Original Article
Inhibition of γ-secretase by retinoic acid chalcone (RAC) induces G2/M arrest and triggers apoptosis in renal cell carcinoma

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Abstract: The present study was devised to investigate the effect of RAC on inhibition of cell proliferation and apoptosis of renal carcinoma cells. MTT assay and flow cytometry analysis were used to determine cell proliferation and apoptosis along with cell cycle examination. Western blot analysis and immunohistochemistry were used for the detection of expression levels of Notch1 and Jagged1 in renal cell carcinoma (RCC) and normal kidney tissues. The results revealed a significant inhibition of cell proliferation, G2/M phase cell cycle arrest and cell apoptosis at 30 μM concentration of RAC after 72 h. In ACHN and 769-P cells, the population in G2/M phase was increased to 45.27, and 54.23% respectively on treatment with 30 μM RAC for 72 h. In 769-P and ACHN renal carcinoma cells treatment with 30 μM RAC caused 69.71 and 59.27% of the cells to undergo apoptosis compared to 5.23 and 4.93% respectively in control cells. The positive staining rates of Notch1 and Jagged1 in renal carcinoma tissues were 95.3 and 93.0% compared to normal kidney tissues 36.4 and 42.4% respectively. Treatment of renal carcinoma tissues caused a significant decrease in staining rates of Notch1 and Jagged1 after 96 h. Thus RAC can be a potent agent in the treatment of renal cell carcinoma.

Keywords: Renal cell carcinoma, apoptosis, Notch1, Jagged1

Introduction

Among all the adult malignant neoplasms, renal cell carcinoma (RCC) alone accounts for 2-3%. There are around one lakh fifty thousand cases detected and seventy eight thousand deaths globally caused by renal cell carcinoma every year [1]. Of the patients diagnosed with renal cell carcinoma, 25-30% is detected at metastatic stage which makes the treatment difficult [2]. It is reported that multikinase inhibitors increase progression-free survival rates, however, the effective therapy for patients with metastatic advanced-stage RCC remains limited [3, 4]. Renal cell carcinoma requires additional tumorigenic events, suggesting the importance of these pathways in the development of novel agents [5].

It is reported that in renal carcinoma cells the Jagged1 is overexpressed which determines

poor outcome in RCC patients [6]. Latter it was shown that Notch plays a role in the tumorigenesis of renal carcinoma cells and γ-secretase inhibitor inhibits cell proliferation and apoptosis in these renal carcinoma cells. Taking into consideration the therapeutic importance of Notch signaling inhibition, we designed an experiment to investigate the effect of RAC on inhibition of Notch signaling.

Retinoic acid and other retinoids (RAs) are used in the prevention and treatment of dermatological diseases [7, 8]. Retinoic acid has been shown to be promising candidate in the treatment of cancer as well [9]. Retinoic acids are known to effect in vitro proliferation, differentiation, and apoptosis of normal and abnormal cells of several cancers. It includes colon [10], prostate [11], lung [12], and leukemia [13]. There are reports that all *trans*-retinoic acid (ATRA) and 9-*cis* RA also influence the

Figure 1. Structure of retinoic acid chalcone (RAC).

morphological differentiation, proliferation, and gene expression of neuroblastoma [14] and astrocytoma cells [15]. Recurrent malignant cerebral gliomas have been treated with ATRA [16, 17] and 13-*cis* RA [18]. Retinoids are known to have anti-proliferation, anti-migration, and anti-invasive activity against human malignant gliomas [19, 20], suggesting that retinoids are suitable anticancer agents to inhibit progression of tumors. In the present study the effect of retinoic acid amide (**Figure 1**) having more bioavailability compared to the parent compound on human liver cancer apoptosis was investigated.

