Complement factor H-related 3 overexpression affects hepatocellular carcinoma proliferation and apoptosis

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Abstract. Complement factor H-related 3 (CFHR3) belongs to the human factor H protein family and is associated with various human diseases, including nephropathy, age-related macular degeneration and atypical hemolytic uremic syndrome. However, to the best of our knowledge, the role of CFHR3 in hepatocellular carcinoma (HCC) remains largely unknown. In the present study, reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and western blot analysis were performed to determine mRNA and protein expression levels of CFHR3 in HCC and normal adjacent tissue. In addition, CFHR3 was overexpressed in Huh-7 cells and cell counting kit-8 assay was used to determine cell viability. Cell proliferation and apoptosis were assessed using flow cytometry, RT-qPCR and western blotting. The results demonstrated that mRNA (2-ΔΔcq) and protein expression levels of CFHR3 were significantly lower in tumor tissue compared with in adjacent tissue. Additionally, CFHR3 overexpression decreased cell viability, inhibited cell proliferation and significantly increased apoptosis. It was also identified that CFHR3 could downregulate the expression of Ki67. The results suggested that CFHR3 induced apoptosis by downregulating the expression of survivin and B cell lymphoma 2, upregulating the expression of Bcl-2-associated X and promoting caspase-3 activity. Western blotting revealed that CFHR3 significantly inhibited the protein expression levels of phosphorylated (p)-phosphoinositide 3-kinase (PI3K), p-protein kinase B (Akt) and p-mammalian target of rapamycin (mTOR). Overexpression of CFHR3 suppressed proliferation and promoted apoptosis of HCC cells by inhibiting the PI3K/Akt/mTOR signaling pathway.

Introduction

Liver cancer is one of the commonest types of cancer, with ~850,000 new cases being diagnosed annually worldwide (1). Hepatocellular carcinoma (HCC) is the most common type of primary liver cancer and accounts for ~90% of all liver cancer cases (2). HCC is the second leading cause of cancer related mortality in China (3). The high rate of mortality could be explained by many risk factors, including hepatitis B and C virus infections as well as excessive alcohol intake and chronic liver injury, all of which can contribute to the development of HCC (4-6). A previous study demonstrated that complement factor H-related (CFHR) 3 is highly expressed in normal liver tissue, but not in other tissues (7). This tissue-specific expression led to the hypothesis that CFHR3 may serve a role in HCC.

CFHR3 belongs to the human factor H protein family, which contains complement regulators, including complement factor H (CFH) and CFH-like protein 1 as well as other CFHR proteins (8). CFH family proteins have individual folding domains that are called short consensus repeats. These are also reflected in the tandem arrangement of the CFH gene and the five CFHR genes. Notably, CFHR3 and CFHR4 are similar, and have overlapping functions (9-11). Previous studies have reported that CFH genes are associated with several human diseases, including age-related macular degeneration, atypical hemolytic uremic syndrome and membranoproliferative glomerulonephritis type II (12-15). These diseases are increasingly thought to be caused by CFH polymorphisms, homozygous or heterozygous mutations and CFHR1/3 deletion (16-18).

Using in-situ hybridization, it was revealed that many tumor cells, including bladder, cervical and renal cancer cell lines, can produce and secrete human CFHR proteins (19,20). However, normal epithelial keratinocytes and colon cancer cell lines do not express CFHR proteins (21). Several studies have suggested that tumor cells evade the immune system by using CFH and its associated proteins (22-24). Cui et al (25), suggested that abnormal expression of the CFHR1 and CFHR3 gene may be related to cisplatin resistance in U251/CP2 gliomas. However, to the best of the authors’ knowledge, no studies have been conducted on the relationship between CFHR3 expression and HCC.

The phosphoinositide 3-kinase/protein kinase B/mamalian target of rapamycin (PI3K/Akt/mTOR) pathway is known
to be regulated in HCC (26). Thus, in the present study, the aim was to determine whether CFHR3 could inhibit liver cancer cells via the PI3K/Akt/mTOR pathway.

