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Opsin3 sensitizes hepatocellular carcinoma cells to 5-fluorouracil treatment by regulating the apoptotic pathway

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ABSTRACT

Hepatocellular carcinoma (HCC) is the third most common cancer worldwide, causing over 0.5 million deaths per year, with approximately half of these in China. Chemotherapy is the optimal treatment for patients with advanced HCC, although chemoresistance has become a significant obstacle to successful anti-cancer therapy. The expression of opsin3 (OPN3), also called encephalopsin or panopsin, is lower in 5-fluorouracil (5-FU)-resistant $Bel7402_{5\text{-FU}}$ cells compared to 5-FU-sensitive Bel7402 cells. To explore the role of OPN3 in 5-FU resistance, OPN3 overexpressing ($Bel7402_{5\text{-FU}}$–OPN3) and knockdown (Bel7402–RNAi–OPN3) cell lines were generated. $Bel7402_{5\text{-FU}}$–OPN3 cells were more sensitive to 5-FU treatment than controls, while OPN3 knockdown resulted in a significant increase in 5-FU resistance. This result was replicated in a second HCC cell line, HepG2. Further investigation of the mechanism revealed that decreased OPN3 levels in $Bel7402_{5\text{-FU}}$ cells activated the anti-apoptotic pathway through increasing phospho-Akt and the Bcl2/Bax ratio, while overexpression of OPN3 inactivated this pathway. Taken together, these results suggest that OPN3 depletion is involved in 5-FU resistance, and that therapeutic strategies targeting OPN3 may improve HCC sensitivity to chemotherapy.

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1. Introduction

Hepatocellular carcinoma (HCC) is an increasingly prevalent problem worldwide. There is a higher incidence of HCC in African and Asian regions, especially in China, and it has become one of the leading causes of cancer-related death in the developing world [1]. Surgical treatment is considered to be the best choice for HCC [2], but only a small proportion of patients are candidates for radical resection at the time of diagnosis. For most patients with non-resectable tumors, effective non-surgical treatments are needed [3]. According to the Barcelona Clinic Liver Cancer (BCLC) diagnostic and treatment strategy, chemoembolization is effective for intermediate stage tumors, while for advanced stage tumors or HCC with extrahepatic disease, chemotherapy is the best option [4].

Chemotherapy is the most common treatment for advanced cancers [5,6]. Compared with local treatments, such as radiation and surgery, chemotherapy is a systemic treatment that may reach cancer cells wherever they have spread [7]. To date, more than 100 drugs are used for chemotherapy, either alone or in combination. 5-Fluorouracil (5-FU) is a chemotherapeutic drug effective against various types of cancer, including colorectal, breast, liver, stomach, and gullet (or esophageal) cancer [8–10]. 5-FU is also the optimal drug for the treatment of HCC [11]. However, the rapid development of acquired resistance to 5-FU has limited its clinical usage [12], and its underlying molecular mechanism remains undefined.

Opsin3 (OPN3), also known as encephalopsin or panopsin, is a member of the opsin family and is widely expressed in mouse tissues, especially in the brain and testes [13]. Human OPN3, the first extraocular opsin to be identified, has also been detected in the liver [14]. The OPN3 gene was localized to chromosome 1q43 and encodes a putative protein containing seven α-helical transmembrane domain, a serine- and threonine-enriched C-terminus, and a glycosylated N-terminus [14]. Increasing evidence suggests that opsin can activate G proteins in a light-dependent or light-independent manner in both visual and non-visual systems [15]. OPN3 is reported to play a role in non-visual photic processes such as asthma [16].

This study was based on the finding that OPN3 is significantly downregulated in a 5-FU resistant HCC cell line compared to a sensitive one. We therefore investigated the role of OPN3 in 5-FU resistance in HCC cells and its regulation of pro-and anti-apoptotic signaling pathways.

