

Original Article

Androgen-STAT3 activation may contribute to gender disparity in human simply renal cysts

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Abstract: Background: Simple renal cysts (SRC) are a common urological disease mostly in elderly, however the male-to-female ratio was 2.81. Androgen receptor (AR) activation was initially proposed as a vital signaling pathway in prostate cancer and consequent signal transducer and activator of transcription 3 (STAT3)-AR complex led an important putative mechanism by which prostate cells are sensitized with growth factor signals. However, in SRC disease, no related study emerged. Methods: 30 patients with SRC and 20 age-matched healthy controls were recruited. Puncture biopsy was performed to acquire cyst-adjacent kidney tissue and normal kidney tissues were from healthy kidney donor who received living-related donor nephrectomy. The expression of STAT3 and androgen receptor was determined by immunohistochemical staining and western blotting. The in-vitro effect of androgen on human HK-2 (an immortalized proximal tubule epithelial cell line from normal adult human kidney) cells' STAT3 expression was analyzed as well. Results: Activated STAT3 was strongly expressed in tubular epithelial cells from kidneys of SRC patients, while it was barely found in normal kidneys. Meanwhile, the androgen receptor positive cyst epithelial cells and adjacent normal renal tubule cells were observed in kidneys from SRC patients, however, AR was weakly expressed in normal healthy male kidneys, statistically significant differences existed. In-vitro experiment demonstrated that when treated with exogenous added androgen, the expression level of STAT3 in HK-2 cells was significantly elevated. Conclusions: Our data raised the possible novel evidence that androgen-STAT3 activation might contribute to gender disparity in human SRC disease and clarification the esoteric mechanisms will provide us attractive therapy target for cystic kidney disease.

Keywords: Kidney, renal cysts, androgen, STAT3, epithelial cells

Introduction

Simple renal cysts (SRC) are common in adults, especially in the elderly population. The bulk of them are asymptomatic and incidentally found by renal imaging including ultrasonography and computed tomography (CT). The overall prevalence of simple renal cysts was about 10.7% in the 7th or later decade of life, the incidence increased dramatically after the 6th decade of life. In a large-scale demographic investigation, SRC were observed in 15.14% of the men and 5.38% of the women, thus the male-to-female ratio was 2.81 [1, 2]. The reason for the gender disparity remains unclear, although the occurrence of sex specificities in kidney structure and glomerular hemodynamics was proposed in recent years [3-5].

Micro-dissection analysis of polycystic kidneys demonstrated that renal cyst formation require an over-proliferation of renal epithelial cells from the walls of kidney tubules. Evidence showed that a "third hit" especially injury of renal epithelial cells accelerates cystogenesis in human adult polycystic kidney disease (ADPKD), the injury include different cytokines, growth factors, interleukins and hormones mediated tubule cells apoptosis or proliferation. Sex differences have been observed in a variety of chronic kidney disease, such as IgA or hypertensive nephropathy, and extensive studies document a role of sex hormones, androgens and estrogens, in the regulation of renal physiology and pathology [6-8]. Generally, the beneficial effect of estrogens was considered

to underlie the protection of women against loss of nephron. The androgen's *in vivo* function was crucially controlled by androgen receptor (AR) distribution, and the regulation of AR is tissue-specific and age-dependent, however, either the AR expression status or androgen signaling pathway has NOT been studied in the kidney cyst formation procedure in human SRC. In the present study, we investigated the androgen receptor expression profile in tissues and experimentally explore androgen related pathway in cystogenesis.

Materials and methods

Patients

We recruited 30 male patients with simple renal cysts admitted to Tongji University Affiliated Tenth hospital, Shanghai, study protocols were approved by the Hospital Ethics Committee and we have obtained the permission of Institutional Review Board for working with the human tissues 15 years ago under the supervision of Tongji University Medical College. All patients were fully informed and signed information-consent-form before operation & tissue-harvesting practice. Since literature reported androgen receptor was hardly detected in female patients' kidney [9], all enrolled cohort were male patients.

