STAT3-Mediated Mcl-1 Protein Expression Involves in Gemcitabine Sensitivity in Human Cholangiocarcinoma Cells

การแสดงออกของโปรตีนเอ็มซีแอล-1 ที่กระตุ้นผ่านทาง STAT3 มีความเกี่ยวข้องกับความไวต่อยาเจมไซทาบีนในเซลล์มะเร็งท่อน้ำดีของมนุษย์

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ABSTRACT

Cholangiocarcinoma (CCA) is a major public health problem in the northeastern region of Thailand. Gemcitabine is an effective chemotherapeutic drug for treating advanced CCA patients, however, many patients eventually develop resistance. Previous studies show that anti-apoptotic protein, myeloid cell leukemia-1 (Mcl-1), is associated with chemotherapeutic drug resistance in many cancers. Thus, the aim of this study is to examine the role of Mcl-1 protein in sensitivity to gemcitabine of human CCA cell lines. The result showed that the low gemcitabine sensitive cell line (KKU-M213) has higher Mcl-1 expression than the high gemcitabine-sensitive cell line (HuCCA-1). Moreover, we found that STAT3 knockdown decreased Mcl-1 expression and enhanced sensitivity to gemcitabine in KKU-M213. This study may provide an additional therapeutic strategy to overcome the low gemcitabine sensitivity for CCA patients.

บทคัดย่อ

มะเร็งท่อน้ำดีเป็นปัญหาที่สำคัญทางสาธารณสุขในแถบภาคตะวันออกเฉียงเหนือของประเทศไทย เจมไซทาบีนเป็นยาเคมีบำบัดที่มีประสิทธิภาพในการรักษาผู้ป่วยโรคนี้ อย่างไรก็ตามการรักษาด้วยยาเคมีบำบัดดังกล่าวมักไม่ได้ผลเนื่องจากผู้ป่วยบางรายเกิดการดื้อต่อยา จากการศึกษาก่อนหน้าพบว่าโปรตีนเอ็มซีแอล-1 มีความเกี่ยวข้องกับการดื้อต่อยาเคมีบำบัดในมะเร็งหลายชนิด ดังนั้นวัตถุประสงค์ของการศึกษานี้คือการตรวจหาบทบาทของโปรตีนเอ็มซีแอล-1ต่อความไวต่อยาเจมไซทาบีนในเซลล์มะเร็งท่อน้ำดี ผลการทดลองแสดงให้เห็นว่าเซลล์ที่มีความไวต่อยาเจมไซทาบีนต่ำ (KKU-M213) มีการแสดงออกของโปรตีนเอ็มซีแอล-1 มากกว่าเซลล์ที่มีความไวต่อยาเจมไซทาบีนสูง (HuCCA-1) นอกจากนี้ เรายังพบว่าการน็อกดาวน์โปรตีน “สแตท3” ทำให้การแสดงออกของโปรตีนเอ็มซีแอล-1 ลดลง รวมทั้งเพิ่มความไวต่อยาเจมไซทาบีนในเซลล์ KKU-M213 การศึกษานี้อาจนำไปสู่แนวทางในการรักษามะเร็งท่อน้ำดีที่มีความไวต่อยาเจมไซทาบีนต่ำต่อไป

Key Words: Cholangiocarcinoma, Mcl-1, STAT3

คำสำคัญ: มะเร็งท่อน้ำดี โปรตีนเอ็มซีแอล-1 โปรตีนสแตท3

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Introduction

Cholangiocarcinoma (CCA) is a malignant tumor derived from bile duct epithelium. It occurs with a higher incidence in tropical countries, especially in northeast of Thailand. The major risk factor of CCA in Thailand is infection with liver fluke, *Opisthorchis viverrini* (Sripa et al., 2007). Different types of treatments are used to cure cancer including surgery, radiation therapy, chemotherapy and palliative therapy. Surgery is the first choice which is used to manage CCA patients (Su et al., 1996). Some patients may receive chemotherapy or radiation therapy after tumor removal by surgery.

