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Omega-3 polyunsaturated fatty acid inhibits the malignant progression of hepatocarcinoma by inhibiting the Wnt/β-catenin pathway

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Abstract. – OBJECTIVE: Omega-3 polyunsaturated fatty acid (ω-3 PUFA) has been found to possess anti-cancer potential in previous studies. However, the underlying mechanism of ω-3 PUFA in protecting hepatocarcinoma has not been fully elucidated. This study aims to explore the function of ω-3 PUFA in the development of hepatocarcinoma and its potential mechanism.

PATIENTS AND METHODS: In this study, human hepatocarcinoma cell line Hep G2 was treated with ω-3 PUFA. Cell counting kit-8 (CCK-8) and cell cloning assay were applied to detect the proliferation of Hep G2 cells. In addition, flow cytometry was performed to analyze the cell cycle and apoptosis rate. At the same time, the effect of ω-3 PUFA on invasion and metastasis of hepatocarcinoma cells were analyzed by transwell assay. Moreover, protein levels of key factors in Wnt/β-catenin pathway were detected by Western blot.

RESULTS: Cell proliferation of Hep G2 cells was decreased after ω-3 PUFA treatment in a time- and dose-dependent manner. CCK-8 assay showed that the IC50 value was 12.8 ± 0.67 μmol/L, 8.8 ± 0.43 μmol/L and 4.6 ± 0.42 μmol/L after ω-3 PUFA treatment for 24 h, 48 h and 72 h, respectively. Besides, ratio of Hep G2 cells blocked at G2/M phase after ω-3 PUFA treatment (5 μmol/L, 10 μmol/L and 20 μmol/L) was increased in a dose-dependent manner ($p<0.05$). Meanwhile, ω-3 PUFA could increase cell apoptosis ($p<0.05$) and inhibit cell proliferation. In addition, ω-3 PUFA reduced protein expressions of total, cytoplasmic and nuclear β-catenin in Hep G2 cells, indicating that the Wnt/β-catenin pathway is inhibited. Decreased expression levels of Dvl-2, Dvl-3, GSK-3β (*p*-ser9), c-myc and survivin, and increased expression levels of GSK-3 (*p*-tyr216) and Axin-2 were observed in Hep G2 cells treated with ω-3 PUFA, but no significant alteration in total GSK-3β protein level was observed ($p>0.05$).

CONCLUSIONS: Omega-3 PUFA regulates the malignant progression of hepatocarcinoma by inhibiting proliferation and promoting apoptosis of hepatocarcinoma cells via Wnt/β-catenin signaling pathway.

Key Words
ω-3-polyunsaturated fatty acids, Wnt/β-catenin signaling pathway, Hepatocarcinoma, Apoptosis.

Introduction

Hepatocarcinoma is one of the most common malignant tumors in clinical practice, which mostly affects people in Asia and Africa[1,2]. Hepatocarcinoma is the fifth common cancer and the third leading cause of tumor death[1], which is characterized as strong invasion, high malignancy and poor prognosis. The insidious onset, fast-growing invasion and occult symptoms of hepatocarcinoma lead to the poor prognosis and severe fatality[3,4]. Only about 10% to 20% hepatocarcinoma patients could receive early diagnosis. Therefore, it is urgent to find effective prevention and treatment for hepatocarcinoma[5].

It has been reported[6-10] that the aberrant activation of Wnt/β-catenin is involved in various cancers, including colon cancer, breast cancer, hepatocarcinoma and pancreatic cancer. Meanwhile, the molecular mechanism of Wnt/β-catenin signaling pathway is highly conserved in different species[11]. Inactivated Wnt/β-catenin signaling pathway leads to the formation of a protein complex by Axin, APC and GSK-3β, which further leads to the formation of phosphorylates cytoplasmic β-catenin. Phosphorylated β-catenin is subsequently ubiquitinated, and finally degraded by proteasome[12]. When the extracellular ligand Wnt binds to frizzled (the transmembrane receptor) and the single transmembrane receptor LRP5/6, the Wnt signaling pathway is activated, affecting the survival, proliferation, invasion and metastasis of cancer cells[13].

