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# Prediction of Angiopoietin-like Protein 4-related Signaling Pathways in Cholangiocarcinoma Cells

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**Abstract.** *Background/Aim: Angiopoietin-like protein 4 (ANGPTL4) is a multifunctional signaling protein implicated in carbohydrate metabolism, inflammation, cancer growth and progression, anoikis resistance, angiogenesis, and metastasis. However, signaling pathways of ANGPTL4 in cholangiocarcinoma (CCA) remain unknown. The aim of this study was to explore ANGPTL4-related signaling proteins and pathways associated with CCA biology. Materials and Methods: ANGPTL4 of CCA cells was silenced by small interfering RNA (siRNA) with scramble control and ANGPTL4-related signaling proteins were investigated using mass spectrometry, bioinformatics tools and molecular docking. Results: Among the 321 differentially expressed proteins, 151 were down-regulated. Among them, bioinformatic analyses revealed that ANGPTL4 interacts with DNA-dependent protein kinase catalytic subunit (PRKDC) and 60S ribosomal protein L21 (RPL21) via AKT serine/threonine kinase 1 (AKT1), mechanistic target of rapamycin kinase (MTOR) and ribosomal protein L5 (RPL5). Online database analysis showed that mRNA and protein expression levels of ANGPTL4-related signaling proteins were significantly higher in CCA than in normal tissues. Moreover, a high mRNA expression level was associated with high tumor grade ($p<0.0001$) and lymph node metastasis ($p<0.0001$). Conclusion: The signaling pathway of ANGPTL4 in CCA progression might be regulated by PRKDC and RPL21. Furthermore, high expression of ANGPTL4-related signaling proteins has potential to be used in clinical prognosis.*

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*Key Words:* ANGPTL4, signaling pathways, mass spectrometry, bioinformatics, CCA.



Cholangiocarcinoma (CCA) is a type of highly aggressive cancer that can develop anywhere in the biliary tree or within the liver parenchyma (1). It is a rare cancer worldwide, but it is highly prevalent in Thailand, with the highest prevalence of 85/100,000 population being recorded in northeast Thailand and in association with the high prevalence of infection with the oncogenic liver fluke (*Opisthorchis viverrini*) (2-4). Although complete surgical resection is the only curative treatment for CCA, the postoperative 5-year survival rate of patients remains low (11.2%) due to late presentation of clinical symptoms (5, 6). Carbohydrate antigen 19-9 (CA19-9) and carcinoembryonic antigen (CEA) are currently used tumor markers for clinical diagnosis/prognosis of CCA. However, elevation of these markers has also been found in benign biliary diseases, pancreatic cancer, and colorectal cancer, amongst others (7-9). Hence, developing reliable biomarkers and exploring the mechanisms underlying CCA progression are essential to improve the outcomes of patients with CCA.

Angiopoietin-like protein 4 (ANGPTL4) is a secreted protein with a highly hydrophobic signal peptide and has an *N*-terminal coiled-coil domain and a *C*-terminal fibrinogen-like fragment (10). It has a variety of biological roles in both normal and

malignant cells, including effects on carbohydrate metabolism, inflammatory processes, differentiation, angiogenesis, and carcinogenesis (11, 12). ANGPTL4 expression is higher in several cancer types, such as esophageal squamous cell carcinoma (13), cervical cancer (14), colorectal cancer (15) and gastric cancer (16). Recently, we found higher ANGPTL4 expression in the sera and cancer tissues of patients with CCA, particularly, those with lymph node metastasis and advanced tumor stage (17). Related to this, San *et al*. showed that ANGPTL4 was markedly up-regulated in anchorage-independently cultured CCA cells, and its knockdown tended to sensitize these cells to cell death and chemotherapy (18). Since the previous research strongly suggested the important roles of ANGPTL4 in CCA cells, in this study, we investigated the putative signaling proteins and pathways of ANGPTL4 in the KKU-213A CCA cell line using gene silencing, mass spectrometry, bioinformatics tools and molecular docking.

## Materials and Methods

*Cell lines and cultivation*. CCA cell lines (KKU-M055, KKU-100 and KKU-213A) were obtained from the Japanese Collection of Research Bioresources Cell Bank, Osaka, Japan. The immortalized human cholangiocyte cell line MMNK1, established and characterized by Maruyama *et al*. at Okayama University, Japan (19), was also obtained from the Japanese Collection of Research Bioresources Cell Bank. All cell lines were grown in Ham's F-12 Nutrient Mixture medium (Life Technologies, Grand Island, NY, USA) with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin (Life Technologies) and maintained at 37˚C in an atmosphere with 5% $CO_2$.

*Transient silencing of ANGPTL4 by siRNA*. Since ANGPTL4 was highly expressed in the KKU-213A cells, we selected this CCA cell line for transient gene silencing. Scramble control siRNA (Invitrogen, Carlsbad, CA, USA) and siRNA against human ANGPTL4 (sc-4464; Santacruz, Dallas, TX, USA) were used for *ANGPTL4* gene silencing. Briefly, $6\times10^5$ cells/well were seeded in a six-well plate and incubated overnight at 37˚C with 5% $CO_2$. KKU-213A cells were transfected at 37˚C for 48 h according to the manufacturer's protocol using Lipofectamine 3000 (Invitrogen). ANGPTL4 expression levels before and after gene silencing were determined using western blot analysis.

*Western blot analysis*. Cell pellets of MMNK1, KKU-M055, KKU-100, KKU-213A as well as *ANGPTL4* siRNA- or scramble control-treated KKU-213A cells were dissolved in RIPA lysis buffer (Cell Signaling Technology, Danvers, MA, USA) with protease and phosphatase inhibitors (Roche, Basel, Switzerland) and proteins were extracted by centrifugation. The protein concentration was measured by Bradford assay. Samples were separated on a 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel at 150 V for 2 h and electrically transferred to a polyvinylidene difluoride membrane at 90 V for 2 h. The membrane was then blocked with 5% skim milk in Tris-buffered saline with 0.1% Tween-20 (1X TBST, pH 7.4) before being treated with rabbit anti-ANGPTL4 (orb228886, 1:2,000; Biorbyt, Cambridge, UK) overnight at 4˚C. After washing with 1X TBST three times, the membrane was incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody (1:10,000; Abcam, Cambridge, UK) for 1 h at room temperature. The membrane was then washed with 1X TBST three times. Finally, peroxidase activity was developed as chemiluminescence using an ECL solution (GE Healthcare, Chalfont St. Giles, UK) and then visualized under an Amersham imager 600 (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). The band intensities were quantified by ImageJ software v.1.52d (National Institute of Health, Bethesda, MD, USA) with β-actin being used as an internal control.

*In-solution tryptic digestion*. Five micrograms of total protein isolated from cell lysates of *ANGPTL4*-silenced and scramble-treated control cells were digested according to the method developed by the Functional Proteomics Technology Laboratory, National Center for Genetic Engineering and Biotechnology, National Science and Technology Development Agency, Thailand (17). Briefly, samples were completely dissolved in 10 mM ammonium bicarbonate then 5 mM dithiothreitol in 10 mM ammonium bicarbonate was used to reduce disulfide bonds at 60˚C for 1 h and 15 mM iodoacetamide in 10 mM ammonium bicarbonate was used for alkylation of sulfhydryl groups at room temperature for 45 min in the dark. Then proteins were digested overnight at 37˚C using sequencing grade trypsin (50 ng/μl) (1:20) (Promega, Mannheim, Germany). The digested samples were then dehydrated and reconstituted in 0.1% formic acid before being analyzed by liquid chromatography with tandem mass spectrometry (LC-MS/MS).