Gemcitabine is a common chemotherapeutic drug for CCA patients. It is used as a single agent (Park et al., 2005) or in a combination with cisplatin (Giuliani et al., 2006), irrinotecan (Bhargava et al., 2003) or capecitabine (Knox et al., 2005). Gemcitabine is a cytidine nucleoside analog in which the hydrogen atoms on the 2' carbon of deoxycytidine are replaced by fluorine atoms. When the cells incorporate these substances into the cellular metabolism, gemcitabine is metabolized into gemcitabine triphosphate by deoxycytidine kinase (dCK). Gemcitabine triphosphate competes deoxycytidine in DNA replication. This process arrests tumor cell growth and induces cell apoptosis. However, gemcitabine resistance is occurred in many CCA patients/cells.

Previous reports show that the inflammatory cytokine interleukin-6 (IL-6) plays a pivotal role in cholangiocarcinoma progression (Wehbe et al., 2006). Generally, IL-6 exerts its biological effects via JAK/STAT signaling pathway and activates many downstream targets to drive the expression of particular genes. Previous studies show that IL-6 regulates the Mcl-1 expression in CCA cell lines via JAK/STAT3 signaling (Isomoto et al., 2005). An anti-apoptotic protein, myeloid cell leukemia-1 (Mcl-1), is associated with chemotherapeutic drug resistance in some cancers (Wei et al., 2008). However, the role of Mcl-1 in gemcitabine sensitivity and the regulation of this protein in CCA cells still unclear.

Objectives

1. To examine the role of Mcl-1 in gemcitabine sensitivity of human CCA cell lines.

2. To examine the role of STAT3 on Mcl-1 expression and gemcitabine sensitivity of human CCA cell lines

Methodology

Cell culture

KKU-M213 and HuCCA-1 were cultured in Ham's F-12 nutrient mixture medium (Gibco BRL, Paisley, Scotland) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, 100 µg/ml streptomycin sulfate, 0.25 µg/ml amphotericin B (Invitrogen Corp., Auckland, NZ) and 15 mM HEPES (Merck, Darmstadt, Germany). Cells were incubated at 37 °C in humidified atmosphere with 5% CO_2.

For subculturing, cells were washed twice with PBS and incubated with 0.25% trypsin-EDTA (Gibco BRL, Paisley, Scotland) for 5 min at 37 ° C. Then cells were subsequently resuspended in fresh medium. Subculturing was performed when cells reached confluent stage.

Cytotoxicity assay

Cells (HuCCA-1 or KKU-M213) were seeded at 1 x10^4 cells per well in 96 well plate, adhered for 24 hrs and incubated with various concentrations of gemcitabine hydrochloride (Sigma, Singapore) or normal saline solution (NSS) as control for 48 hrs. Cell survival was detected by MTT assay.

For MTT assay, MTT (Applichem, Darmstadt, Germany) was added to each well and incubated for 3 hrs. Then, supernatant was removed and 200 µl DMSO was added. The plate was shaken for 1 min until the formazan crystal was dissolved. The absorbance was measured by a microplate reader at 540 nm. Five replicates were performed for each experimental condition. The absorbance of each condition was used to calculate the half maximal inhibitory concentration (IC_{50}) by Chou-Talalay method.

Western blot analysis

Cells were lysed with HEPES lysis buffer, and the total protein concentration was determined by Bradford assay (Bio-Rad, Hercules, California). Equal amounts of protein in leammli buffer (312.5 mM Tris-HCl pH 6.8, 50% Glycerol, 10% SDS, 0.05% Bromophenol blue, 12.5% 2-β-Mercaptoethanol) were heated for 5 min at 96 °C. The proteins (30 µg) were separated on 10% separating gel with 3% stacking gel by constant current (30 mA) using Hoefer apparatus. The precision Plus Protein Standard was used as protein marker.

Following electrophoresis, the proteins in SDS-PAGE were transferred to PVDF membrane by semi-dry electro-blotting. The electroblotting were carried out at constant voltage (25 V) for 60 min.

The PVDF membranes were blocked with 10% BSA in TBS-N buffer for 1 hr. The membranes were washed twice with TBS-N buffer and incubated with STAT3, Mcl-1 or β-actin primary antibody (Santa Cruz, Santa Cruz, California) shaking overnight at 4 ^{O}C. The blots were washed three times for 5 min with TBS-N buffer and incubated with HRP-conjugated mouse secondary antibody for 45 min. Signals were detected using the Clarity western ECL system (Bio-Rad Hercules, California).

