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FGFR2-CCDC6 fusion gene promotes the proliferation of Hucct-1 cells

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ABSTRACT

To investigate the effect of the FGFR2-CCDC6 fusion gene on cell proliferation and its mechanism of action, pCDNA3.1- FGFR2bWT, pCDNA3.1- FGFR2-CCDC6 expression plasmids were transiently transfected into Hucct-1 cells using Lipo-2000 liposomes. The effect of the fusion gene on cell proliferation was examined by MTT and the expression of FGFR2/AKT/signaling pathway proteins was detected by Western blot. Results showed that Hucct-1 cells transfected with the FGFR2-CCDC6 fusion gene showed increased FGFR2 protein expression ($P<0.001$) and significantly higher cell proliferation capacity ($P<0.001$) compared to normal controls. It was concluded that The FGFR2-CCDC6 fusion gene excessively activates the AKT, and ERK signaling pathway downstream of FGFR2 and plays a role in promoting cell proliferation.

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Introduction

Intrahepatic Cholangiocarcinoma (ICC) has a poor prognosis due to its high malignancy and atypical early clinical symptoms, with most patients already in the progressive stage when diagnosed and losing the best chance of surgical treatment (1). Therefore, a deeper understanding of the molecular mechanisms of ICC development is key to the exploitation of effective targeted therapies.

Fibroblast growth factor receptor (FGFR) is widely expressed in a variety of cells, with fibroblast growth factor (FGF) involved in regulating important physiological processes such as cell proliferation, differentiation and growth (2,3). During tumor development, abnormal changes in the FGFR signaling pathway occur in a variety of cancer cells, including mutations, amplifications, gene rearrangements or ectopic and gene fusions (4). The FGFR2 gene is a member of the FGFR family. With the development of high-throughput transcript sequencing technology, the fibroblast growth factor receptor-2 gene was found to be fused to a variety of genes in ICC (5). Early clinical drug trials have also found that patients with FGFR2 fusion genes are more sensitive to tyrosinase inhibitor therapy, suggesting that FGFR2 fusion genes may be potential targets for ICC drug therapy (1,5).

Previous studies of clinical cases have identified the presence of an FGFR2-CCDC6 fusion gene in ICC, which is closely associated with the development of ICC. The fusion gene has been shown to promote tumor growth in an in vivo study of xenograft tumors in nude mice, a process associated with the over-activation of the FGFR2 downstream signaling pathway. However, the mechanism of its oncogenic potential still needs further validation (6). To further investigate the mechanism of the tumorigenic effect of this fusion gene, human bile duct epithelial carcinoma cells (Hucct-1) were used in this study to observe the effect of this fusion protein on cell proliferation and the related mechanism at the cellular level.

Materials and Methods

Main materials

Hucct-1 cells (preserved at the School of Pathology, Qiqihar Medical College), pCDNA3.1- FGFR2b expression plasmid (synthesized by Feng Hui Biological Co., Ltd.), pCDNA3.1- FGFR2-CCDC6 expression plasmid (gift from Prof. Robert), Mouse monoclonal Bek (FGFR2,SC-6930), Rabbit polyclonal to phosphorylated FGFR2 (cell signaling-3471), Rabbit polyclonal to AKT (cell signaling-9272), Rabbit polyclonal to phosphorylated AKT (cell signaling-4058), Mouse polyclonal to ERK1/2 (sc-514302), Mouse polyclonal to phosphorylated ERK1/2 (sc-81492), Mouse anti-actin (sc-8432), Sheep anti-mouse IgG (Invitrogen-35513), Sheep anti-mouse rabbit IgG (Invitrogen-16110), Lipofectamine 2000 (Invitrogen-11668027), OptiMEM low serum medium (Invitrogen-31985070), Extra Ultrasensitive ECL Chemiluminescence Kit (Biyuntian-P0018AS, China), TRIzol™ Reagent (Invitrogen-15596026), CCK8 Cell Proliferation Assay Kit (Biyuntian-C0037,China). All other reagents were domestic analytical purity and imported sub-assemblies. The main instruments included a CO2 cell incubator

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(Thermo 8000), YJ1452 ultra-clean bench (Suzhou Purification Equipment Factory), etc.

Cell culture and transfection

Hucct-1 cells were cultured in RPMI1640 medium containing 10% fetal bovine serum in an incubator at 37°C and 5% CO2 saturated humidity. Transfection experiments were performed on cells in the number growth phase. The cells were inoculated in 24-well plates with $4X10^4$ cells per well. pCDNA3.1- FGFR2 and pCDNA3.1- FGFR2-CCDC6-TOPO expression plasmids were transiently transfected by referring to the Lipofectamine2000 transfection reagent instructions. The cells were collected 48 hours after transfection for the next step of the experiment.