Materials and methods

Patients and tissue. Between May 2005 and July 2017, 42 HCC and adjacent normal tissues samples were collected from patients with HCC (age: 55.1±9.2 years; 23 male, 19 female), undergoing surgical resection at the Yantai Infectious Disease Hospital (Yantai, China). Inclusion criteria were HCC diagnosed according to Clinical diagnosis and staging of Primary Hepatocellular carcinoma (27). Exclusion criteria were patients receiving hormones and/or antineoplastic drugs, radiotherapy or chemotherapy within 2 weeks prior to surgery. Tissues were confirmed as HCC following pathological examination. The normal liver tissues were taken at a distance of >2 cm from the edge of the tumor. All HCC specimens and corresponding adjacent normal tissues were collected within 0.5 h of surgical resection. One section of the paired tissues was used for pathological diagnosis and stored in 4% formaldehyde, while the section was stored in liquid nitrogen for later experimentation. This study was approved by the Ethics Committee of Yantai Infectious Disease Hospital (Yantai, China). Written informed consent was obtained from the patients.

The Cancer Genome Atlas (TCGA) database. Another cohort of patients with HCC was obtained from the TCGA database (https://cancergenome.nih.gov/) via the Genomic Data Commons Data Portal. The expression values of genes from RNA-seq data were scaled with log2 (FPKM + 0.01). The CFHR3 expression in human tissues. Using NCBI database (https://www.ncbi.nlm.nih.gov/gene/10878), RNA-seq was performed on tissue samples from 95 human individuals representing 27 different tissues in order to determine tissue-specificity of all protein-coding genes.

Cell culture. The Huh-7 cell line was purchased from Procell Life Science & Technology Co., Ltd. (Wuhan, China). Cells were cultured in RPMI 1640 medium (HyClone; GE Healthcare Life Sciences, Logan, UT, USA) containing 20% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) at 37°C and 5% CO2. The cells were digested with 0.25% trypsin (Gibco; Thermo Fisher Scientific, Inc.), according to manufacturer’s protocol. Cells were transfected with 20 µM CFHR3 overexpression or NC plasmids using 5 µl Lipofectamine® 3000 (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer’s protocol. Cells were transfected with 20 µM CFHR3 overexpression or NC plasmids using 5 µl Lipofectamine® 3000, in serum-free medium at 25°C for 10 min. Cells treated with 0.1% PBS were used as the control. Following a 6 h incubation at 37°C, the cells were cultured in RPMI 1640 medium containing 10% FBS. Cells were used for subsequent experiments 3 day later.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). RT-qPCR was performed to examine the gene expression levels of CFHR3, Ki67, survivin, caspase-3, Bcl-2-associated X (Bax), B cell lymphoma 2 (Bcl-2), PI3K, Akt and mTOR. Total RNA was extracted from tissues samples or cultured cells using TRIzol® (Invitrogen; Thermo Fisher Scientific, Inc.), according to manufacturer’s instructions and reversed transcribed to cDNA using the QuantiTect Reverse Transcription kit (Qiagen GmbH, Hilden, Germany). The RT conditions were as follows: 37°C for 60 min and 95°C for 5 min. qPCR was carried out using the SYBR qPCR Mix (Toyobo Life Science, Osaka, Japan), with GAPDH as the internal reference. The thermocycling conditions were as follows: 95°C for 10 min, followed by 40 cycles of 95°C for 10 sec, 60°C for 10 sec and 72°C for 10 sec. The primer sequences are listed in Table I. The relative mRNA expression levels were calculated using the 2-ΔΔCq method (28).

