Original Article
Up-regulation of ABCG2 promotes cell proliferation in renal cell carcinoma and associate with clinicopathologic features

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Abstract: ABCG2, also known as breast cancer resistance protein (BCRP) is a drug efflux pump and an important member of the ATP-binding cassette transporter superfamily. Recent studies showed that ABCG2 play an important role in tumor progression. However, the role of ABCG2 in human renal cell carcinoma (RCC) remains largely unclear. In the present study, our data showed that elevated ABCG2 expression were observed in RCC tissues compared to adjacent non-tumor tissues, showing a high correlation with advanced clinical stage, elevated tumor stage, lymph node metastasis and lowered overall survival rates. Multivariate analysis revealed that ABCG2 expression was also an independent prognostic factor for comparison of clinical stage, tumor stage and lymph node metastasis. Furthermore, in vitro assays, we found that ABCG2 expression was lower in poorly-differentiated Caki-1 cells compare to well-differentiated 786-O cells. By respectively up-regulating or depleting ABCG2 expression in RCC cells, our finding suggested that ABCG2 could act as a promotor of RCC cell proliferation and cell apoptosis resistance. Thus, our data demonstrate that ABCG2 is a novel molecule involved in both RCC progression and prognosis. Full elucidation of ABCG2 functionality may supply a potential novel therapeutic agent for the treatment of RCC.

Keywords: Renal cell carcinoma, ABCG2, overall survival, cell proliferation, cell cycle, cell apoptosis

Introduction

Renal cell carcinoma (RCC), the third most common malignancy of the genitourinary system, accounts for 3% of all adult malignancies [1]. Clear cell RCC (ccRCC) is the most common subtype, and it accounts for 70-80% of all kidney neoplasm [2]. Half of RCC patients suffer from metastatic disease, and the 5 year survival rate is less than 10% and long-term remission is infrequent [3]. Distant metastases and local recurrence are the main causes of fatalities following curative resection [4]. Thus, there is an urgent requirement to identify novel biomarkers to predict tumor metastasis and recurrence and to develop more novel treatment strategies to effectively control aggressive renal cell carcinoma.

ABCG2, also called breast cancer resistance protein, was a member of ATP-binding cassette transporter family [5]. ABCG2 was firstly discov-ered as a molecule involved in MDR from doxo-rubicin-resistant human MCF-7 breast cancer cells [6]. Recent study revealed that ABCG2 was over-expressed in hepatocellular carcinoma cells (HCC) and inducing HCC cells insensitive to sorafenib [7]. Interestingly, Golebiewska et al reported that ABCG2 could present as a potential marker of cancer stem cells (CSCs) in diverse human malignances [8]. Cancer stem cells are sub-population of cells induce cell renewal, contributing to initiation of heterogeneous lineage of cancer cells [9]. These sorts of cells play pivotal roles associated with tumor initiation, maintenance, relapse, metastasis and multidrug resistance in human carcinomas, including RCC [10, 11]. Recent research showed that ABCG2 was over-expressed in the side population (SP) cells of bladder cancer cell line. SP cells are cells utilized and identified as putative CSCs, and highly expressed ABCG2 was associated with higher risk of recurrence and mortality associated with poor differentiation of the

cancer cells [12]. Moreover, recent study showed that hsa-miR-520h could down-regulate ABCG2 in pancreatic cancer cells to inhibit migration, invasion, and side populations [13]. However, the role of ABCG2 in human renal cell carcinoma remains unclear.

In the present study, we explored the expression of ABCG2 in both RCC tissues and cell lines, and the relationship between ABCG2 and RCC patients' clinicopathologic features was analyzed. We regulated the expression of ABCG2 in RCC cells to study the effect of ABCG2 on RCC cell proliferation, apoptosis and cell cycle distribution. Our data showed that ABCG2 was highly expressed in both RCC tissues and cell lines, and overexpression of ABCG2 is associated with RCC clinicopathologic features and short overall survival of RCC patients. In addition, our data showed that the poorer RCC cells differentiated the higher ABCG2 expressed, indicating a mighty relationship between ABCG2 and RCC cell differentiation. Moreover, we reported that over-expression of ABCG2 could promote the abilities of RCC cells on cell proliferation and cell apoptosis resistance. Thus, our study suggested that ABCG2 might be involved in renal carcinogenesis and become a potential therapeutic for RCC.

