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Expression of FOXO4 Inhibits Cholangiocarcinoma Cell Proliferation *In Vitro via* Induction of G_0/G_1 Arrest

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Abstract. *Background/Aim: Forkhead box O4 (FOXO4) has been demonstrated to be a tumor suppressor and proposed as target for treatment of a variety of cancer types. However, the role of FOXO4 in cholangiocarcinoma (CCA), a dangerous cancer of bile-duct epithelium, has rarely been explored. Materials and Methods: The proliferative rate of CCA cell lines KKU-213B, KKU-055 and KKK-D068 was investigated using the sulforhodamine B (SRB) assay. Levels of FOXO4, cyclin E1 (CCNE1), CCNE2, cyclin-dependent kinase 2 (CDK2) and cell division cycle 25A (CDC25A) expression were measured using reverse transcription quantitative real-time polymerase chain reaction (RT-qPCR). The cell-cycle profile was explored using flow cytometry. Results: The SRB assay demonstrated that KKU-213B expressed low levels of FOXO4 but its proliferative rate was highest of all cell lines tested. Interestingly, ectopic expression of FOXO4 significantly suppressed proliferation of KKU-213B cells. Cell-cycle analysis revealed that the cell population in the G_0/G_1 phase was significantly higher in FOXO4-transfected KKU-213B cells than in controls. RT-qPCR analysis demonstrated that the levels of expression of genes that play a role in the G_1/S transition, namely CCNE1, CCNE2, CDK2 and CDC25A, were significantly lower in FOXO4-transfected KKU-213B cells compared to controls. Conclusion: FOXO4 suppressed CCA cell proliferation partly via down-regulating the expression of genes involved in the G_1/S transition, leading to G_0/G_1 arrest. Our findings suggest that induction of FOXO4 expression might be an alternative approach for the treatment of CCA.*

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Cholangiocarcinoma (CCA), a cancer originating from bile-duct epithelial cells, used to be a largely neglected disease but its incidence has been increasing in many parts of the world in recent years (1). Although the cause of CCA is obscure, primary sclerosing cholangitis is an important risk factor for CCA development in Western countries, whereas infection with small human liver flukes (*Opisthorchis viverrini* and *Clonorchis sinensis*) is an important risk factor for CCA in Southeast Asia and East Asia, respectively (2). Liver fluke-associated CCA in Asia differs from CCA elsewhere in the world in terms of genetic and epigenetic abnormalities (3-5). However, most patients with CCA have similar clinical characteristics, including poor survival and limited response to standard chemotherapy. Thus, apart from seeking early diagnostic biomarkers, research on novel targets for effective treatment of CCA is also urgently needed.

It has been widely accepted that altered expression of transcription factors is involved in all aspects of cancer biology such as proliferation, apoptosis, progression and metastasis, as well as response to treatment. Forkhead box class O proteins (FOXOs) are a family of transcription factors consisting of FOXO1, FOXO3, FOXO4 and FOXO6. FOXOs are regarded as tumor-suppressor transcription factors (6). Thus it is not a surprise that FOXOs have been suggested as potential targets for cancer therapy (6). The tumor-suppressive properties of FOXO4 in many types of cancer has received the most attention (7). However, little is known about the role of FOXO4 in CCA, especially liver fluke-associated CCA.

In this study, we explored the role of FOXO4 in CCA cells *in vitro* with a view as to the feasibility of modulation of FOXO4 expression as a strategy for treatment of CCA.

Materials and Methods

Human cell lines. Immortalized cholangiocyte MMNK-1 (8) and CCA cell lines KKU-213B (9), KKU-055 (10) and KKK-D068 (11) were used in this study. The latter three cell lines were established from Thai patients with CCA. All cell lines were grown in Dulbecco's modified Eagle's medium (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (Corning, NY, USA), 100 U/ml penicillin (Gibco) and 100 μg/ml streptomycin (Gibco) at 37˚C with 5% CO_2 in a humidified incubator.