*Protein quantitation and identification by LC-MS/MS*. The trypsinized peptides were injected in triplicate into an Ultimate 3000 Nano/Capillary LC System (Thermo Fisher Scientific, Madison, WI, USA) equipped with a Hybrid quadrupole Q-TOF Impact II™ (Bruker Daltonics, Hamburg, Germany). The mobile phase comprised solvents A and B, which were 0.1% formic acid, and 80% acetonitrile in water containing 0.1% formic acid, respectively. Peptides were separated on a nanocolumn with a flow rate of 300 nl/min using an elution period from 3.0-30.0 min with 5-55% solvent B, a washing period from 30.01-35.0 min with 95% solvent B, and a re-equilibration period from 35.01-40.00 min with 1% solvent B. Electrospray ionization was conducted using CaptiveSpray at 1.6 kV. MS and MS/MS spectra were obtained in the positive-ion mode over the range of m/z 150-2200 (Compass 1.9 for OTOF Series software; Bruker Daltonics, Billerica, MA, USA). DeCyder MS Differential Analysis software (DeCyderMS; GE Healthcare, Chalfont St. Giles, UK) was used to quantify the proteins in individual samples. Using Mascot software (Matrix Science, London, UK), the analyzed MS/MS data file was submitted to be searched. The data were searched against the UniProt *Homo sapiens* database for protein identification. At least one unique peptide was required for protein identification.

*Prediction of ANGPTL4-related signaling pathways*. To predict ANGPTL4 signaling pathways, the potential interactions of ANGPTL4 and related proteins were analyzed using STITCH 5.0, a web tool for interactions of genes/proteins (http://stitch.embl.de; last accessed on 21 September 2021) (20). The Kyoto Encyclopedia of Genes and Genomes (KEGG) in STITCH was used to identify signaling pathways of ANGPTL4-related signaling proteins. Briefly,

UniProt ID/gene names were put into multiple name boxes. *Homo sapiens* was then selected as the organism of interest. The page displayed the protein–protein interaction which were presented as solid lines with a confidence score of 0.4. Stronger associations were represented by thick lines, whereas weak associations were represented by thin lines.

*Correlation analysis*. The correlation of gene expression among ANGPTL4 signaling proteins was analyzed by Spearman correlation statistics based on Genotype-Tissue Expression (GTEx) Program and The Cancer Genome Atlas (TCGA) expression data in Gene Expression Profiling Interactive Analysis 2 (GEPIA2, http://gepia.cancer-pku.cn; last accessed on 2 October 2021) (21).

*Molecular docking analysis*. Three-dimensional structural models of the candidate proteins were obtained from the Research Collaboratory for Structural Bioinformatics Protein Data Bank (https://www.rcsb.org; last accessed on 5 October 2021) (22) and Zhang's Iterative Threading Assembly Refinement (I-TASSER) (http://zhanglab.ccmb.med.umich.edu/I-TASSER) web server (23-25). Molecular docking was performed by ClusPro 2.0 web server (https://cluspro.bu.edu) (26-28). It is an automated web server for protein–protein docking that ranks among the top servers in Critical Assessment of Predicted Interactions. It performs rigid-body docking using the fast Fourier transform correlation approach, with root-mean square deviation-based clustering of the structures generated to find the largest cluster that represents the likely models of the complex, and refinement of selected structures. The server-generated docking complex results were ranked and the structure with lowest energy-balanced score was selected. BIOVIA Discovery Studio Visualizer software (Dassault Systèmes, Paris, France) was used for visualizing the protein docking results.

*Evaluation of mRNA and protein expression in CCA tissues*. The mRNA expression levels of ANGPTL4-related proteins in CCA and normal tissues were investigated using GEPIA2 (http://gepia2.cancer-pku.cn/#index; last accessed on 2 October 2021) (21). The association between clinicopathological variables and mRNA expression levels was examined further using the University of Alabama at Birmingham Cancer data analysis portal (UALCAN) based on tumor grade and lymph node metastatic status (http://ualcan.path.uab.edu/index.html; last accessed on 2 October 2021) (29). The results were obtained as the box plot of mRNA expression levels of CCA and normal tissues. To evaluate protein expression, we retrieved immunohistochemical staining data of ANGPTL4-related proteins in CCA and normal tissues from the Human Protein Atlas (https://www.proteinatlas.org; last accessed on 2 October 2021) (30).

*Statistical analysis*. Protein expression of cell lines and siRNA experiments were analyzed by unpaired two-tailed Student's *t*-test. SPSS software version 26 (IBM Corp., Armonk, NY, USA) was used to analyze the data. Associations with values of $p<0.05$ were considered as statistically significant.

## Results

*ANGPTL4 is up-regulated in CCA cell lines*. ANGPTL4 protein expression in KKU-M055, KKU-100 and KKU-213A CCA cells, and an immortalized cholangiocyte cell line MMNK1 was investigated using western blot analysis. The results showed that the ANGPTL4 expression in the three CCA cell lines was significantly higher compared to that in MMNK1, with that of KKU-213A being the highest (Figure 1). Hence, the KKU-213A cell line was selected for gene-silencing experiments.

*ANGPTL4 gene silencing*. KKU-213A CCA cells were transiently transfected with siRNA against ANGPTL4 or with scramble negative control for 48 h. Then we investigated ANGPTL4 expression using western blot analysis. The results showed that ANGPTL4 expression was significantly lower in *ANGPTL4*-siRNA-treated cells compared to control cells ($p<0.0001$) (Figure 2).

*Differential protein expression between ANGPTL4-silenced and control cells*. To identify ANGPTL4-related proteins in CCA cells, protein-expression patterns of *ANGPTL4* gene-silenced and scramble-treated control cells were compared using a proteomics approach. A total of 681 proteins were identified and the fold-change values were calculated as a power of the intensity difference of proteins between control and siANGPTL4 cells by LC-MS/MS. Differentially expressed proteins were selected according to fold changes with the cut-off <0.6 and ≥2.0. In total, 321 differentially expressed proteins were identified, with 151 being down-regulated (cut-off <0.6) and 170 up-regulated (cut-off ≥2.0) in siANGPTL4 cells. To search for ANGPTL4-related signaling proteins and pathways, we selected down-regulated proteins for further study.