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The effect of ω-3 PUFA on the occurrence and development of human malignancies has been well recognized[14]. Previous researchers[14-16] have shown that ω-3 PUFA regulates the growth and apoptosis in many cancer cells, including vascular cancer, breast cancer, lung cancer and colorectal cancer cells. However, the regulatory mechanism of ω-3 PUFA in human hepatocarcinoma has not been fully elucidated[17]. Therefore, we *in vitro* explored the role of ω-3 PUFA in hepatocarcinoma cells. We found that ω-3 PUFA could attenuate growth and promote apoptosis of hepatocarcinoma cells by inhibiting the Wnt/β-catenin pathway. Our findings might provide a solid foundation for the clinical application of ω-3 PUFA in the prevention and treatment of hepatocarcinoma.

Materials and Methods

Chemicals and Reagents

ω-3 PUFA was purchased from Xuchang Yuanhua Biotechnology (Xuchang, China). Human hepatocarcinoma cell line Hep G2 was purchased from Shanghai Rongchuang Biotechnology (Shanghai, China). Dulbecco's Modified Eagle Medium (DMEM) high glucose medium (Invitrogen, Carlsbad, CA, USA) and fetal bovine serum (FBS) were purchased from Thermo Fisher (Waltham, MA, USA). In addition, the cell cycle and apoptosis detection kit, radioimmunoprecipitation assay (RIPA) lysate, bicinchoninic acid (BCA) protein concentration determination kit, antibody dilution, enhanced chemiluminescence (ECL) were all purchased from Beyotime Biotechnology (Shanghai, China). Primary antibodies including β-catenin, c-myc, survivin, Dvl-2, Dvl-3, GSK-3β, GSK-3β, p-tyr216 and β-actin were purchased from the Cell Signaling Technology (Danvers, MA, USA).

Cell Cultures

Hep G2 cells were cultured in DMEM high glucose supplemented with 10% fetal bovine serum, 100 μg/mL penicillin and 100 μg/mL streptomycin in a 5% CO_2 incubator at 37°C. Cells were digested with 0.25% trypsin and passaged when the cell confluence was up to80%.

Cell Proliferation Assay

Cells were inoculated into 96-well plates and cultured overnight. Different doses of ω-3 PUFA (0, 1.25 μmol/L, 2.5 μmol/L, 5.0 μmol/L, 10.0 μmol/L, 20.0 μmol/L and 40.0 μmol/L) were used for treating cells, with 6 replicates of each dose. After incubation for 24 h, 48 h or 72 h, respectively, 20 μL of cell counting kit-8 (CCK-8) solution (5 mg/mL) (Dojindo, Kumamoto, Japan) were added for detecting absorbance (OD value) at the wavelength of 490 nm by the microplate reader.

Colony Formation Assay

Cells were seeded into a 6-well plate (200 cells per well). Culture medium was replaced every 7 days. 2 weeks after the colony formation, cells were washed with phosphate-buffered saline (PBS) twice and fixed with 2 mL of methanol for 20 minutes. Next, the cells were washed with PBS again and 0.1% crystal violet was added for 20-min staining. 3 random fields in each well were selected for capturing images under an inverted microscope.

Transwell Assay

Cells were digested and resuspended in serum-free medium at a density of 2.0×10^5/mL. Transwell chambers with or without matrigel were placed in 24-well plates. Complete culture medium was added into the lower chamber and cell suspension was added into the upper one. After cells were placed in a 37°C incubator for 48 h, they were fixed with 4% paraformaldehyde for 30 min, followed by staining with crystal violet for 15 min. After cleaning the inner surface of the cell basement membrane and removing the inner cells carefully, stained cells in the outer base were observed and counted using a microscope.

Cell Cycle

Cells were inoculated into 6-well plates. 24 h after ω-3 PUFA treatment, cells were harvested and washed with PBS, followed by fixation overnight with 70% cold ethanol. Before cell cycle determination, cells were centrifuged to remove the ethanol. Cells were gently resuspended with 0.6 mL of propidium iodide (PI) and stained at 37°C for 30 min in the dark. Cell cycle was analyzed by a FACS flow cytometer.

Cell Apoptosis

The Hep G2 cells were inoculated into 6-well plates. Cells were harvested 24 h after ω-3 PUFA treatment. After washing with PBS twice, cells were resuspended in the binding solution and incubated for 15 min in the dark. After that cells were added with 5 μL of AnnexinV- FITC and 5 μL of PI and gently mixed. Cell apoptosis rate was analyzed by a FACS flow cytometer.

Table I. The effect of ω-3 PUFA on the proliferation of Hep G2 cells.