Cell transfection. FLAG-FOXO4 plasmid was a gift from Domenico Accili (Addgene plasmid # 17549; http://n2t.net/addgene:17549; RRID: Addgene_17549). Transfection was performed as described previously (12). For instance, KKU-213B cells were seeded into 10-cm cell-culture dishes and grown overnight. Then the cells were transfected with FLAG-FOXO4 plasmid DNA using X-tremeGENE HP transfection reagent (Roche Diagnostics Ltd, Burgess Hill, UK) according to the manufacturer's protocol. Cells were further incubated with transfection complex at 37˚C with 5% CO_2 in a humidified incubator for 48 h. Transfection of KKU-213B cells without FLAG-FOXO4 plasmid was used as the transfection control.

Cell proliferation assay. Cells were seeded in flat-bottomed 96-well plates (Corning) at a density of 2,000 cells/well and grown in culture medium overnight at 37˚C with 5% CO_2 in a humidified incubator. Then the culture medium was removed, the cells were fixed with cold 40% trichloroacetic acid (Sigma Aldrich, St. Louis, MO, USA) and incubated at 4˚C for 1 h. After washing with tap water, the fixed cells were stained with 0.4% (w/v) sulforhodamine B (Sigma Aldrich) dissolved in 1% acetic acid and incubated at room temperature with gentle shaking for 1 h. After washing with 1% acetic acid, cells were dried at room temperature and dissolved with 10 mM Tris buffer pH 10.5. The absorbance at 492 nM was measured using an enzyme-linked immunosorbent assay reader (Tecan group Ltd., Männedorf, Switzerland).

Cell-cycle analysis. Cell-cycle analysis was performed as described elsewhere (13). In brief, 1×10^6 CCA cells were spun down, washed with ice-cold phosphate-buffered saline and fixed with 70% ethanol overnight at -20 ˚C. Then, the cells were spun down, washed twice with ice-cold phosphate-buffered saline and stained with FxCycle™ PI/RNase solution (Thermo Fisher Scientific Inc., Waltham, MA, USA) in accordance with manufacturer's instructions. Finally, stained cells were passed through a BD FACSCanto™ II flow cytometer (BD Biosciences, San Jose, CA, USA) and resulting data analyzed using BD FACS Diva software (BD Biosciences).

RNA isolation and reverse transcription–quantitative real-time polymerase chain reaction (RT-qPCR) analysis. Total RNA was isolated from cells using PureLink™ RNA mini kit (Thermo Fisher Scientific) and cDNA was synthesized using RevertAid First Strand cDNA synthesis kit (Thermo Fisher Scientific) according to the manufacturer's recommendations. The relative mRNA expression was determined by LightCycler® 480 Real-Time PCR System (Roche Applied Science, Mannheim, Germany) using LightCycler® 480 SYBR Green I Master (Roche) with the conditions as follows: 95˚C for 5 min for enzyme activation, followed by 40 cycles of denaturation at 95˚C for 3 s, primer annealing at 58-65˚C for 20 s and amplification at 72˚C for 30 s. The specific oligonucleotide primer sequences were as follows; *FOXO4* forward: 5'-ACG AGT GGA TGG TCC GTA CTG T-3', and *FOXO4* reverse: 5'-CCT TGA TGA ACT TGC TGT GCA GG-3'; cyclin E1 (*CCNE1*) forward: 5'-CCG GTA TAT GGC GAC ACA AG-3', and *CCNE1* reverse: 5'-TAC GCA AAC TGG TGC AAC TT-3'; C*CNE2* forward: 5'-TCT CCT GGC TAA ATC TCT TTC TCC-3' and *CCNE2* reverse: 5'-ACT GTC CCA CTC CAA ACC TG-3'; cyclin-dependent kinase 2 (*CDK2*) forward: 5'-AAG TTG ACG GGA GAG GTG GT-3' and