*Prediction of ANGPTL4-related signaling pathways*. The potential interactions of ANGPTL4 with the 151 down-regulated proteins were predicted using STITCH 5.0. ANGPTL4 was predicted to interact with 29 signaling proteins. Among these 29 proteins, we focused only on DNA-dependent protein kinase catalytic subunit (PRKDC) and 60S ribosomal protein L21 (RPL21), which are known to be associated with cancer progression signaling pathways such as non-homologous end-joining and ribosome signaling in the Kyoto Encyclopedia of Genes and Genomes in STITCH analysis (Table I). As shown in Figure 3, ANGPTL4 interacted with PRKDC and mammalian target of rapamycin (MTOR) *via* serine/threonine kinase 1 (AKT1). In addition, ANGPTL4 interacted with RPL21 *via* AKT1, MTOR and 60S ribosomal protein 5 (RPL5). Furthermore, the association between gene expression of *ANGPTL4* and *AKT1* (R=0.3, $p=0.00019$), *AKT1* and *PRKDC* (R=0.55, $p<0.0001$), *AKT1* and *MTOR* (R=0.48, $p<0.0001$), *MTOR* and *RPL5* (R=0.16, $p=0.05$), *RPL5* and *RPL21* (R=0.9, $p<0.0001$) in CCA were positively correlated by Spearman correlation test in GEPIA2 (Figure 4).

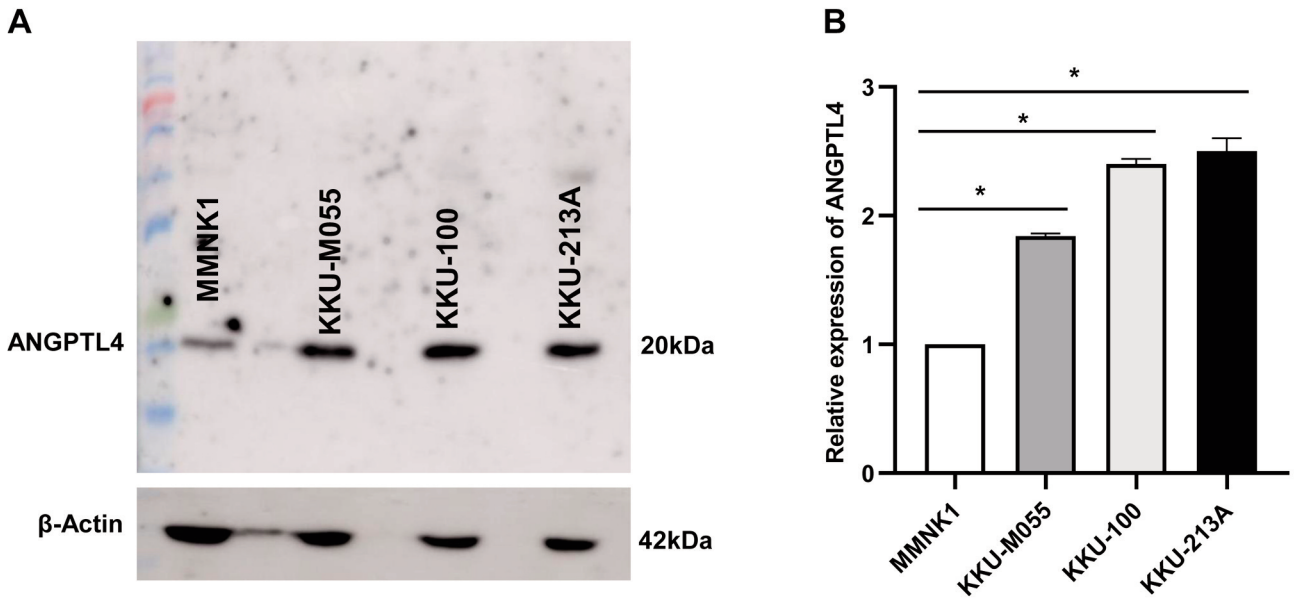


Figure 1. *Angiopoietin-like protein 4 (ANGPTL4) expression of three cholangiocarcinoma cell lines and immortalized cholangiocyte cell line MMNK1. A: Western blotting. β-Actin was used as an internal control for protein loading. B: Quantitative analysis of ANGPTL4 protein expression relative to the β-actin intensity. The values are presented as the mean±standard deviation of three independent experiments. Statistical difference was analyzed by unpaired two-tailed Student's t-test. *Significantly different at p<0.01.*

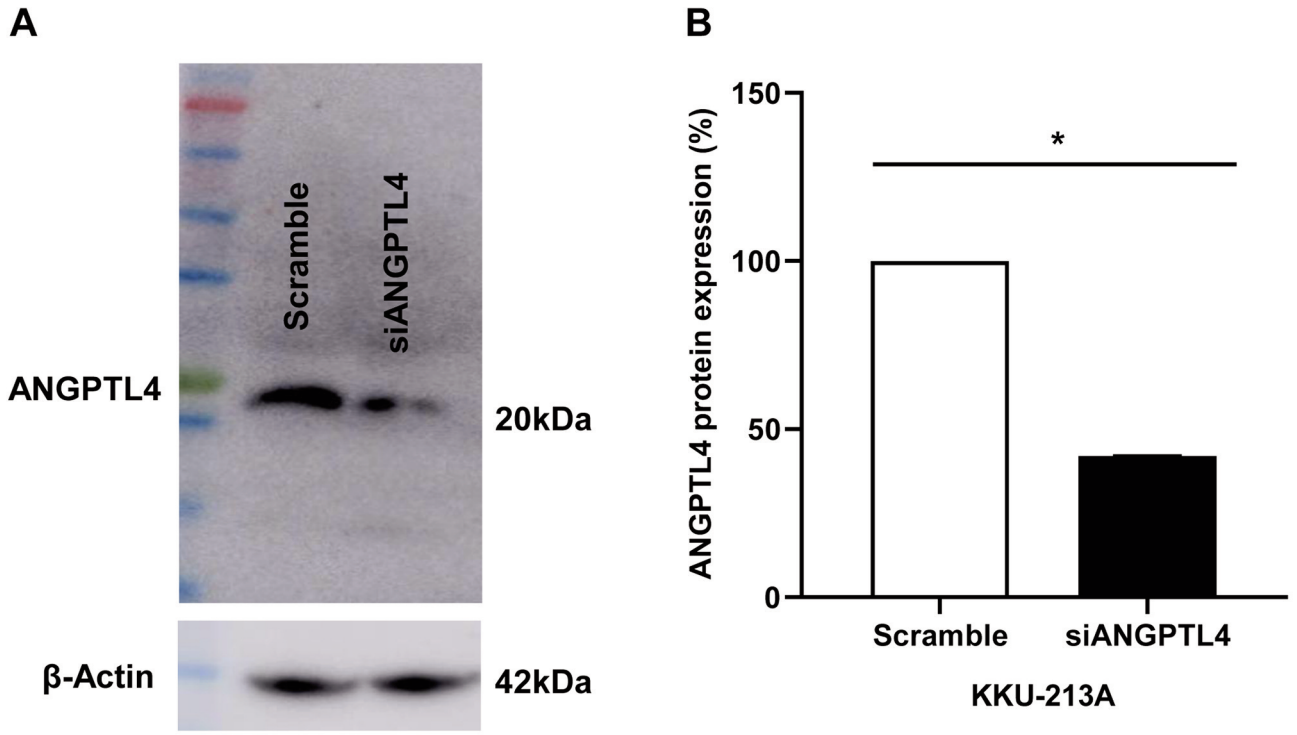


Figure 2. *Expression of angiopoietin-like protein 4 (ANGPTL4) in scramble control-transfected and ANGPTL4-silenced KKU-213A cells. A: Western blotting. β-Actin was used as an internal control for protein loading. B: The expression of ANGPTL4 protein after gene silencing relative to that of β-actin. The values are presented as the mean±standard deviation of three independent experiments. Statistical difference was analyzed by unpaired two-tailed Student's t-test. *Significantly different at p<0.01.*