Concentration (μmol/L)/ Time (h)	0	1.25	2.50	5.0	10.0	20.0	40.0
24 h	100%	95.47%	89.52%	78.32%	70.23%	45.92%	23.35%
48 h	100%	93.26%	79.33%	64.83%	45.67%	39.16%	22.72%
72 h	100%	92.17%	62.51%	43.67%	38.74%	23.63%	21.24%

Western Blotting

After the treatment of ω-3 PUFA for 2 h, cells were collected by RIPA lysis buffer. Total proteins were extracted while protein concentrations were detected by the BCA kit (Beyotime, Shanghai, China). Samples were separated by the sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to a 0.22 μm polyvinylidene difluoride (PVDF) membrane. PVDF membrane (Millipore, Billerica, MA, USA) was then blocked with 5% skim milk. Immunoblots were incubated with the primary antibody at 4°C overnight (dilution 1: 1000, CST, Danvers, MA, USA). On the next day, the immunoblots were washed with Tris-buffered saline and Tween (TBST) for 3 times and then incubated with the secondary antibody (diluted 1:1000, CST, Danvers, MA, USA) for 2 h. Protein bands were detected by enhanced chemiluminescence (ECL).

Statistical Analysis

Statistical analysis was performed using Statistical Product and Service Solutions (SPSS) 19.0 software (SPSS Inc., Chicago, IL, USA) while Graph Pad Prism 5.0 software (La Jolla, CA, USA) was applied for image editing. Data were represented as mean ± S.D. Skewness distribution was expressed as median (quartiles), *t*-test or the Wilcoxon signed-rank test was used to compare differences between groups. $p<0.05$ was considered statistically significant.

Results

ω-3 PUFA attenuates the proliferation and survival of Hep G2 cells.

It has been proved that ω-3 PUFA is related to cell proliferation in many cancer cells. Therefore, we detected the cell proliferation of hepatocarcinoma cell line Hep G2 treated withω-3 PUFA by CCK-8 assay. The results showed that ω-3 PUFA inhibited proliferation of Hep G2 cells in a dose dependent manner. The IC_{50} value was12.8 ± 0.67 μmol/L, 8.8 ± 0.43 μmol/L and 4.6 ± 0.42 μmol/L after ω-3 PUFA treatment for 24 h, 48 h and 72 h, respectively (Table I). Similarly, cell viability and colony formation ability were decreased after treatment with 5.0 μmol/L ω-3 PUFA in a dose-dependent manner (Figure 1A and 1C). In addition, cell proliferation was continuously attenuated after ω-3 PUFA for 24 h in a dose-dependent manner (Figure 1B and 1D). These above findings suggested that ω-3 PUFA inhibits the proliferation and survival of Hep G2 cells.

ω-3 PUFA blocks cell cycle at the G2/M phase in Hep G2 cells

Flow cytometry results found that ω-3 PUFA could block cell cycle at the G2/M phase in a dose dependent manner. In detail, ω-3 PUFA reduced the distribution of hepatocarcinoma cells in G1 phase and increased their distribution in G2/M phase ($p<0.05$). Meanwhile, ω-3 PUFA exerted no significant effect on hepatocarcinoma cell distribution in the S phase (Figure 2A and 2B). In addition, we also found that ω-3 PUFA up-regulated P15 expression and down-regulated Cyclin D1 expression (Figure 2C). These results further indicated that ω-3 PUFA blocks cell cycle of Hep G2 cells at the G2/M phase.

ω-3 PUFA Induces Apoptosis in Hep G2 Cells

As shown in Figure 3A and 3B, ω-3 PUFA increased apoptosis rate of Hep G2 cells in a dose dependent manner ($p<0.05$). Meanwhile, we found that ω-3 PUFA decreased expression levels of anti-apoptotic proteins Bcl-2 and Caspase-3 (Figure 3C). These results demonstrated that ω-3 PUFA induces apoptosis in Hep G2 cells.

