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Methylenetetrahydrofolate Dehydrogenase 1 (MTHFD1) is Underexpressed in Clear Cell Renal Cell Carcinoma Tissue and Transfection and Overexpression in Caki-1 Cells Inhibits Cell Proliferation and Increases Apoptosis

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Background: The aims of this study were to investigate the expression of methylenetetrahydrofolate dehydrogenase 1 (MTHFD1) in human tissue containing clear cell renal cell carcinoma (CCRCC) compared with normal renal tissue, and the effects of upregulating the expression of MTHFD1 in the human CCRCC cell line, Caki-1.

Material/Methods: Tumor and adjacent normal renal tissue were obtained from 44 patients who underwent radical nephrectomy for CCRCC. Caki-1 human CCRCC cells were divided into the control group, the empty vector (EV) group, and the plasmid-treated group that overexpressed MTHFD1. MTHFD1 mRNA and protein levels were measured by quantitative real-time polymerase chain reaction (qRT-PCR) and Western blot, respectively. The cell counting kit-8 (CCK-8) assay measured cell viability. Flow cytometry evaluated apoptosis and the cell cycle. Western blot measured the protein levels of MTHFD1, Bax, Bcl-2, Akt, p53, and cyclin D1, and qRT-PCR determined the gene expression profiles.

Results: MTHFD1 mRNA and protein levels in CCRCC tumor tissues were significantly lower compared with adjacent normal renal tissue. MTHFD1 over-expression in Caki-1 cells inhibited cell proliferation, arrested cells in the G1 phase, increased cell apoptosis, and upregulated gene and protein expression of Bax/Bcl-2 and p53, and inhibited p-Akt, and cyclin D1.

Conclusions: MTHFD1 was underexpressed in CCRCC tissue when compared with normal renal tissue. MTHFD1 transfection of human CCRCC Caki-1 cells *in vitro* inhibited cell proliferation and promoted apoptosis, associated with reduced expression of cyclin D1, reduced Akt phosphorylation, and increased expression of Bax/Bcl-2 and p53.

MeSH Keywords: Apoptosis • Cell Cycle • Kidney Neoplasms • Methylenetetrahydrofolate Dehydrogenase

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Background

Worldwide, primary renal cell carcinoma (RCC) accounts for 2–3% of all malignant tumors in humans, resulting in 180,000 new cases and 90,000 deaths each year, with an increasing incidence [1,2]. The most common histopathological type of RCC is clear cell renal cell carcinoma (CCRCC), which accounts for between 80–90% of all renal cancers. RCC is an epithelial-derived tumor that originates in the renal tubular epithelial system. Currently, several signaling pathways have been reported in CCRCC to be associated with tumor stage and prognosis, but there are no molecular diagnostic, prognostic, or therapeutic biomarkers for CCRCC that are currently used clinical practice [3–5].

Methylenetetrahydrofolate dehydrogenase 1 (MTHFD1) is a hinge enzyme in the folic acid metabolic pathway. Folic acid metabolism involves multiple pathways leading to nucleotide formation and DNA methylation. Specifically, serine hydroxymethyl transferase (SHMT) in the cytoplasm can transfer one-carbon units from serine to tetrahydrofolate (THF) to form glycine and 5,10-methylenetetrahydrofolate (5, 10 methylene-THF). The folate metabolic pathway includes a complicated network of enzymes, including MTHFD1L, MTHFD2, and MTHFD1 [6–10]. Cells mainly use the mitochondrial enzymes, MTHFD2, and MTHFD1L for metabolism, but if these enzymes are inhibited, cells can compensate by using cytoplasmic MTHFD1 [11]. MTHFD1 encodes the activity of three enzymes, including 5,10-methenyltetrahydrofolate cyclohydrolase, 5,10-methenyltetrahydrofolate dehydrogenase, and 10-formyltetrahydrofolate synthetase, and catalyzes three reversible reactions in the pathway of conversion of THF. MTHFD1 can also produce 5,10-methenyltetrahydrofolate indirectly by providing single carbon units for methylation reactions.

In the folate pathway, some important enzymes have been considered as potential cancer-specific therapeutic targets. In esophageal squamous cell carcinoma, the mRNA and protein expression of MTHFD1L have been shown to be overexpressed, and reduced expression of MTHFD1L suppressed the proliferation of the esophageal squamous carcinoma cell line, TE-1, *in vitro* [12]. Similarly, MTHFD2 mRNA and protein have been shown to be overexpressed in human cancer, including breast cancer and is associated with poor survival in breast cancer [7].

MTHFD1 plays a key role in nucleotide synthesis. Previous studies have reported that polymorphisms of MTHFD1 are associated with impaired DNA synthesis, cell division and development, and oncogenesis, but the findings of these studies have been inconsistent [13–15]. The MTHFD1 polymorphic 1958AA variant has been shown to significantly increase the risk of developing gastric cancer, when compared with the 1958GG or 1958AG genotypes [16]. However, Moruzzi et al. showed that the expression of the MTHFD1 1958AA polymorphism was associated with a reduced risk of developing colon cancer, and also showed a significant difference between MTHFD1 1958G>A genotypes in patients with cancer compared with normal subjects [17]. Previous authors have proposed that reduced synthase activity was could be a mechanism for MTHFD1 activity in cancer [18]. The role of MTHFD1 in renal carcinoma remains unknown, as there have been no previous studies on the mechanism of MTHFD1 in renal carcinoma, including CCRCC.