Table I. *List of 29 signaling proteins down-regulated in angiopoietin-like protein 4 (ANGPTL4)-silenced KKU-213A cells and predicted to interact with ANGPTL4. Values represent the fold change of expression relative to scramble control cells.*

| Protein name | Gene symbol | Fold change |
|---|---|---|
| 60S ribosomal protein L21 | *RPL21* | $7\times10^{-7}$ |
| cDNA FLJ75478 | *SUGP1* | $3\times10^{-6}$ |
| E3 Ubiquitin-protein ligase TRIM8 | *TRIM8* | 0.029 |
| Gremlin-1 | *GREM1* | 0.087 |
| cDNA FLJ16482 fis | *SMAD7* | 0.152 |
| DNA-dependent protein kinase catalytic subunit | *PRKDC* | 0.217 |
| Laminin alpha 2 subunit variant | *LAMA2* | 0.295 |
| Polymerase delta interacting protein 46 | *POLDIP3* | 0.337 |
| Sterol carrier protein 2 | *SCP2* | 0.359 |
| Ankyrin repeat domain-containing protein 26 | *ANKRD26* | 0.362 |
| ATP-dependent RNA helicase DDX60 | *DDX60* | 0.366 |
| Peroxisomal proliferator-activated receptor A-interacting complex 285 | *HELZ2* | 0.38 |
| Mutant hemoglobin alpha 2 globin chain | *HBA2* | 0.382 |
| Pre-mRNA-processing factor 6 | *PRPF6* | 0.384 |
| Tripartite motif-containing 25 | *TRIM25* | 0.405 |
| DNA replication ATP-dependent helicase/nuclease | *DNA2* | 0.418 |
| Peptidylprolyl isomerase | *FKBP1A* | 0.432 |
| Histone-lysine *N*-methyltransferase, H3 lysine-36-specific | *NSD1* | 0.448 |
| Alternative protein NSFL1C | *NSFL1C* | 0.448 |
| Zinc finger protein GLI2 | *GLI2* | 0.45 |
| DNA polymerase kappa | *POLK* | 0.471 |
| Ubiquitin carboxyl-terminal hydrolase 15 | *USP15* | 0.498 |
| PREX2 | *PREX2* | 0.511 |
| COMMD3-BMI1 readthrough | *BMI1* | 0.535 |
| Nuclear receptor ROR-alpha | *RORA* | 0.55 |
| Mutant microtubule-associated protein tau | *MAPT* | 0.553 |
| cDNA FLJ16395 fis, clone TRACH2024139, highly similar to DNA polymerase eta | *POLH* | 0.561 |
| Leucyl-tRNA synthetase | *LARS* | 0.575 |
| Reelin | *RELN* | 0.588 |

According to STITCH pathway analysis, *RPL21* was associated with ribosome signaling and *PRKDC* was associated with non-homologous end-joining signaling pathway.

*Molecular docking by ClusPro analysis*. To determine the binding interaction of ANGPTL4 and related signaling proteins identified by STITCH (Figure 3), ClusPro 2.0 was used to investigate the binding geometries of corresponding protein–protein interactions. We ranked protein–protein docking of ANGPTL4 and AKT1, AKT1 and PRKDC, AKT1 and MTOR, MTOR and RPL5, and RPL5 and RPL21 based on proteins analyzed by STITCH. The three-dimensional protein structures of human ANGPTL4 (PDB ID: 6EUB), AKT1 (PDB ID: 4EKL), PRKDC (PDB ID: 5W1R) and MTOR (PDB ID: 4JSV) were downloaded from the Research Collaboratory for Structural Bioinformatics Protein Data Bank. The protein model structures of RPL5 and RPL21 were built in I-TASSER server and validated using PROCHECK's Ramachandra plot (31). Amino acid residues in the most favorable region of RPL5 and RPL21 proteins were determined using I-TASSER modeling. The binding energy of the balanced center and lowest energy scores of the protein–protein models are shown in Table II. The structures of protein–protein interactions were visualized using Discovery Studio Visualizer software (Figure 5). Overall, the results of gene-correlation analysis and molecular docking were comparable to those of STITCH analysis. Hence, ANGPTL4 is assumed to promote proliferation of CCA cells by interacting with PRKDC and RPL21 *via* AKT1, MTOR and RPL5 (Figure 6).

*mRNA overexpression of ANGPTL4-related signaling proteins in CCA tissues*. Since PRKDC and RPL21 were down-regulated, they were identified as potential ANGPTL4-related signaling proteins. Using the GEPIA2 database, we evaluated the mRNA expression levels of ANGPTL4-related signaling proteins in CCA tissues. The results showed that mRNA expression levels of *PRKDC* and *RPL21* in CCA tissues were significantly higher than that of adjacent normal tissues ($p<0.01$) (Figure 7A). Moreover, high levels of mRNA expression were associated with tumor grade II-IV ($p<0.0001$) and lymph node metastasis ($p<0.0001$) in patients with CCA (Figure 7B and C). Furthermore, to assess the protein expression levels, we retrieved the immunohistochemical

