

Down-regulation of c-Myc expression inhibits the invasion of bile duct carcinoma cells

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Abstract

Cholangiocarcinoma is the second most common primary hepatic tumour originating from biliary tract epithelial cells with poor prognosis. Enhanced c-Myc protein expression contributes to many aspects of tumour cell biology. Although the ability of c-Myc to drive unrestricted cell proliferation and to inhibit cell differentiation had been well recognized, whether down-regulated c-Myc expression can inhibit tumour cell invasion still remains to be explored. The c-Myc ASODN (antisense oligodeoxyribonucleotide) and NSODN (nonsense oligodeoxyribonucleotide) were designed, synthesized and transfected into human QBC939 bile duct carcinoma cells using the Lipofectamine 2000 reagent. The protein expression of c-Myc was detected by Western blot. A transwell experiment was applied to evaluate the invasive capacity of the QBC939 cells. c-Myc ASODN could significantly suppress the c-Myc protein expression ($P < 0.05$) and the invasion ($P < 0.01$) of QBC939 cells transfected with c-Myc ASODN compared with that in the control and c-Myc NSODN-transfected group. Thus in the present study we show that down-regulation of c-Myc expression can inhibit the invasion of QBC939 cells *in vitro*.

Keywords: c-Myc; cholangiocarcinoma; invasion

1. Introduction

CCA (cholangiocarcinoma) is a malignant tumour originating from biliary tract epithelial cells (Khan et al., 2005; Sirica, 2005). Among the primary liver tumours, CCA is the second most common primary hepatic tumour, the incidence of which is second only to that of liver cancer (Tang et al., 2004; Shaib et al., 2005), and it is becoming the most common hepatic tumour-induced death (Taylor-Robinson et al., 2001). Invasion, delimitation, insufficient markers for early diagnosis marker and insensitivity to regular radio- and chemotherapy are all causes of poor prognosis of CCA patients (Olmes and Erlich, 2004). Moreover, its worldwide morbidity and mortality have increased rapidly in recent years.

The behaviour of mammalian cells is modulated by many extracellular stimuli, which trigger a variety of intracellular signalling pathways. Those signals induce expression of primary or IE (immediate early) genes. Several IE genes encode transcription factors that, in turn, regulate secondary transcriptional responses. The c-Myc proto-oncogene, located at 8q24, is such an IE gene. Numerous studies have suggested that the c-Myc gene is a regulator of cell cycle and plays a major role in control of cell growth, differentiation, apoptosis and neoplastic transformation (Dang, 1999). c-Myc gene overexpression is a frequent alteration and has been described in several types of human cancer (Little et al., 1983; Escot et al., 1986; Han et al., 1999; Wang et al., 1999; Ishii et al., 2001; Xu et al., 2001; Zhang et al., 2004). An increased

c-Myc gene expression has been found in CCA (Tokumoto et al., 2005; Prakobwong et al., 2011).

Enhanced c-Myc protein expression contributes to many aspects of tumour cell biology. Although the ability of c-Myc to drive unrestricted cell proliferation and to inhibit cell differentiation had been well recognized, a recent work showed that deregulated c-Myc expression can drive cell growth and vasculogenesis, reduce cell adhesion and promote metastasis and genomic instability. c-Myc loss also inhibits cell proliferation, cell growth, accelerates differentiation, increases cell adhesion and leads to an excessive response to DNA damage (Dominguez-Sola et al., 2007). Studies in animal models suggest that c-Myc may be a target for human cancer treatment (Albihn et al., 2010).

However, so far, whether down-regulated c-Myc expression can inhibit tumour cell invasion still remains to be explored. Our present study found that down-regulation of c-Myc expression could suppress the invasion of the QBC939 cells *in vitro*.

2. Materials and methods

2.1. Design and synthesis of c-Myc ASODN [antisense ODN (oligodeoxyribonucleotide)] and NSODN (nonsense ODN)

The gene sequence of c-Myc was retrieved from GenBank, the exon 2 of c-Myc mRNA, and the four codons after it were targeted

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Abbreviations: ASODN, antisense oligodeoxyribonucleotide; CCA, cholangiocarcinoma; IE, immediate early; NSODN, nonsense oligodeoxyribonucleotide.

to synthesize ASODN, and the sense sequence was 5'-ATGCCCTCAACGTT-3', and the antisense sequence was 5'-AACGTTGAGGGGCAT-3', and the self-designed antisense sequence was 5'-CAGAGTCGATGAGCT-3', which had no homology with other human genes. The designed and synthesized oligonucleotides were all treated with sulfate modification and synthesized, modified, purified and packed by the US Invitrogen Corp. Shanghai Office (5 OD in total, 1 OD in each tube, preserved at 4°C for future use). The ODNs (8.0 μmol/l) were introduced into cells using the Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's instructions.

