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World J Gastroenterol 2007 March 14; 13(10): 1561-1568
World Journal of Gastroenterology ISSN 1007-9327

BASIC RESEARCH

Roles of the MEK1/2 and AKT pathways in CXCL12/CXCR4 induced cholangiocarcinoma cell invasion

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Received: 2006-11-13 Accepted: 2007-02-12

Abstract

AIM: To evaluate the expression of C-X-C motif chemokine receptor 4 (CXCR4) and its signaling cascades, which were previously identified as a key factor for cancer cell progression and metastasis, in cholangiocarcinoma cell lines.

METHODS: The expression of CXCR4 and its signaling cascades were determined in the cholangiocarcinoma cell lines (RMCCA1 and KKU100) by Western blotting. The invasion assays and the detection of actin polymerization were tested in these cholangiocarcinoma cells treated with CXC chemokine ligand -12 (CXCL12).

RESULTS: Expression of CXCR4 was detected in both cholangiocarcinoma cell lines and activation of CXCR4 with CXCL12 triggered the signaling *via* the extracellular signal-regulated kinase-1/2 (ERK1/2) and phosphoinositide 3-kinase (PI3K) and induction of cholangiocarcinoma cell invasion, and displayed high levels of actin polymerization. Addition of CXCR4 inhibitor (AMD3100) abrogated CXCL12-induced phosphorylation of MEK1/2 and Akt in these cells. Moreover, treatment with MEK1/2 inhibitor (U0126) or PI3K inhibitor (LY294002) also attenuated the effect of CXCL12-induced cholangiocarcinoma cell invasion.

CONCLUSION: These results indicated that the activation of CXCR4 and its signaling pathways (MEK1/2 and Akt) are essential for CXCL12-induced cholangiocarcinoma cell invasion. This rises Implications on a potential role for the inhibition of CXCR4 or its signal cascades in the treatment of cholangiocarcinoma.

Key words: Cholangiocarcinoma; CXCR4; CXCL12; MEK1/2; PI3K

Leelawat K, Leelawat S, Narong S, Hongeng S. Roles of the MEK1/2 and AKT pathways in CXCL12/CXCR4 induced cholangiocarcinoma cell invasion. *World J Gastroenterol* 2007; 13(10): 1561-1568

http://www.wjgnet.com/1007-9327/13/1561.asp

INTRODUCTION

Cholangiocarcinoma is a malignant tumor composed of cells resembling those of the biliary tract epithelium[1,2]. The incidence of and mortality rate for cholangiocarcinoma varied considerably in different geographic regions, with the incidence highest in Southeast Asia especially in Thailand[3]. Three-year survival rates of 35% to 50% are achieved in only a few numbers of patients when negative histological margins are attained at the time of surgery[2,4-6]. The causes of lethality of this disease are not only its rapid growth but also the tendency to invade adjacent organs and metastasize[4-6].

At present, a number of molecules implicated in the metastasis processes of cholangiocarcinoma cells have been identified[7-9]. However, there have been no studies exploring the precise mechanisms determining the directional of invasion of cholangiocarcinoma cells into specific organs. In this regard, chemokines are a superfamily of small proteins that bind to G protein-coupled receptors on target cells[10,11]. CXC chemokine ligand-12 (CXCL12)-or stromal cell-derived factor-1 (SDF1) is a member of CXC chemokine family, which was initially cloned from murine bone marrow and characterized as a pre-B-cell growth stimulating factor. CXCL12 exerts effects through its cognate receptor C-X-C motif chemokine receptor 4 (CXCR4), which is the only physiological receptor for CXCL12 and is known to play roles in leukocyte homing as well as metastasis of many kinds of cancer cells[12-15]. A previous study demonstrated that CXCL12 released from fibroblasts induced the increase in migration of cholangiocarcinoma cells[16]. However, the signal transduction pathways following CXCR4 activation and stimulation of cholangiocarcinoma cell invasion have not been delineated.

In this study, we have demonstrated the expression of

CXCR4 in human cholangiocarcinoma cell lines. Using *in vitro* model systems, we demonstrated the activation of CXCR4 by CXCL12 induced phosphorylation of the MEK1/2 and Akt and also enhanced cholangiocarcinoma cells invasion. In addition, administration of AMD3100, a bicyclam noncompetitive antagonist of CXCR4 or inhibition of its signal transduction intermediate molecules (MEK1/2 and PI3K) suppressed the invasiveness of cholangiocarcinoma cells.

MATERIALS AND METHODS

Materials

HAM's F12 medium and fetal bovine serum were purchased from Gibco (Grand Island, NY, USA). The recombinant human CXCL12, polyclonal antibodies to human CXCL12 and CXCR4 were purchased from Abcam (Cambridge, MA, USA). Polyclonal antibodies to MEK-1/2 (phosphorylated at Ser217/221 and total), Akt (phosphorylated at Ser473 and total) were purchased from Cell Signaling (Cell Signaling Technology, Beverly, MA, USA). 24-well Biocoat Matrigel invasion chambers (8 μm) were purchased from Becton Dickinson (Becton Dickinson, Franklin Lakes, NJ, USA).