Western Blot detection of protein expression

Total cellular proteins were extracted and quantified, and the proteins were transferred to the PVDF membrane after 12% SDS-PAGE electrophoresis, closed for 2 hours, and then immunoreacted with primary antibody (1:1000) and secondary antibody (1:5000) sequentially. PVDF membranes were subjected to ECL luminescence. The grayscale ratio of the target band to the internal reference band was analyzed semi-quantitatively. The experiment was repeated 3 times.

RT--PCR and sequencing identification of FGFR2-CCDC6 fusion gene

Total RNA was extracted from Hucct1 cells 48 hours after transfection with the fusion expression plasmid using the TRIZOL kit according to the strict procedure of the instruction manual, and the RNA concentration was measured. Primers were synthesized by Takara, Dalian (see Table 1). Reverse transcription was performed according to the cDNA reverse transcription kit instructions. PCR was performed after reverse transcription of cDNA as a backup. The reaction amplification system was 50μl. Fluorescence quantitative PCR was performed using a fluorescence quantitative PCR instrument. The reaction conditions were pre-denaturation at 95°C for 4 min, denaturation at 95°C for 30 s, annealing at 57°C for 30 s, and extension at 72°C for 30 s, for a total of 40 cycles. Electrophoresis was performed on 8% agarose gels. The results were observed under UV light, and the electrophoretic bands were densitometrically scanned using a gel image analysis system. Transfected fusion expression plasmid sets were cut and recovered for sequencing. Each experiment was repeated three times.

CCK8 assay for cell proliferation

The CCK8 assay was performed according to the reagent instructions. Untreated Hucct1 cells and plasmid-transfected cells were added to 96-well plates at $1X10^4$ cells/100mL. The incubation was continued in the incubator and 10μl MTTCCK8 was added to each well after 2 h, 24 h, 36 h, 48 h and 72 h, respectively. After sufficient lysis, the assay was performed using an enzyme marker with a detection wavelength of 570 nm.

Statistical methods

All experimental data were analyzed by mean ± standard deviation, and SPSS17.0 statistical software was applied. The comparison between the means of the two groups was analyzed by t-test, and $P<0.05$ was considered as a statistically significant difference between the groups.

Results

Successful transfection of plasmids into Hucct1 cells

To clarify the successful transfection of the FGFR2-CCDC6 fusion gene into Hucct-1 cells, cellular RNA was extracted 48 hours after transfection of the fusion gene expression plasmid. Primers were designed for sequencing the FGFR2-CCDC6 gene fusion site. The sequencing results showed that the FGFR2-CCDC6 expression plasmid was successfully transfected into Hucc1 at the RNA level (Figure 1A). Compared with the FGFR2 expression in blank control Hucct1 cells, the FGFR2 protein expression in Hucct-1 cells was 0.1936 ± 0.002888 48 h after transient transfection of the FGFR2bWT expression plasmid and was 0.345 ± 0.001411 after transient transfection of FGFR2-CCDC fusion expression plasmid for 48 hours, which was higher than that of the control cells. The difference was statistically significant ($P<0.0001$) (Figure 1 B). The above results showed that FGFR2bWT and FGFR2-CCDC6 expression plasmids were successfully transfec-

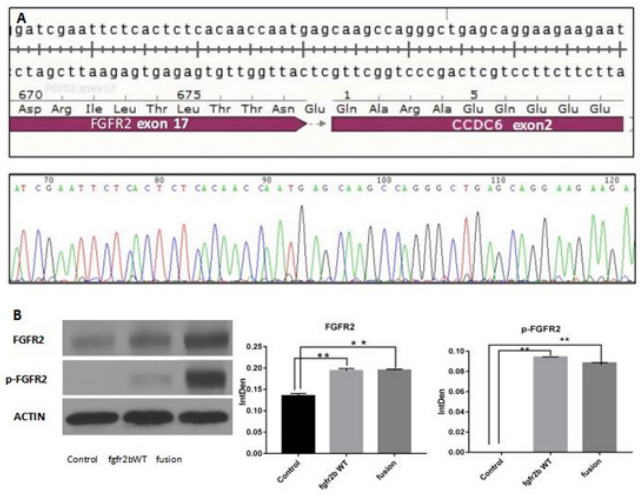

Figure 1. Validation of FGFR2 gene level and protein level expression in each group of cells

Table 1. RT-PCR primer sequences

Gene	Primer sequences
FGFR2-CCDC6	F:5′- CAACGAACTGTACATGATGATGAG -3′
	R:5′- CAGGTGATATAGGCCTGCTTG -3′
	F:5′-TAATACGACTCACTATAGG
	R:5′-TAGAAGGCACAGTCGAGG
GAPDH	F:5′-AGAAGGCTGGGGCTCATTTG-3′
	R:5′-AGGGGCCATCCACAGTCTTC-3′

ted into Hucct1 cells, and other functional assays could be performed 48 hours after transfection.

