INTRODUCTION

Cholangiocarcinoma (CCA) is recognized as a devastating cancer with limited therapeutic options and poor prognosis. Due to lack of effective early diagnostic marker, patients were usually presented in the advanced stage of the disease (Blechacz et al., 2011; Bartella and Dufour, 2015). Thus, an extensively validated biomarker for early disease detection would be clinically beneficial. The knowledge of molecular mechanisms underlying early cellular transformation of CCA is required. Impaired inflammation is well known as an impact factor that plays a critical role in cholangiocarcinogenesis by causing genetic aberrations and activating several oncogenic signaling pathways (Andersen and Thorgeirsson, 2014). According to such knowledge, several potential therapeutic targets for CCA were suggested. However, the dominating molecular signaling which plays an especially early role in the transformation process is largely unclear.

LIN28B is an RNA-binding protein that plays pivotal roles in various cellular functions such as cell differentiation, metabolism, inflammation, and tumorigenesis (Viswanathan et al., 2009; Shyh-Chang and Daley, 2013). LIN28B regulates its targets through modulating mRNA stability and/or enhancing their translation via suppression of let-7 miRNAs biogenesis (Newman et al., 2008; Viswanathan et al., 2008). In the presence of LIN28B, numerous mRNAs would not be degraded, including oncogenes such as cMyc, RAS, HMGA2, IMPs (Wang et al., 2016; Balzeau et al., 2017). An overexpression of LIN28B was found to be reduced in STAT3 inhibition. LIN28B can regulate the inflammatory response and resistance to chemotherapy of cholangiocytes through modulation of STAT3 signaling pathway. A recent study suggests that activated cholangiocytes can be induced by regulation of LIN28B/STAT3 pathway and this may partially contribute to the initiating CCA. Here, LIN28B and its downstream signaling could be considered as an attractive therapeutic target in patients with CCA.

METHODS

Stable LIN28B expression in MMNK-1 cells was generated by infecting with retrovirus-containing LIN28B gene. LIN28B-overexpressing cells were further validated the amount of released cytokines by using human cytokine arrays. After treatment of chemo-drugs, cell viability was subsequently measured using MTT assay. Aldehyde dehydrogenase (ALDH) activity was determined using ALDEFLUOR assay kit and analyzed by flow cytometry. The mRNA and protein expression levels were respectively assayed by RT-qPCR and western blot. Results: Cytokine release results showed that numerous inflammatory cytokines-chemokines related to cancer initiation and development, such as IL-8, IL-6, VEGF, MCP1, TNF-α were significantly increased in LIN28B-overexpressing MMNK-1 cells. Drug sensitivity test showed that LIN28B-overexpressing MMNK-1 treated cells had a high percentage of cell viability compared to MMNK-1-control treated cells. Activity and expression level of a cancer stem cell marker, ALDH was significantly elevated in LIN28B-overexpressing MMNK-1 cells. Moreover, the activation of an oncogenic signaling pathway, signal transducer and activator of transcription 3 (STAT3) was enhanced in LIN28B-overexpressing MMNK-1 cells. Whereas, growth capacity of LIN28B-overexpressing MMNK-1 cells was found to be reduced in STAT3 inhibition. Conclusion: LIN28B can regulate the inflammatory response and resistance to chemotherapy of cholangiocytes through modulation of STAT3 signaling pathway. A recent study suggests that activated cholangiocytes can be induced by regulation of LIN28B/STAT3 pathway and this may partially contribute to the initiating CCA. Here, LIN28B and its downstream signaling could be considered as an attractive therapeutic target in patients with CCA.

Keywords: RNA-binding protein- tumor-initiation- inflammation- drugs resistance- bile duct cancer

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RESEARCH ARTICLE
upregulated in early steps of cholestasis accelerated CCA progression (Yang et al., 2011). Although this evidence indicates that LIN28B can partly contribute to CCA development, but the molecular mechanism of LIN28B in carcinogenesis is still unclear.

In this study, we hypothesized that LIN28B would play a role in the tumorigenesis of CCA. Therefore, we established stable LIN28B expression in cholangiocytes, MMNK-1 cells that closely mimic LIN28B-activating cells in disease progression and investigated the tumorigenic effect of LIN28B on normal cholangiocytes.

Materials and Methods

Cell lines and cell culture

Immortalized cholangiocyte MMNK-1 cells and bile duct cancer cells, HuCCT1 were obtained from JCRB Cell bank and HEK293gp cell line was obtained from Stem Cell and Cell Therapy Research Center, Chulalongkorn University, Thailand. MMNK-1 and HEK293gp cells were maintained in Dulbecco’s Modified Eagle’s Medium (DMEM) (HyClone, USA) and HuCCT1 cells were cultured in RPMI1640 medium (HyClone, USA). The culture medium supplemented with 10% fetal bovine serum, 1% Gluta Max and 1% Antibiotic-Antimycotic reagents (Gibco, USA). Cells were maintained in a humidified incubator at 37°C with 5% CO₂ atmosphere.