Materials and methods

RCC tissue samples and cell cultures

78 paired RCC tissues and adjacent non-tissues were obtained from patients in Zhejiang Cancer Hospital. The tissues were immediately snap-frozen in liquid nitrogen after surgical removal and stored at -80°C. All protocols were approved by the Ethics Committee of Zhengzhou University. RCC cell lines 786-O, Caki-1 and immortalized normal human proximal tubule epithelial cell line HK-2 were purchased from the American Type Culture Collection (ATCC). Renal cancer cells were cultured in RPMI-1640 medium (Gibco) with 10% fetal bovine serum (FBS, Gibco), 50 U/ml penicillin and 50 μg/ml streptomycin. HK-2 cells were cultured in KSFM medium (Gibco). All cells were maintained at 37°C in a 5% CO_2.

Immunohistochemistry

Paraffin sections (4 μm thick) were deparaffinized in xylene and rehydrated in grade alco-

hol, followed by boiling in 10 mmol/L of citrate buffer (pH 6.0) for antigen retrieval. After inhibition of endogenous peroxidase activities for 30 min with methanol containing 0.3% H_2O_2, the sections were blocked with 2% bovine serum albumin for 30 min and incubated overnight at 4°C with primary antibody (Abcam). After washing thrice with PBS, the slides were incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG for 30 min, followed by reaction with diaminobenzidine and counterstaining with Mayer's hematoxylin. The evaluation of the immunohistochemical staining was performed independently by two authors without knowledge of the clinicopathological information. Immunohistochemical staining was scored by using a four-point scale according to the percentage of positive cells: 0, <10% positive; 1+, 11%-25% positive; 2+, 26%-50% positive; 3+, >51% positive. For statistical analysis, the protein expression of ABCG2 in RCC specimens was thus divided into a low-expression group (0 or 1+) and a high-expression group (2+ or 3+).

Total RNA extraction and quantitative real-time PCR

RNA was isolated by TRIZOL reagent (Invitrogen) according to the manufacturer's protocol. Quantitative real-time PCR (qRT-PCR) was performed with an Applied Biosystems 7900HT system (Applied Biosystems) using SYBR Premix Ex Taq (Takara). PCR volume (20 μl) contained 1 μl reverse transcript product. Cycling conditions were 1 cycle of 95°C for 30 sec, 40 cycles of 95°C for 5 sec and 60°C for 30 sec. PCR was performed in triplicate. For measurement of the ABCG2 transcript from total RNA, total cDNA was synthesized using a Takara reverse transcription kit (Takara). qRT-PCR was performed using SYBR Pre-mix ExTaq (Takara). GAPDH were used as an endogenous control. The ΔΔCt method was used to determine relative quantitation of ABCG2 expression, and fold change was determined as $2^{-\Delta\Delta Ct}$. The ABCG2 sense primer was 5'-TGTGTTTATGATGGTCTGT-TGGT-3', and the antisense primer was 5'-CT-GTGCAACAGTGTGATGGC-3'. For the GAPDH gene, the sense primer was 5'-TGCACCACCA-ACTGCTTAGC-3', and the antisense primer was 5'-GGCATGGACTGTGGTCATGAG-3'.

Western blot assay

Cells were lysed using the mammalian protein extraction reagent RIPA (Beyotime) supple-

mented with a protease inhibitor cocktail (Roche) and phenylmethylsulfonylfluoride (Roche). Protein samples were resolved by 10% SDS-PAGE and then transferred to PVDF membranes. The membranes were blocked and probed with antibodies against ABCG2 and GAPDH (Abcam), followed by probed with the secondary antibodies accordingly. Band detection via enzyme-linked chemiluminescence was performed according to the manufacturer's protocol (ECL).

Cell transfection

Recombinant adenovirus Ad5/F35 (Ad5/F35-ABCG2) was constructed for over expressing ABCG2 and Ad5/F35-Null was used as negative control (GenePhrma). Well-differentiated 786-O cells were transfected with Ad5/F35-ABCG2 or Ad5/F35-Null. Poorly-differentiated Caki-1 cells were transfected with pGPU6/GFP/Neo vectors (GenePhrma) containing shRNA against ABCG2 (sh-ABCG2) by using Lipofectamine 2000 (Invitrogen), and non-containing ones were used as negative control. Cell were cultured and selected in medium containing 400 µg/ml G418 (Santa Cruz), and were cultured and maintained in medium containing 200 µg/ml G418. Stable transfected cells above were validated by qRT-PCR and western blot analysis.

