High Annexin A5 expression promotes tumor progression and poor prognosis in renal cell carcinoma

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Abstract. Annexin A5 has been found to act as an oncogenic protein in a variety of cancers. However, its specific biological role and mechanism in renal cell cancer (RCC) remains unknown. Quantitative Real-time PCR and western blotting were used to evaluate the mRNA and protein expression level of Annexin A5 in human RCC cell lines and tissues. Immunohistochemistry was adopted to measure the Annexin A5 expression in 123 cases of RCC tissues. Survival analysis was performed to explore the association between Annexin A5 expression and the prognosis of RCC. The effect of Annexin A5 on RCC growth and metastasis was studied in vitro and in vivo. Annexin A5 was frequently highly expressed in both human RCC cells and tissues. High Annexin A5 expression was associated with higher clinical stage and histological grade. In addition, Annexin A5 might be used as a predictive factor for the prognosis of RCC. Further research suggested that upregulated Annexin A5 in RCC cells could significantly promote tumor cell proliferation, migration and invasion in vitro. Subcutaneous xenograft tumor model displayed that knockdown of Annexin A5 could impede tumorigenesis in vivo. Moreover, mechanism study exhibited that Annexin A5 could activate PI3K/Akt/mTOR signaling pathway, promote epithelial-mesenchymal transition (EMT) and the expression of MMP2 and MMP9. Annexin A5 may be a potential prognostic biomarker in RCC and promotes proliferation, migration and invasion of RCC cells via activating PI3K/Akt/mTOR signaling pathway and regulating EMT process and MMP expression.

Introduction

Renal cell carcinoma (RCC) is the most common type of kidney cancer with approximately 62,700 new RCC cases diagnosed and 14,240 new mortalities in the United States in 2016, accounting for approximately 2-3% of all adult malignancies (1). Clear cell renal cell carcinoma (ccRCC), as the most common histological subtype of RCC, represents approximately 85% of all primary renal neoplasms (2). Due to lack of early warning signs and effective treatments for patients with advanced disease, approximately 20-40% patients were found metastasis at the time of diagnosis and the 5-year survival rate of RCC is approximately 55% (1,3,4). However, the accurate mechanism of the RCC progression remains unclear. Therefore, there is an urgent need to improve the understanding of tumor biology in RCC and identify more effective and highly selective potential therapeutic targets for RCC treatment.

Annexins, as predominantly cytosolic soluble proteins, are classified into five families, including vertebrates, invertebrates, fungi and some groups of unicellular eukaryotes, plants and protists. Annexins can reversibly bind to negatively charged phospholipids in a Ca\(^{2+}\) regulated manner (5). Twelve Annexins common to vertebrates are known as Annexins A1-A11 and A13 (5,6). The non-glycosylated phospholipid binding protein, Annexin A5 is composed of 319 amino acid residues with a molecular mass of ~35.7 kDa (7-9). It is likely that Annexin A5 has a very short unphosphorylated N-terminus compared with other Annexins, contributing to a series of functions, such as cell proliferation and invasion (6,10), signal transduction (11,12), and anticoagulation (13). Previous studies have reported that Annexin A5 promoted tumorigenesis and progression in a variety of cancers, including hepatocarcinoma, colorectal cancer and breast cancer (14,15). However, the possible relationship between Annexin A5 and RCC is not clear. Therefore, we analyzed the expression level of Annexin A5 in RCC tissue samples compared with normal renal tissue samples and explored its potential biomedical functions on RCC cell invasion to confirm whether Annexin A5 is a new molecular biomarker of RCC.

Materials and methods

Patients and RCC samples. All primary RCC and pericarcinorous tissues were obtained from 123 patients with appropriate informed consent at the Department of Urology of the First Affiliated Hospital of Nanjing Medical University from
February 2008 to August 2011. The follow-up deadline was January 2016. The specimens were assessed by immunohistochemistry and the diagnosis was verified by histopathological examination. The study was approved by the Institutional Research Ethics Committee of the First Affiliated Hospital of Nanjing Medical University.