Cell cultures

Two human cholangiocarcinoma cell lines; KKU100 derived from Hilar-cholangiocarcinoma patient[17] (kindly provided by Dr. Banchob Sripa, Department of Pathology, Faculty of Medicine, Khon Kaen University) and RMCCA1 derived from Peripheral-cholangiocarcinoma patient[18] were grown in HAM's F12 medium supplemented with 100 mL/1L fetal bovine serum at 37℃ in a 50 mL/L CO_2 humidified atmosphere. For signal transduction experiments with CXCL12, cells were starved overnight in serum-free medium.

Western blotting analyses

For Western blot analysis, 500 000 cells were seeded in a six-well culture plate, followed by treatment with 100 ng/mL of CXCL12. Cells were collected and then Western blot analyses were performed as previously described[18]. Chemiluminescent detection of antibody–antigen complexes revealed the target proteins on X-ray film.

Cell proliferation assay

Cells were seeded in 96-well culture plates at a density of 10 000 cells per well followed by the addition of CXCL12 in various concentrations. Then cells were incubated for indicated time before applying the WST-1 cell proliferation assay reagent (Roche Diagnostics, Laval, Quebec) according to the recommendation of the manufacturer. The percentage of proliferation was calculated based on the untreated cells.

Cell migration assay

The migration of cholangiocarcinoma cells was assayed using chamber with 8-μm pore filters (Transwell, 24-well cell culture, Coster, Boston, MA). 50 000 cholangiocarcinoma cells were added to the upper chamber. Then 0.5 mL serum-free media with 100 ng/mL of CXCL12 was added to the lower chamber. The chambers were incubated for 12 h at 37℃. After incubation, the filters were fixed and stained with hematoxylin and counted in five random high-power fields under a light microscope[19].

Cell invasion assay

The invasion of cholangiocarcinoma cells was assayed in 24-well Biocoat Matrigel invasion chamber (8 μm; Becton Dickinson, Franklin Lakes, NJ). Similar to the migration assays, 50 000 cells were seeded in the upper chamber while the bottom chamber contained with 100 ng/mL of CXCL12.

Detection of MMP9 activity by gelatin zymography assay

Cholangiocarcinoma cells were starved by culturing in serum-free medium containing with or without CXCL12 for 24 h before collection of the conditioned medium. The conditioned medium was stored at -80℃ and analyzed for MMP9 activity by gelatin zymography.

Detection of actin polymerization

Detection of actin polymerization was performed as described previously[18]. Cholangiocarcinoma cells were treated with AMD3100, U0126, LY294002 or control and incubated for 6 h. Then the cells were incubated in serum-free medium containing with 100 ng/mL of CXCL12 for 4 h. The cells were exposed to Alexa Fluor 488 Phalloidin (Molecular Probes, Eugene, OR) for 30 min and washed with PBS. The cells were examined under a Confocal laser scanning microscope (CLSM), (Olympus SV1000).

Statistical analysis

The experiments were all performed in triplicate and identical results were obtained. Values were expressed as the mean and SD. The student's t-test was used for analysis of the cell proliferation and invasion assay. The P value of less than 0.05 was considered significant.

RESULTS

Expression of CXCR4 and CXCL12 in cholangiocarcinoma cell lines

In order to utilize an *in vitro* system to study the influence of CXCR4 activation, the expression of CXCR4 and CXCL12 in two cholangiocarcinoma cell lines (RMCCA1 and KKU100) needed to be investigated. Western blot analysis demonstrated definite expression of CXCR4 but not CXCL12 in both cholangiocarcinoma cell lines (Figure 1).

The effect of CXCR4 on cholangiocarcinoma cell proliferation

Since the activation of CXCR4 with CXCL12 was known to play an important role in cell proliferation in many kinds of cancer cells, we investigated the role of CXCL12 in cholangiocarcinoma cell proliferation. Cell proliferation assay was performed in RMCCA1 and KKU100 cells treated with CXCL12 at concentrations of 0, 50, 100 and 200 ng/mL. After 48 h of incubation, the results showed that CXCL12 had no effect on cholangiocarcinoma cell

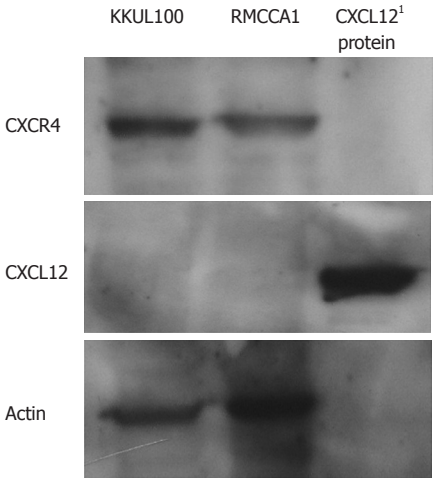

Figure 1 CXCR4 and CXCL12 expression in human colorectal carcinoma cell lines (RMCCA1 and KKU100). Western Blot analysis revealed the presence of CXCR4 specific bands in both cell lines. However, no expression of CXCL12 was identified in both cell lines. [1]The CXCL12 recombinant protein was used as a positive control for detection of CXCL12.