All 30 patients with SRC were symptomatic and diagnosed by Computerized Tomography enhancement scanning. Patients' exclusion criteria includes: history of anomalous kidney, horseshoe kidney, polycystic kidney, neurogenic bladder, medullary sponge kidney or any malignancies, etc. 30 SRC patients received retroperitoneal laparoscopic decortication and at the end of procedure, ultrasonic guided puncture biopsy was performed to acquire the tissues which were near cyst-cavity and the biopsy was taken only for research purpose. All biopsied tissues contained both cortex and medulla near the renal cyst-border and were called "cyst-adjacent renal tissues". All the cyst-walls were collected post-operation for routine pathology and subsequent immunohistological detection.

A control cohort of normal kidney tissues was obtained from 20 male donors who volunteered to receive laparoscopic unilateral-nephrectomy and donate the kidney to their immediate fami-

ly members. We routinely performed protocol biopsies during kidney perfusion and trimming process before transplantation. All the urine samples from the control patients were normal and the pathological data of their kidneys demonstrated no abnormality as well.

Immunohistochemistry

Paraffin-embedded sections (3-5 μm) were dewaxed, rehydrated through graded alcohols and boiled in 10 mM citrate (pH 6.0; VECTOR, Burlingame, CA, USA) for 30 min. The sections were then placed in the staining dish at room temperature and allowed to cool for 1 h. Sections were incubated with 10% goat serum for 30 min and incubated with either antityrosine pSTAT3 antibody (1:200; Cell Signaling, Danvers, MA, USA) or anti-bromodeoxyuridine antibody (1:100; BD Biosciences, San Jose, CA, USA) for 1 h at room temperature. After washing with phosphate buffer solution (PBS), sections were incubated with secondary antibody for 1 h at room temperature. For double staining with the tubule marker Lectin DBA (dihydrodimethyl-benzopyranbutyric acid, VECTOR), a dilution of 1:500 was used. After washing with PBS, sections were mounted with Prolong Gold antifade reagent with 4'-6-diamidino-2-phenylindole (DAPI, Invitrogen, Carlsbad, CA, USA).

For detection of androgen receptor (AR) expression, primary antibody at a concentration of 1:3000 for proliferating cell nuclear antigen (PCNA) and 1:100 for AR (ARPG-1, 306-680; Upstate Cell Signaling Solutions, Lake Placid, NY) overnight at 4°C then incubated with 1:100 goat anti-rabbit IgG (sc-2054, Santa Cruz, CA) and goat anti-mouse IgG-conjugated Alexa Fluor (568, Red A11031, Molecular Probes, Eugene, OR), respectively. Photomicrographs of AR expression were taken, then sections were heated to 100°C for 15 min in 10 mM sodium citrate buffer (pH 6.0) to remove the primary antibodies. After incubation with biotinylated anti-mouse secondary antibody at a concentration of 1:50, peroxidase-conjugated streptavidin (Histofine; Nichirei Biosciences, Tokyo, Japan) immune reaction products were developed using 3,3'-diaminobenzidine as the chromogen, with standardized development times. Protein expression in cystic animals was quantified by co-localization of markers within individual cells. PCNA-positive cells were counted as the reference marker and we counted 110 to

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320 (mean 160) total cells that expressed PCNA in 4 to 5 microscope fields prepared from each group. Then the relative expression of AR was quantified as a percentage of PCNA-expressing cells. In normal control group, PCNA expression was greatly reduced in comparison with simple renal cysts group, therefore only 9 fields were examined to obtain an average of 35 PCNA-positive cells per group.

Western blotting

Kidneys were homogenized with T-PER (Thermo Scientific, Rockford, IL, USA) containing protease inhibitors (Roche, South San Francisco, CA, USA) on ice. Tissue lysates were cleared by centrifugation. Cells were lysed in radio-immunoprecipitation assay buffer (Thermo Scientific) containing protease inhibitors (Roche), and cell lysates were cleared by centrifugation. Equal amounts of lysates (100 mg) were separated on 10 or 12% SDS-PAGE. The proteins were transferred onto nitrocellulose membranes (Amersham, Woburn, MA, USA). The membranes were then blocked with 5% non-fat dry milk in PBS for 30 min incubated overnight at 4°C with antibody to tyrosine pSTAT3 (1:1000; Cell Signaling), STAT3 (1:200; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) or α -tubulin (1:5000; Abcam, Cambridge, MA, USA). After three washings with PBS, the membranes were incubated with peroxidase-conjugated goat anti-mouse immunoglobulin G (IgG) or anti-rabbit IgG (1:5000; Amersham) for 1 h. Finally, the blots were developed by the enhanced chemiluminescence method. Membranes were then stripped of antibodies using Restore™ Western Blot Stripping Buffer (Thermo Scientific) for 30 min at 37°C. Membranes were then re probed with primary antibody and secondary antibodies.