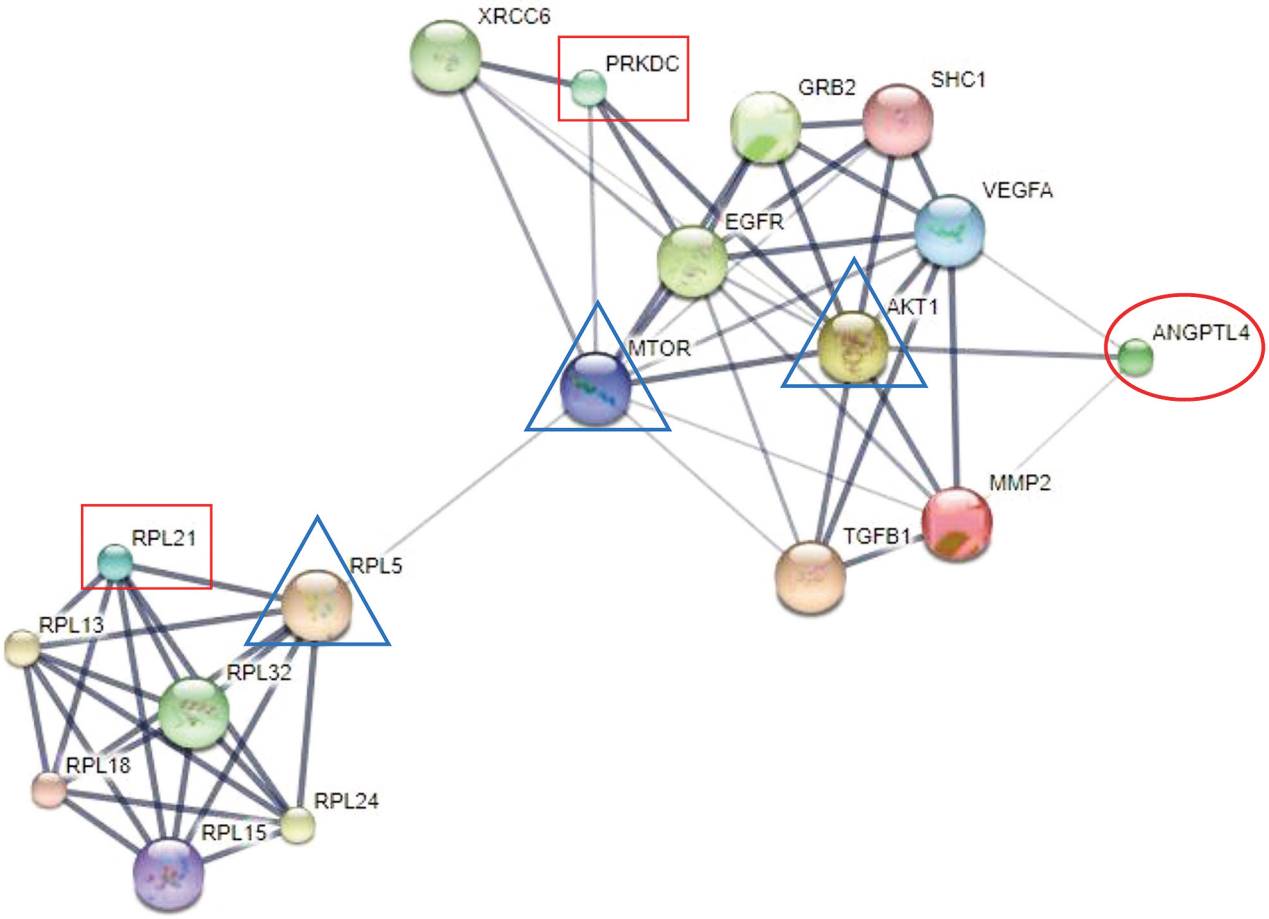


Figure 3. *The potential interaction of angiopoietin-like protein 4 (ANGPTL4)-related proteins in KKU-213A cells predicted by STITCH analysis. ANGPTL4 (red circle) interacts with putative signaling proteins DNA-dependent protein kinase catalytic subunit (PRKDC) and 60S ribosomal protein L21 (RPL21) (red box) via predicted molecules AKT serine/threonine kinase 1 (AKT1), mechanistic target of rapamycin kinase (MTOR) and ribosomal protein L5 (RPL5) (blue triangle). Thicker lines represent stronger associations, whereas thin lines represent weaker associations. EGFR: Epidermal growth factor receptor; GRB2: growth factor receptor-bound protein 2; MMP2: matrix metalloproteinase 2; SHC1: SHC adaptor protein 1; TGFB1: transforming growth factor B1; VEGFA: vascular endothelial growth factor A; XRCC6: X-ray repair cross complementing 6.*

staining data for PRKDC (ten cancerous and three normal tissues) and RPL21 (five cancerous and three normal tissues) from the Human Protein Atlas database. The results demonstrated that both PRKDC and RPL21 staining were obviously positive in cancerous tissues compared with negative staining in normal tissues (Figure 7D). Thus, both PRKDC and RPL21 might be involved in progression of CCA.

## Discussion

The *ANGPTL4* gene is located on chromosome 19p 13.3 and is a member of a family of angiopoietin-like proteins (ANGPTL1 to 8) (10). ANGPTL4 has a strong association with survival, proliferation, apoptosis, migration, invasion, and metastasis of various cancer cell types, including

Table II. *The binding energy score of the protein–protein complex models by ClusPro analysis.*

| Protein–protein complex model | Binding energy (Balanced) | |
|---|---|---|
| | Center (kcal/mol) | Lowest energy (kcal/mol) |
| ANGPTL4–AKT1 | –802.6 | –1,697.5 |
| AKT1–PRKDC | –1,046.6 | –1,204.5 |
| AKT1–MTOR | –813.1 | –1,040.2 |
| MTOR–RPL5 | –962.4 | –1,243.5 |
| RPL5–RPL21 | –974.2 | –1,010.1 |

ANGPTL4: Angiopoietin-like protein 4; AKT1: AKT serine/threonine kinase 1; PRKDC: DNA-dependent protein kinase catalytic subunit; MTOR: mechanistic target of rapamycin kinase; RPL5: ribosomal protein L5; RPL21: 60S ribosomal protein L21.