Cell proliferation assay

To assess cell proliferation ability, 786-O and Caki-1 cells were plated in 96-well plates. After transfection, proliferation was determined by 3-(4,5-dimethylthiazol-2-yl)-2-5 diphenyltetrazolium bromide (MTT, Sigma) assay at 24, 48, 72, and 96 h. The absorbance of samples was measured with a spectrophotometer reader (Molecular Devices) at 570 nm. All experiments were performed in five replicates and were repeated three times independently.

Cell cycle assay

To assess cell cycle distribution, 786-O and Caki-1 cells were plated in 6-well plates. After transfection, the cells were collected by trypsinization, fixed in 70% ice-cold ethanol overnight, then washed with phosphate buffer saline (PBS), and stained with propidium iodide (PI, 50 mg/ml) in PBS supplemented with RNase (50 mg/ml) in the dark at room temperature for 30 min. Tests performed in triplicate for each

sample, and analyses of cell cycle distribution were performed by flow cytometer in accordance with the manufacturer's guidelines (BD Bioscience).

Apoptosis assay

To assess cell apoptosis ability, the Annexin V-FITC Apoptosis Detection kit (BD Biosciences) and PI was used. The transfected 786-O and Caki-1 cells were suspended in RPMI-1640 medium. The cells were resuspended in 500 µl cold Binding Buffer with 1.25 µl Annexin V-FITC, and incubated for 15 min at room temperature in the dark. Cells were resuspended in 500 µl cold Binding Buffer with 10 µl PI, incubated for 4 h and analyzed by flow cytometry (BD Bioscience).

Statistical analysis

All statistical analyses were performed using SPSS version 18.0 software. The data are shown as the mean ± SD from at least three independent experiments. Comparison of continuous data was analyzed using an independent t-test or chi-square test. Overall survival curves were plotted according to the Kaplan-Meier method, with the log-rank test applied for comparison. Variables were used in multivariate analysis on the basis of the Cox proportional hazards model. $P<0.05$ were considered statistically significant.

Results

ABCG2 expression is up-regulated in RCC tissues and cell lines

Using IHC, the ABCG2 expression levels were defined for 78 paired RCC tissues and adjacent non-tumor tissues from patients. In tumor tissues, 67.9% (53/78) of the cases showed "high expression" ofABCG2, whiles case of "low expression" was counted only 25. On the contrary, in adjacent non-tumor tissues, cases of "high expression" were counted only 15, which consisted 19.2% of the 78 cases, and the cases of "low expression" were counted 63. Those data significantly showed that the expression of ABCG2 was increased in RCC tissues compared with adjacent non-tumor tissues (**Figure 1A** and **1B**, $P<0.05$).

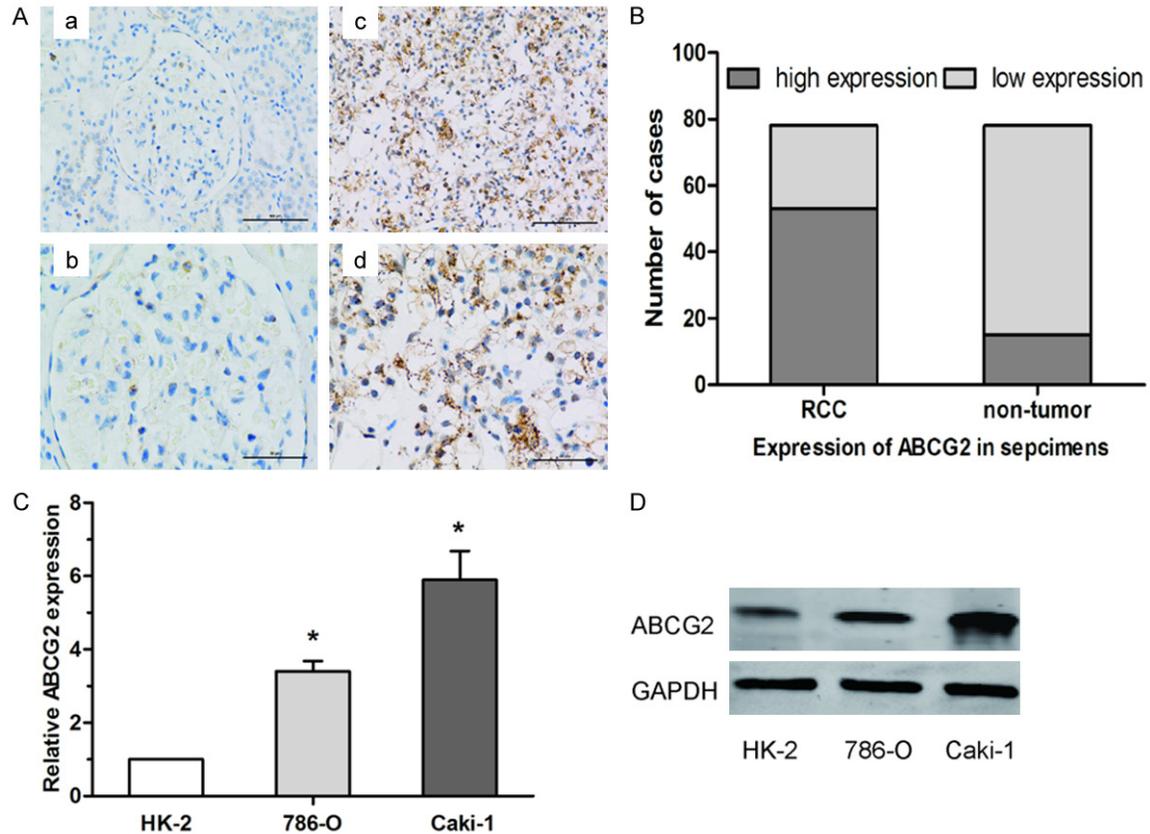Next, we evaluated the expression of ABCG2 in two human RCC cell lines (786-O and Caki-1).