For detection of AR expression, protein was transferred electrophoretically onto nitrocellulose membrane (Hybond ECL, Amersham Biosciences), and blocked with 5 w/v ECL Advance blocking agent (Amersham Biosciences) in 0.05 w/v PBS-Tween for 1 h. Rabbit polyclonal antibodies (AR, Santa Cruz Biotechnology Inc, Santa Cruz, CA, USA) was diluted 1:500. After incubation overnight at room temperature and a series of washes with 0.1 w/v PBSTween, the membranes were exposed to secondary anti-rabbit antibody (horseradish peroxidase conjugate; Amersham

Biosciences) with a dilution of 1:1 000 (AR). Proteins were detected using enhanced chemiluminescence (ECL; Amersham Biosciences) and autoradiography. AR blocking peptide (Santa Cruz Biotechnology Inc) was used to block the AR specific band. Anti-AR antibody was expected to bind to the protein at about 120 kDa according to manufacturer guidelines in our blots. For AR Western Blots 15 μ g of protein from whole HKC-8 cell lysates treated with testosterone was used as positive control (HKC-8 cells are male specimen derived from kidney proximal tubule cells).

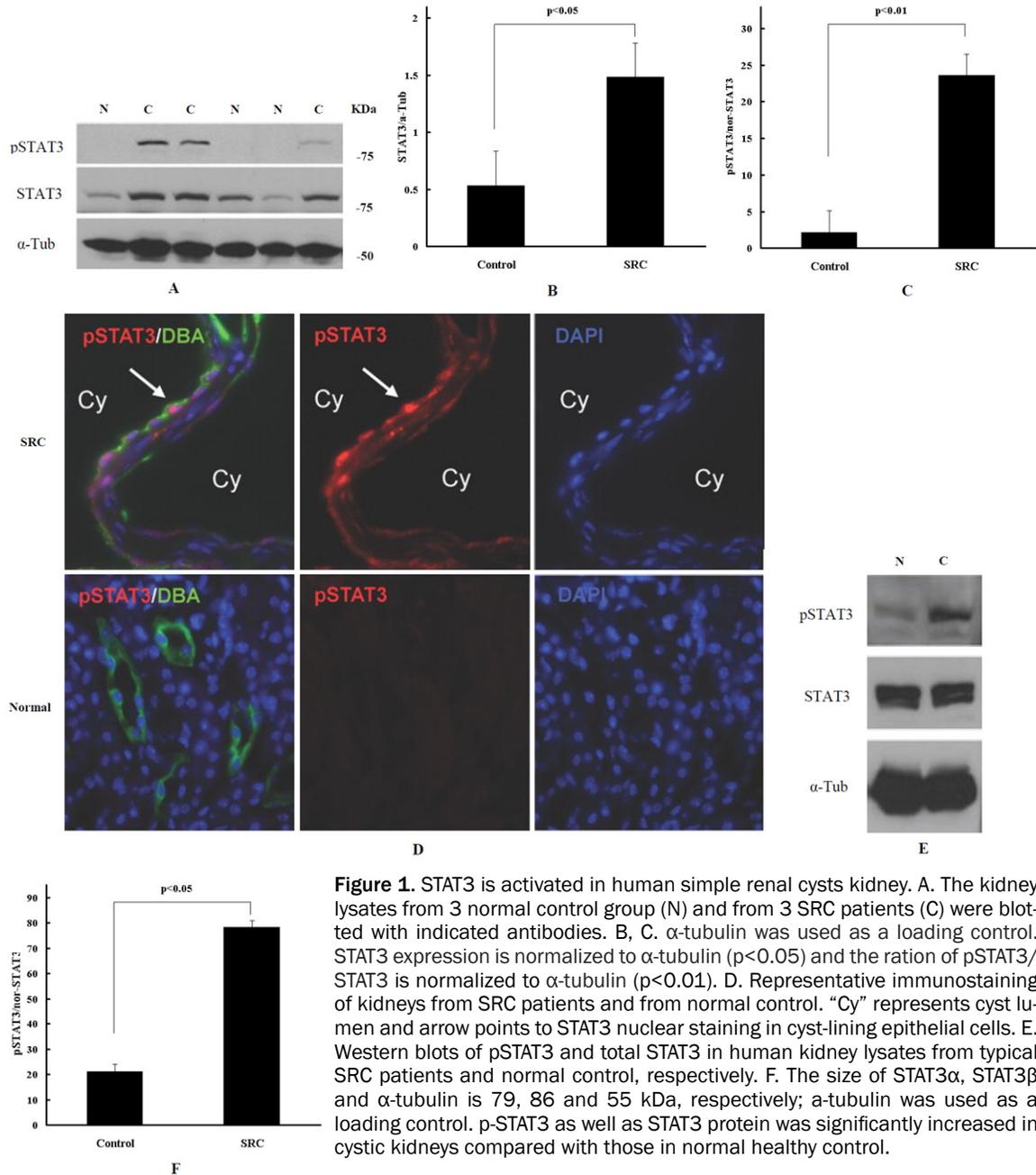
Cell culture and in-vitro stimulation experiment

