An underlying prognosis predictor of hepatocellular carcinoma: Oncoprotein 18

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Abstract. Recent studies have reported the association between the expression of oncoprotein 18 (op18) and hepatocellular carcinoma (HCC). However, any underlying mechanistic connection between op18 expression and hepatocarcinogenesis is poorly understood. In the present study, Flag-pcDNA3.1 vector and Flag-pcDNA3.1-op18 plasmid were stably transfected in SMMC7721 cells, respectively. Stable SMMC7721 control and op18 overexpression SMMC7721 cell lines were constructed and identified by western blot analysis. Using a cell counting kit-8 (CCK8), it was shown that cell proliferation was significantly increased in the op18 overexpression SMMC7721 cell group (0.60±0.05), compared with the control group (0.29±0.03) at an absorbance of 450 nm (P<0.01). Flow cytometry was used to analyze cell apoptosis by FITC-Annexin V and propidium iodide (PI) apoptosis assay kit. The results demonstrated that the percentage of apoptotic cells was inhibited to 5.80±0.33% in the op18 overexpression group, compared with 11.79±1.09% in the control group. Using FACS, single cell analysis data showed that op18 overexpression induced cell cycle arrest by inhibiting progression from G2 to M phase. The results suggest that op18 expression is closely associated with SMMC7721 cell proliferation and apoptosis, which appears to be a potential predictor of prognosis in HCC.

Introduction

Hepatocellular carcinoma (HCC) is the most common primary malignant liver tumor and ranks the fifth most prevalent malignant tumor worldwide. In addition, China has the highest incidence of HCC, accounting for 55% of all new cases globally. Although many risk factors, such as alcohol, aflatoxin, hepatitis, genetic predisposition, obesity and diabetes have been identified, the exact molecular mechanism has yet to be determined (1-3).

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Oncoprotein 18 (op18), also known as op17, op19 and stathmin, a ubiquitous 19-kDa cytosolic phosphoprotein, has been reported to play a critical role in microtubulin destabilizing, spindle assembly, chromosomal stability, cell shape, mitosis, and other cell processes. It has been reported that op18 expression correlates with tumorigenesis and tumor progression (4-7). However, few studies have directly compared cell proliferation and apoptosis in HCC cell lines transfected with op18. The mechanism of op18 in HCC should be elucidated as well as the significance of the upregulated expression of op18 in HCC cell lines. We therefore analyzed cell proliferation, apoptosis and cell cycle in op18 overexpression SMMC7721 cells by stably transfecting Flag-pcDNA3.1-op18 plasmid, compared with control SMMC7721 cells, which were transfected with Flag-pcDNA3.1 vector. The aim of this study was to examine the involvement of op18 in human hepatocarcinogenesis and to evaluate its prognostic significance in HCC.

Materials and methods

Cell lines and plasmids. Human HCC SMMC7721 cells were cultured in DMEM supplemented with 10% fetal bovine serum (Gibco, Carlsbad, CA, USA) at 37°C in a humidified atmosphere of 5% CO_2. The Flag-pcDNA3.1-op18 plasmid was kindly provided by Dr Baldassarre (Texas University, USA).

Stable transfection. The Flag-pcDNA3.1-op18 plasmid or Flag-pcDNA3.1 vector was transfected into SMMC7721 cells with Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Promycin (200 μg/ml) was used to select stably transfected cell lines. The transfection efficiency was then determined by western blot analysis.

Western blot analysis. Western blot analysis was performed as described previously (8). Briefly, total cell proteins were extracted from SMMC7721-op18 and control cells with cell lysis buffer (Beyotime, Jiangsu, China), separated on 10% SDS-PAGE gels and transferred onto PVDF membrane (Millipore, Billerica, MA, USA). The primary antibody (1:10,000; Abcam, Cambridge, MA, USA) was then added at 4°C overnight and bound with HRP-conjugated secondary antibodies at room temperature for 1 h. Chemiluminescent signaling was detected using an ECL kit (Millipore) and autoradiography.
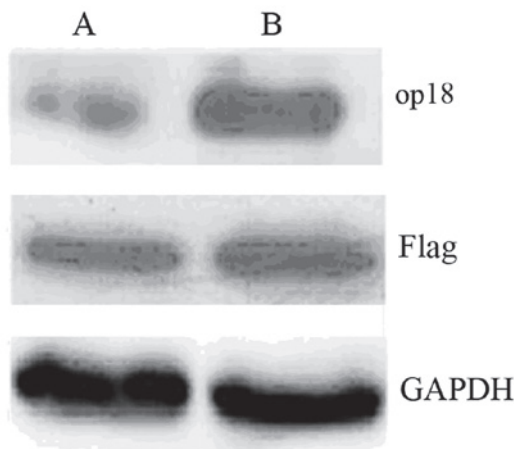
Figure 1. Western blot analysis for expression of oncoprotein 18 (op18) and Flag in the two cell groups. (A) The control group; (B) the op18 overexpression group. GAPDH was detected to normalize the expression of op18.
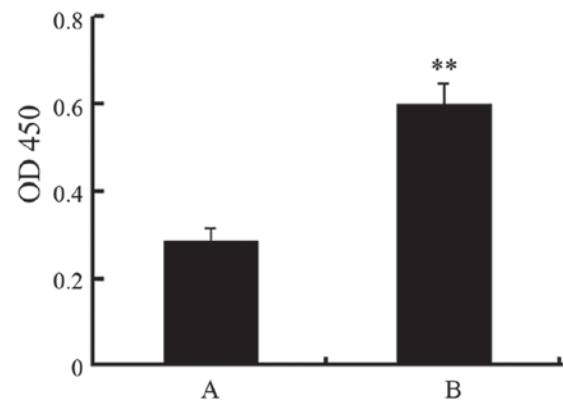