ω-3 PUFA Inhibits the Invasion and Migration of Hep G2 Cells

Next, we explored the effect of ω-3 PUFA on Hep G2 cells by transwell assay. Migration experiments showed that the number of Hep G2 cells penetrating the transwell chamber after ω-3 PUFA treatment at 24 h was significantly reduced, compared with the control group (Figure 4A). In-

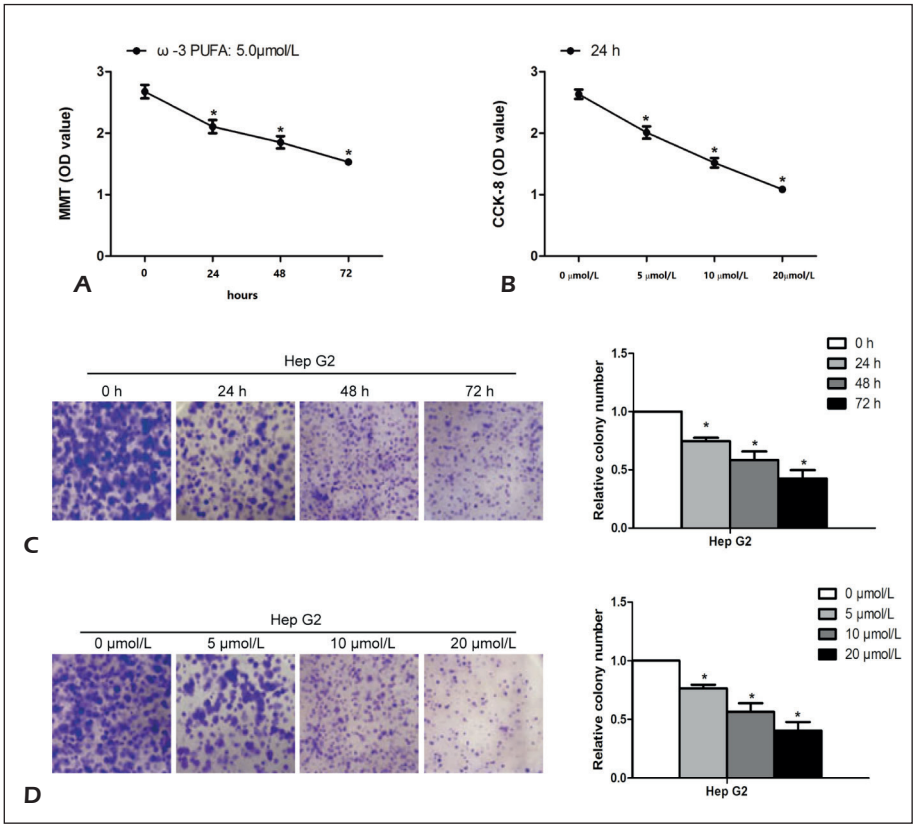

Figure 1. ω-3PUFA induces cell apoptosis in a dose-dependent and time-dependent manner. **A**, Hep G2 cells were treated with 5 μmol/Lω-3PUFA for four different time points (0, 24, 48, 72 h). The effect of ω-3PUFA on cell viability was detected by CCK-8 assay. **B**, Hep G2 cells were treated with different concentrations of ω-3PUFA (0, 5, 10, 20 μmol/L) for 24 h. The effect of ω-3PUFA on cell viability was detected through CCK-8 assay. **C**, The ability of cell colony formation in Hep G2 cells treated with 5 μmol/L ω-3PUFA for different time points (0, 24, 48, 72 h). **D**, Hep G2 cells were treated with different concentrations of ω-3PUFA (0, 5, 10, 20 μmol/L) for 24 h. The effect of ω-3PUFA on cell colony formation was detected by colony formation assay (*p<0.05 compared with control group).

vasion experiments obtained the similar results (Figure 4B). All these results demonstrated that ω-3 PUFA inhibits the invasion and migration of HepG2 cells.

ω-3 PUFA Regulates Protein Level of β-Catenin in HepG2 Cells

Our data showed that omega-3 PUFA decreased protein levels of total, nuclear and cytoplasmic β-catenin in Hep G2 cells in a dose dependent manner (Figure 4C). These results suggested that ω-3 PUFA might affect the Wnt/β-catenin pathway.

ω-3 PUFA Suppresses the Wnt/ β-Catenin Signaling Pathway in Hep G2 Cells

Further studies showed that ω-3 PUFA significantly affected expressions of Wnt/β-catenin pathway-related proteins in Hep G2 cells in a dose dependent manner. In detail, ω-3 PUFA decreased the levels of Dvl-2 and Dvl-3. GSK-3β was phosphorylated at tyr216 while the ser9 phosphorylation site was inactive. Besides, the expression of the degradation complex Axin-2 was increased by ω-3 PUFA treatment (Figure 5). In sum, these results demonstrated that ω-3 PUFA suppresses the Wnt/β-catenin signaling pathway in Hep G2 cells.