2.2. Cell culture

Human QBC939 bile duct carcinoma cell line was established by SG-W, et al. from the Third Military Medical University of China Southwest Hospital and provided by the Cell Center of Xiangya Medical College, Central South University. The cells were incubated in modified RPMI 1640 culture medium (containing 10% inactivated fetal bovine serum and 100 U penicillin+streptomycin) at 37°C in a 5% CO₂ incubator.

2.3. Western blot

Twenty-four hours after transfection, cells were washed with PBS, collected by scraping and centrifuged at 1000 *g* for 5 min, then lysed in ice-cold RIPA buffer containing 150 mM NaCl, 10% Triton X-100, 0.5% deoxycholate, 0.1% SDS and 50 mM Tris (pH 7.5–8.0) for 30 min and centrifuged at 12 000 *g* for 30 min. The supernatant was used for protein determination by the Bradford procedure (Bio-Rad) and Western blot. Forty milligrams of total protein was suspended in 5 × reducing sample buffer, boiled for 10 min, electrophoresed on SDS/PAGE gels and then transferred to PVDF membrane by electroblotting. Blocked membranes were incubated with mouse anti-human c-Myc monoclonal antibody (Sanata) for 1 h, washed and then incubated with horseradish peroxidase-conjugated secondary antibody for 1 h. Antigens were detected with a chemiluminescent substrate system [ECL (enhanced chemiluminescence)]. Three independent cultures for the protein analysis were performed. All data were expressed as the relative differences between control and treated cells after normalization to β-actin expression.

2.4. Transwell experiment

The cell suspension was prepared, the cell concentration was adjusted to 2 × 10⁵/ml and the cell suspension was added to a 24-well culture plate, 500 μl for each well with the cell number of 1 × 10⁵. The experiment was performed in three groups; there were six wells in every group. The cell transfection was carried out, and the final concentrations of both ASODN and NSODN were 8.0 μmol/l. The control group did not undergo transfection. The NSODN group was transfected with NSODN, and the cells in all groups were collected 6 h after the transfection for further experiment. The Transwell chambers were prepared according to the instructions of Transwell Chamber Kit (US Invitrogen Corp.), the basement membrane was enveloped with 1:8 diluent of 50

mg/l Matrigel and then the membrane was hydrated with blood serum-free culture fluid containing 10 g/l BSA. Five hundred microlitres of 1640 culture fluid (containing antibiotics and 10% FBS) was added to wells in the Transwell lower chambers; the cells in the aforesaid experimental groups were, respectively, added to the wells in the Transwell upper chambers, and then were incubated at 37°C for 24 h in 5% CO₂ incubators. The upper chambers were taken out, and the Matrigel and the cells in the upper chambers were wiped out, fixed with 75% alcohol and stained using Trypan Blue. Afterwards, the chambers were placed upside down and observed and photographed under an upright microscope. Five visual fields around the well and in the middle of the well were chosen at random when the cell number was counted. Three independent cultures for the Transwell experiment analysis were performed.

2.5. Statistical analysis

The experimental data were represented as mean ± S.D., SPSS-16.0 Software was used for statistical analysis. Student's *t* test was applied to make comparison between the two groups. *P* < 0.05 constituted a difference and was considered to be statistically significant.

3. Discussion

CCA is the second most common primary malignancy of the liver, which arise from epithelial cells lining the biliary tree (Khan et al., 2005; Sirica, 2005). Current evidence suggests that the prevalence of CCA is increasing worldwide. In the U.S.A., approximately 5000 new cases are diagnosed every year accounting for almost 3% of all tumours of the gastrointestinal tract (Lazaridis and