Figure 2. Cell proliferation was increased by oncoprotein 18 (op18) overexpression. Cell viability was evaluated by using the CCK8 assay following op18 upregulation. The data shown are from three independent experiments, **$P<0.01$. (A) The control group; (B) the op18 overexpression group.

Cell proliferation assay. The cell counting kit-8 (CCK-8; Dojindo, Kumamoto, Japan) colorimetric assay was used to measure cell proliferation and viability with triplicate experiments for each set of conditions. SMMC7721 control and SMMC7721-op18 cells were seeded in 96-well plates at a density of $5x10^3$ cells per well. The cells were cultured for 24 h, the supernatant was removed, and 100 μl of DMEM medium containing 10 μl of CCK8 was added to each well for 1 h at 37°C. The absorbance at 450 nm was measured with a plate reader (Multiskan GO Microplate Spectrophotometer; Thermo Fisher Scientific, Inc., Waltham, MA, USA).

Cell cycle assay. Cells pellet were digested and collected by trypsin, fixed in 70% ethanol on ice and then stained with 50 μg/ml propidium iodide (PI) (Sigma, St. Louis, MO, USA) and 0.1 μg/ml RNase A (Sigma). The cells were detected with flow cytometry using FACStar Plus (FACSCalibur Flow cytometer; Becton-Dickinson, Mountain View, CA, USA). Cell Quest software (BD CellQuest Pro Software, BD Biosciences, San Diego, CA, USA) was used to analyze the percentage of the cell population in each phase.

Cellular apoptosis assay. Apoptosis was assessed with the Annexin V-FITC kit according to the manufacturer's instructions. The cells were washed twice with cold PBS, digested, collected, and resuspended to binding buffer. Annexin V-FITC and PI were added (BioVision, Milpitas, CA, USA), and the cells were incubated for 10 min at room temperature in the dark. Then, 200 μl binding buffer was added, and the cells were calculated with flow cytometry (FACScan, BD, Germany). The percentage of apoptosis was analyzed using the equation: 100 x [experimental apoptosis (%) - spontaneous apoptosis (%)]/[100 - spontaneous apoptosis (%)].

Statistical analysis. Results are expressed as means ± SD of multiple experiments. Statistical analysis was performed with the Student's t-test for comparison between two groups or an analysis of variance (ANOVA) followed by Tukey's t-test for comparison of multiple groups. $P<0.05$ was considered to indicate a statistically significant difference.
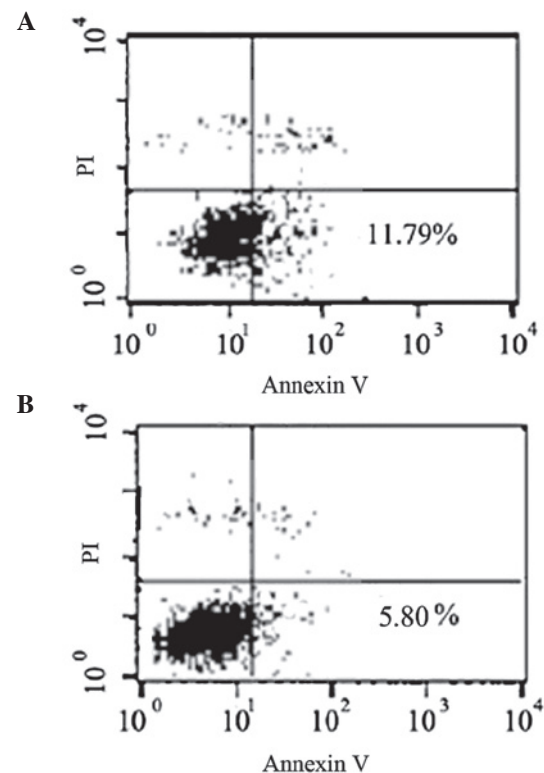

Figure 3. SMMC7721 apoptosis was inhibited by regulation of oncoprotein 18 (op18) expression. op18 overexpression suppressed cell apoptosis in the op18 overexpression group in SMMC7721 cells. Cells were stained with Annexin V (x-axis) and PI (y-axis) to quantify apoptosis and necrosis, respectively. (A) The control group; (B) the op18 overexpression group.