To further investigate the cross talk between STAT3 and AR signaling, we did some androgen pretreatment in vitro experiment. In detail, HK-2 (an immortalized proximal tubule epithelial cell line from normal adult human kidney) cells were maintained in RPMI 1640 (Gibco) containing 10% fetal bovine serum (HyClone), penicillin (100 units/ml), and streptomycin (100 μ g/ml) at 37°C in 5% CO₂. Starvation was performed in phenol red free RPMI 1640 (Gibco). R1881 was a kind of synthetic androgen methyltrienolone; it was purchased from NEN Life Sciences Products Inc. Antibodies to STAT3 were purchased from Cell Signaling, to β -Actin from Sigma.

HK-2 cells were plated in 6-well plates at 3 \times 10⁵ per well and serum starved in the presence or absence of R1881 (1 nM) for 24 h or other indicated times. Trizol (Invitrogen) was used to extract RNA and from this mRNA was isolated (Qiagen). STAT3 primers were as follows: STAT3 sense 5'-CAAGCAGTTTCTTCAGAGCA-3' and STAT3 antisense 5'-CGTCACCACGGCTGCTGT-3' and β -Actin sense 5'-CGGAACCGCTCATTGCC-3' and β -Actin antisense 5'-ACCCACACTGTGCC-CATCTA-3'. Quantitech SYBR Green RT-PCR kit (Qiagen) was used to perform real-time RT-PCR with the LightCycler (Roche) according to manufacture's instructions. Standard curves for each primer set were determined and quantities of mRNA were normalized to β -Actin. Each sample was performed in duplicate and the experiment was performed three times. Quantities are expressed as fold change.

Cells were placed on ice and washed once with cold PBS before being lysed in cold lysis buffer

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(10mM HEPES (pH 7.2), 142mM KCl, 5mM $MgCl_2$, 2mM EGTA, 0.2% NP40, lupeptin, aprotinin, PMSF, NaF, and $NaVO_4$). Lysates were cleared of cellular debris through centrifugation and soluble protein quantified. Equal amounts of soluble protein (30 μ g) were resolved on a SDS-PAGE followed by transfer to nitrocellulose membrane for immuno blot analysis. Immunoblots were blocked for 1 h in 5% non-fat dry milk in PBS and 1% Tween (PBST). Blots were then incubated overnight at 4°C

rocking with anti-STAT3 (1:1000), anti- β -Actin (1:10000). Blots were than washed with PBST three times for a total of 30 min before being incubated rocking at room temperature in appropriate secondary antibody (1:5000) for 1 h. Blots were then washed again three times with PBST before adding ECL reagent (Amersham) and exposed to X-ray film. All experiments were repeated at least three independent times and representative blots are shown.