Discussion

Hepatocarcinoma is one of the most common malignant tumors that cause death. The prevalence and mortality of hepatocarcinoma have been increasing annually with the aging of population and changes of dietary habits[18]. Many factors could lead to the occurrence of hepatocarcinoma, such as genetic factors, dietary habits, viral infections, and so on. However, the exact molecular mechanism of hepatocarcinoma metastasis re-

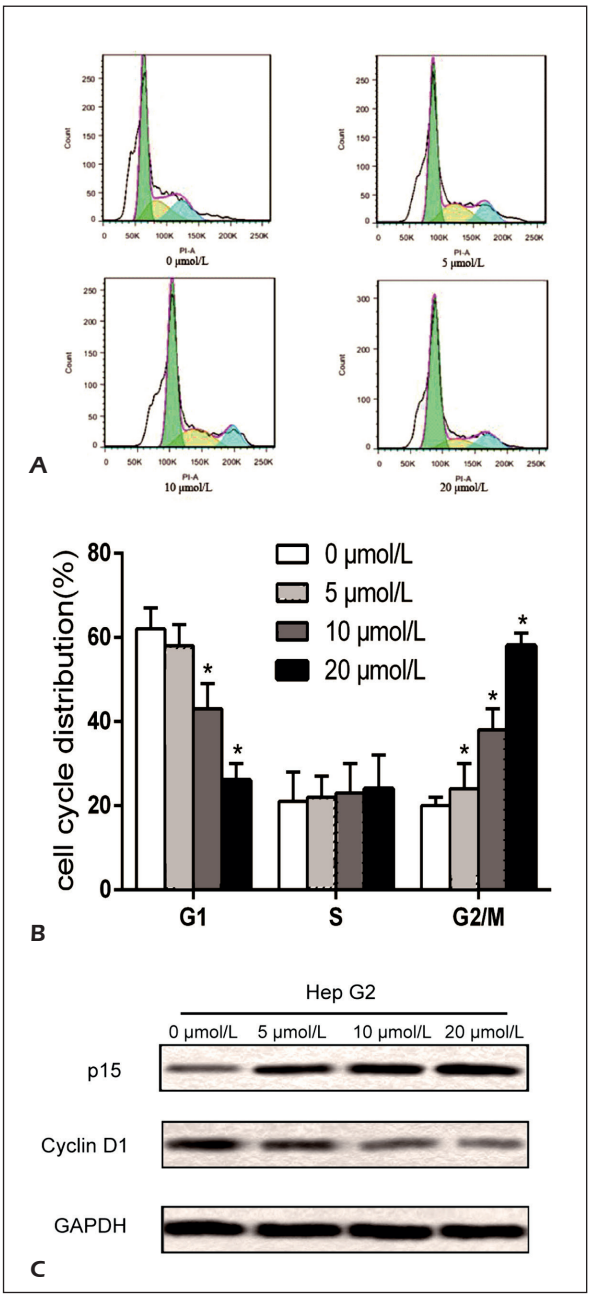

Figure 2. ω-3PUFA induces cell cycle arrest in Hep G2 cells. **A**, After treatment of Hep G2 cells with different doses of ω-3PUFA (0, 5, 10, 20 μmol/L) for 24 h, flow cytometry was performed to investigate the influence of ω-3PUFA on cell cycle. **B**, Cell cycle in each group was analyzed (*p<0.05 vs. 0 μmol/L). **C**, Western blot analysis of P15 and Cyclin D1 expression levels in Hep G2 cells treated with different concentrations of ω-3PUFA (0, 5, 10, 20 μmol/L) for 24 h. Each experiment was performed for three times.