Results

Establishment and identification of op18 overexpression SMMC7721 stable cell lines. Stable transfection method was used to establish stably expressed Flag-pcDNA3.1 vector and Flag-pcDNA3.1-op18 plasmid human hepatocarcinoma SMMC7721 cell lines. Western blot analysis results revealed that op18 expression was increased in SMMC7721-op18 cells transfected with Flag-pcDNA3.1-op18 plasmid, compared

with control SMMC7721-transfected Flag-pcDNA3.1 plasmid (Fig. 1).

Increase of SMMC7721 cell proliferation by op18 over-expression. To determine the effect of the upregulation of op18 expression on SMMC7721 cell proliferation, CCK8 assay was used to analyze cell proliferation. The results showed that cell proliferation was significantly increased in the op18 over-expression SMMC7721 cell group (0.60±0.05), compared with the control group (0.29±0.03) at the absorbance of 450 nm (P<0.01, Fig. 2).

Inhibition of SMMC7721 apoptosis by upregulating op18 expression. Flow cytometry was performed to test the effect of op18 overexpression on SMMC7721 cell apoptosis through FITC-Annexin V and PI labeling. The results revealed that the percentage of apoptotic cells was inhibited to 5.80±0.33% in the op18 overexpression group, compared with 11.79±1.09% in the control group (Fig. 3).

SMMC7721 cell cycle arrested at the phase of G2/M by upregulation of op18 expression. To determine whether op18 plays a key role in the progression of SMMC7721 cell cycle, we demonstrated the effect of op18 overexpression in SMMC7721 cells on the cell cycle by single cell analysis using FACS. The results showed that op18 overexpression induced cell cycle arrest by inhibiting progression from G2 to M phase.

Discussion

op18 plays a crucial role in tumorigenesis and metastasis. Numerous studies have demonstrated the upregulated expression of op18 in several types of cancer, including breast, prostate, lung, ovarian cancer and sarcoma (9-11), which was associated with the malignant biological behavior of cancer cells, as well as a potential predictor of worse prognosis and poor treatment.

Recent studies have reported the association between the expression of op18 and HCC (12-14). Wang *et al* (12) found that op18 distinctly expressed proteins identified in HCC cells treated by gambogic acid (GA) by proteomic approach and western blotting. Furthermore, it was reported that the over-expression of op18 in HCC cells decreased their sensitivity, whereas small interfering RNAs targeting op18 enhanced their sensitivity to GA, suggesting that op18 is a potentially significant target for GA in combating HCC. Results of a study by Chen *et al* (13) revealed that the upregulation of E2F1 and op18 proteins is associated with worse outcomes in patients with HCC, and E2F1 significantly correlates with the op18 protein level in HCC lesions and *in vitro* transactivation assays. It was reported that op18 expression may be associated with HCC metastasis, recurrence and prognosis, by detecting op18 mRNA expression in normal liver, non-metastasis, metastasis and recurrence in HCC tissues.

Our previous findings also suggested that the consecutive upregulation of op18 expression was associated with hepatocarcinogenesis by tissue microarray and IHC technology in normal liver, hepatitis, hepatic cirrhosis and HCC tissue (8). To examine the role of op18 in hepatocarcinogenesis in the present study, we established an op18 overexpression

SMMC7721 cell model and detected the difference in HCC cell proliferation, apoptosis and cell cycle in established cell lines. The results demonstrated that the upregulation of op18 expression induced cell proliferation, inhibited cell apoptosis and arrested the cell cycle from G2 to M phase in SMMC7721 cells, showing that op18 overexpression was closely associated with HCC tumorigenesis.

In an experimental model on HCC and lesions, Singer *et al* (15) indicated that the overexpression of op18 correlated with tumor progression, proliferation and activation of a few pro-tumor factors, such as p53. Results of that study demonstrated that op18 expression was associated with HCC cell viability, migration and was mediated by gain-of-function mutations in p53 (15). Accordingly, in an experimental model of large-size HCC mice and in xenograft models of human hepatoma tumors, Chen *et al* (16) demonstrated an increase in op18-mediated tumor growth by preventing its upregulation of EZH2 gene expression. However, any underlying mechanistic association between op18 expression and hepatocarcinogenesis is poorly understood, suggesting that additional studies are required to investigate the mechanism by which op18 is involved in HCC cell proliferation and apoptosis.

In conclusion, our findings suggest that op18 expression contributes to cell proliferation, represses cell apoptosis and induces cell cycle arrest by inhibiting the progression of G2 to M phase in human HCC SMMC7721-op18 cells. Therefore, op18 is a potential predictor of prognosis in HCC.

Acknowledgements

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