Viral production and establishment of stable gene-expressing cells

The plasmids pBABE-hLIN28B and pBABE-control were gifted from George Daley. HEK293gp cells were transfected with the transfection reagents composing of plasmid encoding LIN28B or control, VSVG and X-tremeGENE HP DNA transfection reagent (Roche, Germany). After 72 h of transfection, the medium-containing viruses were collected and filtrated through 0.45 micron sterile filter. Prepared MMNK-1 cells expressing cells were selected in medium containing 1 mg/ml of puromycin.

Cell viability assay

Whole protein lysate was extracted using RIPA lysis buffer (Cell Signaling Technology, USA) mixing 1X Protease inhibitor cocktail (Thermo Scientific, USA). Cells lysate was centrifuged at 12,000 rpm for 10 minutes at 4°C, and then the supernatant was collected. Protein concentration was measured using BCA Protein Assay by following the assay kit’s protocol (Thermo Scientific, USA). Protein samples (1 ug/ lane) were prepared and processed by using Simple Western assays (Protein Simple, USA), according to the manufacturer’s procedure and data was analyzed by using Compass for SW version 3.1.7 program. The following antibodies were used in this study: Stat3 (D3Z2G) Rabbit mAb, Phospho-Stat3 (Tyr705) Rabbit pAb, and β-actin (13E5) Rabbit mAb, according to the manufacturer’s protocol. Western blot

In brief, 5,000 cells/well were seeded into 96 well-plate. Next day, cells were treated with vary concentrations of chemotherapeutic drugs, 5-FU, Cisplatin, Gemcitabine, Etoposide (obtained from King Chulalongkorn Memorial Hospital) and STAT3 inhibitor; Cryptotanshinone (MedChem Express, USA). After indicated time, cells were incubated with 0.5 mg/ml of MTT (Sigma–Aldrich, USA) for 3 hours at 37 °C. The formazan crystals were solubilized in DMSO and then absorbance at 570 nm. The percentage of cell viability was calculated by following the percentage of cell viability = (OD570 of treatment x 100)/mean OD570 of non-treated cells.

ALDH activity assay

ALDH activity was performed by using...
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ALDEFLUOR™ Kit (StemCell Technologies, USA). As following the manufacturer’s protocol, briefly, 1x10⁶ cells were suspended with 1 ml of ALDEFLUOR™ Assay Buffer. The activated ALDEFLUOR™ reagent was added and immediately transfers 0.5 ml of mixture to the tube containing DEAB solution, and then incubated for 45 minutes at 37°C. ALDH fluorescence signal was detected using flow cytometry and analyzed by FlowJo™ software v10.4 (BD Biosciences, USA).

Cytokine array

After cultured cells for 24 hours, the medium was collected and centrifuged at 1,000 xg, 15 minutes, 4°C, and then the supernatant was collected. The amount of cytokines were measured using Bio-Plex Pro™ Human Cytokine 27-plex Assay (Bio-Rad, USA), according to the manufacturer’s instructions. The cytokine-standard and amount of cytokine in samples were analyzed using Bio-Plex 200 systems (Bio-Rad, USA).

Immunofluorescence staining

Cells-growing on a glass coverslip were then fixed by 4% paraformaldehyde in PBS for 10 minutes, and then incubated with permeabilization solution (0.3% Triton X-100) for 10 minutes. After washed three time in PBS, cells were blocked non-specific binding by blocking buffer (5% normal goat serum in 0.3% Triton X-100 PBS) for 30 minutes. Cells were subsequently incubated with antibody (1:100) overnight at 4°C. Cells were gently washed three times in 0.05% Tween20 PBS (PBST), and then incubated with Goat anti-rabbit IgG (H+L) secondary antibody FITC or Cy3 in a dilution 1:50 (Invitrogen, USA) for 1 hour. Lastly, cells were incubated with 1:100 of DAPI for 5 minutes (Invitrogen, USA). The fluorescently labeled cells were mounted by gold antifade mountant (Invitrogen, USA). The fluorescence signal was observed and captured the picture under the fluorescence microscope (ZEISS, Germany).

Trypan blue exclusion assay

After incubation with chemotherapeutic agents for 72 h, cells were trypsinized and washed with PBS. Cells were mixed at 1:1 ratio with a 0.4% solution of trypsin blue (Gibco, USA). The number of viable cells was measured using an EVE™ automatic cell counter (NanoEnTek Inc.). The percentage of cell viability was calculated, following the percentage of cell viability = (number of viable cells x 100) / number of total cells.