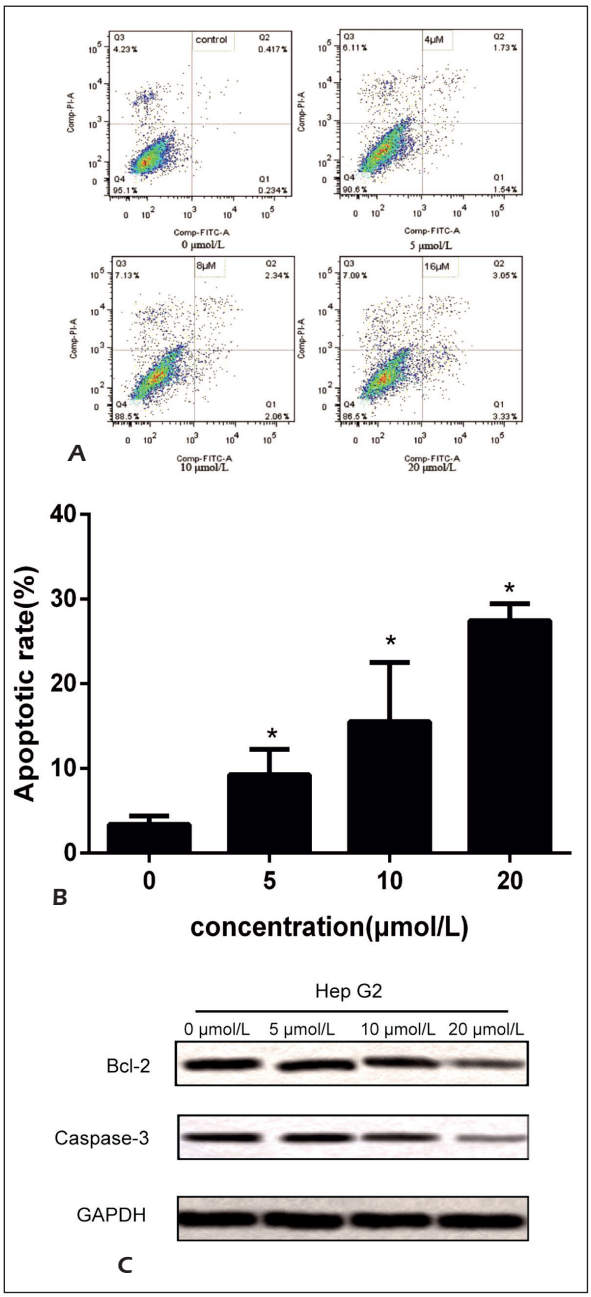

Figure 3. ω-3 PUFA induces apoptosis in Hep G2 cells. **A**, Cell apoptosis was detected by flow cytometry. **B**, Cell apoptosis rate in each group was analyzed (*p<0.05 vs. 0 μmol/L). **C**, Western blot analysis of Bcl-2 and Caspase-3 expression levels in Hep G2 cells treated with different concentrations of ω-3PUFA (0, 5, 10, 20 μmol/L) for 24 h. Each experiment was performed for three times.

mains unclear[19, 20]. Tumor invasion and migration are complex. These complex processes involve the destruction of the basement membrane, generation of local infiltrates, tumor metastasis into blood and lymphatic vessels and rebuilding of intercellular junctions at target organs to form new metastases[21]. In addition, invasion and metastasis of malignant tumor are important causes of tumor recurrence, which are also the major causes of tumor cure failure.

