# Overexpression of CYP11A1 recovers cell cycle distribution in renal cell carcinoma Caki-1

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**Research Article**

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# Abstract

### Background

Clear cell renal carcinoma is commonly known for its metastasis propensity to outspread to other organs and does not exhibit symptoms in the early stage. Recent studies have shown that deficiencies in CYP11A1 expression can lead to fatal adrenal failure if left untreated and are associated with downstream regulation in various cancer types. However, the molecular mechanisms of CYP11A1 and kidney cancer proliferation remain unclear.

### Methods

Normal and renal carcinoma cell lines (Hek293 and Caki-1) were transfected with CYP11A1 to stimulate overexpression. Cell cycle distribution was investigated using flow cytometry. Related signaling pathways were analysed by western blot.

### Results

We observed that overexpression of CYP11A1 suppressed the expression of cyclin B1 and cell-division cycle 2 but cyclin-dependent kinases 2 and 4 were not affected. Cancer cell migration and invasion were suppressed along with epithelial-intermediate metastatic markers snail and vimentin. In addition, CYP11A1 overexpressed Caki-1 cell line resulting in downregulation of cdc2/cyclinB1 while increasing phosphorylation of cdc25c, an upstream signal related to G2/M arrest. The markers for intrinsic-mitochondrial apoptosis pathway were not significantly altered. We also identified that the C-Raf/ERK/JNK/p38 pathway is an important mechanism for pro-apoptosis in CYP11A1 overexpressed cell-based models. Our results suggest that the disturbed cell cycle arrest distribution in renal cell carcinoma Caki-1 was recovered by the overexpression of CYP11A1 through G2/M arrest and C-Raf/ERK/JNK pathway.

### Conclusions

Our findings may suggest a promising new therapeutic target to suppress kidney cancer proliferation without affecting normal cells, thus could be used to improve cancer patients' survival rate.


# Background

Renal cell carcinoma (RCC) is a heterogeneous cancer group including clear cell RCC, papillary RCC, and chromophore RCC in which clear cell RCC occurs in renal tubular epithelial cells of the kidneys and is the most common subtype [1]. Incidence of RCC varies greatly around the world, with approximately 403,000 cases along with 175,000 deaths from kidney cancer in 2018 [2]. Early diagnosis of local diseases results in a survival rate of 70–94% [3]. Recent years have seen a rise in major practice-changing trials for the treatment of RCC with the metastatic stage that completely converted the therapeutic goal for this disease. The vascular endothelial growth factor (VEGF) pathway has been investigated as a key mediator

in the development of RCC [4]. Some types of inhibitors that block immune responses such as nivolumab and ipilimumab are now accepted for primary treatment of RCC patients in the metastasis stage [5]. This has improved overall survival throughout multiple clinical trials and made a far-reaching change in the therapeutic landscape with the metastatic situation. Results from various clinical trials demonstrated valuable advantages of the combination of VEGF therapy and immune response inhibition, renewing hope for the treatment of most-at-risk RCC [6]. The role of surgery for metastatic RCC has been defined and the initial extirpation tumor did show clinical advantages. However, even if detected early, RCC patients almost certainly cannot be cured with nephrectomy [7]. Furthermore, metastases is often the main cause of death in these patients, due to long-term risk to the opposite kidney from local or systemic recurrence. Abnormal lipid metabolism was previously proposed as the clinical outcome of metastatic RCC. Pregnenolone is an up-stream steroid that acts as a precursor or metabolism component in the biosynthesis of other down-stream steroid hormones, including the progestogens, estrogens, androgens, glucocorticoids, and mineralocorticoids [8]. Glucocorticoids are frequently applied as a therapeutic or clinical treatment for a variety of cancers as well as RCC [9]. Glucocorticoid-related compounds including triamcinolone, dexamethasone, and fluorometholone were identified as drug candidates and confirmed to weaken mobility, invasiveness, and persuade mesenchymal to epithelial-like transition of RCC [10].

Cytochrome P450s (CYPs) are the major source of variability in gene expression, pharmacokinetics, and drug delivery. Several previous studies have demonstrated that the CYP11 family plays a key role in steroid biosynthesis associated with numerous cancers. Recent studies have shown that expression of CYP11A1 is downregulated in various cancer types [11]. Clear cell renal carcinoma is commonly known to tend to metastasize to other organs and displays no symptoms in the early stages. However, the molecular mechanisms of CYP11A1 and kidney cancer proliferation remain unclear. Here, we aimed to investigate the associations between CYP11A1 and kidney cancer. We studied the effects of overexpression of CYP11A1 on kidney cancer cell lines (Caki-1) compared to normal epithelial kidney cell lines (HEK293).