Western blotting. Total protein was extracted from tissues or cultured cells using lysis RIPA buffer (Thermo Fisher Scientific, Inc.), followed by centrifugation at 18,000 × g for 5 min at 4°C. Protein concentration was determined using an Enhanced BCA Protein assay kit (Beyotime institute of Biotechnology, Shanghai, China). Proteins (20 µg/lane) were separated by 10% SDS-PAGE and transferred onto polyvinylidene difluoride membranes, which were blocked with 5% milk in Tris-buffered saline containing 0.2% Tween-20 (TBST) at room temperature for 2 h. Proteins were incubated with primary antibodies: Rabbit anti-CFHR3 (1:1,000; cat. no. 16583-1-AP; ProteinTech Group, Inc., Chicago, IL, USA), rabbit anti-Bcl-2 (1:1,000; cat. no. ab32124; Abcam, Cambridge, MA, USA), rabbit anti-Bax (1:1,000; cat. no. ab32503; Abcam), rabbit anti-Ki67 antibody (1:1,000; cat. no. ab16667; Abcam), rabbit anti-survivin (1:5,000; cat. no. ab76424; Abcam), rabbit anti-caspase-3 (1:500; cat. no. ab13847; Abcam), rabbit anti-Pi3K antibody (1:500; cat. no. orb137259; Wuhan Booute Co. Ltd., Wuhan, China), rabbit anti-p-Pi3K antibody (1:1,000; cat. no. 4228; Cell Signaling Technology, Inc., Danvers, MA, USA), rabbit anti-p-Akt (1:500; cat. no. ab8805; Abcam), rabbit anti-p-Akt antibody (1:2,000; cat. no. 4060; Cell Signaling Technology, Inc.), rabbit anti-mTOR (1:1,000; cat. no. abl137341; Abcam), rabbit anti-p-mTOR antibody (1:1,000; cat. no. 5536; Cell Signaling Technology, Inc.) and rabbit anti-GAPDH (1:1,000; cat. no. ab9485; Abcam) at 4°C overnight. Subsequently, membranes were washed with TBST and incubated with goat anti-rabbit horseradish peroxidase-conjugated antibodies (1:2,000; SA00001-2, ProteinTech Group, Inc., Chicago, IL, USA) at room temperature for 90 min. The protein bands were visualized using an ECL system (Amersham; GE Healthcare, Chicago, IL, USA). Densitometric analysis of western blots was performed using Quantity One® software version 2.4 (Bio-Rad, Laboratories, Inc., Hercules, CA, USA).

Cell Counting Kit-8 (CCK-8) cell viability analysis. Cells were dissociated using 0.25% trypsin, 0, 12, 24 and 48 h after transfection. Then, cells were seeded in 96-well plates at a density of 1x104 cells/well and 10 µl CCK-8 solution was added to the cells for 2 h at 37°C. The optical density was measured at a wavelength of 450 nm using a microplate reader (Bio-Rad, Laboratories, Inc.).
Flow cytometry. Cells were harvested 48 h after transfection, and then digested by 0.25% trypsin without EDTA and collected in 1.5 ml Eppendorf tubes. Cells were washed twice using washing buffer, and then incubated with annexin V-FITC and propidium iodide (cat. no. 40302; Yeasen, Shanghai, China) in the dark at 25˚C for 20 min. Binding buffer was added to each tube and apoptosis analyzed within 1 h. The apoptosis rate is derived from the addition of right upper quadrant and right lower quadrant together.

Cell proliferation was measured using carboxyfluorescein succinimidyl ester (CFSE) (29). Cells were labeled with CellTrace™ CFSE kit (cat. no. 34554; Invitrogen; Thermo Fisher Scientific, Inc.) for 10 min at 37˚C and then washed twice with phosphate-buffered saline. The cells were incubated for at least 10 min before analysis to allow the CellTrace™ reagent to undergo acetate hydrolysis. Fluorescence was measured using a flow cytometer and FlowJo version 10.0 software (FlowJo LLC, Ashland, OR, USA).

Statistical analysis. GraphPad Prism software version 6.0 (GraphPad Software, Inc., La Jolla, CA, USA) was used to perform statistical analyses. All data are presented as the mean ± standard deviation. One-way analysis of variance followed by Tukey's post hoc test was used to compare the means of multiple groups. Correlation between CFHR3 expression levels and tumor diameter was analyzed by Pearson's correlation coefficient. P<0.05 was considered to indicate a statistically significant difference.