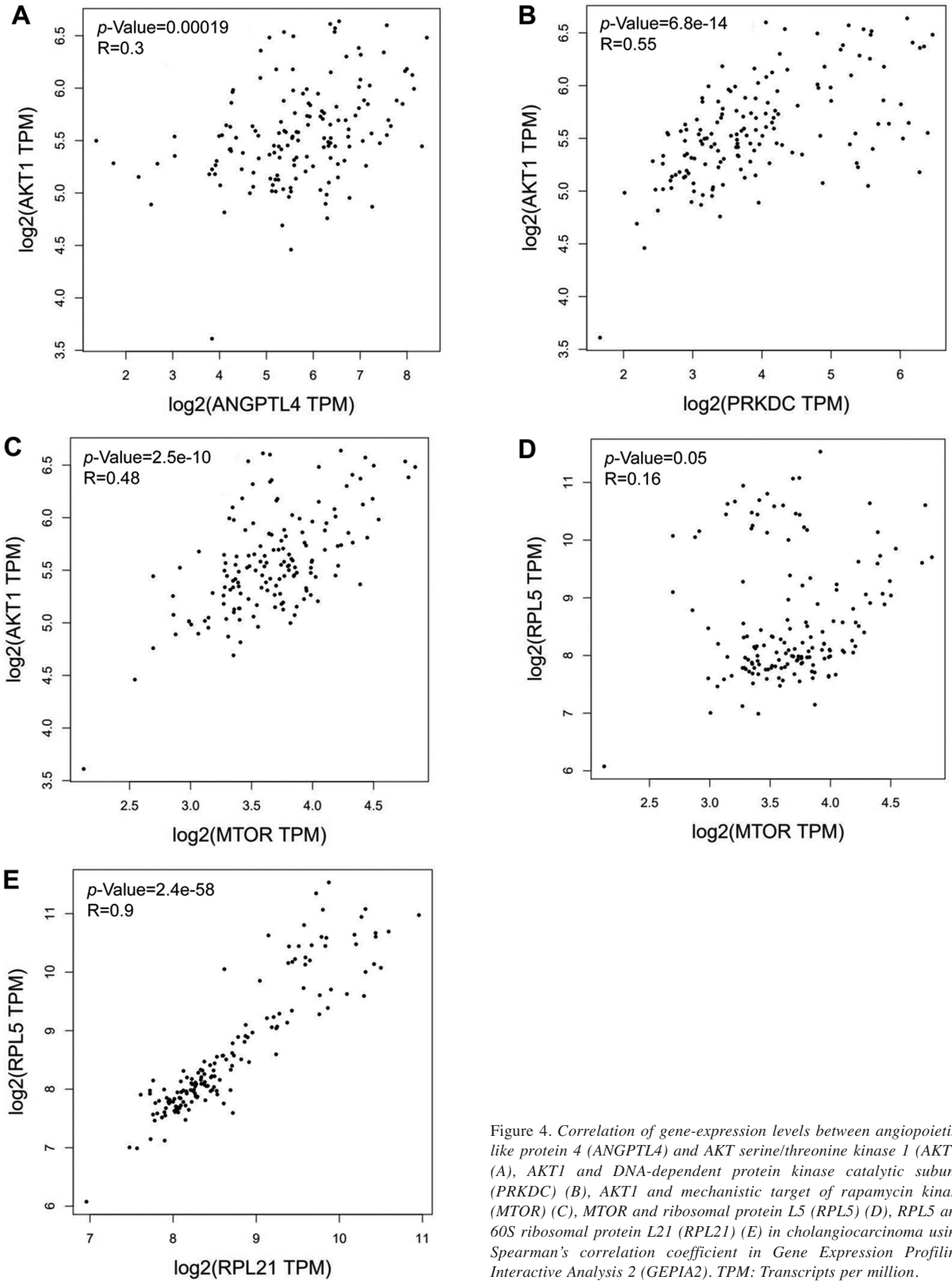


Figure 4. *Correlation of gene-expression levels between angiopoietin-like protein 4 (ANGPTL4) and AKT serine/threonine kinase 1 (AKT1) (A), AKT1 and DNA-dependent protein kinase catalytic subunit (PRKDC) (B), AKT1 and mechanistic target of rapamycin kinase (MTOR) (C), MTOR and ribosomal protein L5 (RPL5) (D), RPL5 and 60S ribosomal protein L21 (RPL21) (E) in cholangiocarcinoma using Spearman's correlation coefficient in Gene Expression Profiling Interactive Analysis 2 (GEPIA2). TPM: Transcripts per million.*

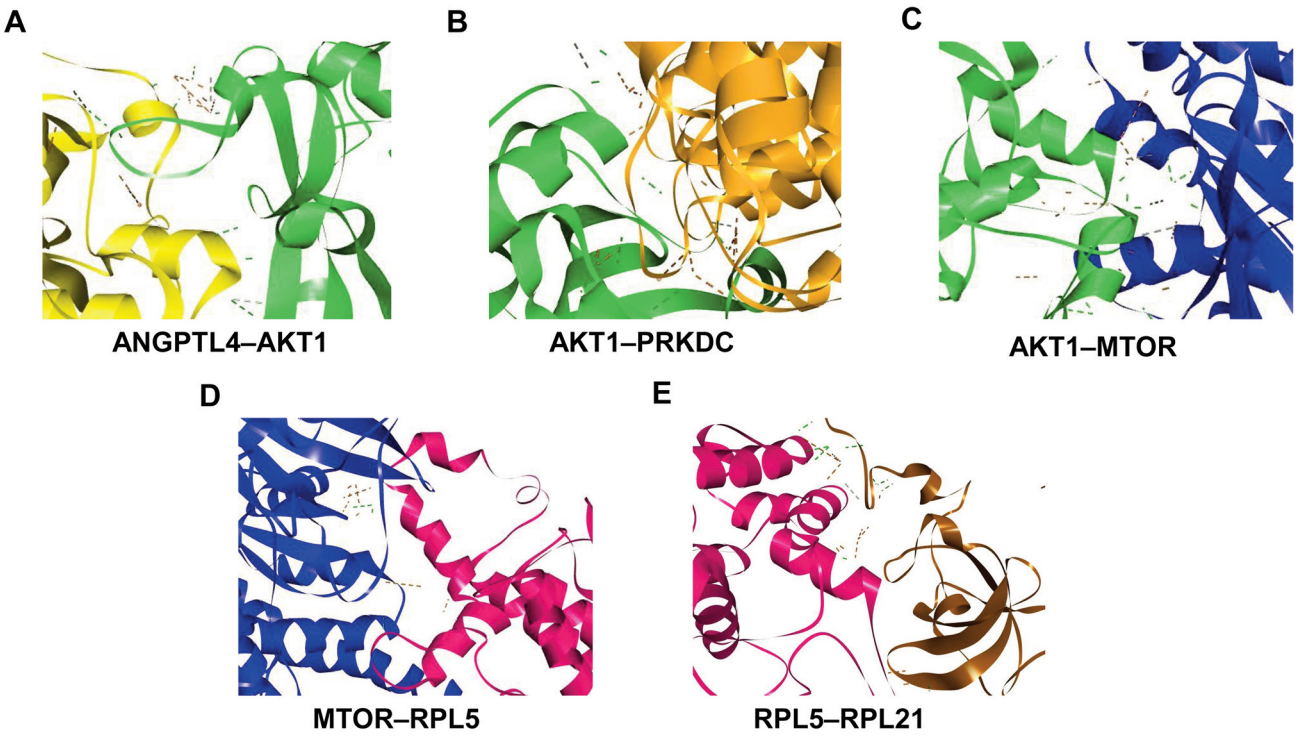


Figure 5. *Molecular interactions between protein–protein complexes by ClusPro 2.0. The dashed lines represent the interaction bonds between two structures. Angiopoietin-like protein 4 (ANGPTL4) is represented by a yellow ribbon interface with AKT serine/threonine kinase 1 (AKT1) (green) (A). AKT1 is represented by a green ribbon interface with DNA-dependent protein kinase catalytic subunit (PRKDC) (orange) (B). AKT1 is represented by a green ribbon interface with mechanistic target of rapamycin kinase (MTOR) (blue) (C). MTOR is represented by a blue ribbon interface with ribosomal protein L5 (RPL5) (pink) (D). RPL5 is represented by a pink ribbon interface with 60S ribosomal protein L21 (RPL21) (brown) (E).*

cervical (14), colorectal (15), gastric (16), and breast (36) cancer. We previously reported overexpression of ANGPTL4 in CCA tissues and serum from patients, suggesting that it may be a marker for poor prognosis of CCA (17). San *et al*. demonstrated that knockdown of *ANGPTL4* reduced the death of CCA cells (18). Proteomics approaches have been applied for determining cellular protein–protein interactions, signaling pathways and functions of proteins in cancer research (37-39). To our knowledge, this study is the first proteomic analysis to identify ANGPTL4-associated downstream proteins and signaling pathways in CCA cells. In this study, we found the highest expression of ANGPTL4 in KKU-213A cells. Thus, proteomic analysis was performed of *ANGPTL4* gene-silenced and scramble control KKU-213A cells. Using bioinformatic analysis, PRKDC and RPL21 were identified as potential ANGPTL4 signaling proteins. GEPIA2 analysis demonstrated that mRNA expression levels of both PRKDC and RPL21 were significantly higher in CCA than those in adjacent normal tissues. Their high levels of mRNA expression were also associated with advanced tumor grade and lymph node metastasis by UALCAN. Moreover, at the protein expression levels, they were overexpressed in CCA tissues retrieved from the Human Protein Atlas database. Therefore, ANGPTL4 may be associated with PRKDC and RPL21 and play a role in CCA progression.