## Materials And Methods

# Plasmid DNA purification and transfection

CYP11A1 cDNA with a pCMV-SPORT5 vector (Clone ID: hMU004796) was used for the transfection. The clone was provided by Korea Human Gene Bank, Medical Genomics Research Center, KRIBB, Korea. LB broth media (25g/L) was prepared by adding an antibiotic including 100 μg/mL ampicillin. According to the manufacturer's instructions, plasmid DNA was isolated using Plasmid Midi Kit (Qiagen, MD, USA) after culturing competent cells overnight. CYP11A1 cDNA concentration was measured by Nano-drop (Thermo Fisher Scientific, MA, USA). Lipofectamine 3000 Transfection Reagent (Invitrogen, MA, USA) was used to transfect cells with CYP11A1, following Invitrogen’s protocol. The transfection efficiency of CYP11A1 was confirmed by western blotting; an increasing amount of plasmid DNA (1000 ng, 2000 ng, and 4000 ng) was tested for the optimal concentration.

# Cell culture and protein quantification

Human normal epithelial kidney (HEK293) and renal cancer (Caki-1) cell lines were purchased from the Korea Cell Line Bank (KCLB) and cultured in Dulbecco's modified Eagle medium (DMEM)-medium-high glucose (GenDEPOT) containing 10% fetal bovine serum (GIBCO) and 1% penicillin/streptomycin (GIBCO). Culture plates were maintained at 37°C with 5% $CO_2$ in a humidified incubator. For protein quantification, cultured cells were rinsed twice with ice-cold phosphate-buffered saline (PBS) and then lysed using Pierce™ RIPA Lysis Buffer (Thermo Fisher Scientific) accompanied with Protease/phosphatase Inhibitor Cocktail (Cell Signaling Technology, MA, USA). Cell lysates were incubated at 4°C for 20 min and centrifuged at 10,000 × *g* for 20 min at 4°C. The supernatant was collected and Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific) was used to measure the protein concentrations according to the manufacturer's protocol.

# Wound healing, invasion, and reactive oxygen species assay

Wound-healing assay was performed for 24 h after transfection. Uniform wounds were created across the confluent cell monolayer using a sterilized 200 µL pipette tip. Cell debris was removed by extensive washing with 1× PBS and cells were allowed to migrate into the wound area for 24 h at 37 ℃. Digital photos were taken at 0 and 24 h after the scratch. ImageJ 1.53a (National Institutes of Health, Bethesda, MD, USA) was used to calculate the width of the wound at four locations within each well. The percentage of wound closure was quantified by dividing the healed wound width at 24 h by the initial width. Each experiment was performed three times using triplicate wells.

Matrigel-invasion assay was performed using Transparent PET Membrane chambers with an 8.0 µm pore size. Cells ($1 \times 10^5$ cells/well) were suspended in 100 µL DMEM serum-free media and added to the upper chamber, coated with Matrigel (BD Biosciences, NJ, USA). The lower cavity of the transwell was filled with 500 µL 5% FBS medium containing fibronectin (5 µg/mL) as a chemoattractant. After 24 h, cells were fixed with 4% formaldehyde and permeabilized with 100% methanol, followed by staining with Giemsa (Merck, NJ, USA) for 15 min at room temperature. The upper chamber was cleaned with a cotton swab. Cells were counted using a fluorescence microscope (Nikon Eclipse TE 2000-U) by randomly selecting multiple fields per membrane. Invasiveness of cells was expressed as the mean number of cells that invaded the lower capacity of the chambers. Each experiment was performed in triplicate.

EZ-Hydrogen Peroxide/Peroxidase assay kit (DooGen Bio, Seoul, Korea) was used for the measurement of oxidative stress. Cell culture supernatant was centrifuged at 10,000 × *g* for 5 min to remove all insoluble particles. The standard curve was generated using the same non-conditioned media according to the manufacturer's instructions. Absorbance emissions were measured by using a spectrophotometer (Bio-Rad, CA, USA) at 560 nm.