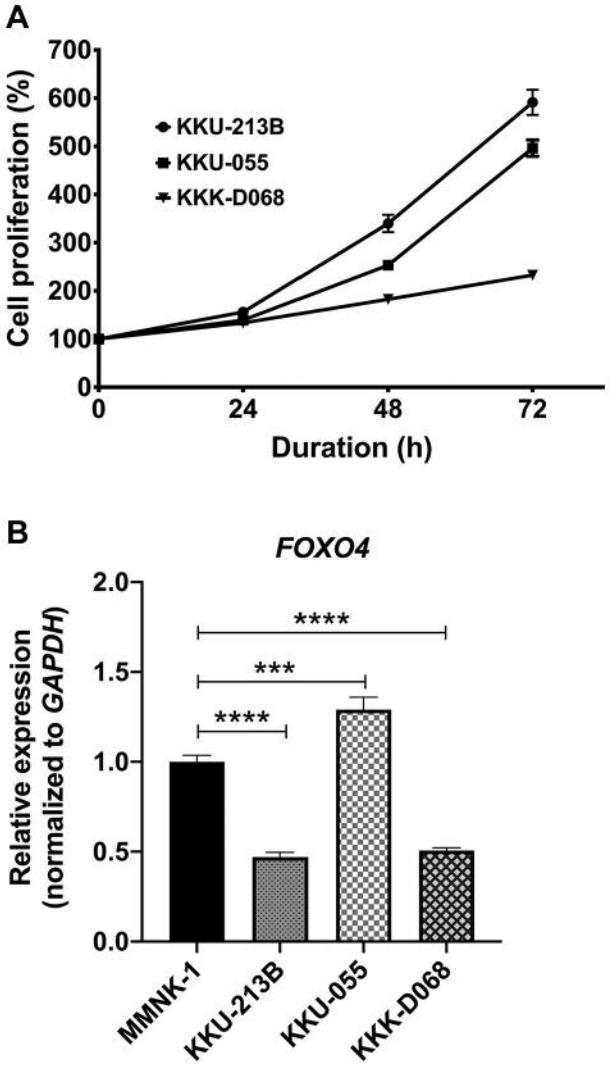

Figure 1. *The proliferative rate of CCA cell lines was inversely correlated with expression level of forkhead box O4 (FOXO4). A: The proliferative rate of CCA cell lines was measured using the sulforhodamine B assay and reported at 24-h intervals. B: The expression of FOXO4 relative to that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) at each time point was also investigated using reverse transcription quantitative real-time polymerase chain reaction (RT-qPCR) assay. For RT-qPCR, the immortalized cholangiocyte cell line MMNK-1 was used as the control. Data are the mean±SD and the experiment was performed in triplicate. Significantly different at ***p<0.001 and ****p<0.0001, respectively.*