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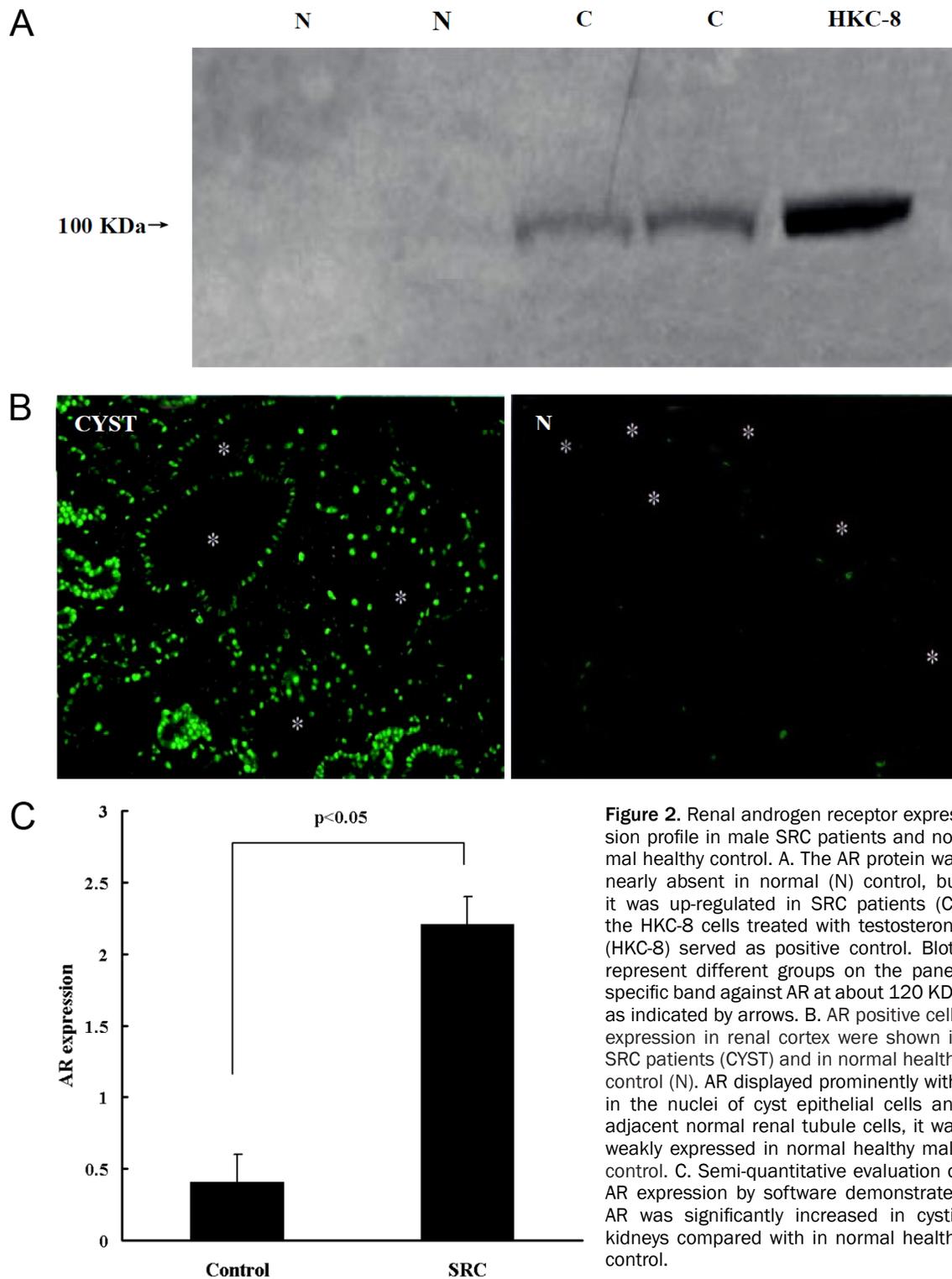


Figure 2. Renal androgen receptor expression profile in male SRC patients and normal healthy control. A. The AR protein was nearly absent in normal (N) control, but it was up-regulated in SRC patients (C), the HKC-8 cells treated with testosterone (HKC-8) served as positive control. Blots represent different groups on the panel, specific band against AR at about 120 KDa as indicated by arrows. B. AR positive cells expression in renal cortex were shown in SRC patients (CYST) and in normal healthy control (N). AR displayed prominently within the nuclei of cyst epithelial cells and adjacent normal renal tubule cells, it was weakly expressed in normal healthy male control. C. Semi-quantitative evaluation of AR expression by software demonstrated AR was significantly increased in cystic kidneys compared with in normal healthy control.