STAT3 siRNA transfection

KKU-M213 cells were cultured in 60 mm plate for 24 hrs and then transfected with STAT3 siRNA or negative control siRNA. STAT3 siRNA and scrambled negative control siRNA were obtained from Santa Cruz biotechnology (Santa Cruz, Santa Cruz, California). Transient transfection of siRNA oligonucleotides were performed by Lipofectamine® transfection reagent (Gibco BRL, Paisley, Scotland) according to the manufacturer's protocol. After 96 hrs, cells were used to determine gemcitabine sensitivity by MTT assay and knockdown efficiency was determined by Western blot analysis

Statistical analysis

Statistical analysis was performed using the Student's t-test with $P < 0.05$ considered to be significant

Results

Mcl-1 protein expression correlates with gemcitabine sensitivity

In this study, we investigated whether Mcl-1 plays a role in gemcitabine sensitivity of CCA cells.

The gemcitabine sensitivity of two human CCA cell lines, HuCCA-1 and KKU-M213 was determined by MTT assay. Cells were treated with various concentrations of gemcitabine and the half maximal inhibitory concentration (IC_{50}) was calculated by Chou-Talalay method. The results show that HuCCA-1 is a high gemcitabine-sensitive cell line (IC_{50} = 478 nM), whereas KKU-M213 is a low gemcitabine-sensitive cell line (IC_{50} = 2467 nM) (Figure 1).

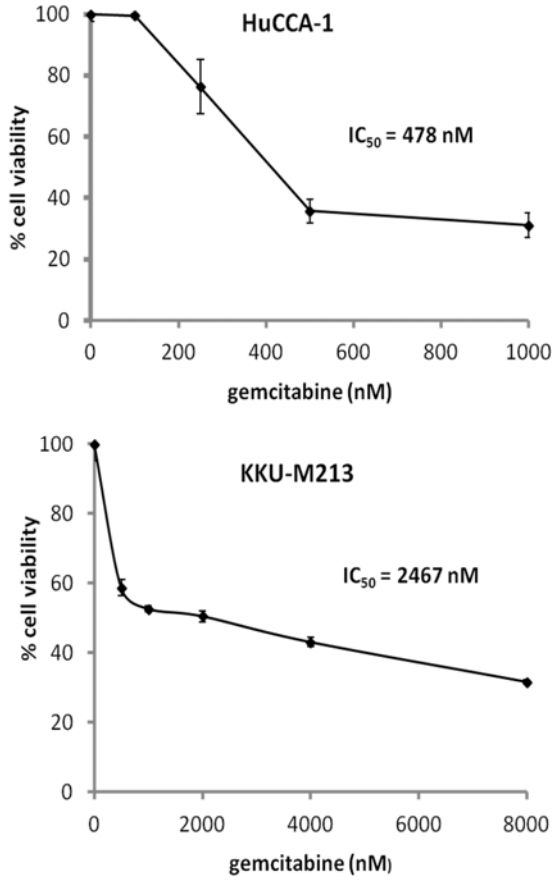

Figure 1 HuCCA-1 and KKU-M213 were treated with different concentration of gemcitabine, and cell viability was determined by MTT assay. Results are presented as the mean of three experiments with standard deviation. Next, we investigated the basal level of Mcl-1 protein expression in both cell lines by Western blot analysis (Figure 2) The results showed that the low gemcitabine-sensitive KKU-M213 cell lines has higher Mcl-1 protein expression than the high gemcitabine sensitive HuCCA-1 cell line suggesting that Mcl-1 may play a key role in gemcitabine sensitivity of CCA.

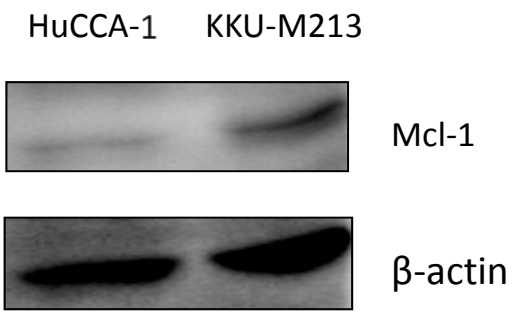

Figure 2 Basal level of Mcl-1 protein expression in KKU-M213 and HuCCA-1 was examined by Western blot.