Therefore, the aims of this study were to investigate the expression of MTHFD1 in human tissue containing clear cell renal cell carcinoma (CCRCC) compared with normal renal tissue, and the effects of upregulating the expression of MTHFD1 in the human CCRCC cell line, Caki-1, *in vitro*, and the possible role of Akt-p53-cyclin D1 signaling.

Material and Methods

Patient samples and tissue preparation

Fresh tissue samples were obtained from 44 patients who underwent radical nephrectomy for histologically diagnosed clear cell renal cell carcinoma (CCRCC) at Chongqing Three Gorges Central Hospital from May 2009 to July 2012. Fresh renal carcinoma tissues and adjacent normal tissues were taken from specimens after radical nephrectomy. Two sets of the paired tissues were obtained from all patients. A piece of tissue was routinely stored in 4% formaldehyde solution for histopathological diagnosis, and other piece of tissue was rapidly frozen by direct immersion in liquid nitrogen and stored at –80°C, then processed for quantitative real-time polymerase chain reaction (qRT-PCR) and Western blot analysis. The Ethics Committee of the Chongqing Three Gorges Central Hospital approved the study. Written informed consent was provided by all patients who participated in the study.

Cell culture

The human clear cell renal cell carcinoma (CCRCC) cell line, Caki-1 was obtained from the Cell Repository of Shanghai Institute of Life Sciences, Chinese Academy of Sciences (Shanghai, China). Cells were cultured in RPMI 1640 medium with 10% (v/v) fetal calf serum (FCS) and 100 U/ml penicillin or 100 µg/ml streptomycin in an incubator at 37°C containing with a humidified atmosphere of 5% CO_2. When the cells in the culture flask reached 80% confluence, the culture solution was discarded, washed once, and 2.5g/L trypsin was added at a dilution of 1: 5.

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Table 1. Primers used in qRT-PCR.

Gene	Primer	Sequence
MTHFD1	Forward	5'-TTTGGCTTGAAGAGGGACATGAGG-3'
	Reverse	5'-AGGACCTTAGAGGACTAGCAGGGT-3'
Bax	Forward	5'-TCATGGGCTGGACATTGGAC-3'
	Reverse	5'-GAGACAGGGACACAGTCGC-3'
Bcl-2	Forward	5'-GTGAAGTCAACATGCCTGCC-3'
	Reverse	5'-ACAGCCTGCAGCTTTGTTTC-3'
p-Akt	Forward	5'-CGAGGAGGAGGTGTATCA-3'
	Reverse	5'-CGGTAAAGGCACGTTCGGTA-3'
p53	Forward	5'-CGCGGATCCATGGAGGAGCCGCAG-3'
	Reverse	5'-CCCAAGCTTGTCAGTCTGAGTCAGGCCC-3'
Cyclin D1	Forward	5'-TCTAAGATGAAGGAGACCATC-3'
	Reverse	5'-GCGGTAGTAGGACAGGAAGTTGTT-3'
GAPDH	Forward	5'-TCACTCACATTTGCCTCCCTC-3'
	Reverse	5'-CTGAAGGGCAAGGCCATGTA-3'

Quantitative real-time polymerase chain reaction (qRT-PCR)

The method of quantitative real-time polymerase chain reaction (qRT-PCR) was performed on samples of different groups to examine the gene expression profiles of MTHFD1, Bax, Bcl-2, Akt, p53, cyclin D1. Total RNA was extracted from tissues or cultured cells with using Trizol (Invitrogen, San Mateo, CA, USA) according to manufacturer's instructions and reversed transcribed to cDNA by PrimeScript™ reverse transcription (RT) reagent kit (TaKaRa, Otsu, Shiga, Japan), performed at 37°C for 14 min and 85°C for 5 s. The PCR reaction system contained 10.0 μL SYBR Fast qPCR Mix, 0.8 μL sense and antisense primers at 10 μM, 2.0 μL and cDNA, and 6.4 μL RNase-free H_2O. The PCR reaction was performed at 95°C for 10 min, followed by 30 cycles of 95°C for a further 10 s, 60°C for 20 s, and 75°C for 15 s. The primer sequences are summarized in Table 1. Amplified products were electrophoresed through 1.2% agarose gels. The amount of RNA was calculated using the $2^{-\Delta\Delta CT}$ method with GAPDH as an internal control [19].

Western blot

Total protein was extracted from tissues or cultured cells using lysis buffer consisting of 10 mM Tris-HCl pH 7.4, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1 mM dithiothreitol (DTT) and 0.2 mM phenylmethylsulfonyl fluoride (PMSF) and protein inhibitor cocktail, then separated on a 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel. Proteins were then transferred onto a polyvinylidene difluoride (PVDF) membrane and were blocked in 5% dried milk powder, with Tris-buffered saline (TBS), and Tween-20. Proteins incubated with primary antibodies, including: rabbit anti-Bcl-2 (ab32124) (1: 1000) (Abcam, Cambridge, MA, USA), anti-Bax (ab32503) (1: 1500) (Abcam, Cambridge, MA, USA), anti-MTHFD1(ab103698) (1: 1000) (Abcam, Cambridge, MA, USA), anti-p53 (ab32049) (1: 1000) (Abcam, Cambridge, MA, USA), anti-cyclin D1 (ab134175) (1: 1000) (Abcam, Cambridge, MA, USA), anti-GAPDH (ab8245) (1: 1000) (Abcam, Cambridge, MA, USA), anti-p-Akt (ab38449) (1: 1000) (Abcam, Cambridge, MA, USA), washed with TBST, and then incubated with HRP-conjugated goat anti-rabbit IgG secondary antibodies (Proteintech, Rosemont, IL, USA). The blot was visualized via enhanced chemiluminescence (ECL) (Thermo Fisher Scientific, Inc., Waltham, MA, USA). An ECL system (Amersham, GE Healthcare, Chicago, IL, USA) was used to detect the bands. The density of the blots was read with the Quantity One software version 2.4 (Bio-Rad, Hercules, CA, USA) [20].