In this study, using STITCH analysis, we found that ANGPTL4 interacted with PRKDC and RPL21 *via* AKT1, MTOR and RPL5. GEPIA2 analysis indicated a positive correlation between gene expression of *ANGPTL4* and *AKT1*, *AKT1* and *PRKDC*, *AKT1* and *MTOR*, *MTOR* and *RPL5*, *RPL5* and *RPL21* by Spearman correlation test. Additionally, ClusPro molecular docking showed the energy binding between ANGPTL4 and AKT1, AKT1 and PRKDC, AKT1 and MTOR, MTOR and RPL5, RPL5 and RPL21.

The *PRKDC* gene is located on chromosome 8q11. It encodes for a protein which is one of the core factors involved in non-homologous end-joining which is implicated in cancer progression (40). *PRKDC* was overexpressed in hepatocellular carcinoma cells and its silencing caused significant reduction of cell proliferation and induced massive apoptosis (41). In other malignancies, including gastric cancer (42) and breast cancer (43), high expression of PRKDC was associated with poor survival.

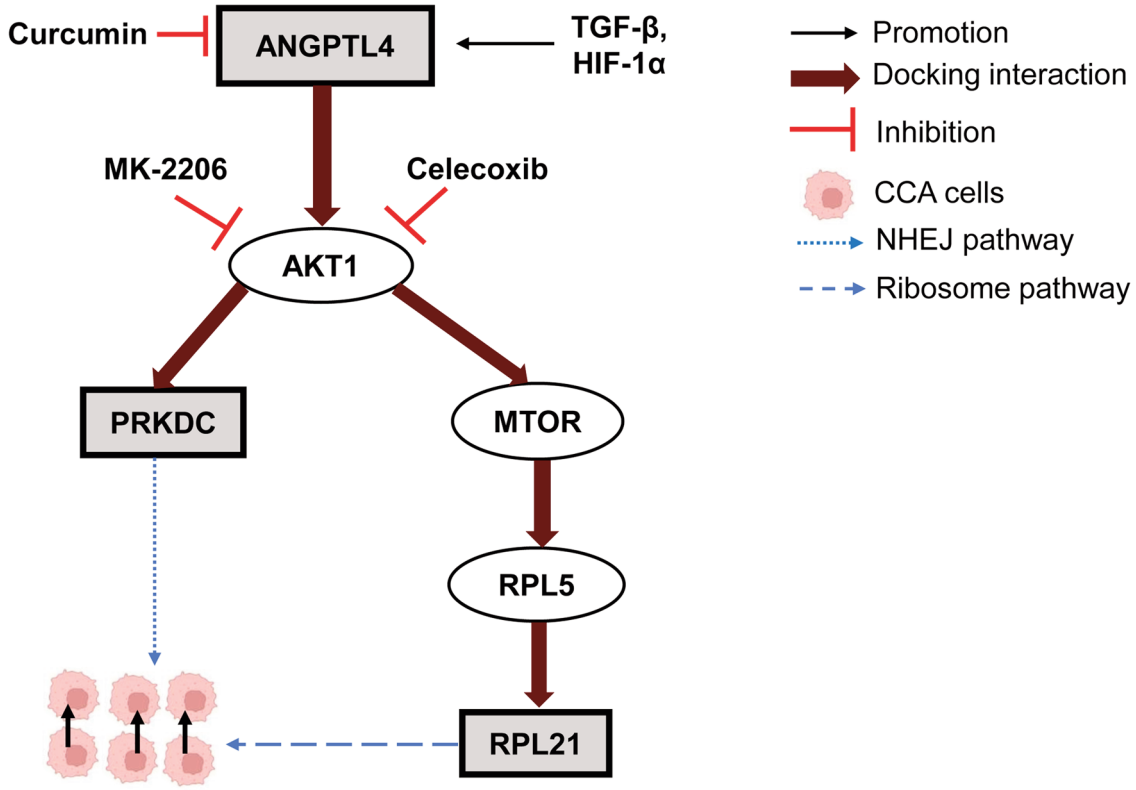


Figure 6. *Schematic diagram of prediction of angiopoietin-like protein 4 (ANGPTL4)-related signaling proteins and pathways in cholangiocarcinoma (CCA). The major signaling actor ANGPTL4 interacts with putative signaling proteins DNA-dependent protein kinase catalytic subunit (PRKDC) and 60S ribosomal protein L21 (RPL21) via predicted molecules AKT serine/threonine kinase 1 (AKT1), mechanistic target of rapamycin kinase (MTOR), and ribosomal protein L5 (RPL5), and is involved in CCA cells proliferation by the non-homologous end-joining (NHEJ) pathway and ribosome signaling pathway. Previous studies showed that transforming growth factor β (TGF-β) (32) and hypoxia-inducible factor-1α (HIF-1α) (11) induced ANGPTL4 expression. Curcumin inhibited ANGPTL4 expression in anoikis-resistant CCA cells (18). MK-2206 (AKT inhibitor) (33) and celecoxib (cyclooxygenase-2 inhibitor) (34, 35) inhibited CCA cell proliferation by inhibiting AKT1 protein.*

Depletion of PRKDC resulted in the reducing of proliferation and cell-cycle arrest. Ewald *et al*. showed that AKT1 is responsible for cell proliferation in CCA cells (44). It was reported that ANGPTL4 also promoted cell proliferation and reduced cell-cycle arrest in papillary thyroid cancer cells through activation of AKT phosphorylation (45). Toulany *et al*. showed that AKT1 interacts with PRKDC, promoting survival of tumor cells after irradiation (46). Therefore, ANGPTL4 might interact with PRKDC *via* AKT1 in the progression of CCA. The *RPL21* gene is located on chromosome 13q12.2. It encodes a ribosomal protein of a component of the 60S subunit, ribosomal protein L21 and is a member of the ribosomal protein L21e family (47). A recent study by Li *et al*. revealed that inhibition of RPL21 in pancreatic cancer induced cell-cycle arrest and apoptosis (48). MTOR, a member of phosphatidylinositol 3 kinase-related kinase family, acts as signal transducer in cell-cycle progression, cell metabolism, cell proliferation and survival (49). MTOR is a downstream molecule of AKT1 and the AKT/MTOR signaling pathway is one of the classical pathways for tumor growth and metastasis (50). Additionally, AKT1/MTOR activates ribosomal protein synthesis for survival, growth, and proliferation of cells (51). Jung *et al*. demonstrated that RPL5 is a MYC proto-oncogene bHLH transcription factor-mediated oncogene associated with midline 1-interacting protein 1 (MID1IP1) and is involved in hepatocellular carcinoma growth (52). Thus, we assume that ANGPTL4 might interact with RPL21 through AKT1, MTOR and RPL5 and is implicated in CCA development. Finally, the ANGPTL4 signaling pathways associated with PRKDC and RPL21 should be explored further *in vitro* and in *in vivo* models in order to develop new therapeutic strategies for CCA to improve poor prognosis.