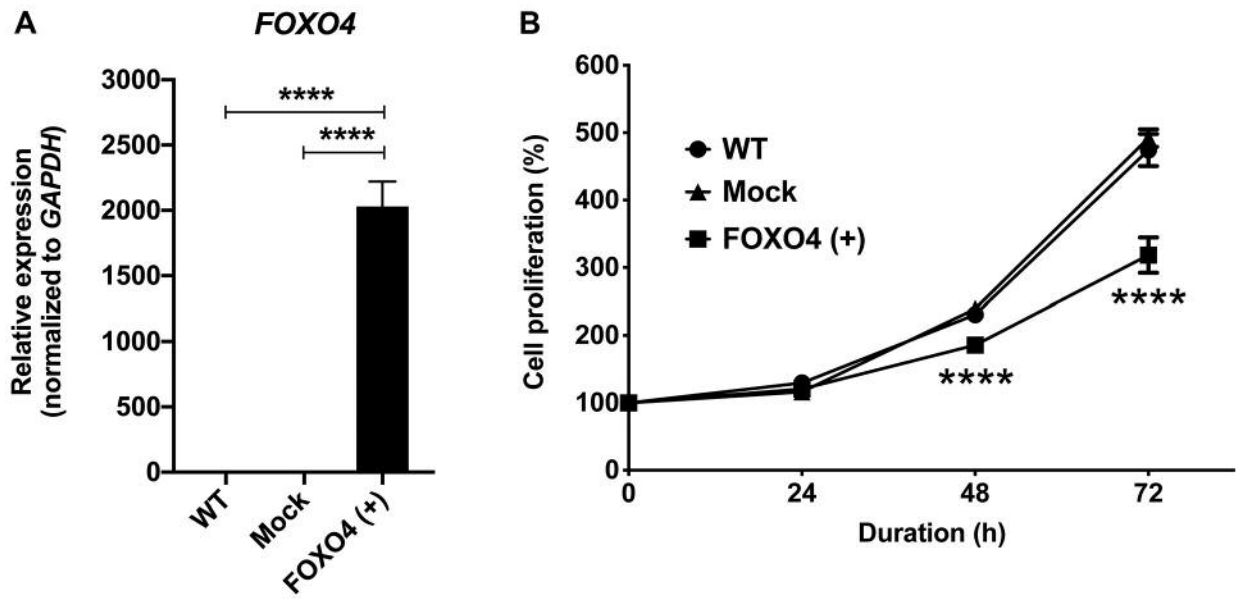

Figure 2. *Ectopic expression of forkhead box O4 (FOXO4) suppressed KKU-213B cell proliferation. A: Overexpression of FOXO4 in KKU-213B cells [FOXO4(+)] was induced by transfection with FLAG-FOXO4 plasmid DNA. The expression level of FOXO4 relative to that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was confirmed by reverse transcription quantitative real-time polymerase chain reaction. Non-transfected (WT) and mock-transfected (Mock) cells were used as controls. B: The proliferative rate of KKU-213B cells in each group was measured using the sulforhodamine B assay. Data are the mean±SD and the experiment was performed in triplicate. ****Significantly different at p<0.0001 (compared to WT in B).*

CDK2 reverse: 5'-TGA TGA GGG GAA GAG GAA TG-3'; cell division cycle 25A (*CDC25A*) forward: 5'-GTC GCC TGT CAC CAA CCT-3' and *CDC25A* reverse: 5'-CGG AGG AGC CCA TTC TCT-3'; glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) forward: 5'-GTC TCC TCT GAC TTC AAC AGC G-3' and *GAPDH* reverse: 5'-ACC ACC CTG TTG CTG TAG CCA A-3'. Relative gene expression was calculated by the $2^{-\Delta\Delta Ct}$ method (14) using *GAPDH* as a calibrator.

Statistical analysis. Data are expressed as mean±SD. Either Student's *t*-test or analysis of variance was used to test differences between experimental groups. A value of $p<0.05$ was considered statistically significant. All statistical analyses were carried out using Graphpad Prism 8.0 for Mac (GraphPad Software, Inc., CA, USA).

Results

FOXO4 expression was inversely correlated with the proliferative rate of CCA cells. In order to investigate whether *FOXO4* expression is correlated with CCA cell proliferation, a proliferative assay and RT-qPCR were used. The SRB assay demonstrated that all CCA cell lines proliferated rapidly, especially from 48 h onwards. The proliferative rate of KKU-213B was highest and that of KKK-D068 was lowest (Figure 1A). Interestingly, RT-qPCR analysis revealed that the relative expression of *FOXO4* in KKU-213B was significantly lower ($p<0.0001$) than in MMNK-1 control cells but was significantly higher ($p<0.001$) than that in KKU-055 cells (Figure 1B). Notably, although the proliferative rate of KKK-D068 was the lowest, the relative expression level of *FOXO4* was also significantly lower ($p<0.0001$) than in MMNK-1 cells (Figure 1B). The inverse correlation between CCA proliferative rate and FOXO4 expression indicated that FOXO4 possibly exhibits a tumor-suppressor role in CCA.

Ectopic expression of FOXO4 inhibited CCA cell proliferation. In order to investigate the growth-inhibitory function of FOXO4 in CCA cells, KKU-213B cells were transfected with FLAG-FOXO4 plasmid DNA. RT-qPCR analysis confirmed that the expression of *FOXO4* was significantly elevated ($p<0.0001$) by 48 h after transfection (Figure 2A). The SRB assay demonstrated that the proliferation rate of KKU-213B in control cells was similar to that in mock-transfected cells (Figure 2A). By contrast, the proliferative rate of KKU-213B was significantly reduced ($p<0.0001$) in the FOXO4-transfected compared to the control and mock-transfected cells. These results indicate that FOXO4 exhibits growth-suppressive effects in CCA cells.

