STUDIES ON THE MOLECULAR MECHANISMS INVOLVED IN SLOW VIRUS-INDUCED MUC16 GENE EFFECT ON CELL INVASION AND METASTASIS IN GALLBLADDER CARCINOMA

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ABSTRACT

Introduction*: To study the effect of lentivirus-induced mucin16 (MUC16) gene on cell invasion and metastasis in gallbladder carcinoma, and the molecular mechanisms involved.*

Method*s: Gallbladder cell-Shandong (GBC-SD) of over-expression MUC16 and empty viral plasmid-transfected cells were produced through slow virus transfection system. The effect of MUC16 on proliferation of GBC-SC cells in vitro was determined using 3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2-H-tetrazolium bromide (MTT) assay. Scratch test and Transwell chambers were used to measure the effect of MUC16 over-expression on in vitro cell migration and invasion. The effect of MUC16 over-expression on GBC-SD cell adhesion was determined with adhesion test, while real time-polymerase chain reaction (RT-PCR) was used to ascertain the pathway involved in MUC16-induced regulation of GBC-SD cell biology.*

Results*: The growth rate of MUC16 over-expression cells was significantly higher than that of the control group ($p < 0.05$). MUC16 over-expression increased the migration, scratch recovery, and extracellular matrix (ECM) adhesion abilities of GBC-SD cells in vitro. MUC16 significantly enhanced the expression of matrix metalloproteinase-2 (MMP2) and MMP7 mRNAs, relative to control ($p < 0.05$).*

Conclusion*: MUC16 enhances the proliferation, invasion and migration of gallbladder carcinoma cells in vitro by activating the PI3K/Akt signal pathway.*

Keywords*: Mucoprotein, Gallbladder carcinoma, Proliferation, Invasion, PI3K/Akt signal pathway.*

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Introduction

In China, the incidence of gallbladder carcinoma, particularly female gallbladder carcinoma, is high and increasing yearly[(1)]. The early clinical symptom of gallbladder is slur. The diagnosis is difficult and error-prone most of the time. Gall bladder carcinoma develops rapidly, and is associated with a high degree of malignancy and poor prognosis. Many patients are diagnosed at advanced stage of the disease when they can no longer benefit from surgical intervention. Thus, the five-year survival of gallbladder carcinoma patients is very low (5 to 10 %) and the mortality is extremely high[(2)]. The mechanisms involved in the pathogenesis of gallbladder carcinoma are unclear: the clinical symptoms are insidious, and there are no specific clinical markers. Thus, the diagnosis and treatment of gallbladder carcinoma are difficult[(3).]

Several studies suggest that mucin (MUC) plays an important role in the pathogenesis of tumors[(4)]. Mucin (MUC) is a high molecular volume glycoprotein secreted by epithelial cells. It participates in physiological processes such as signal transduction and cell adhesion[(5)]. Indeed, many multiple tumors abnormally express MUC16. However, not much is known about the relationship between gallbladder cells and MUC16. The present study used MUC16 over-expression and intervening cell line to investigate the influence of MUC16 on

GBC-SD cell invasion and migration, and the mechanism involved.

Materials and methods

Cell line

Human GBC-SD gallbladder cells were bought from the cell bank of Chinese Academic of Sciences.

The Ethical Committee of Department of General Surgery. The Second People's Hospital of Hubei Province, China, approved this research according to the declaration of Helsinki promulgated in 1964 as amended in 1996 (approval number is 20170101[6]

Reagents

PCR premiers were synthesized and provided by Shanghai Sangon Biotech Engineering CP., Ltd; BCA protein concentration detection kits were products of China Boster Biological Engineering CO., Ltd.; while cDNA synthesis kit, fluorescent quantitation PCR kits, and cell apoptosis kits were purchased from OMEGA Biological Company. Small interfering PNA was synthesized and provided by Shanghai Sangon Biotech Engineering Co., Ltd.; DMEM hyperglucose cell medium was bought from America GIBCO Company; Trypsin-EDTA cell dissociation solution (0.25%) was purchased from Beijing Neuronbc Technology Development Co., Ltd; FBS and streptomycin-penicillin double antibody were products of America ThermoFisher Company. Trizol was obtained from America Invitrogen Company; DEPC was from Sigma; 30% H2O2 was product of Shanghai Beyotime Biotechnology Co., Ltd; hydrochloride buffer solution, chloroform, isopropanol and absolute ethyl alcohol were purchased from Sinopharm Chemical Reagent Co., Ltd. Normal saline was got from Anhui Double-crane Pharmaceutical Co., Ltd. Plasmid and slow virus package: plasmid pc DNA3.1-MUC16-myc, over-expression slow virus plasmid p GC-FU-MUC16-GFP and MUC16 over-expression, interfering slow virus were constructed and confirmed by Shanghai Jikai Gene Chemical Co., Ltd.