Statistical analysis

Mean and SEM were calculated as usual. The significance of differences between groups was determined by Student's t-test. A p -value of <0.05 was considered statistically significant.

Results

The activation of STAT3 was increased in human SRC kidney

The mean age of the SRC patients and normal control was 56.4 ± 12.5 and 57.3 ± 13.0 years,

respectively. The patients' age in two groups was not statically different. We firstly looked at the activation of STAT3 in SRC patients and normal healthy group. As expected, the levels of tyrosine phosphorylated active STAT3 (p-STAT3) as well as STAT3 protein significantly increased in cystic kidneys compared with those in normal healthy control (**Figure 1A-C**). In addition, we also observed the p-STAT3 nuclear localization in the kidney epithelial cells. And we found there were barely p-STAT3 signals in the normal control kidneys, however, in the SRC patients, there were striking nuclear localization of p-STAT3 in a subset of cyst-lining epithelial cells (**Figure 1D-F**).

AR expression was elevated in human SRC kidney

Next we examined the expression profile of androgen receptor in the kidneys from SRC patients and normal healthy subjects. Western blot demonstrated that AR protein expression was significantly increased in SRC group compared with normal control group (**Figure 2A**); Immunohistochemistry revealed that AR was weakly expressed in male control; however, AR expression prominently elevated in the SRC patients (**Figure 2B**). We made a semi-quantitative assessment of AR expression using LUZEX FS software (Kideko Co. Ltd., Tokyo, Japan), and 15 microscope filed ($\times 4$ magnification) were examined per kidney section. AR displayed prominently within the nuclei of cyst epithelial cells and adjacent normal renal tubule cells, it was weakly expressed in normal healthy male, which is statically different (**Figure 2C**). AR expression was elevated in the cystic kidneys compared with normal control subjects.

Androgen increased STAT3 mRNA and protein expression in vitro

Eventually we need to investigate the potential cross link between androgen and STAT3 signaling pathway. Here we used human-derived immortalized proximal tubule epithelial cell line HK-2. An important factor determining response in biological systems is the change in concentration of a particular component within the system. When HK-2 cells were treated with androgen for 24 h there was a six-fold increase in STAT3 mRNA (**Figure 3A**) and a two-fold increase in protein levels (**Figure 3B and 3C**). This increase in STAT3 remained elevated for

up to 24 h after withdrawal of androgen (**Figure 3D**). For both the mRNA and protein measurements actin levels were used as control and do not change throughout.

Discussion

Although various factors may be attributable to renal cysts formation in human beings, simple renal cysts etiology remained unclear to date, a definite mechanism for the initiation and progression of this complex process has not been elucidated. In our present study, the sex distribution difference and male-susceptibility which was mentioned by previous evidence was concerned [10]. In our present data, IHC analysis demonstrated that the expression of STAT3 activation is up-regulated in SRC patients' kidney, as well as the strong activation of androgen receptor; by in-vitro experiment, the effect of androgen on proximal tubule epithelial cell line HK-2 STAT3 expression was investigated, and gave us some novel clues for gender disparity in human simply renal cysts. Thus, we concluded that STAT3 activation is up-regulated in human SRC kidney.

The Janus kinase/signal transducer and activator of transcription (JAK/STAT) pathway is critical for developmental regulation, growth control and homeostasis in organs [11, 12]. Phosphorylation of STAT3 at tyrosine 705 occurs in response to a variety of cytokines and growth factors including interleukin-6 (IL-6), epidermal growth factor and hepatocyte growth factor [13, 14]. Previous study revealed the expression of these cytokine and growth factors is associated with polycystic kidney disease (ADPKD) progression [15, 16]. Interestingly, ciliary neurotrophic factor-induced phosphorylation at serine 727 of STAT3 is reported to be mediated by the mTOR pathway [17], which is also up-regulated in both ADPKD kidneys and ischemic injured rat kidneys [18]. And latest literature revealed that ischemic injury was a newly-found factor that contributed to human kidney cyst formation [19]. Constitutively activated STAT3 and the over-expression of its downstream target gene cyclin D1 has been found in many solid tumors including head and neck squamous cell carcinoma [20] and renal cell carcinoma [21]. The relationship between STAT3-mediated signaling pathway and human SRC etiology, however, has not been investigated. And Leonhard et al. report-