Ectopic FOXO4 expression induced G_0/G_1 arrest of CCA cells. Using the SRB assay, we showed above that cell proliferation was inhibited in CCA cells after transfection with FLAG-FOXO4 plasmid DNA. We therefore measured the cell-cycle distribution of CCA cells using flow cytometry. There were no significant differences in cell-cycle profiles between control

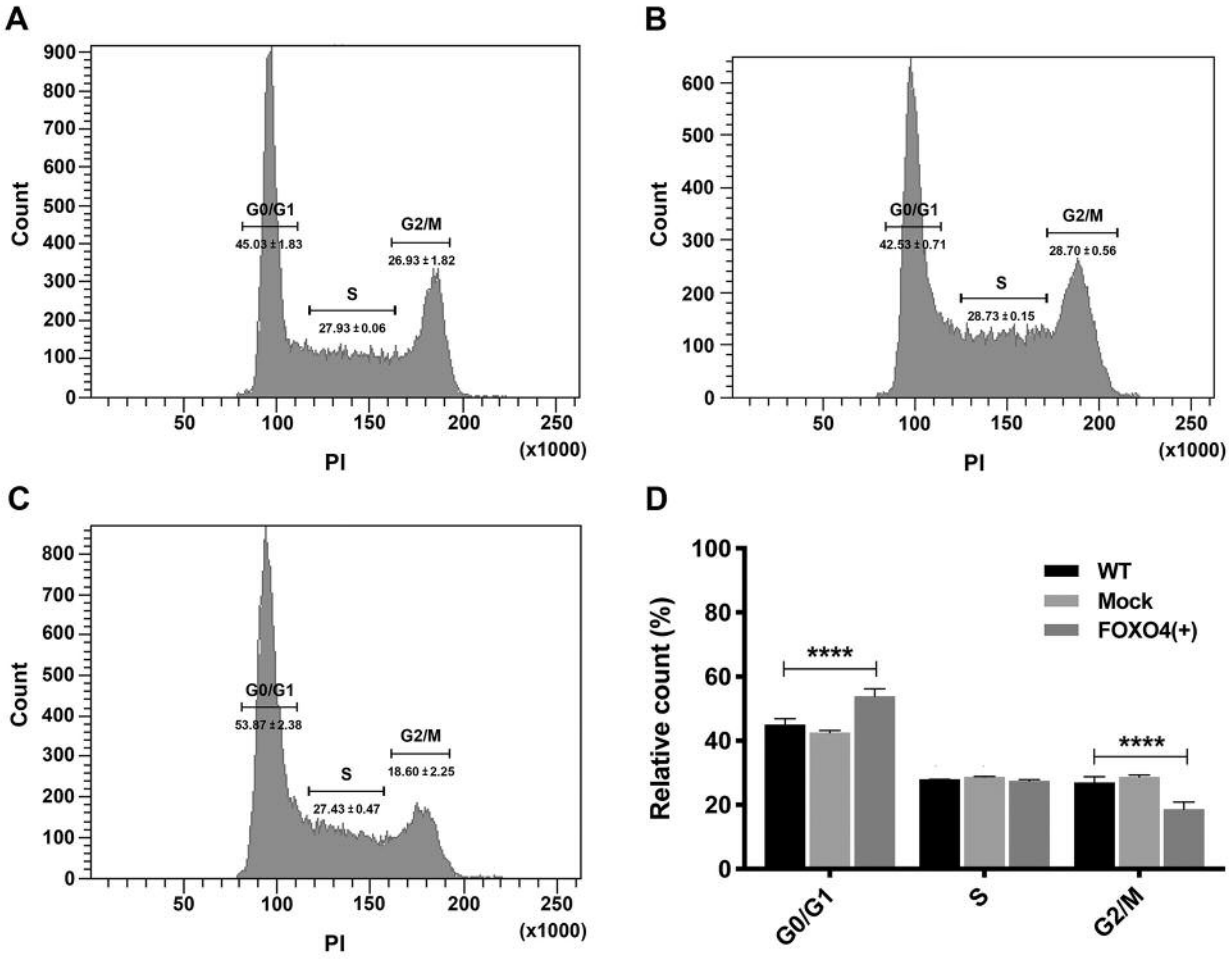

Figure 3. *Overexpression of forkhead box O4 (FOXO4) caused G0/G1 cell-cycle arrest of KKU-213B cells. Cell-cycle analysis of non-transfected (wild-type, WT) (A), mock-transfected (Mock) (B), and FLAG-FOXO4-transfected [FOXO4(+)] (C) KKU-213B cells was performed using flow cytometry. Quantification of results is shown in D. Data are the mean±SD and the experiment was performed in triplicate. ****Significantly different at p<0.0001.*

and mock transfection groups ($p>0.05$, Figure 3A, B and D). However, in the cells with FOXO4 overexpression, the cell population at the G_0/G_1 phase was significantly higher than in control and mock transfection cells ($p<0.0001$, Figure 3). This indicates that the growth-inhibitory effect of FOXO4 in CCA cells is partly mediated by induction of G_0/G_1 arrest.