FGFR2-CCDC6 overexpression promoted the proliferation of Hucct1

The cell proliferation activity was measured and growth curves were produced at 2 h, 24 h, 36 h, 48 h and 72 h after transfection with FGFR2bWT and FGFR2-CCDC6 fusion gene expression plasmids. Compared with the normal Hucct1 control, the results showed that overexpression of FGFR2 had a proliferative effect on cell proliferation compared to normal Hucct1 control. Similarly, overexpression of the FGFR6-CCDC6 fusion gene also had a proliferative effect and was greater than overexpression of the FGFR2 gene (P<0.001) (Figure 2).

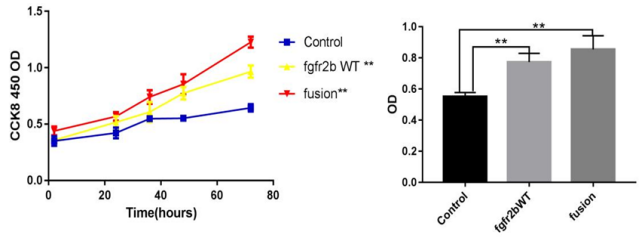

Figure 2. Comparison of cell proliferation ability among groups.

The FGFR2-CCDC6 fusion protein can activate AKT and ERK signaling pathways

After overexpression of FGFR2bWT and FGFR2-CCDC6 fusion expression plasmids in Hucct1 cells for 48 h, the protein expression was detected by western blot to detect the protein expression of FGFR2/p-FGFR2, AKT/p-AKT and ERK1/2/p-1/2. The results revealed that compared with the human intrahepatic cholangiocarcinoma cell line Hucct1, the expression of p-FGFR2/FGFR2, p-AKT/AKT and p-/ERK was significantly higher in the group transfected with the FGFR2bWT expression plasmid, and p-FGFR2/FGFR2, p-AKT/AKT and p-ERK/ERK protein expression in the group transfected with the FGFR2-CCDC6 expression plasmid fusion plasmid were significantly higher than those in the control and FGFR3bWT groups (Figure 3, all P<0.001). It is suggested that FGFR2-CCDC6 fusion protein can activate the AKT, ERK signaling pathway, which is related to its role in promoting cell proliferation.

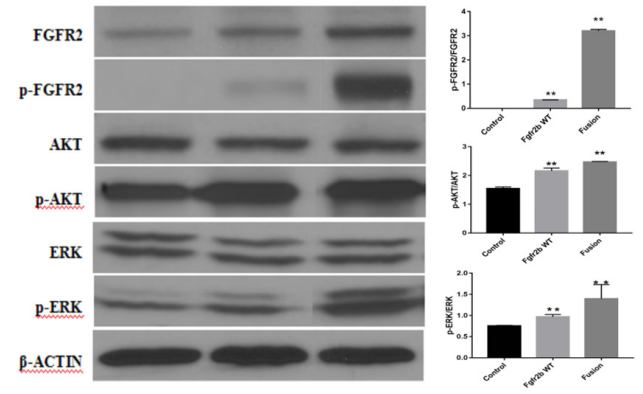

Figure 3. FGFR2-CCDC6 fusion protein acts through activation of AKT signaling pathway

Discussion

The FGFR2 gene is located on chromosome 10 and encodes a protein consisting of extracellular, transmembrane and intracellular regions with tyrosine kinase activity (2). The FGFR2 family is one of the members of the proto-oncology family, which has the role of promoting tumor growth. For example, the FGFR2 positive rate in breast cancer was 68.67% (57/83), which was higher than that of normal controls. Moreover, the high expression of FGFR2 was closely correlated with tumor progression and clinical stage (P<0.05). Guy reported that FGFR2 gene amplification was present in 2% of gastric cancer tissues and that FGFR2 gene amplification was closely associated with poor patient prognosis. These results suggest that FGFR2 gene amplification is a key node in the development of tumors (7,8). The YAP gene was found to promote cell proliferation by activating the FGFR2 gene in Hucct1 cells, suggesting that FGFR2 is involved in the progression of ICC (9).

A deeper study of the molecular mechanisms of ICC is key to the development of effective targeted therapies (9). Gene fusions have also been reported in many solid tumors such as prostate, lung and breast cancers (10-12). Certain fusion genes have been clinically targeted for drug therapy (e.g., EML-ALK fusion in lung cancer) and treatment with drugs targeting the fusion gene can prolong patient survival (11).