Main instruments

GTR16-2 type high-speed desktop cold centrifugal machine was bought from Beijing Shidaibeili Centrifugation Co. Ltd; real-time quantitative PCR instrument was from America MJ Research Company, while Shanghai Precision Instruments Company supplied UV-8000 ultraviolet spectrophotometer. Multifunctional Enzyme Instrument was purchased from Japan Bio-Rad; CX41 inverted optical microscope and CX41 laser scanning confocal microscope were obtained from Japan Olympus Medical Company; electronic analytical balance was purchased from Shanghai Yuyan Scientific Instruments Co., Ltd; Amersham electrophoresis apparatus was from Sweden Bioscience Company; shaking bath and YCZ-40D migration electrophoresis chamber were products of Beijing liuyi Instrument Factory, while FluorChem FC3 gel imaging digital analysis system was supplied by America ProteinSimple Company.

Methods

Cell cultivation

GBC-SD cells were cultivated in Dulbecco's Modified Eagle Medium (DMEM) with 10% fetal bovine serum (FBS) in a 5%-CO2 incubator at 37 oC. The culture medium was changed every 24 to 48 h, and 48 h was allowed for passage.

Slow virus carrier and cell transfection

Entrust Shanghai Jikai Gene Chemical Company provided pcDNA3.1-MUC16-myc and lenti-MUC16-GFP. Cells (5 × 108) were inoculated in a six-well transfection plate and cultivated for 24 - 48 h. Adhesion reached 50 to 60%. Appropriate volumes of normal saline, polyetherimide (PEI) reagent and plasmid were put in 2-mL sterile Eppendorf tube in line with instructions on the assay kits. Then, DNA solution was added and the mixture was vortexed and incubated at room temperature for 15 to 20 min. Liposome-plasmid DNA composites were instilled into the cell surface and cultivated in a 5 % CO2 cell incubator for 24 to 48 h at 37 oC. Cellular RNA and protein were extracted, and the conditions for the target gene expression and transfection efficiency were determined.

Slow virus infection

GBC-SD cells were inoculated in 6-well plates to 50 - 60% confluence. Then, slow virus liquid (MOI = 10-30) diluted with non-serum DMEM cultivation liquid was added. After 8 h, the culture medium was replaced with DMEM containing 10% FBS.

Determination of effect of MUC16 over-expression on proliferation and activity of GBC-SD cells with MTT

MUC16-stable transferring cells and control GFP cells were inoculated into 96-well plate containing 100 ul slow virus (3×10^3 cells/well). Each well contained 100 ul DMEM cultivation medium and 10% FBS. Every 24 h, 20 ul MTT was added to 6 wells at 37 oC, and the wells were incubated in a 5% CO2 cell cultivation incubator for 4 h. Thereafter, the supernatant was discarded, and 200 ul DMSO was added to each well. The wells were shaken for 5 min, and observed under the microscope for non-purple crystals. A total of 96-well plates were subjected to absorbance readings at 450 nm in a 450 ELISA reader.

Scratch test

GBC-SD cells were inoculated into 12-well plates until the cells reached 50 - 60% convergence. The medium was then discarded, and a 200-ul yellow gun tip was used to scratch the cells, prior to addition of a fresh medium. Every 24 h, the cells were observed under an inverted microscope and photographed.

Migration and invasion tests

Trypsin peptic cells were centrifuged and the supernatant was discarded. Then, DMEM without serum was added for adjustment of the cell concentration to 3×10^3 cells/ml, and 1ml human GBC-SD gallbladder carcinoma monolayer was inoculated into a Transwell cell enveloped by collagen in a 6-well plate. DMEM without serum was put in the inner cell, while MWEM with 10% FBS was placed in the outer cell. The Transwell cells were incubated at 37 °C and 5% CO2 for 72 h. The invasion cells were fixed with 4% triformol for 30 min, and stained with 0.1 % purple crystals for 30min. Cell population was observed through the membrane under the microscope, and migration was calculated.

Fibronection assay

The Fn stock solution was diluted to 20 ug/ml with sterile PBS, and put in 96-well plates incubated at 37 oC for 2 h in a 5% CO2. incubator. Then, 100 ul BSA solution was added to each well and incubated under the same conditions for another period of 2 h. Thereafter, trypsin-digested cells were centrifuged, and the supernatant was discarded. DMEM without serum was sued to adjust the cell concentration to 3×10^5 cell/ml.

Then, 100 ul of cells was added to each well at 37 oC for 2 h in a 5% CO2 incubator. The 96-well plates were fetched at 30 min and 90 min separately. Four parallel control wells were selected in each group, and the cells were washed with PBS prior examination of cell adhesion under an inverted microscope.