Figure 1. Expression of ABCG2 in RCC tissues and cell lines. A. Immunohistochemical (IHC) staining of ABCG2 expression in RCC. a. IHC expression of ABCG2 in adjacent non-tumor tissues (×200). b. IHC expression of ABCG2 in adjacent non-tumor tissue (×400). c. IHC expression of ABCG2 in RCC tissues (×200). d. IHC expression of ABCG2 in RCC tissues (×400). B. ABCG2 expression was over-expressed in most of RCC tissues (53/78) than adjacent non-tumor tissues (25/78). C, D. The expression level of ABCG2 in two RCC cell lines (786-O and Caki-1) and human proximal tubule epithelial cell line HK-2 were examined by qRT-PCR and western blot. *$P<0.05$.

Table 1. Relationship between ABCG2 expression level and clinicopathologic features

Parameters	Group	Total	ABCG2 expression High	ABCG2 expression Low	P value
Gender	Male	38	26	12	0.931
	Female	40	27	13	
Age (years)	<65	29	20	9	0882
	≥65	49	33	16	
Tumor size (cm)	<4 cm	37	24	13	0.579
	≥4 cm	41	29	12	
Clinical stage	I-II	34	18	16	0.013
	III-IV	44	35	9	
Histological grade	G1-G2	32	21	11	0.714
	G3-G4	46	32	14	
Tumor stage	T1-T2	33	18	15	0.030
	T3-T4	45	35	10	
Lymph node metastasis	Absence	59	36	23	0.012
	Presence	19	17	2	

We found that ABCG2 was significantly highly expressed in RCC cells both on mRNA level and on protein level compared with human proximal tubule epithelial cell line HK-2 (**Figure 1C** and **1D**, $P<0.05$). Interestingly, we found a higher ABCG2 expression in poorly-differentiated RCC cell line Caki-1 than the well-differentiated RCC cell line 786-O. Thus, it was concluded that the increased expression of ABCG2 might play an important role in RCC progression and development.