Quantitative real-time PCR (qRT-PCR). Total RNA was extracted from cultured cell lines and clinical samples using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and cDNA was synthesized using Primerscript RT Reagent (Takara, Otsu, Japan) according to the manufacturer's instructions. The qRT-PCR was performed by using StepOne Plus Real-time PCR system (Applied Biosystems, Foster City, CA, USA) with SYBR® Premix Ex Taq™ Reagent (Takara). The following primers were used for qRT-PCR: Annexin A5, forward: 5'-AGCGGCTGATGCAGAACC-3', reverse: 5'-ACTTCGGATGTCAACAGAGT-3'; β-actin, forward: 5'-CCTggCCTTCACAGTCTGCTTG-3'. Data analysis was performed with ABI Step One Software version 2.1 and the relative mRNA level was calculated using 2 \(^{-\Delta\Delta Ct}\) method.

Western blotting. Cells or frozen tissues were lysed in cell lysis buffer for 30 min on ice and centrifuged at 14,000 x g at 4°C for 15 min. The total protein concentration was calculated by the BCA Protein Assay kit (Pierce, Rockford, IL, USA). Proteins were separated by 10% SDS-PAGE gel and transferred onto a polyvinylidene difluoride membrane (Millipore, Billerica, MA, USA). Western blot analysis followed a standard procedure. The primary antibody Annexin A5 was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). N-cadherin, vimentin, β-catenin, MMP2, MMP9, PI3K, phospho-PI3K, AKT, phospho-AKT, mTOR, phospho-mTOR were obtained from Cell Signaling Technology, Danvers, MA, USA. The anti-mouse and anti-rabbit secondary antibodies were also from Cell Signaling Technology.

Cell lines and reagents. The human renal cancer cell lines (Caki-1, Caki-2, ACHN, 769P) and normal epithelial cells of renal tubule (HK2) were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). The cells were cultured in McCoy's 5A or DMEM media supplemented with 10% fetal bovine serum (gibco, Carlsbad, CA, USA) and 1% penicillin/streptomycin (Invitrogen) at 5% CO₂ and 37°C incubator. LY294002, a phosphatidylinositol 3-kinase (PI3K) inhibitor, was obtained from Selleck Chemical (Houston, TX, USA) (no. S1105).

Transfection. Lentivirus packaging cells were transfected with LV3-pGVL-h1-GFP-puro vector (GenePharma, Shanghai, China) containing either the Annexin A5 knockdown (shA5-1 and shA5-2) or Annexin A5 overexpression (A5) and a negative control sequence (NC), respectively. Lentiviral transduction was performed in Caki-1 and Caki-2 cell lines. Pools of stable transductants were generated by selection using puromycin (4 μg/ml) for 2 weeks.

Cell proliferation assay. A Cell Counting Kit-8 assay (Dojindo Laboratories, Kumamoto, Japan) was used to estimate the proliferation potential. Cells were seeded in 96-well plates with 3000 cells/well. CCK-8 reagents were added into wells after cells grew for 1, 2, 3, and 4 days, respectively, and the absorbance was measured at 450 nm using a micro-plate reader at 2 h after CCK-8 addition.

Colonies formation assay. Cells were seeded into 6-well plates (600 cells/well) and cultured in media containing 10% FBS for 2 weeks. Then the colonies were fixed with paraform and stained with 0.1% crystal violet. The colonies were counted and each group were repeated three times.

Transwell cell migration and invasion assay. Cell migration or invasion assay were performed using a 24-well Transwell chamber (Costar, Corning, NY, USA) with or without Matrigel (Invitrogen). Cells (2x10⁵) were suspended in serum-free medium and seeded into the upper chambers which were inserted in the 24-well plate. Medium containing 10% FBS was added to the lower chamber. Cells were incubated at 37°C for 48 h and then cells on the surface of the upper chambers that did not migrate through the pores were removed with a cotton swab. Cells which migrated to the bottom surface of the chamber were stained with 0.1% crystal violet. Numbers of migratory and invasive cells were counted in five randomly selected fields and the presented data represent three individual experiments.