Results

CFHR3 mRNA and protein expression levels in HCC and adjacent tissue. Survival analysis of patients with HCC from the TCGA database is shown in Fig. 1A. The overall survival of patients with HCC was significantly improved in the CFHR3 high expression group compared with the low expression group (P=9.62×10⁻⁵). CFHR3 expression levels in 27 different normal tissues were also compared (Fig. 1B), using the HPA RNA-seq normal tissues from NCBI (https://www.ncbi.nlm.nih.gov/gene/10878). The data demonstrated that CFHR3 was only highly expressed in liver tissues, suggesting that CFHR3 may be tissue-specific and related to the progression of liver cancer.

In the present study, 42 patients with HCC were recruited, and CFHR3 mRNA expression levels in HCC and adjacent tissues were detected by RT-qPCR. Western blot analysis of CFHR3 protein expression levels was performed on tissue samples from six patients. The results revealed that CFHR3 mRNA expression levels were significantly decreased in HCC tissue compared with adjacent normal liver tissue (P<0.05; Fig. 2A). This was consistent with the western blot results, which demonstrated that CFHR3 protein expression levels were significantly decreased in HCC tissue (P<0.01; Fig. 2B). However, the correlation between CFHR3 expression levels and tumor diameter in the 42 HCC cases was not significant (P>0.05; Fig. 2C).

CFHR3 expression in HCC cells. Huh-7 cells were transfected with the NC plasmid, and the RT-qPCR and western blot results

Table I. Primers used for reverse transcription-quantitative polymerase chain reaction.

| Gene       | Primer | Sequence                  |
|------------|--------|---------------------------|
| CFHR3      | Forward | 5'-TGGGCATTAGTCAGAATACAGTAAA-3' |
|            | Reverse | 5'-ATTAATGGCCGCTTCTACATTGACTT-3' |
| Ki67       | Forward | 5'-AAAGTGCCAAGCCATAGA-3'    |
|            | Reverse | 5'-CACCATTGCGATGTCCCTC-3'   |
| Survivin   | Forward | 5'-TCATCTCCTAGGCGGTGTC-3'   |
|            | Reverse | 5'-AGGTCATCTCGGCTGGTCCGTG-3'|
| Caspase-3  | Forward | 5'-ACTTGACTGTGCGATTAG-3'    |
| Bax        | Forward | 5'-GCGAATTGGGAGTAACT-3'     |
|            | Reverse | 5'-GTGACAGGCGGCGGTAGGAC-3'  |
| Bcl-2      | Forward | 5'-GTTGAGCTTATGATTTTGTGG-3' |
|            | Reverse | 5'-CCTCCGCAATTGCAAAG-3'     |
| PI3K       | Forward | 5'-ATGCGCTGCTCTGATGCGG-3'   |
| Akt        | Forward | 5'-TTGTCAATGGAGTACGCCAACG-3'|
|            | Reverse | 5'-ACAGCCGGAGCTGTGACTCCTT-3'|
| mTOR       | Forward | 5'-CCAATCATCCGATTACGCT-3'   |
| GAPDH      | Forward | 5'-AATCTCATCACCATTCA-3'     |
|            | Reverse | 5'-CTGCTTCACCACCCTTGGTG-3'  |

Akt, protein kinase B; Bax, Bcl-2-associated X; Bcl-2, B cell lymphoma 2; CFHR3, complement factor H-related 3; mTOR, mammalian target of rapamycin; PI3K, phosphoinositide 3-kinase.
demonstrated that transfection had no effect on CFHR3 expression levels compared with the control group. Cells transfected with the CFHR3 overexpression plasmid had significantly higher CFHR3 mRNA and protein expression levels compared with the control or NC group (P<0.01; Fig. 3A and B).

**CFHR3 inhibits cell viability.** Cell viability was detected using CCK-8 assay. The results revealed that at 24 and 48 h post-transfection cell viability was significantly decreased in the CFHR3 overexpression group compared with in the control or NC group (P<0.05; Fig. 3C). Therefore, the results suggested that cell viability can be inhibited by CFHR3.