Suppression of STAT3 expression reduces Mcl-1 expression and enhances gemcitabine sensitivity

Previous studies show that STAT3 regulates the Mcl-1 expression in CCA cell lines (Isomoto et al., 2005). Therefore, STAT3 expression in KKU-M213 cells was knockdown using siRNA approach. 96 hrs after transfection, STAT3 and Mcl-1 expression were examined by Western blot analysis. The result showed that STAT3 knockdown cells had lower Mcl-1 expression than untransfected cells and scrambled

negative siRNA transfected cells (Figure 3). These demonstrate Mcl-1 is a targeting gene of JAK/STAT3 signaling.

We also examined gemcitabine sensitivity in STAT3 knockdown cells. As expected, IC_{50} value of STAT3 knockdown cells was decreased about 30% (IC_{50} = 1042 nM) whereas the IC_{50} values of untransfected cells and scrambled negative control siRNA transfected cells remain unchanged (Figure 4).

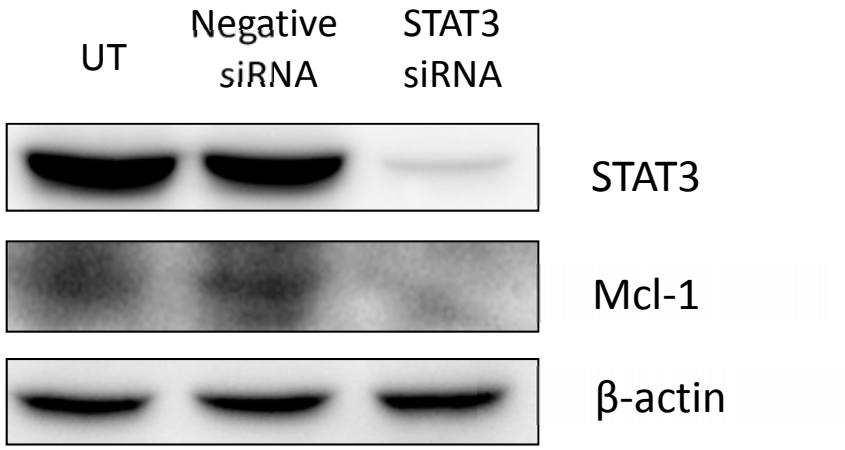

Figure 3 STAT3 and Mcl-1 expression in untransfected (UT), negative siRNA and STAT3 siRNA transfected KKU-M213 cells were determined by Western blot

Discussion and conclusions

The aim of this study is to examine the role of Mcl-1 in gemcitabine resistance of human CCA cell lines. Previous studies showed that Mcl-1 is associated with chemotherapeutic drug resistance in pancreatic cancer (Wei et al., 2008). However, there is no study about the relationship between Mcl-1 expression and chemotherapeutic drug resistance in CCA. In this study, we showed that the protein level of Mcl-1 expressed in gemcitabine-sensitive CCA cell line was lower than gemcitabine-resistant CCA cell line, suggesting that Mcl-1 may be a key player in gemcitabine or chemotherapeutic drug resistance in CCA cells.

The inflammatory cytokine IL-6 plays pivotal roles in cholangiocarcinoma progression. Previous reports suggested that IL-6 activates Mcl-1 expression in CCA cells (Isomoto et al., 2005). Preliminary data in our group showed that IL-6 also activated Mcl-1 expression in our CCA cell lines (data not shown). Generally, IL-6 exerts its biological effects via JAK/STAT signaling pathway. Therefore, we knockdown STAT3 in KKU-M213 which is a gemcitabine-resistant cell line and the results showed that Mcl-1 expression was decreased in STAT3 knockdowned cells, demonstrating that Mcl-1 is also a targeting gene of JAK/STAT3 signaling in our CCA cell line. Moreover, suppression of STAT3 expression enhances gemcitabine sensitivity.