Extraction of total RNA

Cells (1×10^{10}) were collected and placed in an EP tube; and 1ml pre-cooled Trizol was added, with gentle mixing for about 10 min. Then, 200 ul chloroform was added, with gentle mixing for 5 -10 min, followed by centrifugation at 12000 rpm. The supernatant was added to 500 ul of isopropanol, and mixed for 5 - 10 min, prior to centrifugation at 12000 rpm. The supernatant was discarded. Then, 1 ml of 75% ethanol was added, with vortexing for 30 sec. After centrifuging at 12000 rpm, the supernatant was discarded. The EP tube was inverted on a filter paper, and the extracted RNA was dried fully. Finally, the RNA was dissolved in 20 ul of DEPC and preserved at -80 oC prior to use. The molecular weight of the RNA was determined using gel electrophoresis, while its concentration was determined spectrophotometrically.

RNA reverse transcription

The RNA reverse transcription was carried out in line with kit instructions. cDNA was stored at -20 oC prior to use.

Real-time quantitative PCR

The 20-ul reaction system was placed in a thermostatic water bath at 37 oC for 60 min, and at 85 oC for 5 sec. Deionized water was added to make it up to 100 ul, and 2 ul was fetched from each reaction well for PCR. The 20-ul PCR system was prepared in ice bath at 95 oC for 30 sec of pre-degeneration; 95 oC for 5 sec, and 60 oC for 30 sec. There were 45 cycles. The sequences of premiers were:

MMP2 (premier in up-stream: 5'-TGGC-GATGGCAGTGTCTTAG-3';

MMP2 premier in down-stream: 5'-GTGCAGGGTCCGAGGT-3');

MMP7 (premier in up-stream: 5'-CAGCTTTGAGGTTCGTGTTTGT-3';

MMP7 premier in down-stream: 5'-ATGCTCTTCTTTTTTGCGGAAA -3');

Statistical analylis

SPSS19.0 statistical software[6] was used to do data analyses; homoscedasticity was done with single factor ANOVA analysis. Comparison between groups was done with t-test. Heterogeneity of variance was done with rank sum test. Statistical significance was assumed at $p < 0.05$.

Results

Effect of over-expression MUC on proliferation of GBC-SD cell in vitro

The results of MTT assay showed that, with increase in time, the growth rate of MUC16 over-expression cell line was significantly higher than that of the control group, especially after 96 h ($p < 0.05$; Table 1).

OD	Time (h)					
	24	48	72	96	120	144
Over-expression group	0.135±0.012	0.319±0.025	0.581±0.037	1.122±0.039	1.485±0.048	
The control group	0.142±0.009	0.306±0.017	0.548±0.023	0.984±0.021	1.176±0.032	

Table 1: Effect of MUC16 on proliferation of GBC-SD cells at different time points.

Effect of overexpression MUC16 on migration ability of GBC-SD cells

MUC16 overexpression significantly promoted the migration ability of GBC-SD cells in vitro, when compared with the control group ($p < 0.05$; Figure 1).

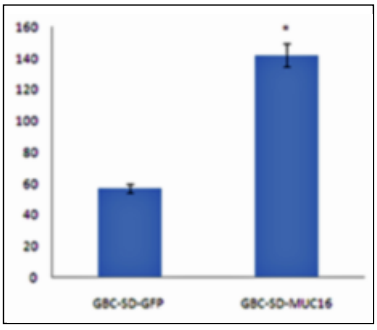

Fig. 1: Effect of MUC16 over-expression on migration ability of GBC-SD cells.
*(*compared with the control group, $p < 0.05$)*

Effect of over-expression MUC16 on invasion ability of GBC-SD cells

Results from scratch test showed that over-expression of MUC 16 led to significant increase in scratch recovery ability of GBC-SD cells in vitro, when compared with the control group ($p < 0.05$; Figure 2).

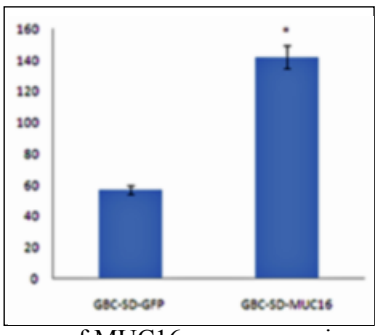

Fig. 2: Influence of MUC16 over-expression on invasion ability of GBC-SD cells.
*(*compared with the control group, $p < 0.05$).*

Effect of MUC16 over-expression on adhesion ability of GBC-SD cells

Results from Fn adhesion test showed that MUC 16 over-expression significantly enhanced the adhesion ability of GBC-SD cells on ECM in vitro, relative to the control group ($p < 0.05$; Figure 3).