Materials and methods

Reagents and antibodies

RAC, antibodies used against Notch1 (polyclonal rabbit anti-human Notch1; ab27526) and Jagged1 (polyclonal goat anti-human Jagged1; sc-6011) were purchased from Sigma-Aldrich (St. Louis, MO, USA). The peroxidase-conjugated mouse anti-goat IgG antibody and glyceraldehydes-3-phosphate dehydrogenase (GAPDH) was purchased from Santa Cruz Biotechnology, Inc. and the γ-secretase inhibitor, N-[N-(3,5-difluorophenacetyl)-I-alanyl]-S-phenylglycine t-butyl ester (DAPT), was purchased from Merck Biosciences (Darmstadt, Germany). The Annexin V-fluorescein isothiocyanate (FITC) apoptosis detection kit was purchased from Beckman Coulter (Fullerton, CA, USA).

Cell culture

Human renal carcinoma cell lines, 786-O, 769-P, ACHN, Caki-1, HEK293, OS-RC-2, and TUHR-14TKB were obtained from The Cell Bank of Type Culture Collection of Chinese Academy of Sciences, Shanghai Institute of Cell Biology (Shanghai, China). The cells were cultured in

DMEM containing penicillin 100 units/ml, streptomycin 100 mg/ml and 10% FBS (PAA). The cells were maintained in an incubator at 37°C with 5% CO_2 atmospheric condition.

MTT assay

Cell proliferation was assessed at various time points by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) assay. Briefly, 1 × 10^5 cells were seeded in each well of a 96-well plate (BD Falcon, Franklin, NJ) and allowed to adhere for 8 h. Then, 5 mg/mL MTT (Sigma, Germen) was added to each well and incubated for 4 h. The cells were lysed by adding 150 μL/well of dimethyl sulfoxide and read at 490 nm absorbance wavelength in microplate reader (MPR-A4i; Tosoh Corporation, Tokyo, Japan). The experiments were repeated at least three times independently.

Flow cytometry apoptosis assay

Analysis of apoptosis was accessed by an AnnexinV-FITC/propidium iodide double staining kit (Genmed Bioscience, China) following the manufacturer's protocols. Briefly, the cells were plated in six wells. Cells were continuously cultured for 48 and 72 h, and then to be harvested. Before reading on the flow cytometer, cell suspensions were washed in PBS, resuspended with a 1 × binding buffer and exposed to 5 μL of Annexin V-FITC (20 μg/mL) and 10 μL of propidium iodide (PI; 50 μg/mL). After incubation of 20 min in the dark, the samples were subjected to a FACScan flow cytometer [equipped with CellQuest and ModFITLT for Mac V1.01 software (Becton Dickinson, San Jose, CA)]. The experiments were repeated at least three times independently.

Cell cycle analysis

The cells were treated with various concentrations of RAC (10, 20, 30 and 50 μM) for 72 h at 37°C. After 72 h RAC treatment cells were harvested, washed in PBS and then fixed in cold 70% ethanol. The cells were then stained with propidium iodide while treating with RNase and analysed with Beckman Coulter EPICS XL-MCL cytometer (Beckman Coulter). Multi-Cycle DNA Content and Cell Cycle Analysis Software (Phoenix FlowSystems, Inc.) were used for analysis of cell cycle distribution.

Ethical statement

The present study was approved by the ethical committee of China-Japan Union Hospital of Jilin University (no. 2010-98; Changchun, China). Each patient was involved after providing informed written consent.

Tissue samples

For western blot analysis, fresh tumor tissues (later verified as clear cell RCC) and normal (non-tumor) kidney tissues were obtained intraoperatively from eight patients who underwent radical nephrectomy at the Department of Urology, China-Japan Union Hospital of Jilin University. The tissue samples were then snap-frozen in liquid nitrogen and stored at -80°C prior to analysis. For immunostaining, a total of 129 patients with pathologically verified clear cell RCC were enrolled consecutively. All patients underwent nephrectomy (partial or radical) performed at the Department of Urology, China-Japan Union Hospital of Jilin University, between 2010 and 2014.