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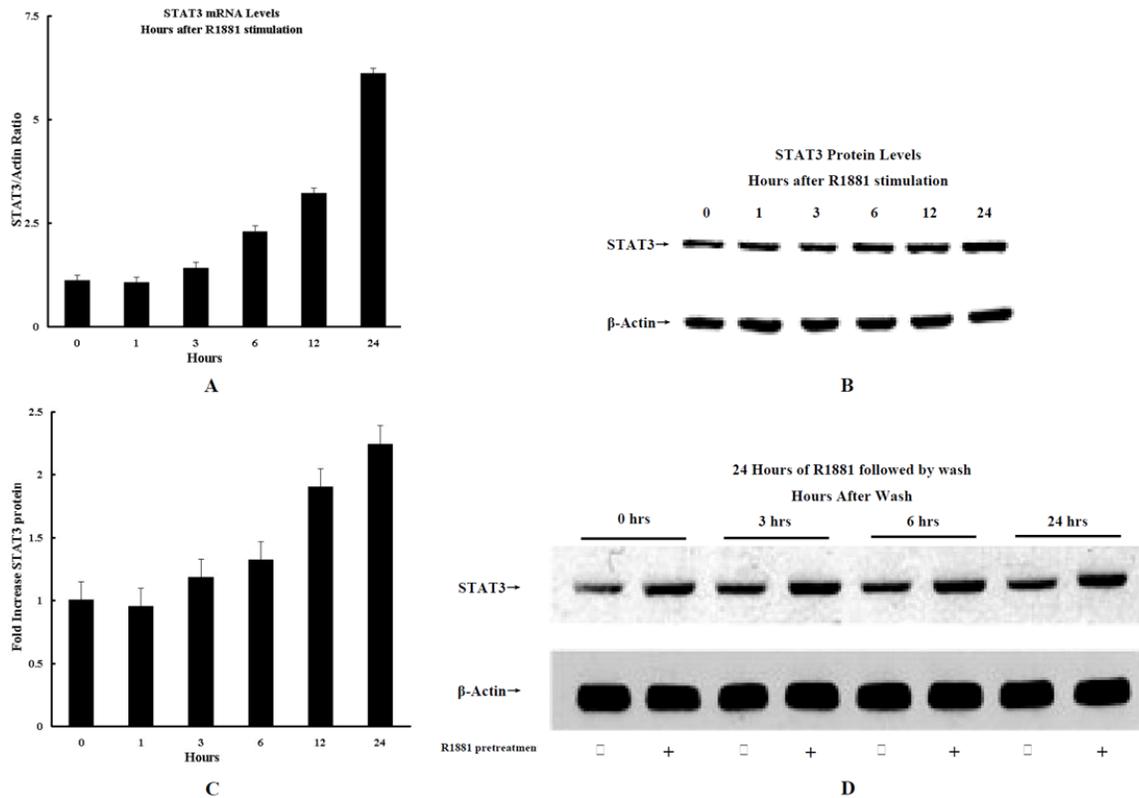


Figure 3. Androgen treatment increases STAT3 mRNA and protein levels and increased complex formation between the androgen receptor and STAT3. HK-2 cells were plated in 6-well plate at 60% confluence overnight. Cells were serum starved and treated with 1 nM R1881 for the times indicated. A. Quantitative RT-PCR was performed on STAT3 and β -Actin using the LightCycler. mRNA levels are shown as a ratio of STAT3/ β -Actin. Values are mean \pm S.E.M. of triplicate samples. B. Cell were stimulated with R1881 for the times indicated and the cell lysates were resolved on an SDS polyacrylamide gel and immunoblotted for STAT3 and β -Actin. C. Graph of the densitometric scan of the STAT3 and β -Actin bands showing a two-fold increase in STAT3 protein after normalization for β -Actin 24 after stimulation with R1881. D. Cells were stimulated with R1881 for 24 h and were washed and incubated with fresh medium with no R1881 for the times indicated. The cell lysates were probed for STAT3 and Actin as shown.