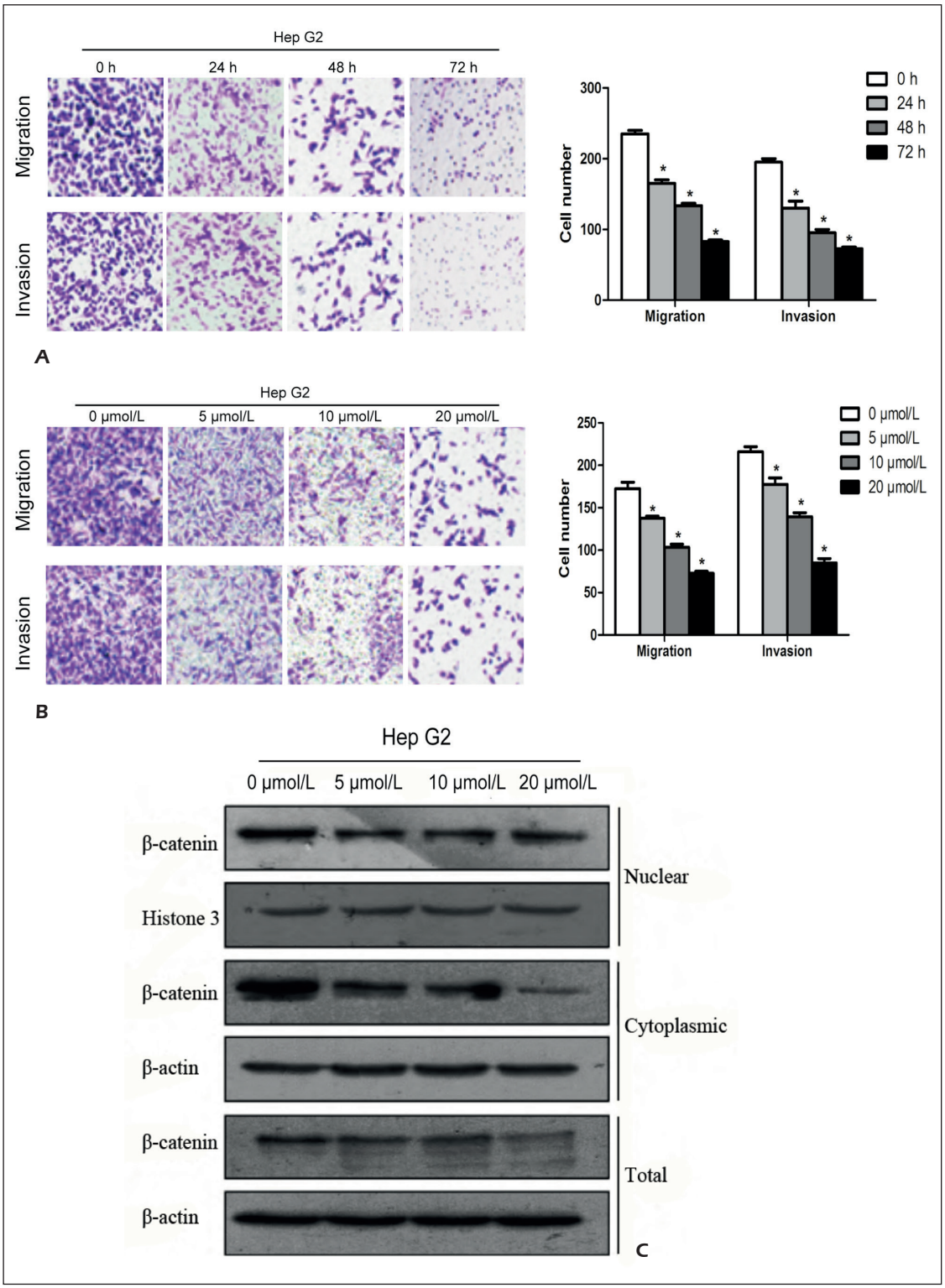

Figure 4. ω-3 PUFA inhibits the invasion and migration of Hep G2 cells. **A**, Migration abilities of Hep G2 cells treated with 5 μmol/Lω-3PUFA for different time points (0, 24, 48, 72 h). **B**, Invasion abilities of Hep G2 cells treated with 5 μmol/Lω-3PUFA for different time points (0, 24, 48, 72 h) were detected (*p<0.05 compared with control group). **C**, Western blot analysis of β-catenin expression levels in Hep G2 cells treated with different concentrations of ω-3PUFA (0, 5, 10, 20 μmol/L) for 24 h. Each experiment was performed for three times.

Effective inhibition of tumor cell migration and invasion is the key to improve the prognosis of cancer patients[22, 23].

ω-3PUFA is a polyunsaturated fatty acid, the first unsaturated bond of which appears at the third position of the methyl end of the carbon chain. It is the essential fatty acid in human body and the main component of the cellular membrane phospholipid[24]. There is growing evidence[25,26] suggesting that ω-3PUFA inhibits many malignancies such as breast cancer, colon cancer, uterine cancer, and prostate cancer by inducing cancer cell apoptosis. Related studies have shown that ω-3PUFA could increase the fluidity of tumor cell membrane and reduce tumor invasiveness. Besides, ω-3PUFA could disturb the tumor cell protein metabolism, cell cycle regulation and signal conduction though affecting the enzyme activity, ion channel and receptor expression of the membrane surface, thereby inhibiting or killing cancer cells[25,26]. ω-3PUFA could also competitively antagonize COX-2, making disordered intercellular adhesion in tumor epithelial cells. In addition, ω-3PUFA could induce the production of anti-inflammatory and anti-tumor prostaglandin E1, inhibit tumor growth and promote apoptosis, thereby inhibiting tumor invasion and metastasis[27]. Angiogenesis is crucial for tumor growth, invasion and metastasis. It has been shown[28] that ω-3 PUFA could reduce expressions of VEGF-α and VEGF receptor Kdr, and inhibit the adhesion of cancer cells and endothelial cells, thereby reducing the formation of tumor neovascularization.