Correlations between ABCG2 expression and clinicopathological features

Table 1 summarized the association between ABCG2 expression and clinico-pathological features of RCC patients. High ABCG2 expression was observed to be associated with clinical stage, tumor
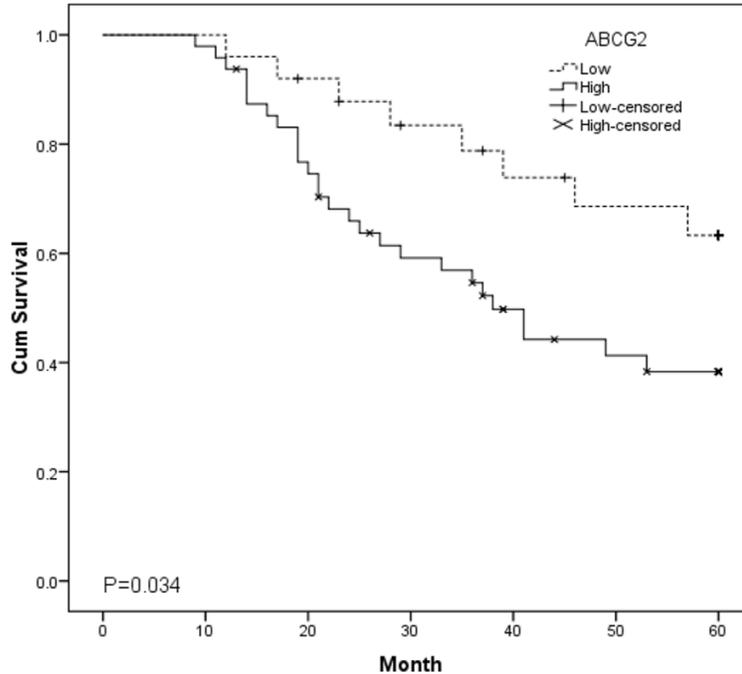
Figure 2. Kaplan-Meiercurves of the overall survival of 78RCC patients. Overall survival rate in patients with highABCG2 expression was significantly lower than that in patients with low ABCG2 expression. P=0.034.

Table 2. Multivariate analyses of prognostic features for overall survival in RCC patients

Parameters	Multivariate analysis		
	Risk ratio	95% CI	P-value
Clinical stage III-IV vs I-II	2.074	1.165-4.618	0.011
Tumor stage T3-T4 vs T1-T2	2.518	1.836-5.628	0.008
Lymph node metastasis Presence vs Absence	3.927	2.124-7.632	0.001
ABCG2 High vs Low	2.842	1.914-6.915	0.003

stage and lymph node metastasis. However, there was no association between ABCG2 expression and other clinical factors, such as gender, age, tumor size and histological grade (P>0.05).

Association between ABCG2 expression and patients' survival

Kaplan-Meier survival analysis and log-rank tests using patient postoperative survival was conducted to further evaluate the correlation between ABCG2 expression and prognosis of RCC patients. From the Kaplan-Meier survival curve, we observed that patients with high ABCG2 expression had significantly shorter overall survival than those patients with low AB-CG2 expression (**Figure 2**; P<0.05, log-rank test). Furthermore, multivariate analysis indicated that relative ABCG2 expression level, clinical stage, tumor stage and lymph node metastasis were each determined to be independent prognostic indicators for the overall survival rate of RCC patients (**Table 2**). These results revealed that ABCG2 expression level can be aplied as a powerful independent prognostic factor.

Up-regulated expression of ABCG2 promotes cell proliferation in 786-O cells

We used recombinant adenovirus Ad5/F35 as a vector to overexpression ABCG2 in well-differentiated 786-O cells, the successful overexpression experiments of ABCG2 in 786-O cells was confirmed by qRT-PCR and western blot (**Figure 3A**, P<0.05). MTT assays were performed to investigate the effect of ABCG2on RCC cell proliferation. The results showed that increased expression of ABCG2 significantly promoted the proliferation of 786-O cells (**Figure 3B**, P<0.05). We further explored the effect of ABCG2 on cell cycle progression. Cell cycle assay indicated that overexpression of ABCG2 resulted a significant decreased in the cellular population in G0/G1 phase but an increase in S and G2/M phase (**Figure 3C**, P<0.05). In addition, we explored the effect of ABCG2 on cell apoptosis. Our results suggested that up-regulated expression of ABCG2 inhibited the apoptosis of 786-O cells (**Figure 3D**, P<0.05).