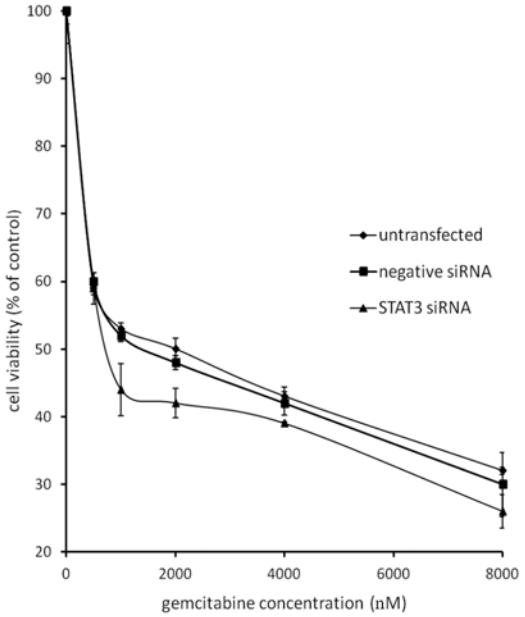

Figure 4 KKU-M213 with untransfected, negative siRNA or STAT3 siRNA were treated with different concentration of gemcitabine, and cell viability was determined by MTT assay. Results are presented as the mean of three experiments with standard deviation.

In conclusion, Mcl-1, a key protein in chemotherapeutic drug resistance, is regulated by JAK/STAT3 signaling and is involved in gemcitabine sensitivity of CCA cells. This knowledge may provide an additional therapeutic strategy that is helpful to overcome the low gemcitabine sensitivity for CCA patients.

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References

Bhargava P, Jani CR, Savarese DM, O'Donnell JL, Stuart KE, Rocha Lima CM. Gemcitabine and irinotecan in locally advanced or metastatic biliary cancer: preliminary report. Oncology 2003. 17(9): 23–6.

Giuliani F, Gebba V, Maiello E, Borsellino N, Bajardi E, Colluci G. Gemcitabine and cisplatin for inoperable and/or metastatic biliary tree carcinomas: a multicenter phase II study of the Gruppo Oncologico dell'Italia Meridionale (GOIM). Ann Oncol 2006. 17(Suppl 7): vii73–7.

Isomoto H, Kobayashi S, Wernburg NW, Bronk SF, Guicciardi ME, Frank DA, et al., Interleukin-6 Upregulates Myeloid Cell Leukemia-1 Expression Through a STAT3 Pathway in Cholangiocarcinoma Cells. Hepatology 2005. 42(6): 1329-38.

Knox JJ, Hadley D, Oza A, Feld R, Siu LL, Chen E, et al. Combining gemcitabine and capecitabine in patients with advanced biliary cancer: a phase II trial Combining gemcitabine and capecitabine in patients with advanced biliary cancer: a phase II trial. Jpn J Clin Oncol 2005. 23(10): 2332-8.

Park JS, Oh SY, Kim SH, Kwon HC, Kim JS, Kim HJ, et al. Single-agent gemcitabine in the treatment of advanced biliary tract cancers: a phase II study. Jpn J Clin Oncol 2005. 35(2): 68-73.

Sripa B, Kaewkes S, Sithithaworn P, Mairiang E, Laha T, Smout M, et al. Liver fluke induces cholangiocarcinoma. PLOS medicine 2007. 4(7): 1148-55

Su CH, Tsay SH, Wu CC, Shyr YM, King KL, Lee CH, et al. Factors influencing postoperative morbidity, mortality, and survival after resection for hilar carcinoma. Ann Surg 1996. 223(4): 384-94

Wehbe H, Henson R, Meng F, Mize-Berge J, Patel T. Interleukin-6 contributes to growth in cholangiocarcinoma cells by aberrant promoter methylation and gene expression. Cancer Res 2006. 66(21): 10517-24.

Wei SH, Dong K, Lin F, Wang X, Li B, Shen JJ, et al. Inducing apoptosis and enhancing chemosensitivity to Gemcitabine via RNA interference targeting Mcl-1 gene in pancreatic carcinoma cell. Cancer chemother pharmacol 2008. 62: 1055-64.