**Effect of CFHR3 on cell proliferation and apoptosis.** Cell proliferation and apoptosis were measured using flow cytometry. CFHR3 overexpression significantly decreased the cell fluorescence intensity (M1 value; P<0.01; Fig. 4A-D), indicating that CFHR3 inhibited cell proliferation. In addition, the results also demonstrated that CFHR3 overexpression significantly increased the apoptosis rate compared with the control or NC group (P<0.01; Fig. 4E-H).

**CFHR3 regulates the expression of proliferation- and apoptosis-related genes.** In order to confirm the effects of CFHR3 on cell proliferation and apoptosis, protein and mRNA expression levels of proliferation- and apoptosis-related proteins and genes were detected using western blotting and RT-qPCR, respectively. The western blot results revealed that the protein expression levels of Ki67, survivin and Bcl-2 were significantly downregulated in the CFHR3 overexpression group (P<0.05; Fig. 5A and B). However, protein expression levels of active-caspase-3 and Bax were significantly increased in the CFHR3 group compared with in the control or NC group (P<0.01; Fig. 5A and B). A similar pattern was observed for mRNA expression levels. CFHR3 overexpression significantly decreased the mRNA expression levels of Ki67, survivin and Bcl-2 (P<0.05; Fig. 5C), whereas active-caspase-3 and Bax were significantly increased (P<0.05; Fig. 5C).
Figure 2. CFHR3 expression levels in HCC and adjacent tissue. (A) A total of 42 patients with HCC were recruited and relative CFHR3 mRNA expression levels were detected in HCC and adjacent tissue using reverse transcription-quantitative polymerase chain reaction. *P<0.05. (B) CFHR3 protein expression levels were evaluated in the HCC and adjacent tissue of six patients with HCC by western blot analysis. (C) Correlation between CFHR3 expression levels and tumor diameter in 42 HCC cases. Data are presented as the mean ± standard deviation from three independent experiments. **P<0.01 vs. Adjacent. A, adjacent; C, cancer; CFHR3; complement factor H-related 3; HCC, hepatocellular carcinoma.

Figure 3. Effect of CFHR3 on the cell viability of Huh-7 cells. Huh-7 cells were transfected with the CFHR3 overexpression plasmid and transfection efficiency was confirmed by (A) western blot analysis and (B) reverse transcription-quantitative polymerase chain reaction. (C) Cell viability was determined using the cell counting kit-8 assay. Data are presented as the mean ± standard deviation from three independent experiments. *P<0.05, **P<0.01 vs. Con. CFHR3, complement factor H-related 3; Con, control; NC, negative control.
CFHR3 inhibits PI3K/Akt/mTOR signaling. The protein expression levels of PI3K, Akt and mTOR were detected in order to determine the potential involvement of the PI3K/Akt/mTOR signaling pathway in HCC. The results demonstrated
no noticeable differences in the protein expression levels of PI3K, Akt and mTOR among the control, NC and CFHR3 groups. However, CFHR3 significantly inhibited the phosphorylation of PI3K, Akt and mTOR (P<0.01; Fig. 6). Taken together, the results suggested that CFHR3 can inhibit liver cancer cell growth via the PI3K/Akt/mTOR pathway.

Discussion

CFHR3 is mainly studied in the context of IgA nephropathy, age-related macular degeneration and atypical hemolytic uremic syndrome (30-32). Previous studies have demonstrated that CFH family proteins may be important in cancer (19,20,33); however, little is known about the role of CFHR3 in cancer. Researchers have suggested that high expression levels of CFHR3 could increase the risk of neuroblastoma and glioma (19,34). CFHR3 is tissue-specific and is related to the progression of liver cancer. In the present study, the expression of CFHR3 in different normal human tissues was investigated and it was demonstrated that CFHR3 was highly expressed in normal liver tissues. In addition, high expression of CFHR3 in patients with HCC resulted in an improvement in overall survival. Therefore, it was hypothesized that CFHR3 may inhibit the occurrence and development of HCC. To further validate this, 42 patients with HCC were recruited, and expression of CFHR3 in HCC and adjacent normal tissues were detected. The results demonstrated that liver cancer tissue had a lower CFHR3 mRNA (2-ΔΔCt) and protein levels compared with normal tissue. However, no significant correlation between CFHR3 expression level and tumor diameter was identified in the 42 HCC cases. The sample size in this study was relatively small, which may affect the results obtained.