Western blot analyses

The renal carcinoma cells were washed with ice-cold phosphate-buffered saline and then lysed in lysis buffer containing 10 mM Tris (pH 7.5), 150 mM NaCl, 10 mM ethylenediamine-tetraacetic acid (EDTA), 1% sodium dodecyl sulfate (SDS), 1 mM sodium orthovanadate, and a mixture of protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 1 μg/mL pepstatin A, 2 μg/ mL aprotinin). The lysates were sonicated for 10 s, centrifuged for 20 min at 20,000 × g and then stored at -70°C. Equal amounts (25 μg) of the cell lysates were resolved by 12% SDS-PAGE and transferred to polyvinylidene fluoride membranes. After blocking, blots were incubated with Notch1 (1:200) or Jagged1 (1:500) antibodies overnight at 4°C and followed by each corresponding second antibody at room temperature for 1 h at 37°C. Then, the results developed by ECL (Pierce Biotechnology, USA). The protein bands were then analysed using the BioImaging System (UVP, USA). The gray scale values were normalized to the values of the corresponding GAPDH band to determine the expression level of the protein. The experiments were repeated at least three times independently.

Immunohistochemistry

Fifty renal carcinoma cancer tissues were obtained from the Department of Urology, China-Japan Union Hospital of Jilin University frozen in liquid nitrogen and stored at -80°C prior to analysis. The paraffin-embedded tissues were cut into 2 mm sections followed by deparaffinization and rehydration. The tissues after 3% hydrogen peroxide treatment were boiled in 10 mM citrate buffer (pH 6.0) for 15 min. The sections were incubated with goat serum for 1 hand rinsed in PBS. The slides treated with Notch1 (1:200) or Jagged1 (1:100) antibodies for 1 h were rinsed with PBS and incubated with horse radish peroxidase-conjugated secondary antibody. Again slides were rinsed in PBS, incubated with 3,3'-diamino-benzidine staining and counterstaining with hematoxylin blue. Negative controls were performed by substituting the primary antibody with a non-immune serum. Control sections were treated in parallel with the samples. The proportion score was determined semi-quantitatively by assessing the whole tumor section at low magnification and each sample was scored on the following scale of 0-3: 0, no positive cells; 1, 1-20% of positive cells; 2, 21-60% of positive cells; and 3, 61-100% of positive cells. The intensity score was determined at high magnification as follows: 0, negative staining; 1, weakly positive staining; 2, moderately positive staining; and 3, markedly positive staining. Then, the total score of each section was calculated by sum of the two parameters.

Statistical analysis

SPSS software (Inc., Chicago, IL, USA) was used for the statistical analyses. The χ^2 test was used for detection of immunostaining differences and the study of correlations. The difference was considered statistically significant at $P < 0.05$.
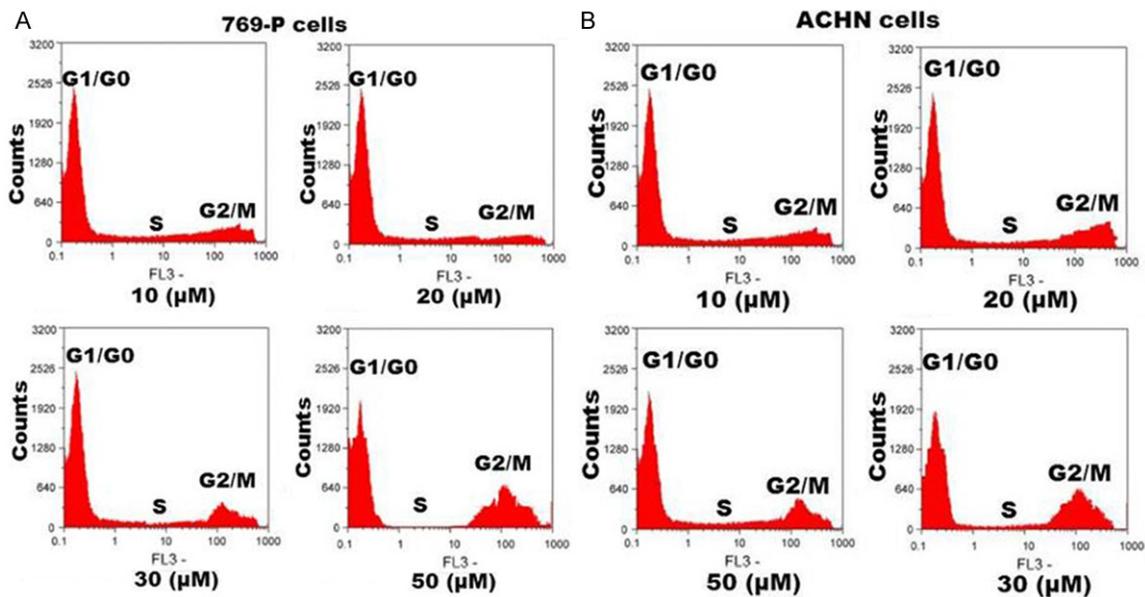
Results