Down-regulated expression of ABCG2 suppresses cell proliferation in Caki-1 cells

We used pGPU6/GFP/Neo vectors to down-regulated expression of ABCG2 in poorly-differentiated Caki-1 cell, the successful knockdown
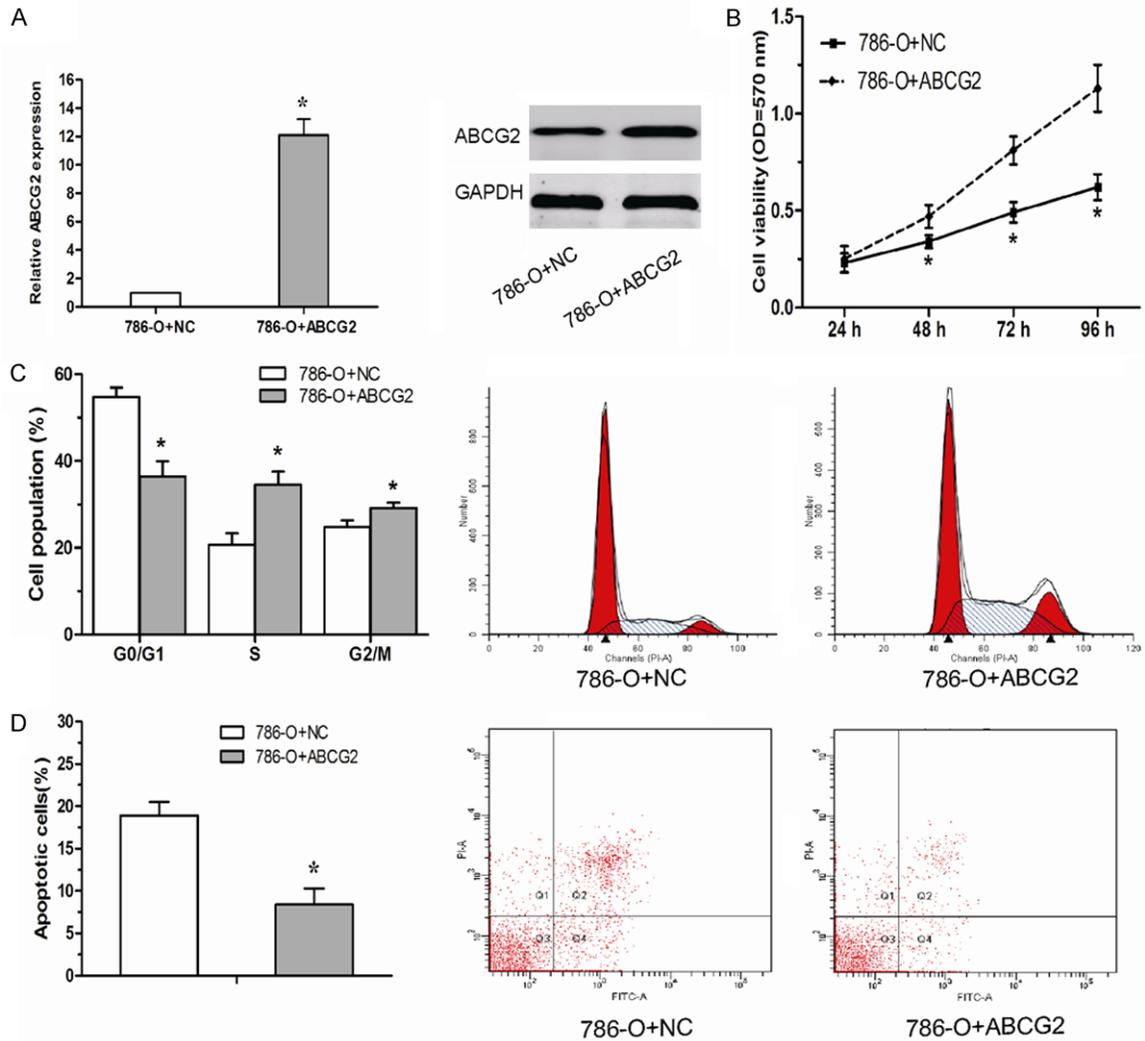
Figure 3. Up-regulated expression of ABCG2 promotes cellproliferation in 786-O cells. A. Recombinant adenovirus Ad5/F35 (Ad5/F35-ABCG2) was transfected for over expressing ABCG2 in well-differentiated 786-O cells. Transfected cells with significantly higher ABCG2 expression were validated by qRT-PCR and western blot. B. MTT assay showed that the ability of cell proliferation of 786-O cells was significantly enhanced by over-expressing ABCG2. C. Flow cytometry showed that ABCG2 overexpression in 786-O cells resulted in a significant decreased in the cellular population in G0/G1 phase but a increased in S and G2/M phase. D. Apoptosis assay found that the apoptosis rate was declined when ABCG2 up-regulated in 786-O cells. *P<0.05.

experiments of ABCG2 in Caki-1 cells was confirmed by qRT-PCR and western blot assay. (**Figure 4A**, P<0.05). MTT assays showed that decreased expression of ABCG2 remarkably inhibited the proliferation of Caki-1 cells (**Figure 4B**, P<0.05). Cell cycle assay indicated that silencing of ABCG2 noticeably induced G1 phase arrest of Caki-1 cells (**Figure 4C**, P<0.05). Furthermore, apoptosis assay showed that decreased expression of ABCG2 might promote apoptosis of Caki-1 cells (**Figure 4D**, P<0.05).