Xenograft studies. Mouse studies were approved by the Animal Research Ethics Committee of Nanjing Medical University. The 5-week-old female nude mice were randomly divided into two groups consisting of five mice each. The stable cells (7x10⁶) shA5-Caki-1 and the control cells (NC-Caki-1) were suspended in 150 μl PBS and injected subcutaneously into the flank of each mouse. Tumor size was calculated (length x width² x0.52) once a week. After 6 weeks, tumors were removed, weighed, fixed, and embedded for immunohistochemical staining.

Immunohistochemistry. Immunohistochemistry was performed on tissue microarray to evaluate Annexin A5 protein expression. Tissue microarray was incubated with a primary antibody against Annexin A5 at 4°C overnight and incubated with HRP conjugated secondary antibody followed by DAB staining. Immunohistochemistry staining was assessed by two experienced pathologists. To evaluate the expression of Annexin A5 in RCC tissues, a semi-quantitative scoring system (0-3) was used based on the staining intensity of the tumor tissue: 0, negative; 1, weak positive; 2, moderate positive; 3, strong positive. Annexin A5 high expression refers to scores 2-3 and Annexin A5 low expression refers to scores 0-1. For assessing the association of Annexin A5 expression with clinicopathological characteristics of the RCC patients, following parameters were included: age (≤60 and >60), gender, tumor size (≤4 and >4), Histology (clear cell carcinoma and others), histological stage (grades I, II, III, and IV) and tumor stage (TNM stages I, II, III, and IV).

Statistical analysis. Statistical analyses were performed with SPSS 22.0 software. All the data were presented as the mean ± SD from three independent experiments. Student's
t-test and the Chi-square test were used to analyze the differences between groups and survival curves were drawn by the Kaplan-Meier method. P<0.05 was considered to indicate a statistical significant difference.

Results

Annexin A5 is highly expressed in RCC tissues and cell lines. To explore the protein and mRNA expression of Annexin A5 in RCC cell lines, western blot and qRT-PCR were performed in four human RCC cell lines (Caki-1, Caki-2, ACHN and 769P) and one normal epithelial cell of renal tubule (HK2). As shown in Fig. 1A, Annexin A5 mRNA was upregulated by 61.2- to 92.4-fold in all RCC cell lines compared to HK2 and western blot analysis showed similar results. To assess whether Annexin A5 was also highly expressed in RCC tissues, 22 pairs of RCC tissues and matched adjacent non-cancerous tissue were selected for qRT-PCR and western blot, the results suggested that Annexin A5 expression was markedly upregulated in both protein and mRNA level compared to the adjacent tissue (Fig. 1B-D).

Annexin A5 expression is correlated with clinical stage, histological grade and overall survival in RCC patients. To validate the association between Annexin A5 expression and clinicopathologic features, 123 cases of RCC were evaluated by IHC. Patients were divided into a low expression group (n=56) and a high expression group (n=67) according to the Annexin A5 expression. As shown in Table I, although Annexin A5 expression and age (P= 0.628), gender (P= 0.576), tumor size (P= 0.192) or tumor histology (P=0.637) did not significantly correlate, Annexin A5 exhibited a significantly positive correlation with histological grade (P=0.007) and TNM stage (P=0.013). High expression group was correlated with higher histological grade and TNM stage compared with the low expression group. As shown in Fig. 2, RCC patients with high Annexin A5 expression had a significantly shorter overall survival time than those with low Annexin A5 expression (P=0.031). These data suggested...
that the upregulated expression of Annexin A5 might have an important role during RCC progression.