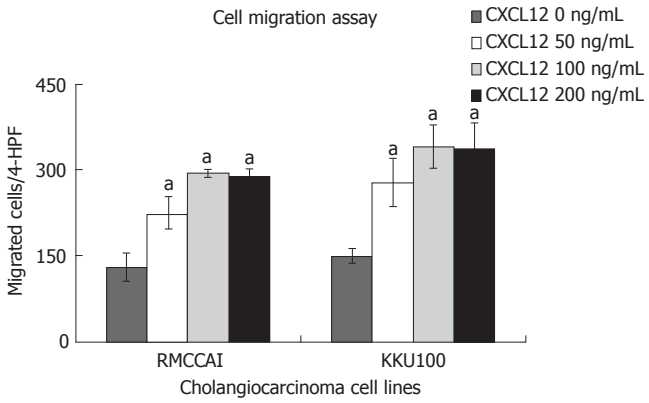

Figure 3 Effect of CXCL12 on the migration of cholangiocarcinoma cells. RMCCA1 and KKU100 were seeded in the 8-μm pore filters (Transwell, 24-well cell culture, Coster, Boston, MA). The bottom chamber contained 0 or 100 ng/mL of CXCL12. After 24 h, the cells on the lower surface were counted under a microscope at five random 100 x power fields. The experiment was repeated for 3 times and the data represent the average results from 3 individual experiments. CXCL12 induced the migration of cholangiocarcinoma cells ($^aP < 0.05$ *vs* 0 ng/mL of CXCL12).

proliferation (Figure 2).

The effect of CXCL12 on cholangiocarcinoma cell migration and invasion

We found that CXCL12 induced the migration of RMCCA1 and KKU100. Their maximum effect was identified at 100 ng/mL of CXCL12 (Figure 3). Therefore, the following cell invasion experiments were performed by using CXCL12 at a concentration of 100 ng/mL. CXCL12 significantly enhanced cholangiocarcinoma cell invasion when compared with untreated cells (Figure 4). To confirm the mechanism by which CXCR4 induced invasion of cholangiocarcinoma cells, RMCCA1 and KKU100 cells were pre-treated with AMD3100, a specific inhigitor for CXCR4, and then treated with CXCL12 for evaluation of cell invasion activity. The invasion induced by CXCL12

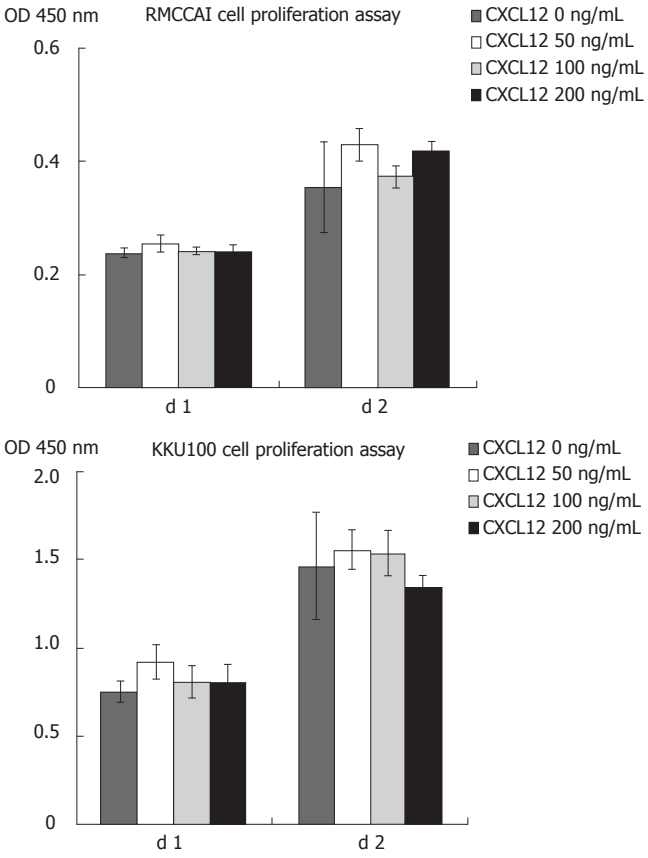

Figure 2 Effect of CXCL12 on the proliferation of cholangiocarcinoma cells. RMCCA1 and KKU100 were treated with CXCL12 at various concentrations (0, 50, 100 and 200 ng/mL). Cell proliferation assay was performed after 2 d by using WST-1. The absorbance at 450 nm, against a reference wavelength of 650 nm, was determined.