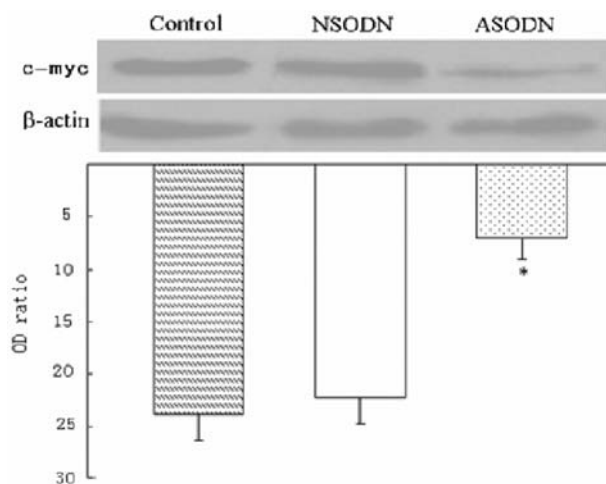


Figure 1 Effects of c-Myc ASODN in c-Myc protein expression in QBC939 cells. Protein levels of c-Myc after transfected with c-Myc ASODN and c-Myc NSODN in QBC939 cells were detected by Western blot. c-Myc ASODN could significantly suppress the c-Myc protein expression in QBC939 cells transfected with c-Myc ASODN compared with the control and c-Myc NSODN-transfected group. **P* < 0.05, compared with the control and NSODN-transfected group.

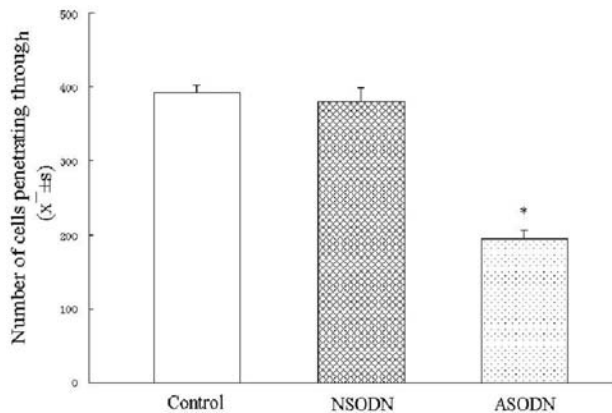


Figure 2 Down-regulation of c-Myc expression inhibits QBC939 cell invasion. QBC939 cell invasion was significantly suppressed in c-Myc ASODN-transfected cells compared with the control and c-Myc NSODN-transfected group. * $P < 0.01$, compared with the control and c-Myc NSODN-transfected group.

Gores, 2005). The prevalence of CCA varies markedly from one geographic region to another, with the highest prevalence in Southeast Asia (Shaib et al., 2004; Patel, 2006).

In recent years, studies on oncomolecular biology and molecular genetics have shown that activation of proto-oncogene, inactivation of anti-oncogene and dysfunction of apoptosis gene are important mechanisms for the development of many kinds of tumours. c-Myc is an oncogene involved in cell cycle regulation, cell growth arrest, cell adhesion, metabolism, ribosome biogenesis, protein synthesis and mitochondrial function (Dominguez-Sola et al., 2007). It has been described as a key element of several carcinogenesis processes in humans. c-Myc gene overexpression is a frequent alteration and has been described in several types of human cancer, and enhanced c-Myc protein expression contributes to many aspects of tumour cell biology (Little et al., 1983; Escot et al., 1986; Wang et al., 1999; Han et al., 1999; Ishii et al., 2001; Xu et al., 2001; Zhang et al., 2004).

An increased c-Myc gene expression has been found in CCA. Consistent with the previous reports (Tokumoto et al., 2005; Prakobwong et al., 2011), in our study, an increased c-Myc protein expression was found in human QBC939 bile duct carcinoma cells. Kimura et al. (1995) also demonstrated that c-Myc ASODN could treat malignancies by means of acting on the three stages of tumorigenesis. Meanwhile, to evaluate the effects of c-Myc ASODN in c-Myc protein expression in QBC939 cells, Western blot was performed, and our present results showed that c-Myc ASODN could significantly suppress c-Myc protein expression in QBC939 cells transfected with c-Myc ASODN compared with that in the control and the c-Myc NSODN-transfected group ($P < 0.05$, Figure 1). Furthermore, the results of Transwell assay showed that the invasion through Matrigel was significantly decreased in c-Myc ASODN-transfected QBC939 cells, compared with that in the control and c-Myc NSODN-transfected group ($P < 0.01$, Figure 2), indicating c-Myc is involved in the process of QBC939 cell invasion.

In conclusion, down-regulation of c-Myc expression can inhibit the invasion of the QBC939 cells *in vitro*. However, further researches are required to explore whether c-Myc ASODN has

the same effect on cholangiocarcinoma cells *in vivo* as it did *in vitro* and what the concentration is for c-Myc ASODN to achieve the maximum inhibition effects.

Author contribution

Zhuo-Ri Li was in charge of the instruction of the whole experiment. Yi-Fei Wu was responsible for the cell culture and Western blot. Chun-Yang Ma was in charge of the cell culture and writing of the paper. Shen-Dan Nie was responsible for the cell culture. Xian-Hai Mao was in charge of the statistical analysis. Yong-Zhong Shi was responsible for the Western blot.

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