Cell transfection and grouping

Overexpression of MTHFD1 and unspecific empty vector (EV) plasmids were purchased from (Genepharma, Shanghai, China). Caki-1 human CCRCC cells were seeded in 6-well plate on the previous day before transfection and divided into three groups, including the control group (0.1% PBS), the empty vector (EV) group, and the MTHFD1 group with overexpression using the MTHFD1 plasmid. Transient transfection was performed using Lipofectamine 2000 (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's protocol. A total of 20 μM over-/EV/control RNA and 1 μL Lipofectamine 2000 were added to serum-free medium and incubated at 25°C for 20 min and then mixed with the cells cultured in serum-free RPMI 1640 medium (Thermo Fisher Scientific, Waltham, MA, USA). After 4 hours in culture, the fluid medium was changed back to RPMI 1640 medium containing 10% fetal bovine serum (FBS) (Thermo Fisher Scientific, Waltham, MA, USA) [21].

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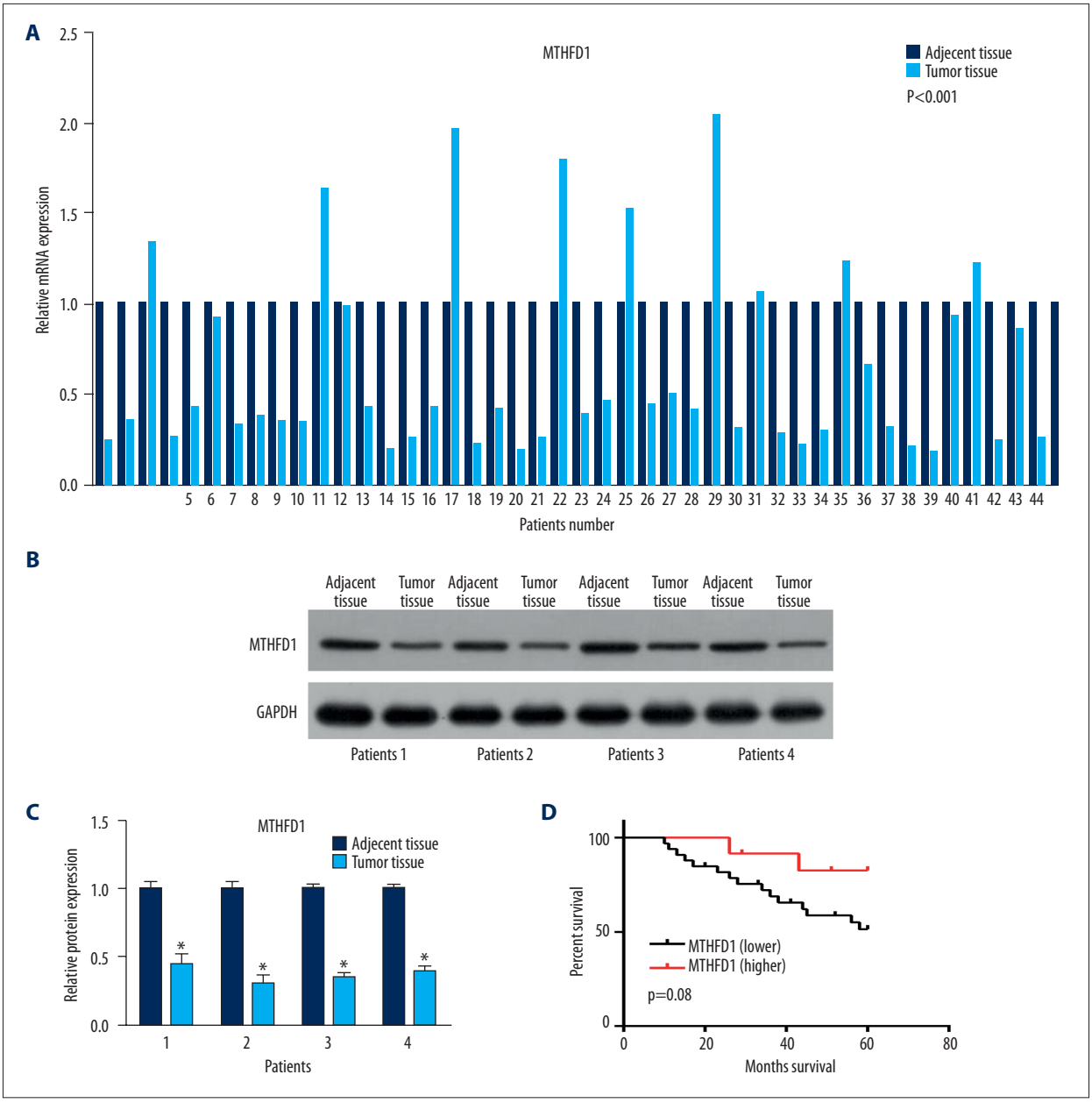

Figure 1. Expression levels of methylenetetrahydrofolate dehydrogenase 1 (MTHFD1) in clear cell renal cell carcinoma (CCRCC) tissues and adjacent normal renal tissues and survival curve analysis. (**A**) For the 44 patients who underwent radical nephrectomy for clear cell renal cell carcinoma (CCRCC), the relative mRNA levels of MTHFD1 in CCRCC tissue and adjacent normal renal tissue are shown by quantitative real-time polymerase chain reaction (qRT-PCR). (**B**) Western blot shows that MTHFD1 protein levels are decreased in the four groups of CCRCC tissue when compared with matched adjacent normal renal tissue. (**C**) The relative protein expression levels are shown as bar diagrams. (**D**) Kaplan-Meier curve analysis of patient survival rates in the lower expression and higher expression groups MTHFD1 in patients with CCRCC. Data are expressed as the mean ±SD from three independent experiments. * Compared with adjacent tissues. * P<0.05.