Effect of RAC on inhibition of renal carcinoma cell growth

The results from MTT assay revealed inhibition of renal carcinoma cells on treatment with RAC in a dose- and time-dependent manner. All the
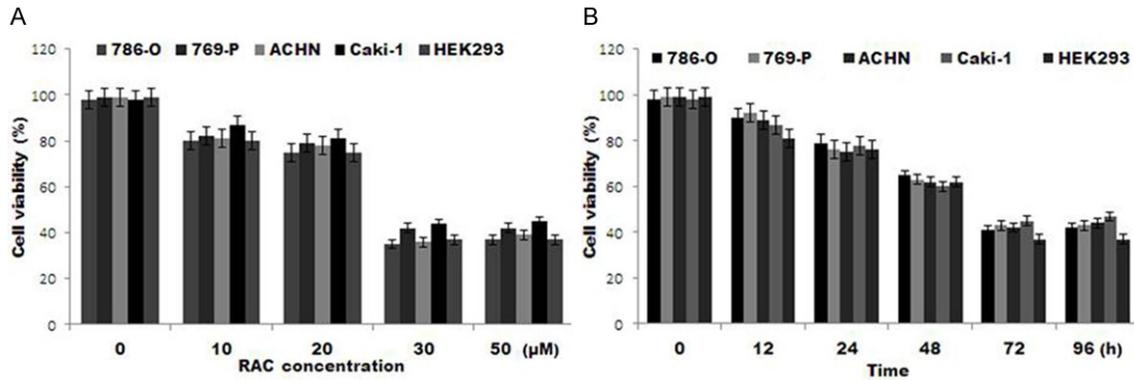
Figure 2. RAC Inhibits cell viability in renal carcinoma cells in a dose and time dependent manner.

Figure 3. RAC-induced G2/M phase cell cycle arrest in human renal carcinoma cell lines. The cells were treated with various concentrations of RAC for 72 h and analyzed by flow cytometry.

tested renal carcinoma cell lines 786-O, 769-P, ACHN, Caki-1, and HEK293 exhibited similar cytotoxicities on treatment with a range of RAC concentrations (10, 20, 30, 40 and 50 μM). Although the decrease in cell proliferation started at 10 μM concentration of RAC but the inhibition was significant at 30 μM after 72 h (**Figure 2**).