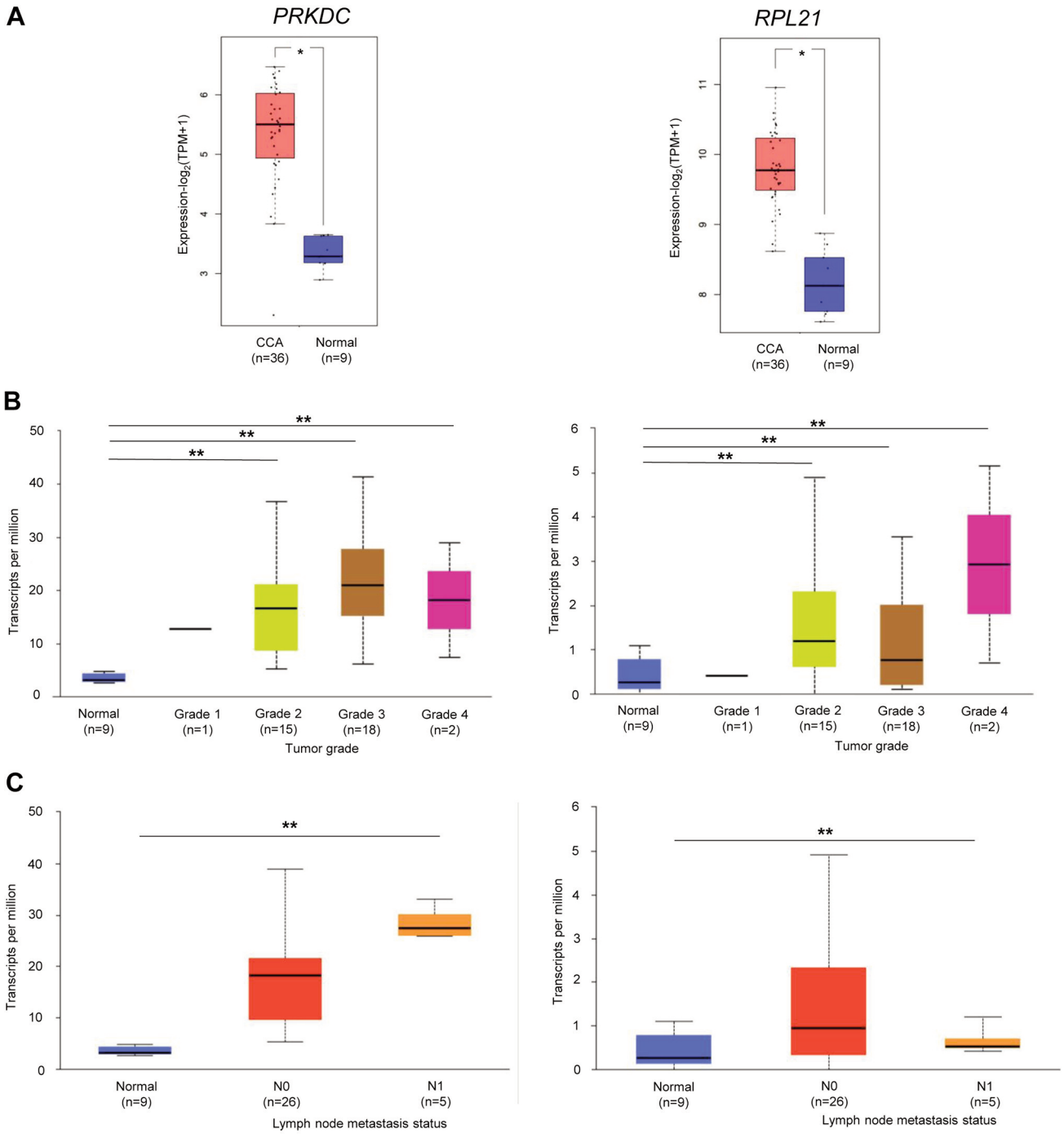


Figure 7. *Continued*

## Conclusion

In the present study, we demonstrated that ANGPTL4 in CCA development was possibly related to PRKDC through AKT1, and RPL21 through AKT1, MTOR and RPL5 by STITCH tools and ClusPro docking analysis. Moreover, by GEPIA, Human Protein Atlas and UALCAN, high expression of PRKDC and RPL21 was found in CCA tissues and associated with tumor grade and lymph node metastasis. Thus, PRKDC and RPL21 might be potential biomarkers in clinical prognosis of CCA.

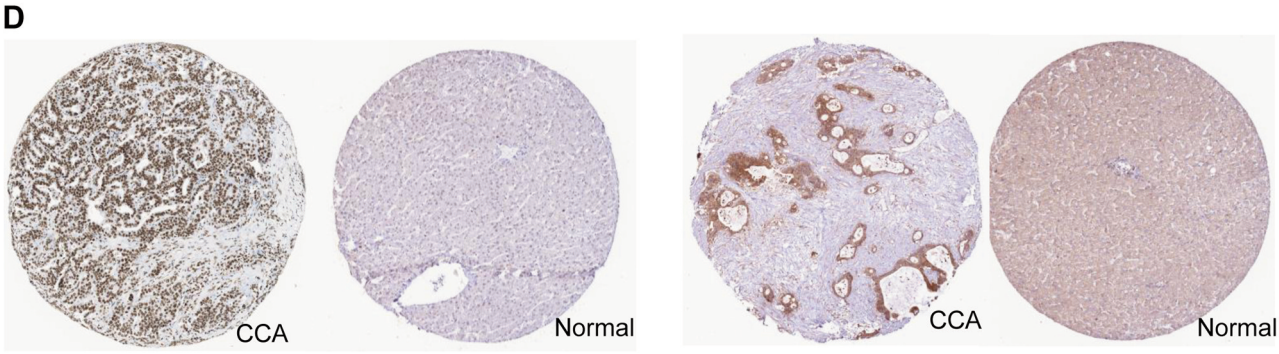


Figure 7. *Comparisons of mRNA expression of DNA-dependent protein kinase catalytic subunit (PRKDC) (left panel) and 60S ribosomal protein L21 (RPL21) (right panel) between cholangiocarcinoma (CCA) and normal tissues as shown by Gene Expression Profiling Interactive Analysis 2 GEPIA2 (A), and according to tumor grade (B) and lymph node metastasis status (C) using the University of Alabama at Birmingham Cancer portal. Comparison of protein expression of PRKDC and RPL21 between CCA and normal tissues (D) using data from the Human Protein Atlas. Grade 1: Well-differentiated (low grade); Grade 2: moderately differentiated (intermediate grade); Grade 3: poorly differentiated (high grade); Grade 4: undifferentiated (high grade); N0: no regional lymph node metastasis; N1: metastases in 1-3 axillary lymph nodes. Data are presented as transcripts per million (TPM) values with the median and interquartile range. The significance of difference in mRNA expression levels between groups was evaluated. Statistically significant at: *p<0.01 and **p<0.0001.*

## Conflicts of Interest

The Authors state that they do not have any conflicts of interest.

## Authors' Contributions

TMA: Conceptualization, methodology, investigation, data curation, writing-original draft; AS: Conceptualization, methodology, data curation; AJ: Conceptualization, methodology, data curation; WC: Conceptualization, methodology, data curation; TP: Conceptualization, methodology, data curation, review and editing; SR: Conceptualization, methodology, software, investigation and data curation; SP: Conceptualization, methodology, investigation, data curation, writing, review, editing and funding acquisition. The final version of the article has been read and approved by all Authors.

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