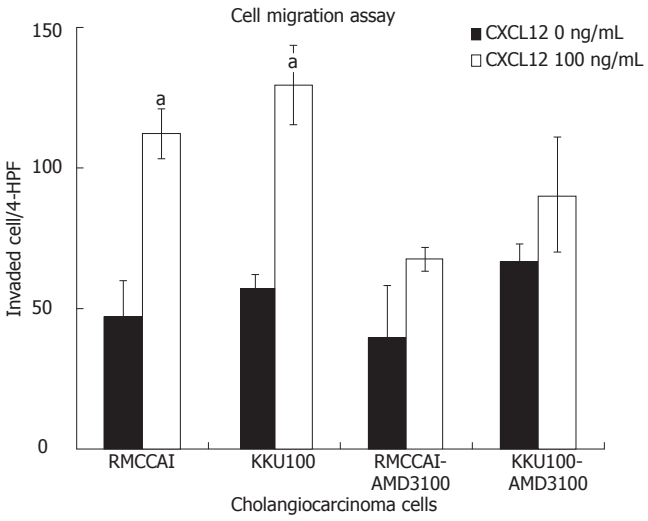

Figure 4 Effect of CXCL12 on the invasion of cholangiocarcinoma cells. RMCCA1 and KKU100 were pre-treated with or without AMD3100 for 12 h then were seeded in the 24-well Biocoat Matrigel invasion chamber. The bottom chamber contained 0 or 100 ng/mL of CXCL12. After 24 h, the cells on the lower surface were assayed as described previously. CXCL12 induced the invasion of cholangiocarcinoma cells. The effect of CXCL12 was decreased when the cells were pre-treated with AMD3100 ($^aP < 0.05$).

was inhibited by AMD3100 in both cholangiocarcinoma cell lines (Figure 4).