# Flow cytometry analysis for cell cycle and apoptosis

The cultured cells ($3 \times 10^6$ cells/mL) were harvested and washed with PBS before exposure to staining buffer. Annexin V and PI staining were performed using the Alexa Fluor® 488 Annexin V/Dead Cell Apoptosis Kit (Invitrogen), according to the manufacturer's instructions. For cell cycle analysis, harvested cells were fixed with 70% ethanol, washed twice with cold PBS, and centrifuged to discard the supernatant. The pellet was re-suspended in PI staining buffer, containing a mixture of 50 µg/mL PI and RNase A/T1 (0.5 µg/mL) and incubated at 4 ℃ overnight in the dark. The data were analyzed using a FACS Calibur instrument and Cell Quest software (BD Biosciences). Flow cytometry analysis was performed at excitation wavelengths of 488 and 535 nm to detect apoptosis; 5,000 cells HEK293 and Caki-1 cells were used to analyze apoptosis. DNA content and apoptosis of HEK293 and Caki-1 cell were analyzed using FlowJo Software (Tree Star Inc., Ashland, OR, USA). The threshold trigger was set on forward and side scatters to eliminate background noise and to analyze cells only. The fluorescence signal was calculated as the mean intensity in arbitrary units (au).

# Western blot analysis

Protein samples were separated by one-dimensional 12% Tris-glycine SDS-PAGE, then transferred to nitrocellulose membranes (Bio-Rad). For membrane blocking, 1× TBST containing 5% skim milk was incubated for 1 h following the washing with 1× TBST. Nitrocellulose membranes were incubated overnight with the primary antibodies 1:1000 in 5% BSA at 4 ℃ in the dark. After washing three times with TBST for 5 min, 1:5000 horseradish peroxidase-conjugated secondary antibodies (Cell Signaling Technology) were added to 5% skim milk and membranes were incubated at room temperature for 1 h. Blots were detected using a chemiluminescence SignalFire™ ECL Reagent (Cell Signaling Technology) by Ez-Capture MG system (ATTO, NY, USA) and relative intensity of the western blot bands was measured using ImageJ 1.53a (National Institutes of Health). GAPDH antibody was used as loading control and purchased from GeneTex (CA, USA). The antibodies used in this study included anti-CYP11A1, anti-Snail, anti-Vimentin, anti-Cdk2, anti-Cdk4, anti-phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204), anti-pp44/42 MAPK (Erk1/2), anti-cdc25c, and anti-phospho-cdc25c Ser216. Cell Cycle/Checkpoint Antibody Sampler Kit, Apoptosis Antibody Sampler Kit, and Pro-Apoptosis Bcl-2 Family Antibody Sampler Kit were also purchased from Cell Signaling Technology.

# Statistical analysis

All data were replicated at least three times and were represented by the mean ± SD with Origin 2022 (OriginLab, MA, USA). One-way ANOVA and Student's t-test were performed to compare the data between groups. Western blot results were reported as fold changes compared to control samples and GAPDH was used as the loading control. Differences were considered significant at $*P \leq 0.05$, $**P \leq 0.01$, and $***P \leq 0.005$.

## Results

# Overexpression of CYP11A1 inhibits the EMT process and cancer cell mobility

HEK293 cell line is a normal kidney epithelial cell model used as control, while the Caki-1 cell line represents an in vitro model system for a specific type of kidney cancer that exhibits extensive vasculature and the common RCC. To investigate the effects of CYP11A1 overexpression on kidney cancer cells, cell proliferation, invasion, and viability were measured. Western blotting was used to confirm the overexpressed level of CYP11A1 and the expression level of EMT protein markers (Fig. 1A, 1B). Our results revealed a significant reduction in Vimentin (Fig. 1C) and Snail (Fig. 1D) expression in Caki-1 cells after CYP11A1 transfection, while the normal kidney HEK293 cells showed a reversible process with the increase in protein levels. A rapid decrease in mesenchymal protein level in the cancer cell line indicates that CYP11A1 overexpression in cancer cells is associated with the inhibition of cell mobility. This in vitro transfection system has been used for the molecular mechanism study of CYP11A1 effect on inhibition of cancer cell mobility and proliferation. Migration distances were displayed separately during periods 0, 5 and 24 h (Fig. 1E). The wound-healing assay showed no significant changes in the HEK293 cell line with CYP11A1-transfection. However, the non-transfected Caki-1 cell line migrated and covered approximately 90% of the wound region. Open space observed was approximately 75% between the wound edges in CYP11A1-transfected Caki-1 cells (Fig. 1F), indicating that cell migration was highly inhibited after CYP11A1 overexpression in cancer cells only. The matrix-gel invasion assay showed that invasion of Caki-1 cells was significantly reduced from $1.87 \times 10^3$ cells/field in the control group to $0.03 \times 10^3$ cells/field after CYP11A1 overexpression (Fig. 1G). CYP11A1-overexpressed Caki-1 cell line was found to have a rapidly decreasing number of invasive cells compared to the non-transfected control group (Fig. 1H). To determine the growth inhibitory effect of CYP11A1 in cells, cytotoxicity was detected. Our results showed that CYP11A1 did not inhibit HEK293 cell proliferation, while significant reduction in Caki-1 cell viability was evident after 24-h transfection (Fig. 1I). Together with the migration, invasion and viability assay findings, these results confirm that CYP11A1 overexpression inhibits cancer cell proliferation in kidney cancer cells.