Wnt/β-catenin signaling pathway is involved in the pathological process of many malignant tumors. It plays a key role in cell proliferation and apoptosis, the abnormal activation of which is closely concerned to the occurrence and progression of hepatocarcinoma[29]. Abnormally expressed proteins in this signaling pathway, such as LRP5/6, Axin, Dvl and GSK3β, would lead to nuclear β-catenin accumulation, which could form into transcriptional cofactors to elevate expressions of C-myc and survivin, eventually leading to the occurrence and development of hepatocarcinoma[30]. Activation of Wnt signaling pathway might inhibit the activity of Gsk 3β and increase the intracellular β-catenin level. Moreover, the increased β-catenin level could lead to its translocation into the nucleus, where it might bind to the T-cell factor/lymphocyte augmentation factor receptor (TCF/LEF) and induces expressions of its downstream target genes, such as cyclin D1 and c-myc, thereby regulating cell cycle, growth and progression[31].

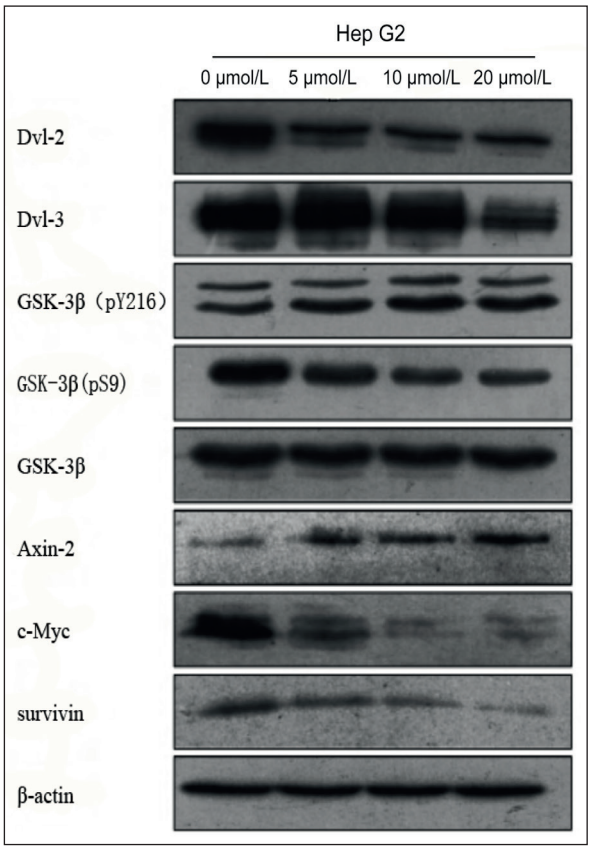

Figure 5. ω-3 PUFA suppresses the Wnt/β-catenin signaling pathway in Hep G2 cells. Western blot analysis of Wnt/β-catenin signaling pathway related protein levels in Hep G2 cells treated with different concentrations of ω-3PUFA (0, 5, 10, 20 and 25 μmol/L) for 24 h. Each experiment was performed for three times.

In this investigation, we explored the effect of ω-3PUFA on the total, cytoplasmic and nuclear β-catenin in Hep G2 cells. The results showed that β-catenin in these three sites was significantly decreased, indicating that expression and intracellular distribution of β-catenin were regulated by ω-3PUFA. Subsequently, the expressions of C-myc and survivin, two target proteins of β-catenin, were also decreased after ω-3PUFA treatment, demonstrating that ω-3-PUFA indeed inhibits the Wnt/β-catenin pathway. Dvl could inhibit the function of GSK-3β, thereby blocking β-catenin phosphorylation and preventing its degradation. We examined protein expressions of Dvl-2, Dvl-3, GSK-3β and GSK-3β), GSK-3β (p-tyr216), Axin-1, Axin-2 and CK1α by Western blotting. The results of Western blotting suggested that ω-3 PUFA may reduce β-catenin expression by Dvl-2 and Dvl-3, thereby promoting the phosphorylation of GSK-3β (p-tyr216) and inac-

tivating the GSK-3β (p-ser9). Meanwhile, ω-3PUFA reduced the expression of β-catenin though increasing the expression of degradation complex Axin-2.

Conclusions

We showed that ω-3PUFA not only regulates lipid metabolism and immune function of the body, but also plays a key role in tumor prevention and treatment. We suggest that ω-3PUFA exerts significant cytotoxicity to hepatocellular carcinoma cell line Hep G2 *via* Wnt/β-catenin signaling pathway. Our study provides the important evidence for the therapeutic prospect of ω-3 PUFA in hepatocellular carcinoma.

Conflict of interest

The authors declared no conflict of interest.

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