**Annexin A5 promotes cell proliferation in vitro.** To further investigate the functions of Annexin A5 in RCC cell lines, we transfected Caki-1 and Caki-2 cells with lentivirus to silence or overexpress the expression of Annexin A5. The silenced cells were named as shA5-1 and shA5-2 and the overexpressed cell lines were called A5, while the matched control cells were named as NC. The expression levels were confirmed by western blotting (Fig. 3A and E) and qRT-PCR (Fig. 3B and F, P<0.01).

The growth curve analysis demonstrated that the downregulation of Annexin A5 in Caki-1 and Caki-2 cells significantly inhibited cell growth compared with the control cells (Fig. 3C and D, P<0.05). Whereas, upregulated Annexin A5 expression enhanced cell growth markedly in Caki-1 and Caki-2 cells (Fig. 3G and H, P<0.05).

To further study the mechanism by which Annexin A5 overexpression or knockdown affected proliferation, we investigated the effects of Annexin A5 overexpression or knockdown on the PI3K/AKT/mTOR pathway. Western blot analysis suggested that the levels of phospho-PI3K, phospho-AKT and phospho-mTOR decreased in the Annexin A5 knockdown cells, while increased in the Annexin A5 over-expressed cells. However, the total levels of PI3K, AKT and mTOR had no obvious change in Annexin A5 over expression or knockdown cells (Fig. 4C and D, P<0.05).

To further confirm our hypothesis, we used PI3K inhibitor (LY294002) at a dose of 20 µM to observe the role of PI3K/AKT/mTOR pathway in Annexin A5-regulated cell proliferation. The dose of LY294002 selected was the dose of IC$_{50}$ to Caki-1 cells. After treated with LY294002 for 48 h, Caki-1 and Caki-2 with Annexin A5 overexpression (A5) and control cells (NC) were used for CCK8 analysis. As shown in Fig. 4E, Annexin A5 overexpression increased the cell proliferation while LY294002 reversed the cell growth promoting trend by Annexin A5. Overall, these results suggested that Annexin A5 positively regulated cell proliferation in RCC and might be mediated by the phosphorylation of PI3K/AKT/mTOR pathway.

**Annexin A5 promotes cell migratory and invasive potential.** To assess the potential role of Annexin A5 in regulating the migration and invasion ability of RCC cells, Transwell migration and invasion assays were performed in Caki-1 and Caki-2 cells with Annexin A5 overexpression or knockdown. Transwell migration assays revealed that Annexin A5 knockdown reduced the migration capability of Caki-1 and Caki-2 cells and overexpressing Annexin A5 significantly increased the ability of migration. The Transwell invasion assays showed similar results (Fig. 5A-D, P<0.01).

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### Table I. Association of Annexin A5 expression with clinicopathological characteristics of the renal cancer patients.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Annexin A5 expression</th>
<th></th>
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</tr>
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<tbody>
<tr>
<td></td>
<td>No. of cases (%)</td>
<td>Low (%)</td>
<td>High (%)</td>
</tr>
<tr>
<td>Age (years)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤60</td>
<td>74 (60.2)</td>
<td>35 (47.3)</td>
<td>39 (52.7)</td>
</tr>
<tr>
<td>&gt;60</td>
<td>49 (39.8)</td>
<td>21 (42.9)</td>
<td>28 (57.1)</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>78 (63.4)</td>
<td>37 (47.4)</td>
<td>41 (52.6)</td>
</tr>
<tr>
<td>Female</td>
<td>45 (36.6)</td>
<td>19 (42.2)</td>
<td>26 (57.8)</td>
</tr>
<tr>
<td>Tumor size (cm)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤4</td>
<td>58 (47.2)</td>
<td>30 (51.7)</td>
<td>28 (48.3)</td>
</tr>
<tr>
<td>&gt;4</td>
<td>65 (52.8)</td>
<td>26 (40.0)</td>
<td>39 (60.0)</td>
</tr>
<tr>
<td>Histology</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Clear cell carcinoma</td>
<td>115 (93.5)</td>
<td>53 (46.1)</td>
<td>62 (53.9)</td>
</tr>
<tr>
<td>Others</td>
<td>8 (6.5)</td>
<td>3 (37.5)</td>
<td>5 (62.5)</td>
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<td>Histological grade</td>
<td></td>
<td></td>
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<tr>
<td>I-II</td>
<td>102 (82.9)</td>
<td>52 (51.0)</td>
<td>50 (49.0)</td>
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<tr>
<td>III-IV</td>
<td>21 (17.1)</td>
<td>4 (19.0)</td>
<td>17 (81.0)</td>
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<td>TNM stage</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>95 (77.2)</td>
<td>49 (51.6)</td>
<td>46 (48.4)</td>
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<tr>
<td>II-IV</td>
<td>28 (22.8)</td>
<td>7 (25.0)</td>
<td>21 (75.0)</td>
</tr>
</tbody>
</table>