# CYP11A1 overexpression recover cell cycle distribution in cancer cell line

To explore the effects of CYP11A1 overexpression on cancer cell mobility and proliferation, cell cycle distribution profiles were analyzed using flow cytometry (Fig. 2A, 2B). In the Caki-1 group, the majority of cells were increased at G2/M phase after 24-h CYP11A1 transfection, while the percentage of HEK293 cells in all phases displayed no significant difference (Fig. 2C). These results indicated that CYP11A1 overexpression arrested the cell cycle at G2/M phase in cancer cells and results in similar distribution of cell cycle to the control HEK293 cell line. The distribution of non-transfected Caki-1 cells was reduced compared with that of normal kidney epithelial cells and was recovered by CYP11A1 transfection.

During cell cycle progression, the G2/M phase is triggered by activation of the cyclin B1/Cdc2 kinase complex, which is controlled by various phosphorylation-dephosphorylation factors. To study the mechanism of CYP11A1-overexpression induction on G2/M phase arrest, the cyclin-dependent kinases (CDK) family and cell cycle regulatory proteins were examined using western blotting (Fig. 2D, 2E). The

results showed that CDK2 and CDK4 expressions were not outstandingly changed in either group. In the CYP11A1-overexpressed HEK293 cells, cdc25C expression level was decreased, resulting in the upregulation of p-cdc25c and an increased amount of cylinB1/cdc2 was detected. However, cyclin B1 and cdc2 expression levels were downregulated in Caki-1 cells after CYP11A1 transfection, which indicated that the cyclin B1/Cdc2 complex could be suppressed and cell cycle arrested in the G2/M phase. All these observations confirmed that CYP11A1 overexpression induces G2/M phase arrest in kidney cancer cells by regulating cell cycle–related kinases. The regulated cell cycle distribution is interestingly recovered to that of normal kidney epithelial cells (HEK293).

# CYP11A1 promoted ROS and activated apoptosis

Cytochrome P450 is known to comprise a series of cellular oxidation reactions that facilitate oxidative transformation and contribute to the production of intracellular ROS. Whereas, ROS generated by CYP11A1 in mitochondria could induce oxidative stress and promote cell death [12]. Our study demonstrated a slight rise in the level of ROS by CYP11A1 overexpression in both HEK293 and Caki-1 cell lines (Fig. 3A). Following transfection with CYP11A1 for 24 h, the apoptotic rate was assessed by FACS using Annexin V/PI double staining (Fig. 3B). The results revealed that overexpression of CYP11A1 significantly increased the apoptotic rate of Caki-1 cells from 2.66–12.8%, while the apoptotic rate of control HEK293 cells was 5.91% and 5.05% in CYP11A1-overexpressed HEK293 cells (Fig. 3C). To identify the mechanism of CYP11A1-induced apoptosis, we performed western blot analysis of apoptosis-associated proteins 24 h after transfection. The activity of caspase 3 and 7 remained stable, whereas caspase 9 and PARP were downregulated in Caki-1 cells (Supplementary Data Fig. 1). However, the expression level of cleaved caspase 3-7-9, PARP and Bcl-2 families could not be determined in any of the groups. These results confirmed that the association between CYP11A1 and apoptosis was not subjected to caspase-related death receptor (extrinsic) and mitochondria-dependent (intrinsic) pathways. We next explored the MAPK pathway that plays a main role in the regulation of proliferation, differentiation and survival of CYP11A1-driven ROS in apoptosis. The phosphorylation levels of MAPK family members including p-C-Raf, p-ERK, ERK, JNK, and p38 were examined between CYP11A1-transfected and non-transfected groups (Fig. 3D, 3E). CYP11A1-overexpressed Caki-1 cells showed maintained activation of JNK and p38 along with inhibition of p-ERK and p-C-Raf, which supports our hypothesis that CYP11A1-induced oxidative stress may activate JNK-p38 pathways and promote apoptosis in the cancer cell line. The inhibition of p-ERK and p-C-Raf may be related with the reduced cell proliferation in kidney cancer cells.