Cell counting kit-8 (CCK-8) assay for cell viability

The viability of Caki-1 cells transfected with MTHFD1, the control group, and the EV group was measured using the cell counting kit-8 (CCK-8) assay (Beyotime, Shanghai, China). Cells were plated into 96-well plates at a seeding density of 5×10^3 cells per well. After incubation for 24 h, the culture medium was changed and fresh medium and cells were cultured for 6, 12, and 24 hours. Then, 10 µL of CCK-8 solution was added to the cells for an additional 2 h at 37°C. The optical density (OD) was

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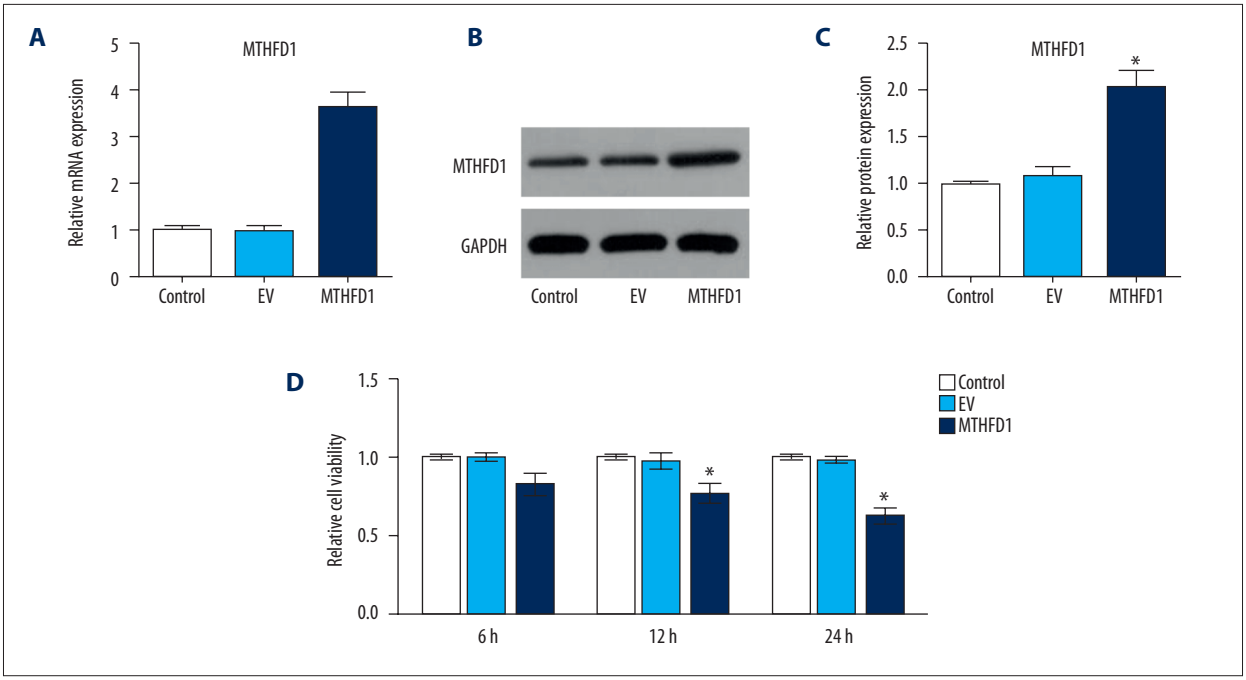

Figure 2. Transfection efficiency of methylenetetrahydrofolate dehydrogenase 1 (MTHFD1) and the effect on cell viability of Caki-1 cells. (**A**) mRNA levels of MTHFD1 in Caki-1 cells. (**B**) Methylenetetrahydrofolate dehydrogenase 1 (MTHFD1) protein levels are elevated in Caki-1 cells. (**C**) The relative protein expression levels are shown as bar diagrams. (**D**) Caki-1 cell viability, assessed by cell counting kit-8 (CCK-8) assay shows that MTHFD1 inhibited Caki-1 cell proliferation. Data are expressed as the mean ±SD from three independent experiments. * Compared with control. * P<0.05.

measured at wavelengths of 450 nm and 630 nm using a microplate reader (Thermo Fisher Scientific, Waltham, MA, USA).

Flow cytometry for evaluation of apoptosis and cell cycle

Apoptosis and cell cycle were detected using flow cytometric analysis [20]. The transfected Caki-1 cells were collected by trypsinization, washed twice in cold PBS, fixed in ethanol at –20°C for cell permeabilization. After 18 h, the cells were washed and resuspended in PBS with RNase and propidium iodide (PI) (Lianshu, Shanghai, China) at 37°C for 30 min. For the apoptosis assay, the three groups of Caki-1 cells were washed twice using the washing buffer, and the suspension was cultured with Annexin-V-PE and PI in the dark at 25°C for 20 min. Binding buffer was added to each well. The cell cycle profile of Caki-1 cells was analyzed using a Becton Dickinson FACScan (Becton Dickinson, Franklin Lakes, NJ, USA), and data were interpreted by ModFit LT™ 3.1 software.