These data suggested that ABCG2 may promote the development and progression of RCC.

Discussion

RCC is one of the leading causes of cancer-related death worldwide, with high possibilities of recurrence and metastasis [14]. Although there has some despite advances in the treatment of RCC over the last decades, the prognosis for RCC patients remains very poor [15]. Recent studies revealed some key elements in
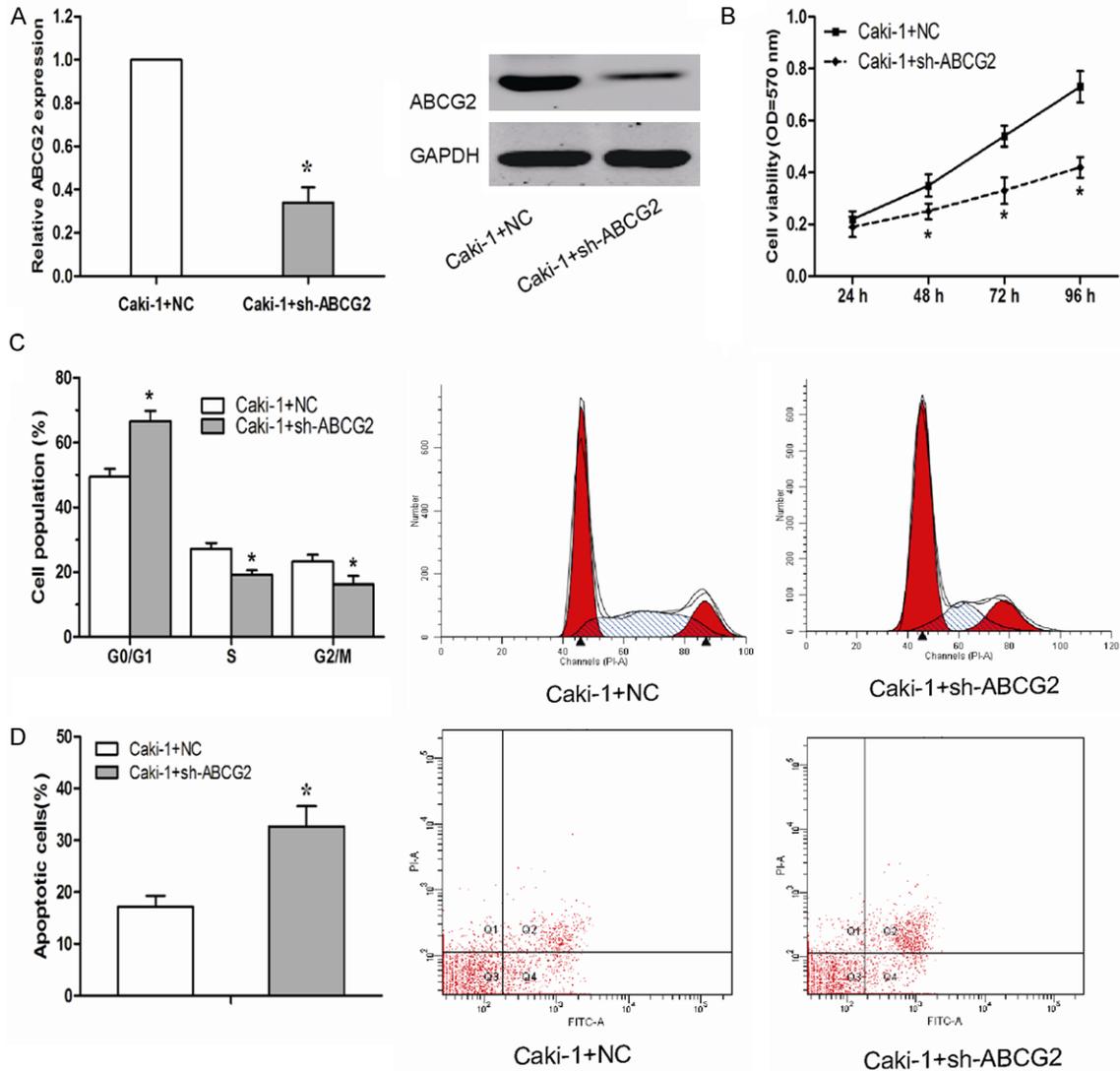
Figure 4. Down-regulated expression of ABCG2 suppresses cell proliferation in Caki-1 cells. A. sh-ABCG2 were trans-fected for knocking down ABCG2 in poorly-differentiated Caki-1 cells. Transfected cells with markedly lower ABCG2 expression were validated by qRT-PCR and western blot. B. MTT assay showed that the capacity of cell proliferation of Caki-1 cells was significantly suppressed by decreased expression of ABCG2. C. Flow cytometry showed that ABCG2 downregulation in Caki-1 cells resulted in a significant increased in the cellular population in G0/G1 phase but a decreased in S and G2/M phase. D. Apoptosis assay revealed that the apoptosis rate was increased when ABCG2 down-regulated in Caki-1 cells. *P<0.05.