Effect of RAC on cell cycle arrest

We used flow cytometry to investigate the effect of various concentrations of RAC on cell cycle distribution in renal carcinoma cells. The examination of cell cycle showed a significant increase in cell population in G2/M phase on treatment with various concentrations of RAC for 72 h compared to untreated control cells. In 769-P cells, the population in G2/M phase was increased by 5.34, 9.16, 54.23 and 44.56% on treatment with 10, 20, 30 and 50 μM RAC for 72 h respectively (**Figure 3A**). Similarly in ACHN cells, the population was increased by 4.93, 8.24, 45.27 and 32.46%, (**Figure 3B**) whereas for Caki cells, the population was increased by 6.45, 10.19, 49.21 and 48.92%, on treatment with 10, 20, 30 and 50 μM RAC for 72 h respectively. These results suggested that RAC induces G2/M phase cell cycle arrest in renal carcinoma cells.
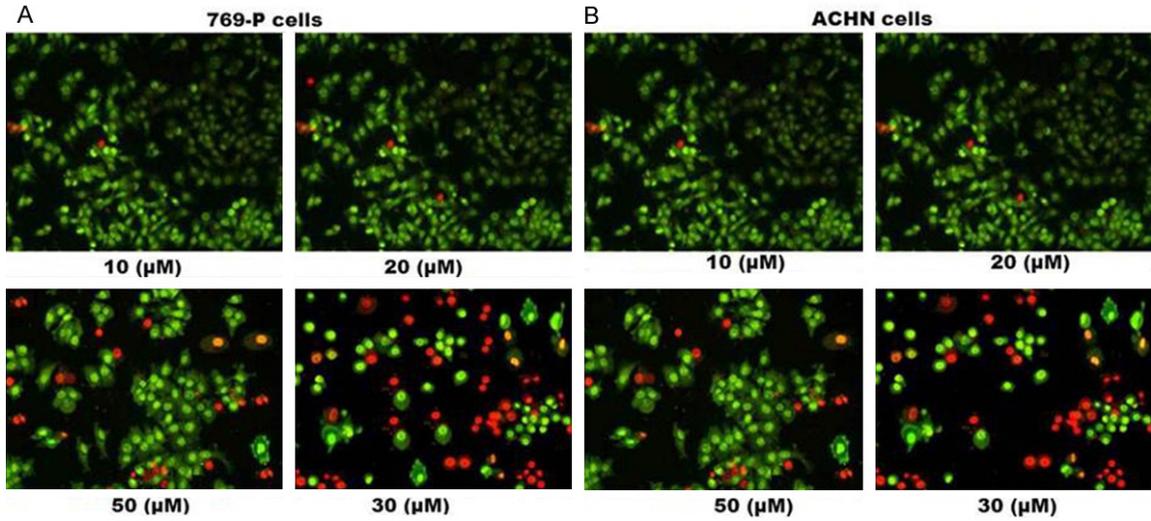
Figure 4. RAC treatment-induced dose-dependent apoptosis in human renal carcinoma cell lines using a double-staining method with Annexin V-FITC/PI.
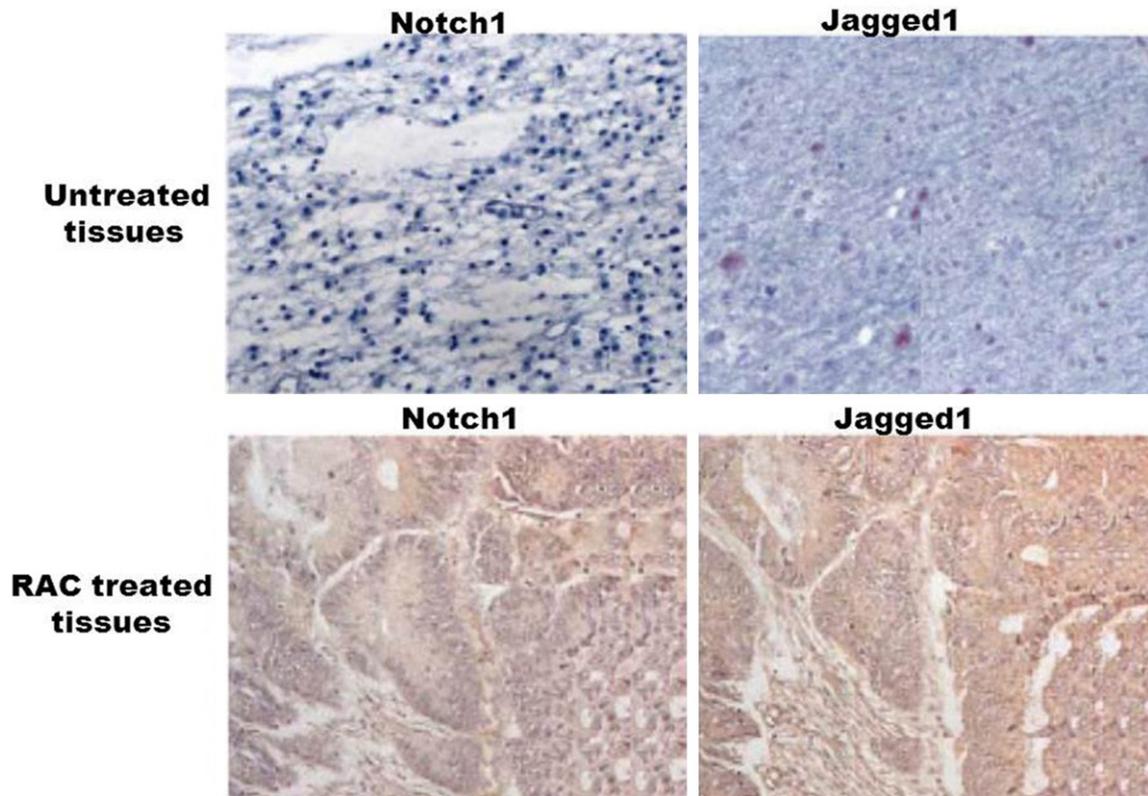