Statistical analysis

Prism Graphpad version 6.0 software was used for statistical analysis. All data were presented as the mean ± standard deviation (SD). Differences were analyzed using one-way analysis of variance (ANOVA) following Tukey's test for multiple comparisons. A P-value <0.05 was considered to be statistically significant.

Results

Methylenetetrahydrofolate dehydrogenase 1 (MTHFD1) RNA and protein expression in clear cell renal cell carcinoma (CCRCC) tissues and adjacent normal renal tissues

Forty-four patients were recruited in this study, and the expression of methylenetetrahydrofolate dehydrogenase 1 (MTHFD1) in clear cell renal cell carcinoma (CCRCC) tissues and adjacent tissues were detected by both quantitative real-time polymerase chain reaction (qRT-PCR) and Western blot. The result showed a significantly lower level of MTHFD1 mRNA in CCRCC renal tumor tissues when compared with the normal adjacent renal tissue (P<0.001) (Figure 1A). The expression of MTHFD1 was upregulated in the CCRCC tissue in nine cases, and downregulated in 34 cases and only one case did not show a significant difference when compared with normal renal tissue. Data from the Western blot results showed that the expression of the MTHFD1 protein was significantly decreased in CCRCC tissues compared with normal adjacent renal tissue (P<0.05) (Figure 1B, 1C). The 5-year survival rate of 44 patients with renal carcinoma showed that patients with increased expression levels of MTHFD1 had an increased 5-year survival rate (P=0.08) (Figure 1D).

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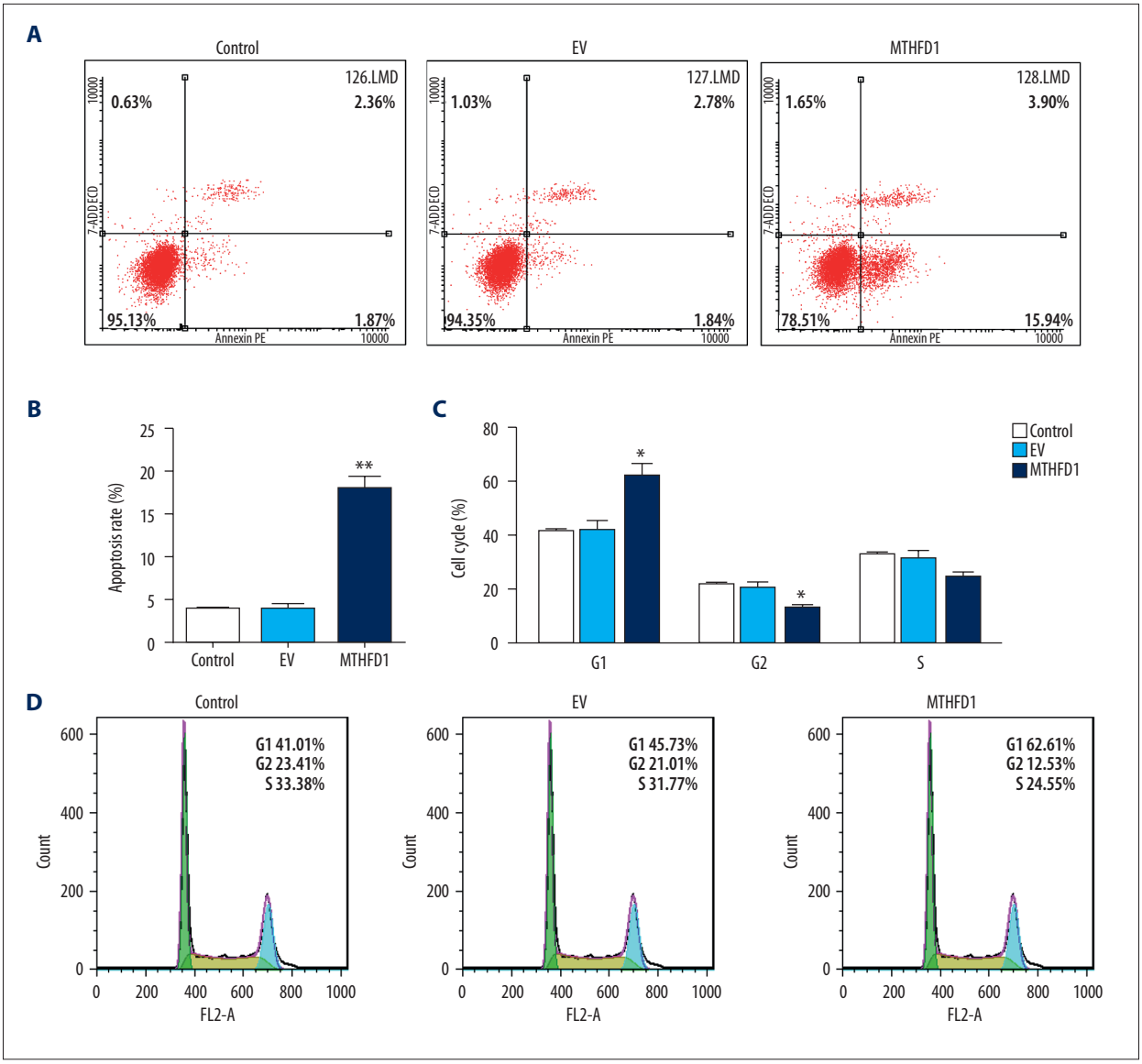

Figure 3. Effect of methylenetetrahydrofolate dehydrogenase 1 (MTHFD1) transfection on the regulation of Caki-1 apoptosis and cell cycle distribution *in vitro*. (**A**) Apoptosis assay was detected using flow cytometric analysis in Caki-1 cells. (**B**) The apoptosis rate shown as bar diagrams. (**C**) Cell cycle analysis was assessed by flow cytometry. (**D**) Quantified data from **C**. Data are expressed as the mean ±SD from three independent experiments. * Compared with control. * P<0.05; ** P<0.01.