# Discussion

Decreased expression of CYP11A1 influences the biosynthesis of steroid hormones and is related to several cancers. In recent years, the majority of studies have investigated the role of steroid hormones in various cancer types such as breast [13, 14], prostate [15], lung [16], liver [17], and colon cancer [18]. However, the molecular mechanisms by which CYP11A1 exerts its anticancer effects on RCC are largely

unknown. CYP11A1 has been demonstrated to trigger excessive oxidative stress in mitochondria against the human placental cell line originating from choriocarcinoma [19].

Our results suggested that CYP11A1 can suppress the EMT process by downregulating Snail and Vimentin levels. This effect was more pronounced in the Caki-1 cell line as this is a model of metastasis in RCC, which can pattern a polarized layer with biological functional and admirably-differentiated kidney tissue [20].

The transition from G2 to M phase was achieved by activating the cdc2/cyclin B complex by upregulation of cdc2 induced by phosphorylating cdc25c, while accreted cdc25c phosphorylation could lead to cell cycle arrest in the G2 phase [21]. Our study found that CYP11A1 induced a remarkable increase in p-cdc25c, and a decrease in cyclin B1 and cdc2 in the Caki-1 cell line, while HEK293 cell lines exhibited reversible expression of these kinases. These results may imply that CYP11A1 is involved in G2/M phase arrest by positively suppressing the cdc2/cyclin B complex pathway with the induction of cdc25c phosphorylation. The regulation of cell cycle distribution after overexpression of CYP11A1 resulted in recovery of cell cycle distribution to normal kidney epithelial cells (HEK293). In RCC, transglutaminase 2 intersects with p53, leading to the depletion of p53 and apoptosis avoidance [22]. Although the loss of p53 function is frequently related to induction of cell cycle arrest [23], we did not observe p53 expression in Caki-1 cell lines. Programmed cell death or apoptosis, is mainly regulated by intrinsic, extrinsic, and MAPK pathways, which are activated by the disruption of intracellular homeostasis, DNA damage, and stress response. The Bcl-2 family includes various pro-apoptotic proteins such as Bax, Bad, and Bim; is the main regulator of the intrinsic pathway. In RCC, incongruity in Bcl-2 expression was reported according to cancer cell type and the majority were negatively expressed [24]. Normally, activation of Bax and Bak proteins can promote the release of cytochrome C following the extrinsic activation of caspase-3, 7, 9, and PARP, thus leading to apoptosis [25]. Our study further observed a rapid decrease in caspase-9 and PARP expression despite the lack of any significant difference in caspase-3 and 7 after CYP11A1 transfection. These results revealed that Bcl-2 and caspase family activity was not closely related to CYP11A1-induced apoptosis. Recent studies have shown that the cellular signal transduction pathway C-Raf/MEK/ERK can act on some key effectors to regulate cell proliferation [26, 27]. ERK1/2 activation usually leads to cell proliferation, resulting in initial hallmarks of various cancer activities. Consequently, factors involved in the ERK pathway are considered prospective therapeutic targets for cancer. A large number of regulators and inhibitors of this pathway are already being used in clinical trials. Notably, ERK1/2 activation can cause cell death, resulting in pro-apoptotic functions [28]. We found that CYP11A1-overexpressed Caki-1 cells blockaded C-Raf/ERK pathway by downregulating the phosphorylation of both C-Raf and ERK1/2. In response to oxidative stress, MAPK family members are crucial contributors to apoptosis by various signaling pathways. For example, upregulation of JNK and p38 expression was considered stress responsive and thus promote programmed cell death [29].

In conclusion, we demonstrated for the first time that CYP11A1 overexpression reversed the EMT process in Caki-1 cells, induced G2 phase arrest, generated ROS, and promoted apoptosis via the MAPK pathway (Fig. 4). All these findings suggest that CYP11A1 might have an anti-tumor effect on RCC, which could be

an essential target leading to the removal of cancerous cells without affecting or minimalizing stress on surrounding normal cells. However, the main regulator of the CYP11A1-mediated signaling pathway suppressing cancer cell proliferation still requires further study.