Liver cancer is one of the most common forms of cancer. Surgical resection and chemotherapy are the most common strategies to treat HCC (35-38). However, the prognosis for patients with liver cancer has not been improved significantly. Thus, it is necessary to develop new approaches to treat liver cancer. In the present study, CFHR3 was overexpressed in HCC cells. This resulted in a significant inhibition of cell viability and proliferation, whereas apoptosis was significantly upregulated. The protein and mRNA expression levels of proliferation- and apoptosis-related genes were also detected. The results suggested that CFHR3 inhibited proliferation and induced apoptosis by downregulating the expression of Ki67, Bel-2 and survivin, and upregulating the expression of Bax and active-caspase-3. Ki67 is a cell proliferation marker (39). Survivin is an important member of the inhibitor of apoptosis protein family and serves a critical role in the inhibition of apoptosis (40,41). Caspase-3 is a key protein of the caspase family and has a major role in the execution-phase of apoptosis (42).

The occurrence and development of primary liver cancer is closely related to cell signaling pathways. The signal transduction pathways in HCC form a complex network, which involves multi-factor and multi-protein pathways. Cell growth is associated with several major molecular pathways, including Ras/
mitogen-activated protein kinase kinase, PI3K/Akt/mTOR, Wnt/β-catenin, Janus kinase/signal transducer and activator of transcription (43-47). Among these four pathways, the PI3K/Akt/mTOR pathway is most closely related to HCC (48). PI3K is a heterodimer that is composed of a regulatory p85 subunit and a catalytic p110 subunit. PI3K is found in the cytoplasm, and when activated, aggregates to the cell membrane and interacts with the pleckstrin homology domain of Akt, leading to its activation. mTOR is a downstream molecule of the PI3K/Akt pathway, which controls cell growth and proliferation by regulating the cell cycle. Dysregulation of the cell cycle can lead to cell transformation and tumor deterioration (49,50).

In the present study, the results revealed that CFHR3 significantly inhibited the phosphorylation and thereby inactivation of PI3K, Akt and mTOR. However, only the effects of CFHR3 overexpression were demonstrated in the present study. Therefore, using a knock-down of CFHR3 could strengthen the results. In addition, the TCGA database contains an abundant of clinical information. It would be useful to conduct multivariate Cox regression analysis to identify the effect of other important prognostic factors, including serum α-fetoprotein, tumor stage, cirrhosis, vascular tumors and inflammation on overall survival rates, using blood samples. This analysis would help evaluate the independent prognostic value of CFHR3 mRNA expression.

In addition, an association between CFHR3 expression and tumor diameter could further confirm the results obtained from the in vitro experiments. Future experiments in vivo will also aid in validating the results and investigations probing into the definite mechanism underlying the effect of CFHR3 will be of interest.

In conclusion, the results from the present study suggested that CFHR3 functions as a tumor suppressor. CFHR3 may suppress proliferation and promote apoptosis via the PI3K/Akt/mTOR signaling pathway.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

HL made substantial contributions to the conception and design of the present study. LZ and PW performed data acquisition, analysis and interpretation. PW and HL drafted the article and critically revised it for important intellectual content. All authors approved the final version of the published article. LZ agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of the study are appropriately investigated and resolved.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of Yantai Infectious Disease Hospital (Yantai, China). Written informed consent was obtained from patients.

Patient consent for publication

Written informed consent was obtained from patients.

Competing interests

The authors declare that they have no competing interests.

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