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Figure 2. Kaplan-Meier survival curves of patients with RCC based on Annexin A5 expression. Patients in the high expression group had a significantly more unfavorable prognosis than those in low expression group (P=0.031, log-rank test).
To further explore the mechanism by which Annexin A5 affects cell migration and invasion, western blotting was performed to determine the expression levels of N-cadherin, vimentin, β-catenin, MMP2 and MMP9, which are related to regulating the cell adhesion and metastasis. The results demonstrated that the expression of these proteins was significantly downregulated in Annexin A5 knockdown cells while they were upregulated in Annexin A5 overexpressing cells (Fig. 5E and F). These data suggested that Annexin A5 could promote migration and invasion potential in RCC cells and the underlying mechanism might be the upregulation of adhesion and metastasis-related molecules.

Knockdown of Annexin A5 impedes tumorigenesis in vivo. To study the effect of Annexin A5 expression on tumor growth in vivo, we constructed subcutaneous xenograft tumor model using Caki-1 cells with Annexin A5 knockdown and control cells in the female nude mice. As shown in Fig. 6A and B, tumors from the Annexin A5 knockdown group (shA5) grew markedly slower than those from control group (NC). The mean tumor weight of shA5 group was lower compared to the NC group (Fig. 6C) and Ki-67, a proliferation marker of tumor, was markedly decreased in tumors from shA5 group (Fig. 6D and E). Collectively, these results showed that Annexin A5 knockdown could significantly impede tumor growth in vivo.

**Discussion**

RCC remains a major clinical challenge due to its poor long-term prognosis. Various oncogenes and suppressor genes
are involved in the tumorigenesis of RCC (15). Therefore, it is urgent to find appropriate RCC biomarkers which are essential to tumor progression. Recent studies have suggested that Annexin A5, a calcium-dependent phospholipid-binding protein, could promote tumorigenesis and progression in certain types of cancers (14,15,17). This study focused on the biological functions of Annexin A5 and its potential clinical value in RCC.

Among the RCC cell lines analyzed, Annexin A5 was found to be highly expressed in RCC cells compared to HK2. Consistent with this, Annexin A5 was frequently upregulated in RCC tissues and acted as a predictor of clinical stage.
histological grade and overall survival in RCC. Therefore, Annexin A5 might be used as a clinical marker for RCC, although more pathological data are required to confirm this conclusion. Moreover, two RCC cell lines Caki-1 and Caki-2 were used as cell models to demonstrate the potential role of Annexin A5 in RCC cells. In both cell lines, knockdown of Annexin A5 markedly reduced cell growth rate and colony formation efficiency, while overexpression of Annexin A5 significantly accelerated cell proliferation. Similarly, knockdown of Annexin A5 increased, whereas overexpression of Annexin A5 decreased the migratory and invasive potential in RCC cells. In addition, xenograft studies showed Annexin A5 downregulated cells formed smaller tumors compared to the control cells. These data suggested that Annexin A5 played as
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It has been reported that Annexin A5 was upregulated in various cancers including hepatocarcinoma (16), breast cancer (17), cervical cancer (18), colorectal adenocarcinoma (19) and glioma (20). In this study, Annexin A5 was also highly expressed in RCC which was in accord with the previous studies. However, Annexin A5 was negatively related with tumorigenesis in thyroid cancer and diffuse large B-cell lymphoma (21,22). Therefore, Annexin A5 might play a dual role in cancer depending on tissue specificity.