# Abbreviations

**Bcl-2** B-cell lymphoma 2

**BSA** bovine serum albumin

**CA** California

**Caspases** cysteine–aspartic proteases

**CDC2** cell-division cycle 2

**CDK2** cyclin-dependent kinase 2

**CDK4** cyclin-dependent kinase 4

**EMT** epithelial to mesenchymal transition

**ER** endoplasmic reticulum

**ERK** extracellular signal-regulated kinases

**JNK** c-Jun N-terminal kinases

**MA** Massachusetts

**MAPK** mitogen-activated protein kinase

**MD** Maryland

**NJ** New Jersey

**PBS** phosphate-buffered saline

**RCC** renal cell carcinoma

**TBS** tris-buffered saline

**TBST** TBS with 0.5% Tween 20

**WST** water soluble tetrazolium salt

# Declarations


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### Conflicting interests

The authors declare that they have no conflict of interest.

### Ethics approval and consent to participate

Not Applicable

### Consent for publication

All authors reviewed the manuscript and consent for publication.

### Availability of data and materials

Not Applicable

### Competing interests

Not Applicable

### Author's contributions

M-J.K. and H.T.M.O. designed and conceptualized the study; H.T.M.O., T-H.K., and E.A. performed the experiments and analysis; M-J.K., H.T.M.O. and T-H.K. wrote the manuscript; M-J.K. and J-C.P. supervised the study and edited manuscript.

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# Figures

### Figure 1

Effects of overexpression of CYP11A1 on cancer (Caki-1) and normal kidney (HEK293) cell lines. (**A)** Western blot of CYP11A1, Vimentin, and Snail. (**B, C, D)** Protein expression levels, normalized to GAPDH.

Inhibition of cell migration (**E, F)**, invasion (**G, H)**, and cell viability proportion (**I)** after CYP11A1 transfection.

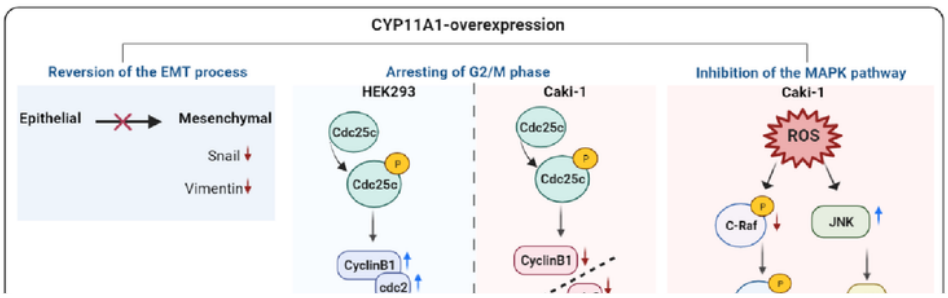
## Figure 2

G2/M phase arrest by CYP11A1-overexpressed cancer cell line. (**A)** DNA content of PI-stained cells detected using flow cytometry. (**B)** The percentage of cells in each phase and (**C)** the total cell proportion in G2/M phase. (**D)** Western blotting of the expression of CDK2, CDK4, cdc25c, p-cdc25c, cyclinB1, and cdc2. (**E)** The expression levels of these cyclin-dependent kinases were normalized to GAPDH.

## Figure 3

CYP11A1 promotes apoptosis by inhibition of MAPK signaling. (**A)** ROS levels determined by the concentration of hydrogen peroxide, generated under conditions of oxidative stress. (**B)** Apoptosis of HEK293 and Caki-1 cells transfected with CYP11A1 for 24 h was detected using Annexin V-PI double staining followed by flow cytometry. (**C)** The percentage of dead cells in each group. (**D)** Western blotting of the expression of p-C-Raf, ERK, p-ERK, JNK, and p38. (**E**) The expression levels of these proteins were normalized to GAPDH.

**Figure. 4**




## Figure 4

Schematic illustration displaying the mechanism of anti-cancer effect of CYP11A1: CYP11A1 reverses the EMT process, induces the G2/M phase arrest by suppression of cdc2/cyclinB1 complex, and inhibits the MAPK pathway by generating ROS.

# Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- BMCSupplementaryMJK.pdf