Ectopic FOXO4 expression suppressed the expression of genes involved in the G_1/S transition of CCA cells. Given the G_0/G_1 arrest of CCA cells after FOXO4 transfection, we investigated the expression of *CCNE1*, *CCNE2*, *CDK2* and *CDC25A* genes involved in the G_1/S transition. RT-qPCR analysis revealed that the expression of these genes in mock-transfected cells did not differ from those in control cells ($p>0.05$, Figure 4). In contrast, all four genes were significantly down-regulated in cells with FOXO4 overexpression ($p<0.05$, Figure 4). This finding indicates that FOXO4 induces G_0/G_1 arrest of CCA cells *via* down-regulation of genes involved in G_1/S transition.

Discussion

The failure of CCA to respond to most chemotherapeutic drugs and the lack of suitable biomarkers for early screening have been recognized as the major problems for CCA management. Much effort has been made to overcome these problems, including seeking novel targets for CCA treatment. Previous study uncovered the role of FOXO4 in suppression of growth factor-driven CCA progression and metastasis (15). In this study, we investigated the role of the transcription factor FOXO4 in CCA and its potential as a novel target for CCA treatment. We found that the expression of *FOXO4* was low in

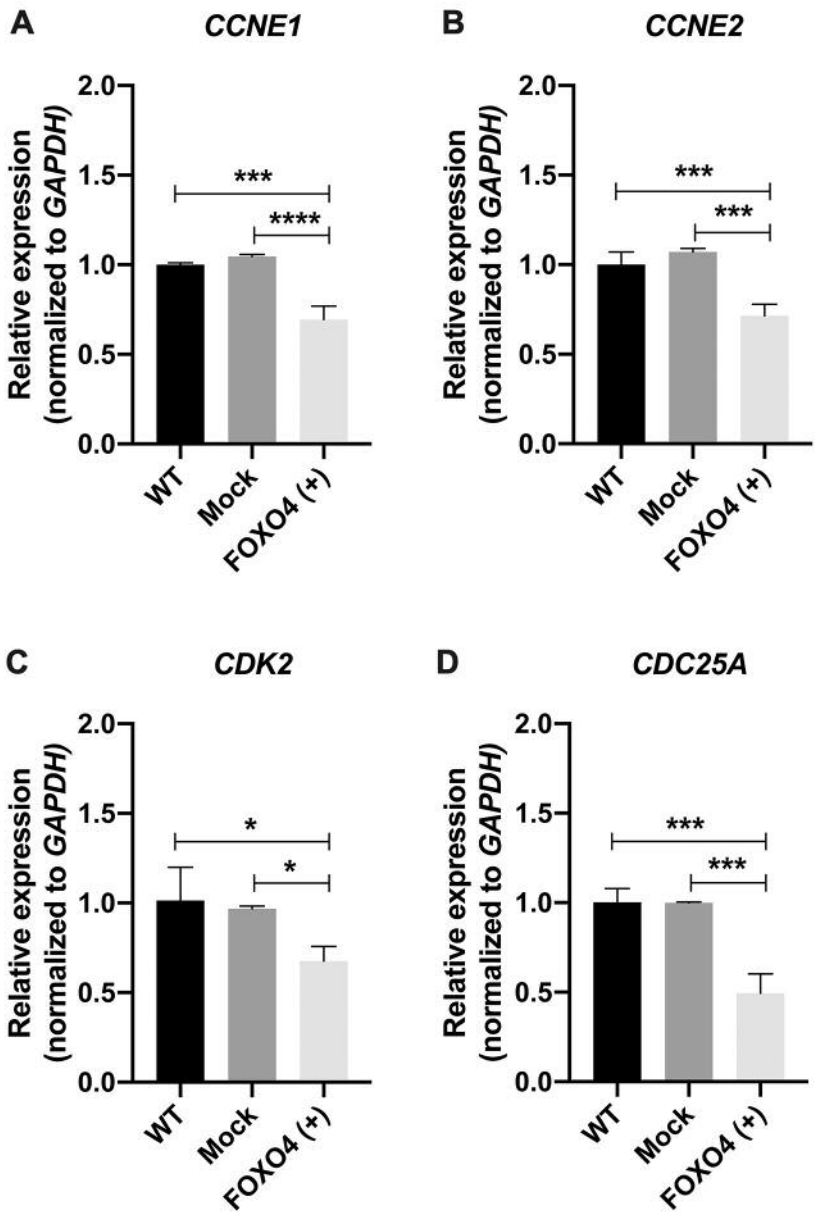

Figure 4. *Overexpression of forkhead box O4 (FOXO4) down-regulated the expression of genes involved in the G1/S transition of KKU-213B cells. The relative expression of genes involved in G1/S transition, namely cyclin E1 (CCNE1) (A), CCNE2 (B), cyclin-dependent kinase 2 (CDK2) (C) and cell division cycle 25A (CDC25A) (D) of non-transfected (wild-type, WT), mock-transfected (Mock), and FLAG-FOXO4-transfected [FOXO4(+)] KKU-213B cells was performed using reverse transcription quantitative real-time polymerase chain reaction. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as calibrator. Data are the mean±SD and the experiment was performed in triplicate. Significantly different at *p<0.05, ***p<0.001 and ****p<0.0001, respectively.*

some CCA cell lines with high proliferative rates. Notably, both the SRB assay and RT-qPCR showed that KKK-D068 cells expressed low levels of *FOXO4* and their proliferative rate was also lower than that of KKU-213B and KKU-055 CCA cells. This might partly be due to the fact that KKK-D068 cells harbor wild-type tumor protein 53 (*TP53*) (11), one of the most important tumor suppressors that inhibits cancer cell growth. KKU-213B and KKU-055, on the other hand, have mutant *TP53* (10). Interestingly, the introduction of FOXO4 into KKU-213B cells inhibited KKU-213B cell proliferation, partly *via* modulation of the expression of genes involved in the G_1/S transition, resulting in G_0/G_1 arrest. Our results suggest that modulation of FOXO4 might be an attractive approach for effective treatment of CCA.