Uncontrolled proliferation and inappropriate cell survival are one of the characteristics in cancer and these processes are commonly regulated by PI3K/Akt/mTOR pathway (23). The PI3K/Akt/mTOR signaling pathway is activated by several tyrosine kinase receptors and plays an important role in the regulation of many aspects of cell function including metabolism, proliferation, protein synthesis and survival (24). It has been reported that the abnormal expression of Annexin A5 may lead to aberrant activation of PKC and cellular signal transduction, which may contribute to tumorigenesis (14). In glioblastoma multiforme (gBM), Annexin A5 promoted gBM progression and chemoresistance to temozolomide through a PI3K-dependent mechanism (25). Our study demonstrated that overexpression of Annexin A5 activated the PI3K/Akt/mTOR signaling pathway which might be involved in RCC cell proliferation. Moreover, PI3K inhibitor (LY294002) could reverse the cell growth promoting trend by Annexin A5.

Migration and invasion are the major events in the metastasis of cancer (26). The EMT refers to the conversion of epithelial cells to mesenchymal cells, which is crucial in the progress of tumor metastasis (27). EMT is characterized by a loss of E-cadherin and an increase in non-epithelial cadherins including N-cadherin and vimentin (28). MMPs belong to a family of zinc-dependent endopeptidases which can degrade the extracellular matrix (29). Especially, MMPs have remarkable effects on tumor invasion and metastasis (30). To explore the underlying mechanism by which Annexin A5 contributes to cell migration and invasion of RCC, we investigated the expression of EMT-related proteins and MMPs. Among the tested cell lines, N-cadherin, vimentin and β-catenin were significantly upregulated in Annexin A5 overexpression group and Annexin A5 markedly increased MMP2 and MMP9 expression. Therefore, we propose that Annexin A5 may promote migration and invasion of RCC by regulating EMT and extracellular matrix degradation. However, the role of these metastasis-related markers in Annexin A5-induced progression of RCC should be further studied in vivo in subsequent studies.

In addition, Annexin A5 also enhanced chemoresistance in gastric cancer and nasopharyngeal carcinoma which might be associated with the multidrug resistance protein (MRP) (14). A study showed that the protein expression of MRP and Annexin A5 were concurrently elevated in drug-resistance of gastric cancer cells SGC-7901/DDP compared to SGC-7901. Downregulation of Annexin A5 in SGC-7901/DDP cells decreased the expression of MRP and increase the sensitivity to cisplatin, paclitaxel and 5-Fu (31). Future studies are required to explore the role of Annexin A5 in the chemosensitivity of RCC. Moreover, Annexin A1, A2, A4, and A5 played a vital role in breast cancer, pancreatic cancer, and laryngeal carcinoma, alone and/or synergistically (32). It would be of interest to investigate whether other Annexin family members play a role, alone or coupled with Annexin A5 in RCC.

In conclusion, our study suggested that Annexin A5 was frequently highly expressed in RCC and might be used as a novel prognostic indicator for RCC. In vitro and in vivo
experiments validated the promotion function of Annexin A5 in RCC proliferation and metastasis via activating the PI3K/Akt/mTOR signaling pathway and regulating EMT process and MMPs expression. Annexin A5 may become a novel therapeutic target and prognosis factor for the future treatment of RCC.

Acknowledgements

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References