Expression of MTHFD1 in human CCRCC Caki-1 cells

Caki-1 human CCRCC cells were divided into three groups, the control group (0.1% PBS), the empty vector (EV) group, and the group with MTHFD1 overexpression using the MTHFD1 plasmid. The transfection efficiency of MTHFD1 in Caki-1 cells was evaluated by quantitative real-time polymerase chain reaction (qRT-PCR) and Western blot method. The findings showed that mRNA and protein expression levels were significantly increased compared with the control and empty vector (EV) (P<0.05) (Figure 2A–2C).

MTHFD1 inhibited Caki-1 cells proliferation

After 6, 12, and 24 hours, Caki-1 cell viability was assessed in transfected cells, control cells, and cells treated with EV. The results from the cell counting kit-8 (CCK-8) assay showed the infected cell significantly decreased when incubated for 12 h or 24 h compared with empty vector (EV) (P<0.05) (Figure 2D). Cells cultured for 6 h did not show a significant difference (P>0.05).

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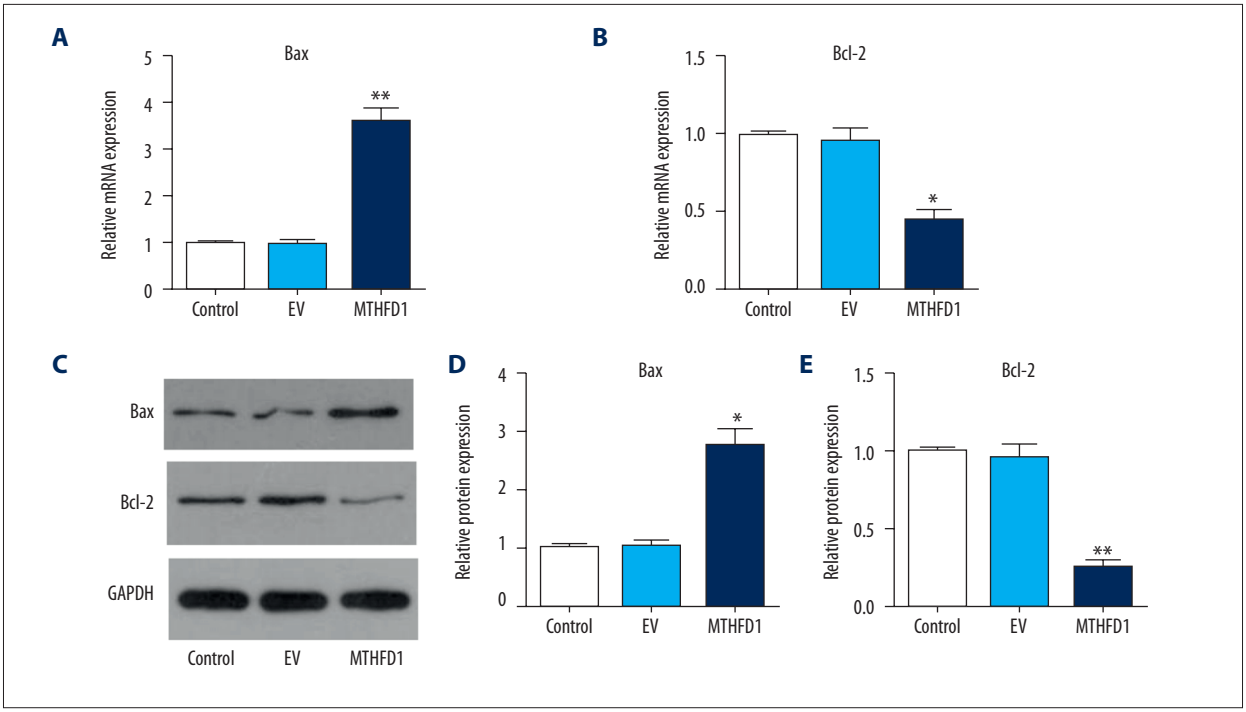

Figure 4. Effects of the mRNA and proteins levels of Bax and Bcl-2 on Caki-1 cells. (**A, B**) Quantitative real-time polymerase chain reaction (qRT-PCR) shows the mRNA expression of Bax and Bcl-2. (**C–E**) Western blot results and relative units of protein levels. Expression of each protein in the control, empty vector (EV) or MTHFD1 transfected Caki-1 cells, following normalization with the loading control GAPDH. Data are expressed as the mean ±SD from three independent experiments. * Compared with control. * P<0.05; ** P<0.01.

MTHFD1 promoted Caki-1 cells apoptosis

There was no noticeable difference in apoptosis between control or EV (P>0.05) (Figure 3B). Apoptosis in Caki-1 cells was assessed by flow cytometry. A significant increase in cell apoptosis (P<0.01) (Figure 3B) was found in Caki-1 cell transfected with MTHFD1 (19.84%) compared with either the EV group (4.62%) or the control group (4.23%) (Figure 3A).