With the development of high-throughput transcript sequencing technology, the FGFR2 gene has been found to be fused with multiple genes in ICC (13). Gene fusions can be caused when chromosomal translocations, intermediate deletions or chromosome inversions occur. Multiple FGFR2 gene fusions have been identified in ICC, and these fusions include FGFR2-BICC1 (14), FGFR2-TACC3 (15), etc., with oncogenic potential (13-18). Genomic mutations involving FGFR2 activation account for nearly 20 % of all ICC cases (19). Indeed, a large number of FGFR2 translocations have been identified as oncogenic drivers in ICC (20-22). These FGFR2 translocations encode functional fusion proteins with FGFR2 as an N-terminal chaperone gene. Importantly, they are fused to the mate gene at the C-terminus with a strong dimerization/oligomerization capacity (10,12,23,24). The discovery of FGFR2 fusion proteins as kinesins offers a new and exciting therapeutic opportunity against ICC through molecular kinase targeting.

The results of previous animal experiments showed that the FGFR2-CCDC6 gene fusion promoted the growth of xenograft intrahepatic bile duct tumors, indicating that the fusion gene was tumorigenic. The mechanism of action may be related to the activation of the FGFR2 signaling pathway. And small molecule tyrosinase inhibitors were able to inhibit tumor growth. The specific mechanism of action is related to the activation of the FGFR2 downstream signaling pathway but has not been verified by further studies under in vitro cellular assays (3). To further investigate the specific tumorigenic mechanism of this fusion gene, Hucct-1 cells were selected for this experiment to observe the effect of the FGFR2-CCDC6 fusion gene on the biological function of cholangiocarcinoma cells. The experimental results showed that the expression level of p-FGFR2 protein in Hucct-1 cells was significantly increased after transient transfection of this fusion gene, and the difference was statistically significant compared

with the normal control group without treatment. The results showed that the expression level of FGFR2 protein was elevated and the FGFR2-CCDC6 fusion gene plasmid was successfully expressed in Hucct-1 cells.

Recent studies have shown that the FGFR pathway is a driver gene for certain cancers, with roles in facilitating cell mitosis, enhancing tumor cell invasion, and promoting epithelial-mesenchymal transition, which is involved in the process of different tumor development as an oncogene. Nakamura et al (18) showed that FGFR2 presented high expression in ICCA. The above findings suggest that the FGFR2 signaling pathway plays an important role in intrahepatic cholangiocarcinoma. To further clarify the effect of the FGFR2-CCDC6 fusion gene on cell biological behavior, we expressed the fusion gene through Hucct-1 cells, and the results showed an increase in cell proliferation capacity compared with normal control cells, with statistically significant differences. This indicates that the fusion gene has a role in promoting tumor cell proliferation, and the mechanism of its action may be related to the over-activation of the FGFR2 signaling pathway.

The fusion of FGFR2 mRNA N-terminal exon 17 to CCDC6 mRNA C-terminal exon 2 in the FGFR2-CCDC6 fusion gene retains the FGFR2 protein phosphorylation region predicting that these FGFR2 fusion proteins exhibit sensitivity to TKIs. The results showed that FGFR2-BICC1 constitutively dimerizes and that the BICC1 structural domain alone (which was identical to that in the fusion) was able to oligomerize. This dimerization fusion caused downstream activation of RAS/MAPK, which in turn exerted a tumor growth-promoting effect (23). In our study, the proliferation ability of cells after overexpression of FGFR2-CCDC6 fusion protein in Hucct1 cells was higher than that of overexpression of FGFR2bWT, indicating that the fusion protein has a proliferation-promoting effect on cell proliferation, and its proliferation-promoting effect was higher than that of overexpression of FGFR-2bWT. Over-activation of AKT signaling pathway through autophosphorylation of fusion proteins was associated with inhibition of apoptosis and enhancement of the signaling pathway to promote cell proliferation, thus promoting the proliferation of tumor cells (24). The downstream constitutive phosphorylation and activation of the signaling pathway was similarly demonstrated in pairs of FGFR2-PPHLN1 overexpressed in HEK293. Treatment of HUCCT1 cells expressing this fusion protein with a pan-FGFR inhibitor significantly inhibited cell migration (25).

In summary, the FGFR2 fusion gene can be a potential target for the treatment of ICC patients, and the study of the mechanism of action of different fusion partners can help in the development and selection of clinical drugs. Our study showed that the FGFR2-CCDC6 fusion gene promoted the growth of tumor cells at the cellular level, and this effect was associated with the over-activation of the PI3K/AKT and MAPK/ERK signaling pathways downstream of FGFR2, but the specific mechanism of its action still needs to be further explored. Further studies on the downstream signaling pathways will help to clarify the role and mechanism of fusion genes in the development of ICC and provide the basis for experimental studies on targeted gene therapy and regulation of FGFR2 and downstream signaling pathways in clinical ICC patients.

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Interest conflict
The authors declare that they have no conflict of interest.

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