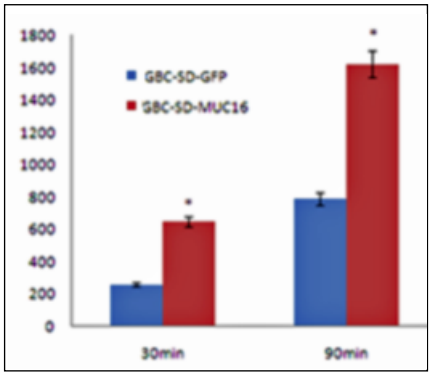

Fig. 3: Effect of MUC16 over-expression on adhesion ability of GBC-SD cells.
*(*compared with the control group, $p < 0.05$).*

Effect of MUC16 on mRNA expressions of MMP2 and MMP7

Previous studies reported that the MMP family plays an important role in invasion and migration of tumor by degrading extracellular matrix component. Results from RT-PCR assay showed that MUC16 significantly increased the mRNA expressions of MMP2 and MMP7

Discussion

Gallbladder carcinoma is a common malignant tumor of the digestive system. It has insidious onset, high degree of malignancy, rapid progress and poor prognosis. Therefore, the affected patients have poor quality of life, and death rate is relatively high[2]. At present, surgery is the best method for treating gallbladder carcinoma because the neo-

plasm lacks sensitive early diagnostic and specific molecular markers. Thus, early and accurate diagnosis is relatively low, causing many patients to lose the best time for surgery. Therefore, it is necessary to find early clinical diagnostic markers and new treatment direction for the disease.

In recent years, studies on the relationship between mucoprotein and occurrence of diseases in China and elsewhere have become quite popular among researchers[4, 7]. Mucin is a transmembrane glycoprotein with high molecular weight. It has abnormal expression in transformed cells. However, at present, there are no studies on the relationship between MUC and gallbladder carcinoma[8]. The present study was aimed at exploring the influence of MUC16 on the proliferation, invasion and migration of human gallbladder carcinoma cell GBC-SD. It has been demonstrated that MUC16 is highly expressed in oviduct endothelial cells, epithelial cells of upper alimentary tract and endometrium of fetus. Thus, MUC16 is widely applied in the diagnosis of ovarian cancer[9]. However, more and more studies have reported that MUC16 is closely linked with the incidence of extrahepatic cholangiocarcinoma. The MUC16 expression level in serum of patients decreases after extrahepatic cholangiocarcinoma surgery[10], but it increases significantly after recurrence. Thus, MUC16 can be applied in the evaluation of prognosis and diagnosis of recurrence after extrahepatic cholangiocarcinoma surgery. Studies have shown that extrahepatic cholangiocarcinoma cells can express MUC16 highly, and this hints poor prognosis[11]. The MUC16 is not expressed in normal liver tissue and intrahepatic bile duct tissue. To further explore the effect and possible molecular mechanism of MUC16 gene induced by slow virus on cell invasion and metastasis of gallbladder carcinoma, we first built a slow virus transfection system.

The results of MTT assay showed that, with increase in time, the growth rate of MUC16 overexpression cell line was significantly higher than that of the control group, particularly after 96 h. The results of migration, scratch test and adhesion tests showed that over-expression MUC 16 can increase migration ability, scratch recovery ability and adhesion of GBC-SD cells to ECM cells in vitro.

Previous studies showed that many membranous mucoproteins can interact with tyrosine kinase receptor to regulate MARK and PI3K and Akt signal pathways[12, 13]. The PI3K and AKT pathways are associated with the expressions of MMPs[14]. Serum MUC16, also called CA125, is a transmembrane glycoprotein with one base pair. Its structure includes a region of amino terminal, a region with wide tandem repeats, and a carboxyl terminal region including the caudal region. Phosphorylation of MUC16 terminal tyrosine can enhance the interaction between Scr homologous regions of MUC16 and PI3Kp85 regulatory subunit, and block inhibition of PI3Kp85 subunit on P110 catalytic subunit, thus activating PI3Kor Akt signal pathways[15].

In addition, MMP9 promoter region has binding site for NK-κB. Previous studies also reported that MUC is linked to the activation of the NK-κB signal pathway[16]. Results from RT-PCR suggest that MUC16 promotes mRNA expressions of MMP2 and MMP7. However, the specific molecular mechanism involved in the MUC16-induced promotion of invasion and migration of gallbladder carcinoma cells is still unclear. It needs further studies.

The present study obtained GBC-SD with stable expression of MUC16 through slow virus transfection, which increased the proliferation, invasion and migration ability of the cells in vitro. These studies suggest that MUC16 may be related to the regulation and control of PI3K or AKT signal pathway. However, the specific mechanism responsible for inducing increases in expressions of MMP-2 and MMP9 still need to be elucidated. Overall, the present study provides a new approach in the treatment of gallbladder carcinoma. The findings indicate that colon cancer cell apoptosis may be promoted, and its invasion and migration controlled by inhibiting MUC16 expression. This provides a new study target and direction for early treatment of gallbladder carcinoma.

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