MTHFD1 arrested cell the cycle in Caki-1 cells

The results of flow cytometry analysis to assess cell cycle showed that the G2-phase of the cell cycle was significantly reduced in the Caki-1 cells that were transfected with MTHFD1, compared with the control group or the EV group (12.53% *vs.* 23.41% *vs.* 21.01%, respectively) (P<0.05) (Figure 3D). Compared with the control group or the EV group, the cells in the G1 phase cells that were transfected with MTHFD1 were significantly increased from 41.01% to 45.73% to 62.61% (P<0.05) (Figure 3D). MTHFD1 arrested cells in the G1 phase of the cell cycle (Figure 3C). There was no observable difference in the S phase between the three different groups (P>0.05) (Figure 3C, 3D).

MTHFD1 regulated the expression of Bax and Bcl-2 at both the mRNA and protein levels in Caki-1 cells

The expression of Bax and Bcl-2 protein and mRNA were measured using both Western blot and qRT-PCR analysis in Caki-1 cells. As shown in Figure 4, compared with the control group or the EV group, MTHFD1 transfection significantly increased the expression of Bax both in mRNA and protein levels (protein, P<0.05; mRNA, P<0.01) (Figure 4A, 4C, 4D). The expression of Bcl-2 was significantly reduced at both the mRNA and protein levels in Caki-1 cells (protein, P<0.01; mRNA, P<0.05) (Figure 4B, 4C, 4E).

MTHFD1 regulated the inhibition of Akt-p53-cyclin D1 signaling at both mRNA and protein levels in Caki-1 cells

To evaluate the molecular mechanism of MTHFD1 in human CCRCC Caki-1 cells the mRNA and protein expression of p-Akt/Akt, p53, cyclin D1 were detected. The results showed that tumor the suppressor p53 was significantly upregulated in Caki-1 cells compared with the control group or EV group of Caki-1 cells at both the mRNA and protein levels (P<0.01) (Figure 5A, 5C, 5D). The results of qRT-PCR and Western blot showed that cyclin D1 was significantly down-regulated in Caki-1 cells (mRNA, P<0.01; protein, P<0.05) (Figure 5B, 5C, 5E).

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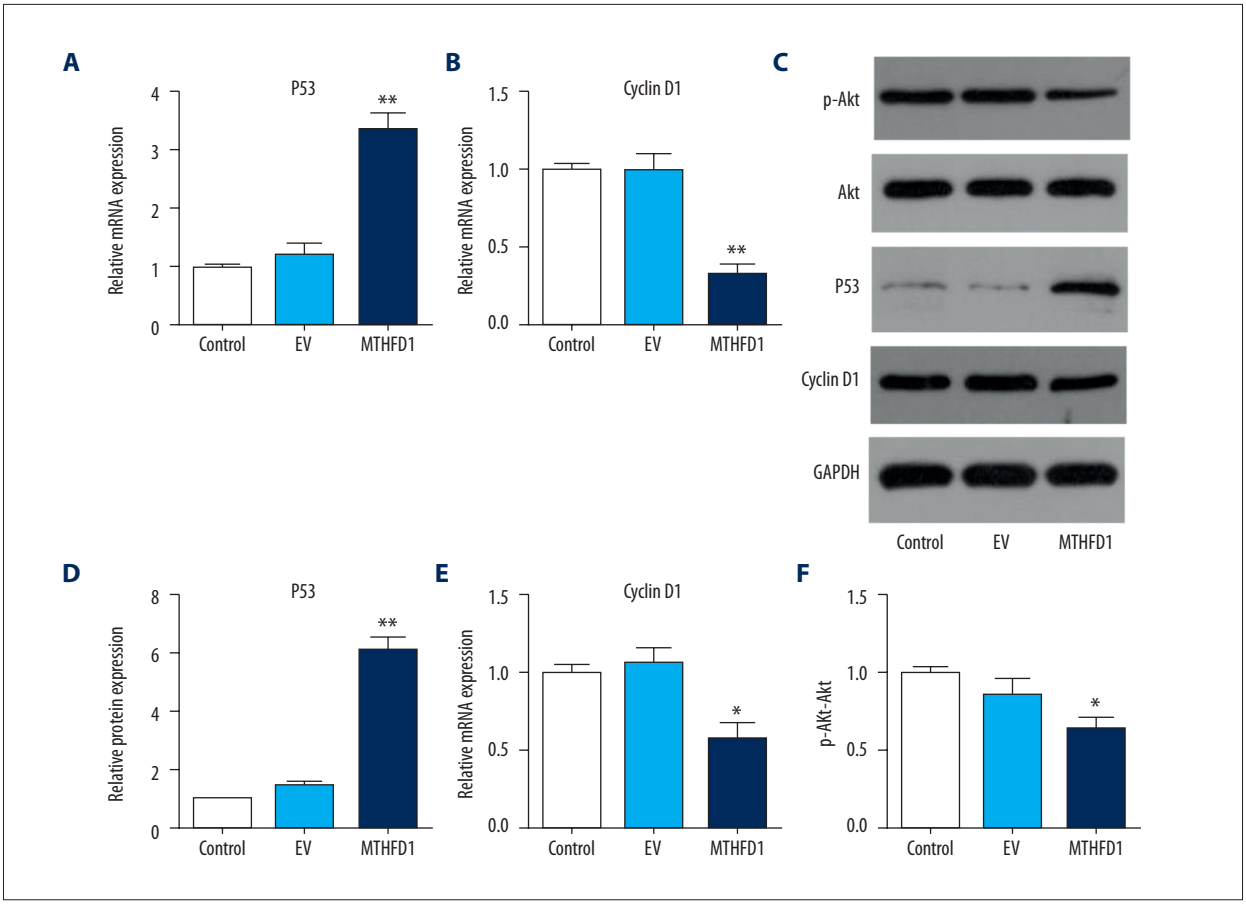

Figure 5. Effects of the mRNA and proteins levels of Akt-p53-cyclin D1 signaling on Caki-1 cells, (**A**) Quantitative real-time polymerase chain reaction (qRT-PCR) shows the mRNA expression level of p53. (**B**) qRT-PCR shows the mRNA expression level of cyclin D1. (**C**) The proteins were measured using Western blot for p-Akt, Akt, p53, cyclin D1 and with normalization using a loading control of GAPDH. The relative levels of protein expression are shown as bar diagrams for P53 (**D**), cyclin D1 (**E**), and p-Akt/Akt (**F**). Data are expressed as the mean ±SD from three independent experiments. * Compared with control. * P<0.05, ** P<0.01.