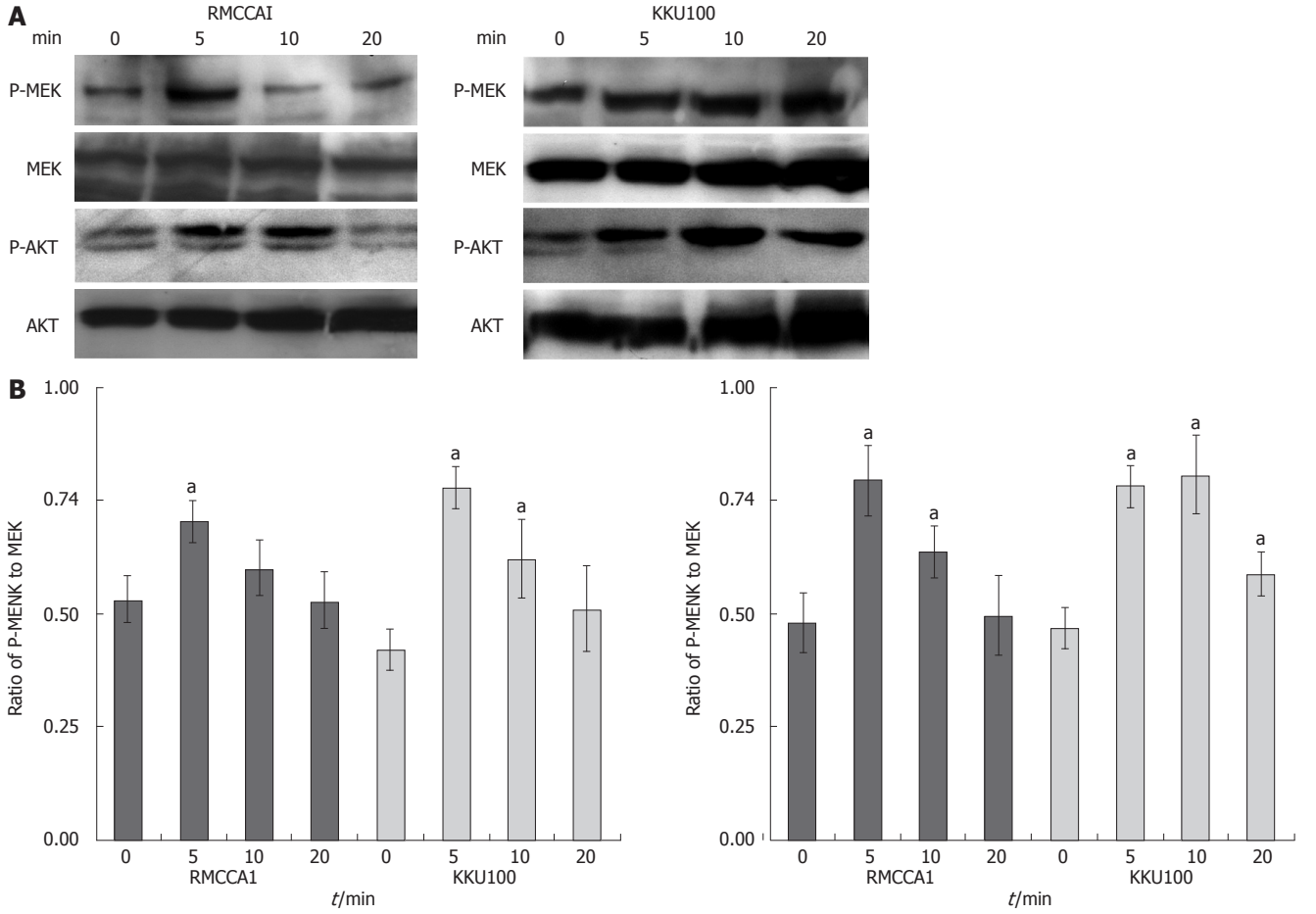

Figure 5 **A**: Western blot analysis of MEK1/2 and Akt phosphorylation in CXCL12-treated cholangiocarcinoma cells. RMCCA1 and KKU 100 were treated with 100 ng/mL of CXCL12 for indicated time. MEK1/2 and Akt phosphorylation were determined by Western blot as described; **B**: The average band intensity based on 3 biologically separate experiments, showing levels of MEK1/2 and Akt phosphorylation relative to the levels of MEK1/2 and Akt expression from the same experiment ($^aP < 0.05$).

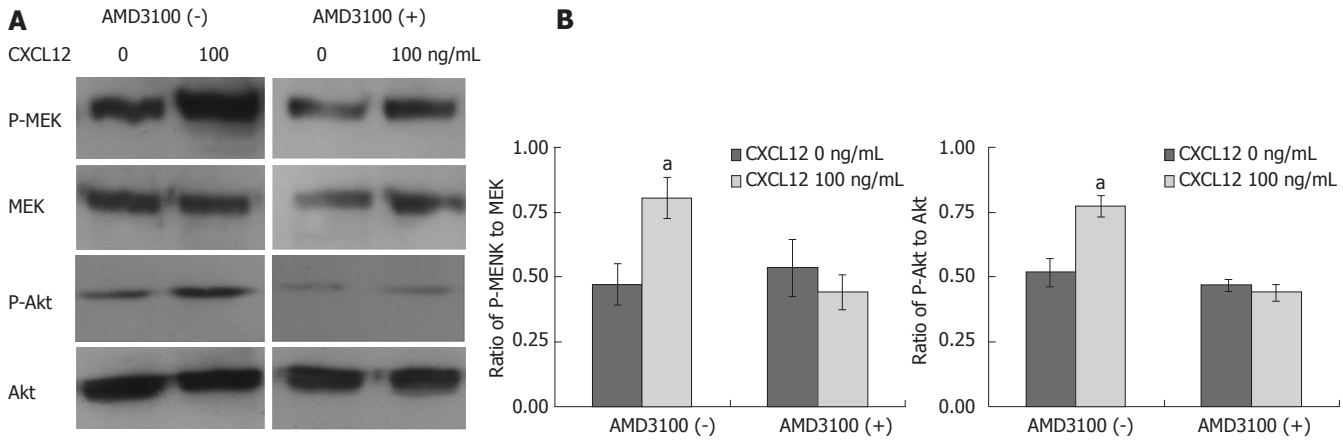

Figure 6 **A**: Effect of AMD3100 on CXCL12-induced phosphorylation of MEK1/2 and Akt in cholangiocarcinoma cells. RMCCA1 cells were pre-treated with or without AMD3100 before added CXCL12. Cells were collected at 5 min and MEK1/2 and Akt phosphorylation were determined by Western blot as described; **B**: The average band intensity based on 3 biologically separate experiments, showing levels of MEK1/2 and Akt phosphorylation relative to the levels of MEK1/2 and Akt expression from the same experiment ($^aP < 0.05$ *vs* 0 ng/mL of CXCL12).

The effect of CXCL12 on the phosphorylation of MEK1/2 and Akt in cholangiocarcinoma cells

We attempted to evaluate the signaling pathways relevant to the CXCL12 induction of cholangiocarcinoma cell invasion. The phosphorylation of molecules, which were previously demonstrated as CXCR4, mediated signaling molecules, was assayed by Western blot analysis. CXCL12-treated cells demonstrated a higher extent of the phosphorylated MEK1/2 and Akt than untreated cells (Figure 5). To determine whether the activation of CXCR4 induced phosphorylation of these signal transduction molecules, cells were pre-treated with AMD3100. The phosphorylation of MEK1/2, and Akt in AMD3100 pre-treated cells was significantly lower than in untreated cells (Figure 6).