FOXO4 is a member of the Forkhead box transcription-factor family known to be tumor suppressors (16, 17). In the case of FOXO4, one of its most important roles is inhibition of cancer cell growth. For instance, ectopic expression of constitutively active FOXO4 was found to suppress breast cancer cell growth *in vitro* and also retarded the development and progression of tumors in nude mice (17). The growth-inhibitory property of FOXO4 has also been demonstrated in gastric (18), cervical (19) liver (20) and nasopharyngeal (21) cancer. Our finding in which overexpression of FOXO4 suppressed CCA cell proliferation *in vitro* is in general agreement with these reports, even for other cancer types. Our results suggest the growth-inhibitory effect of FOXO4 was mediated by cell-cycle arrest but not apoptosis. This view is supported by the significant increase of the cell population at the G_0/G_1 phase along with the absence of a sub-G_0/G_1 population, an indicator of cellular apoptosis (22), in the cell-cycle analysis. Cell-cycle progression requires the cooperation of several cyclin and CDK proteins (23). Specifically, cell-cycle progression from G_1 to the S phase is regulated by the cyclin E–CDK2 protein complex (23), which is activated after dephosphorylation of CDK2 by CDC25A phosphatase (24). We found that FOXO4 overexpression led to down-regulation of *CCNE1*, *CCNE2*, *CDK2* and *CDC25A* genes in CCA cells. Thus, we suggest that FOXO4 induces G_0/G_1 arrest of CCA cells partly *via* downregulation of *CCNE1*, *CCNE2*, *CDK2* and *CDC25A* genes. However, further study of how FOXO4 modulates the expression of these genes is required. Taken together, our findings demonstrated that FOXO4 exhibits anti-proliferative activity against CCA cells and thus represents an alternative and promising target for CCA treatment. Further study on the roles of FOXO4 in other aspects of CCA, especially development and drug treatment response, will be helpful for CCA management.

Conflicts of Interest

The Authors have no conflicts of interest to declare.

Author's Contributions

KI, SC, PT, KW, CP, PP and SP designed the experiments. KI, SC and PT performed the experiments. KI, SC, PT, KW, CP, PP and SP performed the data analysis. KI and SP drafted the article. All Authors approved the final version of article for publication.

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