Western blot analysis showed that MTHFD1 significantly inhibited the expression of p-Akt (P<0.05) (Figure 5C 5F). These results supported that Akt-p53-cyclin D1 signaling may be related to the effect of MTHFD1 in Caki-1 CCRCC cells *in vitro*.

Discussion

The gene that encodes methylenetetrahydrofolate dehydrogenase 1 (MTHFD1) is located in chromosome 14 (14q24). MTHFD1 has catalytic activity, which plays an indirect role in the developing embryo, where purines and pyrimidines are required for *de novo* DNA synthesis [22]. The MTHFD1 gene has several potentially single nucleotide polymorphisms including T401C (R134K) and G1958A (R653Q), which are associated with methotrexate sensitivity in acute lymphoblastic leukemia (ALL), congenital heart disease, neural tube defect, and an increased risk of breast cancer and colorectal cancer [22–28].

The aims of this study were to investigate the expression of MTHFD1 in human tissue containing clear cell renal cell carcinoma (CCRCC) compared with normal renal tissue, and the effects of upregulating the expression of MTHFD1 in the human CCRCC cell line, Caki-1, *in vitro*. To explore the role of MTHFD1 in CCRCC, MTHFD1 was transfected into the human CCRCC cell line, Caki-1. The initial findings of this study showed that the expression of MTHFD was reduced in CCRCC tissues compared with normal adjacent renal tissue and that patients with increased expression of MTHFD1 had improved survival rates. These findings are supported by a previously published study that showed that the MTHFD1 1958AA genotype had similar expression and patient survival profiles in patients with colon cancer patients [17]. Therefore, from these studies, MTHFD1 may have a role as a tumor suppressor gene in CCRCC.

The findings of the present study showed that overexpression of MTHFD1 inhibited cell proliferation. Cancer cells with

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an increased proliferation rate, have a high demand for carbon units and nucleotides, and an increase in the levels of critical enzymes, such as MTHFD1, and MTHFD3, which are involved in folate metabolism, can stimulate the proliferation of tumor cells [8,29]. However, the findings of the present study showed the opposite findings, which may be explained by the functional nucleotide polymorphisms [17,30]. These findings require further study.

Previous studies have reported that apoptosis in CCRCC occurs via several pathways, including following induction of cell cycle arrest at the G0/G1 phase, activation of the pro-apoptotic JNK pathway, and inhibition of PI3K/Akt signaling [31–33]. To explore the effects on apoptosis and the cell cycle, MTHFD1 was transfected into Caki-1 cells, which showed that overexpression of MTHFD1 promoted cell apoptosis by regulating the expressions of Bcl-2 and Bax. Also, G1/S cell cycle arrest was induced by overexpression of MTHFD1. These results demonstrated the anti-tumor effect of MTHFD1 in CCRCC cells *in vitro*.

The P13K/Akt and p53 pathways are two important regulatory pathways that balance cell growth and apoptosis. The p53 tumor suppressor plays a role in the maintenance of genome stability and provides protection from malignant transformation, and is regulated via multiple signaling mechanisms, including murine double minute 2 (Mdm2), which binds to p53 and functions as a ubiquitin E3 ligase to promote p53 ubiquitin-dependent degradation by the proteasome [3,34]. In the present study, the protein expression levels of p-Akt, Akt and p53 were evaluated, and p-Akt expression was inhibited in Caki-1 cells transfected with MTHFD1, indicating that MTHFD1 inhibited the activity of Akt. Also, p53 is a protein responsible for regulating the cell cycle, and several previous studies have shown that the inhibition of p53 can be mediated by the activation of Akt [34–37]. During cell cycle progression, cyclin D1 forms complexes with CDK4 and CDK6 to transform the G1 phase into the S-phase. The findings from this study showed that the expression of cyclin D1 was significantly reduced, both at the mRNA level and protein levels, by overexpression of MTHFD1 and p53 expression was enhanced by MTHFD1. The decreased expression of cyclin D1 may have blocked the G1/S cell transition. Also, the results of this study indicated the possibility that overexpression of MTHFD1 inactivated Akt, which led to increased expression of p53, to induce cell apoptosis.

Conclusions

The findings of this study showed that overexpression of methylenetetrahydrofolate dehydrogenase 1 (MTHFD1) in clear cell renal cell carcinoma (CCRCC) cells of the Caki-1 cell line resulted in an anti-tumor effect *in vitro*. MTHFD1 transfection of Caki-1 cells inhibited cell proliferation, promoted cell apoptosis, and induced cell cycle arrest. MTHFD1 inhibited Akt phosphorylation, which may have stimulated the increased expression of p53, which then regulated apoptosis-related proteins. These results support that MTHFD1 should be investigated further for its role in the progression of CCRCC, as a potential diagnostic, prognostic, or therapeutic biomarker.

Conflict of interest

None.

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