2. Materials and methods

2.1. Cell culture

The HCC cell line Bel7402 and its 5-FU resistant derivative, $Bel\text{-}7402_{5\text{-FU}}$, were purchased from Nanjing KeyGen Biotech. Co. Ltd. (China). A second HCC cell line, HepG2, and the normal liver cell line, HL-7702, were kindly provided by Professor

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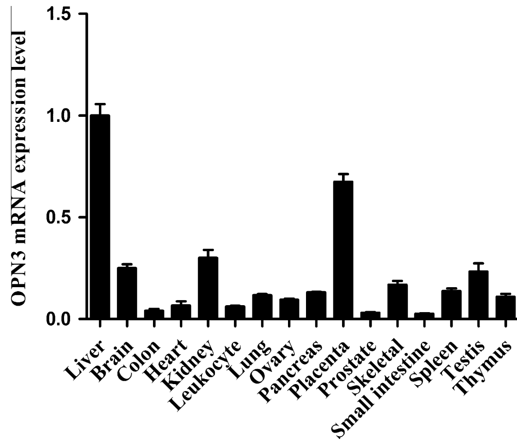

Fig. 1. *OPN3* expression in 16 human tissues was detected by quantitative RT-PCR. *GAPDH* was used as control. The relative *OPN3* expression was calculated using the $2^{-\Delta\Delta}$Ct method.

Binghua Jiang (Nanjing Medical University, China). Cells were cultured in RPMI-1640 supplemented with 10% fetal bovine serum (Sijiqing, Hangzhou, China), 100 IU/mL penicillin, and 100 μg/mL streptomycin (Gibco, Carlsbad, CA, USA) at 37 °C in a humidified atmosphere containing 5% CO_2. The 5-FU resistant cell line Bel-$7402_{5\text{-FU}}$ was grown in medium with step-wise increasing concentrations of 5-FU, then maintained in medium containing 153.8 μM 5-FU (Kingyork, Tianjin, China).

2.2. OPN3 transfection into $Bel7402_{5\text{-FU}}$ and HepG2 cells

The pCMV–SPORT–OPN3 plasmid harboring human OPN3 full-length cDNA was purchased from ATCC (MGC-46103, VA, USA), and the pcDNA3.1/myc-His(-)A plasmid was kindly provided by Professor Jihong Meng (Southeast University, China). OPN3 cDNA was amplified from the pCMV-SPORT-OPN3 plasmid using the forward primer 5′-GAT<u>CTCGAG</u>ATGTACTCGGGGAACCGCAG-3′ and the reverse primer 5′-CCG<u>GAATTC</u>ACAAAGGACGAACTTGGATT-3′. Restriction sites for *Xho*I enzyme (underlined in the forward primer) and *EcoR*I enzyme (underlined in the reverse primer) were introduced using these primers. OPN3 cDNA was subcloned into pcDNA3.1/myc-His(-)A and PEGFP-N2 plasmids and verified by DNA sequencing. $Bel7402_{5\text{-FU}}$ cells were transfected with pcDNA3.1/myc-His(-)A-OPN3 or pcDNA3.1/myc-His(-)A control plasmid using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) following the manufacture's protocol. Seventy-two hours later, G418 (Merck, NJ, USA) was added to cells at a concentration of 400 μg/mL. Stable transfectants containing $Bel7402_{5\text{-FU}}$–OPN3 or $Bel7402_{5\text{-FU}}$–Control were obtained after two weeks culture with G418 and confirmed by quantitative PCR and Western blotting. HepG2 cells were transiently transfected with the PEGFP–N2–OPN3 plasmid (containing the OPN3 gene) or the PEGFP–N2 control plasmid using FuGENE HD (Roche). Transfection efficiency of HepG2–PEGFP–OPN3 and HepG2–PEGFP–Control was analyzed by GFP fluorescence using an Axiovert Zeiss 510 confocal microscope.