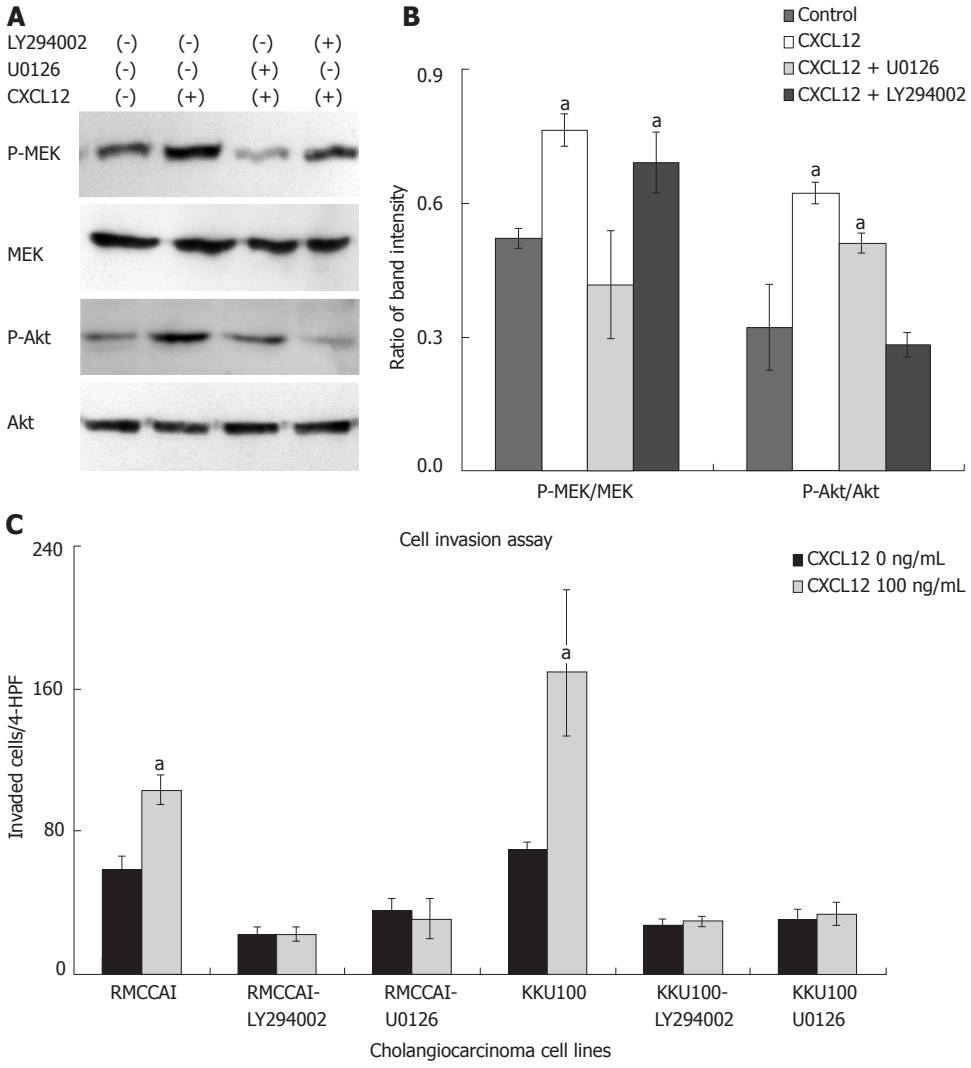

Figure 7 **A**: Effect of U0126 and LY294002 on CXCL12-induced phosphorylation of MEK1/2 and Akt in cholangiocarcinoma cells. RMCCA1 cells were pre-treated with U0126 or LY294002 before added CXCL12. Cells were collected at 5 min and MEK1/2 and Akt phosphorylation were determined by Western blot as described; **B**: The average band intensity based on 3 biologically separate experiments, showing levels of MEK1/2 and Akt phosphorylation relative to the levels of MEK1/2 and Akt expression from the same experiment ($^aP < 0.05$ compared with control); **C**: Effect of MEK1/2 and Akt phosphorylation induced by CXCL12 on the invasion of cholangiocarcinoma cells. RMCCA1 and KKU100 were pre-treated with or without LY294002 and U0126 for 12 h then were seeded in the 24-well Biocoat Matrigel invasion chamber. The bottom chamber contained 0 or 100 ng/mL of CXCL12. After 24 h, the cells on the lower surface were assayed as described previously. LY294002 and U0126 inhibited the effect of CXCL12 induced cholangiocarcinoma cells invasion ($^aP < 0.05$).

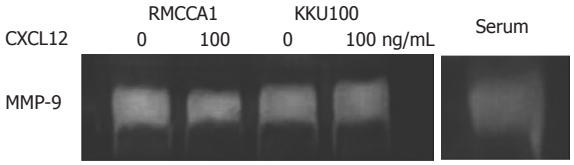

Figure 8 Gelatin zymography of the conditioned medium from cholangiocarcinoma cell lines revealed the proteolytic bands at molecular weight indicating them to be MMP-9. Levels of the proteolytic activity are not different between each sample.

Inhibition of the MEK1/2 or PI3K pathway attenuates CXCL12-induced cholangiocarcinoma cell invasion

The ability of MEK1/2 inhibitor (U0126) and PI3k inhibitor (LY294002) to decrease the effect of CXCL12-induced phosphorylation of MEK1/2 and Akt was assessed. The MEK1/2 inhibitor (U0126) suppressed CXCL12-induced MEK1/2 phosphorylation and the PI-3K inhibitor (LY294002) suppressed CXCL12-induced Akt phosphorylation (Figure 7A and B). To evaluate the contribution of the MEK1/2 or PI3K pathways to CXCL12-induced cholangiocarcinoma cell invasion, RMCCA1 and KKU100 cells were pre-treated with U0126 or LY294002 and then treated with CXCL12. The invasion induced by CXCL12 was inhibited by U0126 or LY294002 in both cholangiocarcinoma cell lines (Figure 7C). These results strongly suggest that the activation of MEK1/2 and PI3K signaling pathways is essential for CXCL12-induced cholangiocarcinoma cell invasion.