Figure 5. Western blot analysis showing expression of Notch1 and Jagged1 in non-neoplastic renal tissue and renal cell carcinoma tissue.

RAC induces apoptosis in renal carcinoma cells

The results from AnnexinV-FITC/propidium iodide double staining revealed typical apoptotic changes with chromatin condensation and nuclear fragmentation in 769-P and ACHN renal carcinoma cells after 72 h of RAC treatment. Annexin V-FITC/PI staining and FACS demonstrated that in 769-P renal carcinoma

Table 1. Pathological characteristics of renal cell carcinoma cases

Characteristics	IHC (n = 100)	Western blotting (n = 10)
Gender, n (%)		
Male	79 (79)	7 (70)
Female	21 (21)	3 (30)
Age, years		
Mean	59.8	58.4
Range	23-89	47-79
Surgery, n (%)		
Radical nephrectomy	95 (95)	9 (90)
Partial nephrectomy	5 (5)	1 (10)
Tumor size (cm)		
Mean	6	5.4
Range	2-19	1.9-14
TNM stage, n (%)		
I	65 (5)	3 (30)
II	15 (15)	4 (40)
III	15 (15)	2 (20)
IV	5 (5)	1 (10)
Fuhrman grade, n (%)		
1	38 (38)	3 (30)
2	42 (42)	4 (40)
3	14 (14)	2 (20)
4	6 (6)	1 (10)
Relapse, n (%)		
Yes	21 (21)	2 (20)
No	79 (79)	8 (80)

IHC, immunohistochemistry.

cells treatment with 30 μM RAC for 72 h caused 69.71% of the cells to undergo apoptosis compared to 5.23% in control. Similarly treatment with 30 μM RAC for 72 hours caused 59.27% ACHN cells to undergo apoptosis compared to 4.93% in control (**Figure 4**).

Notch1 and Jagged1 expression is up-regulated in renal carcinoma tissues

Results from Western blot analysis showed that the protein of Notch1 was highly expressed in RCC tissues (**Figure 5**). Among 10 RCC tissues Notch1 was found to be highly expressed in nine cases of RCC (9/10; 90%) compared with paired non-neoplastic tissues. Similarly, out of 10 RCC tissues, 9 showed higher expression of Jagged1 (9/10, 90%).

