCC. From other point of view, combined with the little HA expression in human RCCC tissues, it clews that, different from many other malignant tumors, HAS or HA may have no important function in the biological process of human RCCC.

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