Activation of CXCR4 by CXCL12 had no influence on MMP-9 activation

Previous studies have demonstrated that CXCL12 induced MMP-9 activation. Therefore, we investigated the effect of CXCL12 on MMP-9 activation by gelatin zymography from condition medium of cholangiocarcinoma cells. The results showed that prominent constitutive MMP-9 activation was observed in both cholangiocarcinoma cell lines. However, activation of CXCR4 by CXCL12 had no influence on MMP-9 activation (Figure 8).

The effect of CXCL12 on the actin cytoskeleton of cholangiocarcinoma cells

The ability of cancer cell invasion requires coordinated activation of extracellular matrix degradation and cell motility mechanism. The cell motility was assessed by checking the actin polymerization. Cholangiocarcinoma cells were stained with phalloidin for detection of actin polymerization. Serum-starved cells showed low levels of actin polymerization. After the treatment with 100 ng/mL of CXCL12, cholangiocarcinoma cells displayed

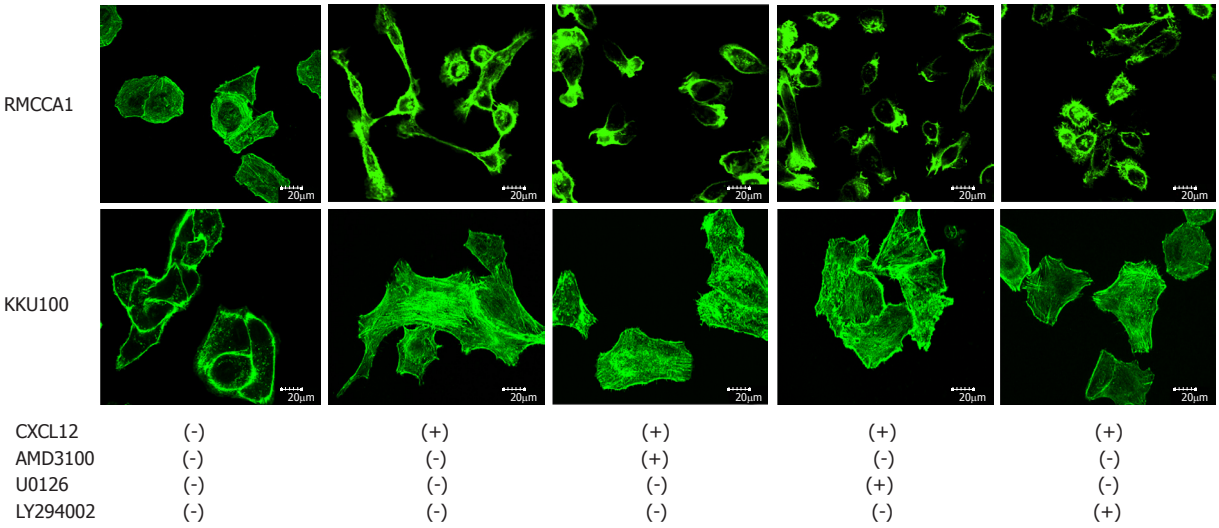

Figure 9 Effect of CXCL12 on the polymerization of actin cytoskeleton. Cholangiocarcinoma cells were pre-treated with vehicle, AMD3100, U0126 or LY294002 and incubated for 6 h in medium containing 0 or 100 ng/mL CXCL12. The cells were stained with Alexa Fluor 488 Phalloidin to visualize actin cytoskeleton under a Confocal laser scanning microscope (Olympus SV1000).

high levels of actin polymerization in the periphery of the cells and a distinct pseudopodia formation. Treatment of cholangiocarcinoma cells with AMD3100, U0126 or LY294002 before addition of CXCL12 caused eradication of actin polymerization (Figure 9).

DISCUSSION

Cholangiocarcinoma is a disease with dismal prognosis characterized by early vascular invasion and metastasis. Therapeutic options for cholangiocarcinoma have been limited due to poor response to chemotherapy and radiation therapy. Surgery is perhaps the only effective treatment for cholangiocarcinoma[2,4]. Previous studies suggested that the most important prognostic factor is a tumor-free surgical margin while other features that were associated with a poor prognosis include factors connected to the extent of disease that is caused by cancer cell invasion, such as bilobar distribution, lymph node involvement, vascular invasion and distant metastases[4,5]. Therefore, an understanding of the mechanism of cholangiocarcinoma cell invasion will be a decisive step towards the development of targeted tumor-specific therapies.