2.3. OPN3 knockdown in Bel7402 cells

The pGCsilencerTM U6/neo/GFP/RNAi–OPN3 plasmid was constructed by the Genechem Co. (Shanghai, China) using the OPN3 shRNA sequence ATGCCTTA TATCGTGATCTGCTTCAAGAGAGCAGATCACGATATAAGGCAT. The pGCsilencer™U6/neo/GFP/RNAi–OPN3 or pGCsilencer™U6/neo/GFP plasmid was transfected into Bel-7402 cells using Lipofectamine 2000. Positive colonies (for both Bel7402–RNAi–OPN3 and Bel7402–RNAi–Control) were obtained following G418 selection and confirmed by quantitative RT-PCR and Western blotting. Plasmids were transfected into HepG2 cells using FuGENE HD (Roche) and verified by GFP fluorescence.

2.4. Quantitative RT-PCR analysis

Total RNA was extracted from cells using TRIzol (Invitrogen, Carlsbad, CA, USA). RNA concentration was measured using a BioPhotometer (Eppendorf, Hamburg, Germany) and 2 μg total RNA was used for RT-PCR. OPN3 expression in Bel7402 and $Bel7402_{5\text{-Fu}}$ cells was measured by real-time PCR using a SYBR Prime Script RT-PCR Kit (Takara Biotec, Shiga, Japan) and an ABI Prism 7300 HT Sequence Detection system (Applied Biosystems, Foster City, CA, USA). The parameters for PCR were set as following: 95 °C for 30 s, followed by 40 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s, and the dissociation curve was inspected for quality control purposes. *GAPDH* was used as a normalization control and relative gene expression was calculated using the $2^{-\Delta\Delta}$Ct method. Real-time PCR was repeated three times for three independently purified RNA samples. Real-time PCR primers were:

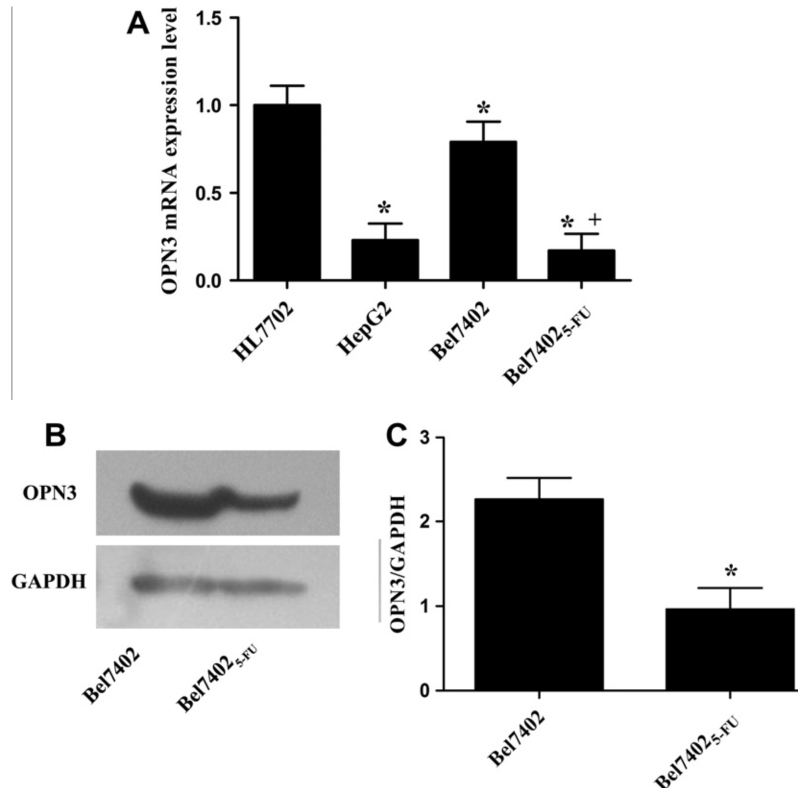

Fig. 2. *OPN3* expression was significantly downregulated in HCC cells. (A) OPN3 expression in normal liver cells compared to HepG2, Bel7402, and $Bel7402_{5\text{-Fu}}$ cells, $^{*}P < 0.05$. "+"indicates relative to the 5-FU sensitive cell line, Bel7402. (B, C) Level of OPN3 protein expression was determined by Western blotting.

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