RCC initiation and procession, however, the mechanism of the biological and molecular basis in RCC is still unclear.

ABCG2 is one of the functional members of ATP-binding cassette (ABC) transporter family. This series of proteins exist in multiple cellular membranes from plasma membranes to in-tracellular components, including endosome, Golgi, endoplasmic reticulum and mitochondria [16]. One important function of ABC transport-ers is applying intracellular ATP hydrolysis and mediating drug efflux and protecting cells from the damages induced by xenobiotic and toxin [17]. This characteristic carries out a critical mechanism of MDR in human malignancy, and over-expression of ABC transporters is frequent in cancer.

ABC transporters are classified into eight groups (ABCA to ABCH) according to the tran-scriptional sequence and protein structure

[18]. There exist 50 members of ATP transporter genes in human genome, and the functional transcription requires two nucleotide-binding domains and two six-pass trans-membrane domains [19]. As research indicated, in human, only ABCA, ABCB, ABCD and ABCG classes are composed with these functional domains, or at least exist as half-transporters which can play full transporter function when homo- or hetero-dimers formed [20]. Especially, ABCB1, ABCC1, ABCC2 and ABCG2 are notable functional molecules [21]. Previous studies showed that ABCG2 is expressed in human cancer stem cells, and participates in human malignancy process.

In the present study, our results showed the ABCG2 expression was significantly higher than that of in non-tumor tissues. By comparing with the clinicopathologic features of these 78 RCC patients, we found that high ABCG2 expression has a significant associate with advanced clinical stage, elevated tumor stage, lymph node metastasis. Kaplan-Meier analysis showed patients with high ABCG2 expression had significantly shorter overall survival than those patients with low ABCG2 expression. Those data suggested that ABCG2 could be a target correlative with RCC process and prognosis.

We further tested the expression of ABCG2 in three RCC cell lines presenting different differentiation stages. We found that ABCG2 was significantly highly expressed in both two RCC cell lines compared with HK-2 cells. However, the level of ABCG2 was digressive from poorly-differentiated RCC cells (Caki-1) to well-differentiated RCC cells (786-O). This result indicated that ABCG2 as an aberrant over-expressed molecule in RCC, and was concerning with poor cell differentiation.

On basis of our findings, we selectively depleted ABCG2 in poorly-differentiated Caki-1 cells and up-regulated ABCG2 in well-differentiated 786-O cells. As we expected, when ABCG2 expression was significantly inhibited, the cell proliferation was suppressed with a significant arrest of cell cycle in G0/G1 phase. On contrast, 786-O cells with ABCG2 up-regulated were promoted in the capacity of cell proliferation, and the cell cycle was more active that the ratio of S phase and G2/M phase were significantly increased. These results show that ABCG2 is exactly participating in the cell gen-

eration of RCC. By interfering ABCG2 could be a mighty diagnostic and therapeutic method against RCC.

Additionally, when ABCG2 was knocked-down in Caki-1 cells, the apoptosis rate was significantly increased, which indicated a cripple of cell apoptosis resistance by depleting ABCG2. On the contrary, in 786-O cells, the cell apoptosis rate was decreased evidently when ABCG2 up-regulated, which supported that ABCG2 promoted RCC cell apoptosis resistance.

Conclusion

This study showed that ABCG2 was a molecule aberrantly up-regulated in RCC tissues and cell lines, and the higher level of ABCG2 expression in RCC patients was associated with progression and prognosis of RCC patients. Furthermore, our data showed that ABCG2 was a promoter in RCC affecting cell proliferation, and high expression of ABCG2 could provide RCC cells stronger resistance to cell apoptosis. Thus, our study suggested that ABCG2 might provide a novel and promising alternative therapeutic approach for the treatment of RCC.

Acknowledgements

Disclosure of conflict of interest

None.

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