ed that curcumin treatment reduced the elevated STAT3 and S6 phosphorylation in iKsp-Pkd-1^{del} mouse kidneys and delayed the onset of renal failure by 10 days in mouse model [22]. Whether STAT3 would be an effective drug target for the treatment of renal cystic disease remains a myth. Here, we showed that STAT3 is activated in human SRC patients, which might predict a novel intervene target. Future studies will determine whether STAT3 could regulate kidney rodent cell apoptosis, necrosis and proliferation through modulating cell cycle and cell survival.

Male gender seems to accentuate the acquirement and progression of human SRC, as already known, increased kidney epithelial proliferation and trans-epithelial fluid secretion promoted the cyst formation and kidney neph-

ron loss [23]. And recent evidences especially focused on the determining roles of the epithelial cell proliferation. Here we made immunohistochemical evaluation of AR expression in SRC patients as well as normal healthy control, and results showed AR abundance was significantly greater in SRC patients compared with normal compeer. We hypothesized, therefore, that the major contributor of gender-positive status in male subjects might be an effect of androgen (testosterone) on the proliferation of cyst epithelial cells.

In purpose of understanding the interactions and changes in androgen signal and the signaling networks of STAT3, we performed the in-vitro HK-2 cell culture and intervention experiment. As we expected, when treated with androgen for 24 hours, the STAT3 mRNA and

protein were both elevated in HK-2 cells, which showed us the substantial evidence. Taken together from our data showed that androgen treatment of human tubular epithelial cells definitely increase STAT3 levels. Previous study has demonstrated the extracellular stimuli of androgen can affect the intra-cellular signaling through several mechanisms in prostate cancer cell. Increasing expression of AR can modulate the STAT3 protein, and the formation of AR-STAT3 complex was regulated by the cytokines of interleukin 6 (IL-6) and epidermal growth factor (EGF). In this theory, the increase in STAT3 is a positive autocrine/paracrine feedback loop of IL-6 secretion by prostate cancer cells [24]. In addition, recent evidence found that the newly-discovered protein inhibitor of activated STAT3 (PIAS3) binds directly to STAT3 and blocks transcriptional activation, PIAS3 directly interacted with androgen receptor (AR) and affected AR-mediated gene activation, AR was associated with STAT3 and enhanced activity in human embryonic kidney carcinoma cell line 293T cells, as well [25]. However, the above mentioned did not involved the profile of normal human tubular epithelial cells, which was investigated in our nature study.

Determining the signal routing within the cell is important to understanding how the over expression of STAT3 protein in SRC state can alter system response to stimuli that might does less effect in normal state. It should be mentioned, that the beneath mechanism is much more complex than we expected. By using system biology approaches, scholars can study how feedback loops and expression level of a component in one pathway can alter the response of another signaling pathway. As novel evidence showed that the cell senescence might contribute to the occurrence of a renal cyst; in fact, human SRC is a retrogressive process accompanied with individual senium process [26, 27]. Whether the AR-STAT3 cross link yield some potential patho-physiological regulatory roles in geriatrics, it is an unsolved mystery and the limitation of our present study. Another limitation to mention is that we did not enroll female subjects in our cohort, since literature reported androgen receptor was hardly detected in female, and latest article suggested the protective effect of progesterone through promoting mesenchymal differentiation and regulating polycystin-1 expression in ADPKD mice [28]. However, limited to fund and

human resources, we could not make further investigation now.

Future work need to determine the how the androgen receptor-STAT3 complex influence proliferation or apoptosis of kidney resident cell, and if such mechanisms are operative in vivo and in-vitro, how we can make some artificial intervention so as to protect human from unpleasant cyst formation for therapeutic purpose.

Conclusions

Our data initially suggest the emerging evidence of androgen-STAT3 activation in human simple renal cyst disease, enhanced expression of STAT3 and AR in renal cortex is related with male-susceptibility. These results might help us to understand the development of SRC pathogenesis and blockade of this alteration might be a novel therapeutic target for patients with renal cysts.

Declaration of conflicting interests

The authors declared no potential conflicts of interests with respect to the authorship and/or publication of this article.

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