Chemokines and their receptors are involved in the process of cell migration during inflammation. Recently, studies implicated CXCR4 in chemotaxis, invasiveness and metastasis of tumors, particularly in metastasis of breast cancer, in an organ-specific manner[13,20]. In this present work, we report the results of our studies of CXCR4 and CXCL12 expression in two kinds of human cholangiocarcinoma cell lines; KKU100 derived from the hilar-cholangiocarcinoma patient and RMCCA1 derived from the peripheral-cholangiocarcinoma patient. Both cell lines are expressed CXCR4 but not CXCL12. These findings imply a paracrine effect of CXCR4/CXCL12 rather than an autocrine such in both cholangiocarcinoma cell lines.

In the present study, the effect of CXCL12 on CXCR4 in two cholangiocarcinoma cell lines was tested *in vitro* by using cell proliferation, cell migration and cell invasion assays. The findings provided interesting data on the possible molecules of significance involved in promoting cholangiocarcinoma cell invasion. Despite suggestions in previous reports that CXCL12 was a potent stimulator for small cell lung cancer cell proliferation[21], this present study included others arrived to exactly the opposite conclusions[22,23]. In cholangiocarcinoma cell lines, we identified that CXCL12 had no direct effect on cell proliferation. We suggested that these differences might be due to the different culture system or to different target cells. We identified that both cholangiocarcinoma cell lines expressed CXCR4 and stimulation of CXCR4 with CXCL12 promotes cancer cell migration and invasion. Moreover, we also found that KKU100 had a higher invasiveness property than RMCCA1. This result was related with the high expression of CXCR4 in KKU100. Our studies suggested that these events may involve the activation of the ERK1/2 and PI3K. Previous studies have demonstrated that activation of ERK1/2 by G-protein-coupled receptors occurred *via* the Raf/MEK1/2/ERK1/2 cascade while activated PI3K converted phosphatidylinositol 4, 5 phosphate (PIP2) into phosphatidylinositol 3, 4, 5 phosphate (PIP3), which results in the membrane localization of Akt[24,25]. The latter assertion is based on the finding that inhibition of CXCR4 by AMD3100 suppressed the phosphorylation of MEK1/2 and Akt and also inhibited the invasiveness properties of cholangiocarcinoma cells. Moreover, the addition of MEK1/2 inhibitor (U0126) or PI3K inhibitor (LY294002) also attenuated the effect of CXCL12-induced cholangiocarcinoma cell invasion. To the best of our knowledge, this present study is the first report demonstrating the signal transduction pathways of CXCR4 in cholangiocarcinoma. The targets of AMD3100, U0126 and LY294002 (CXCR4, MEK1/2 and PI3K, respectively) are shown in Figure 10. These results are consistent with the previous studies that demonstrating the activation of ERK and Akt signaling after stimulation of cancer cells

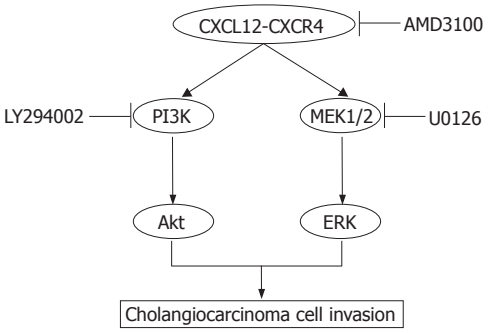

Figure 10 The pathway diagram identified the targets of AMD3100, U0126 and LY294002 (CXCR4, MEK1/2 and PI3K respectively). Inhibition of these pathways abrogates the invasion of cholangiocarcinoma cells.

with CXCL12[22,26,27].

In cancer cells, high level of actin polymerization is a key for the formation of pseudopodia, which in turn are implicated in the enhancement of cancer cell migration and invasion[28]. Treatment of cholangiocarcinoma cells with CXCL12 resulted in our study in the increase in actin polymerization. In addition, inhibiting CXCR4 with AMD3100 in our study resulted in a dramatic decrease in actin polymerization. Our findings suggest that CXCL12 and CXCR4 play an important role in the invasion as well as the metastasis in cholangiocarcinoma. Previous studies have demonstrated the influence of CXCL12 on MMP-9 secretion[26,27]. In cholangiocarcinoma cell lines, we showed that CXCL12 had no effect on MMP-9 secretion. The mechanisms responsible for MMP-9 activation in cholangiocarcinoma cells remain unclear. We suggested that the mechanism of cholangiocarcinoma cell invasion is not dependent on a single oncogenic pathway but possible complex networks of ligands and their receptors are implicated in cancer invasion such as c-Met/HGF[18], focal adhesion kinase[29] and TNFα/TNF receptor as well[30].

In conclusion, in this present experimental study, we show that the stimulation of CXCL12/CXCR4 plays an important role in cholangiocarcinoma cell invasion by the induction of MEK1/2 and Akt signal transductions and stimulation of actin polymerization. Inhibition of CXCL12/CXCR4 and its pathway may represent one of the potential approaches in cholangiocarcinoma therapy.

ACKNOWLEDGMENTS

The authors wish to thank Miss Yuvadee Siriyasub, Center of Nanoimaging (CNI), Faculty of Science, Mahidol University for her excellent technical support in the Confocal laser scanning microscopy analysis.

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