Clinical and pathological characteristics

100 cases with renal cell carcinoma were subjected to immunostaining of Jagged1 and 10 cases to western blot analysis (**Table 1**).
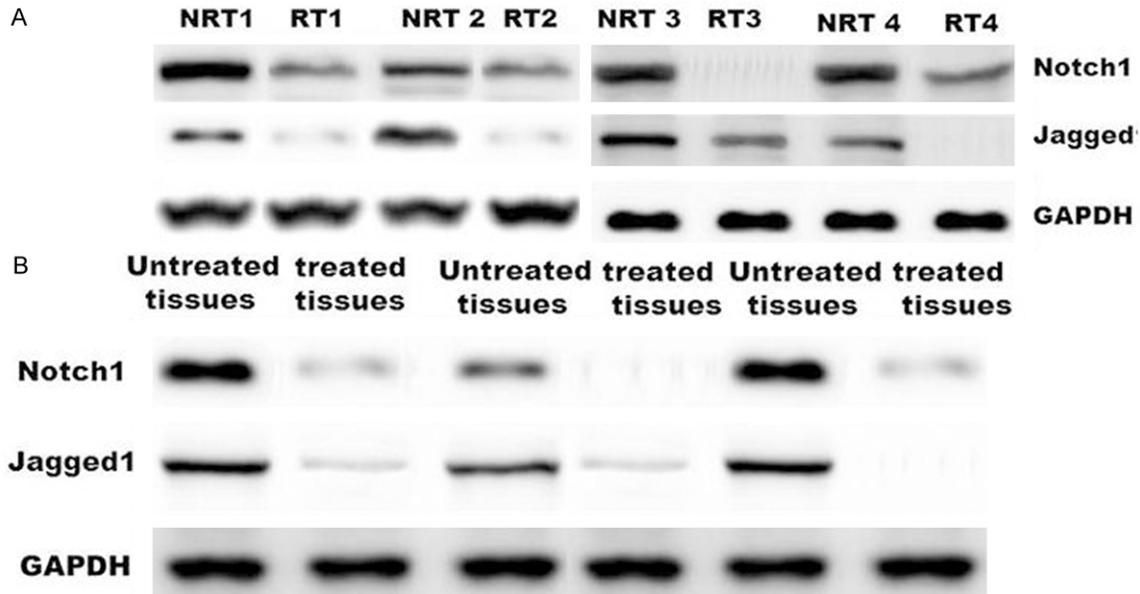
Immunohistochemistry

In renal carcinoma tissues the positive staining rate of Notch1 and Jagged1 was 97 and 96%, respectively compared with 19.8 and 35% in normal tissues (**Figure 6**). Notch1 and Jagged1 exhibited a significantly higher expression in RCC tissues than in normal kidney tissues. Treatment with 30 μM concentration of RAC caused a significant decrease in the expression of Notch1 and Jagged1 after 96 h. In renal carcinoma tissues treated with RAC the positive staining rate of Notch1 and Jagged1 was decreased to 23.4 and 41.5% respectively compared to 97 and 96% in untreated carcinoma tissues.

Discussion

The Notch pathway plays a dual role by either acting as tumor suppressive or oncogenic pathway depending on various factors involved [21]. It has a key role in controlling cell proliferation and apoptosis [22]. In the current study, we observed overexpression of the Notch1 and Jagged1 in neoplastic tissue compared to that in normal kidney tissue. The expression of Notch1 and Jagged1 was related to tumor size and grade indicating that Notch pathway has oncogenic role in the renal cell carcinoma. Renal carcinoma tissues on treatment with RAC showed a significant decrease in expression levels of Notch1 and Jagged1 in neoplastic tissue.

The results from our study showed that RAC significantly inhibits the cell proliferation in the renal carcinoma cells, induces G2/M-phase cell cycle arrest and apoptosis. The oncogenic effect of Notch in renal cell carcinoma may involve diverse mechanisms. There are reports that Notch signaling plays a vital role in tumor angiogenesis [23-25]. It is also reported that Notch signaling suppresses expression of p21Cip1 and p27Kip1, two cyclin-dependent kinase inhibitory proteins regulating the cell cycle control [26]. This suggests that the inhibitory effect of RAC on cell proliferation and induction of apoptosis is due to inhibition of Notch signaling pathway. Although kinase inhibitors like sorafenib and sunitinib increase the progression-free survival rate for patients but the side effects of kinase inhibitors hinder their clinical application [27].

Figure 6. Expression of (A) Notch1 and (B) Jagged1 by immunohistochemistry (magnification, × 200).

The mechanism of these drugs is believed to involve suppression of hypoxia-inducible factor-mediated autocrine growth factor signaling and proangiogenic effects. The therapeutic effect of RAC inhibition on clear cell RCC tumor growth indicates that the inhibition of Notch signaling presents at least a complementary therapeutic approach for treatment of clear cell RCC. In the present study, inhibition of clear cell RCC cells with RAC led to a considerable decrease of cell proliferation and increased apoptosis.

In conclusion, the current study indicated that Notch signaling is important in the tumorigenesis of RCC. The RAC has the potential of being a novel therapeutic regimen towards RCC, although, further investigation is required.

Disclosure of conflict of interest

None.

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