Rhodamine 123 dye exclusion assay

A total of 1x10⁶ suspend cells were incubated with 200 ng/ml of Rhodamine 123 dye (Sigma-Aldrich, USA) for 30 minutes on ice. Cells were then centrifuged at 1,200 rpm, three times for 5 minutes each in PBS to remove the excess of Rho123. Aliquots 500 μl of cell suspension (5x10⁵ cells) and further analyze the retention of intracellular Rho123 using flow cytometry. Another part of cell suspension were then centrifuged and further resuspended with warmed Rho123-free culture medium supplemented with 10% FBS, and subsequently incubated at 37°C for 1 hour. After washing, the fluorescence Rho123 signals were then detected by using flow cytometry and data was analyzed by FlowJo™ software v10.4 (BD Biosciences, USA).

Statistical analysis

Data were displayed as mean ± SD using GraphPad Prism software version 8.4.0 (USA). Student’s t-test was performed and P-value was considered statistically significant, indicating by *P≤0.05, **P≤0.01 and ***P≤0.001.

Results

Overexpression of LIN28B enhances the production of inflammatory cytokines in MMNK-1 cells

To explore the tumorigenic potential of LIN28B in normal cholangiocytes, we then generated the stably overexpressing LIN28B in immortalized normal cholangiocyte, MMNK-1 cell lines. The successful LIN28B transfection was demonstrated by increasing LIN28B expression at the mRNA (Supplementary Figure 1a) and protein levels (Supplementary Figure 1b) in MMNK-1 cells. As such the knowledges, an inflammation...
is an important process that particularly occurs during CCA development and shown to be a critical role in the initiation and progression of CCA. Thus, we aim to investigate the effects of LIN28B on inflammatory response of cholangiocytes. As revealed by cytokine array, we found that the amount of release inflammatory cytokines such as IL-8, IL-6, MCP-1, VEGF, IFN-γ and TNF-α were significantly increased in LIN28B-overexpressing MMNK-1 cells (Figure 1). In the different contexts of LIN28B-expressing cells, we also found that the expression levels of IL-6 and IL-8 genes were increased in LIN28B-overexpressing HuCCT1 cells (Supplementary Figure 2a-b). The results indicate that LIN28B can induce inflammatory cytokine release, which this effect appear to be related with inflammatory response of cholangiocytes.

**LIN28B increases ALDH activity and their resistance to chemotherapy**

As we found LIN28B can induce the expression of several inflammatory cytokines. It is likely that stably LIN28B-overexpressing MMNK-1 cells exhibit the fundamental cancer properties. Thus, we further validated other characteristics of cancer cells. According to data of mRNA microarray analysis by our colleague revealed that the ALDH1A2 gene was significantly upregulated in the context of LIN28B expression (data not shown). As a result, we also validated in our study and found that ALDH1A2 gene was significantly elevated in LIN28B-overexpressing cells (Figure 2a, Supplementary Figure 2c). We subsequently determined the ALDH activity by using ALDEFLUOR assay, and found 4.7% ALDH-positive in MMNK-1 control cells whereas 42.4% ALDH-positive was found in LIN28B-overexpressing MMNK-1 cells (Figure 2b), and increasing of ALDH activity was also found in LIN28B-overexpressing HuCCT1 cells (Supplementary Figure 2d). According to role of the ALDH, its expression were specifically expressed and promoted anti-cancer drugs resistance in cancer stem cells (CSCs) (Clark and Palle, 2016). Hence, we then determined the inhibition effect of chemotherapeutic drugs on LIN28B-overexpressing cells. As revealed by MTT assay, we found that the percentage of cell viability of LIN28B-overexpressing MMNK-1 cells had significantly higher than control cells in Cisplatin, Gemcitabine, and Etoposide, but not 5-FU treatments (Figure 2c), whereas LIN28B-overexpressing HuCCT1 cells were shown to be resisted to Cisplatin and Etoposide (Supplementary Figure 2e). As demonstrated by trypan blue exclusion assay, confirmed that more viable cells...
were found in LIN28B-expressing cells (Supplementary Figure 3). These data suggest that LIN28B promotes chemotherapeutic resistance, which may partly be due to the responsibility of ALDH function.

**LIN28B enhances the activation of STAT3 signaling**

We next investigated the possible mechanisms involved in LIN28B promoting inflammation and survival of cholangiocytes. STAT3 signaling pathway is well known as a transcription factor that contributes to inflammation by regulating the expression of multiple pro-inflammatory cytokines (Yu et al., 2009). Importantly, aberrant STAT3 activation is associated with tumor promotion and progression of CCA (Sia et al., 2013). Thus, we then evaluated the STAT3 activation in LIN28B-overexpressing MMNK-1 cells and found that the phosphorylated STAT3 (Tyr705) was increased (Figure 3a-b). In contrast, the expression of pSTAT3 (Tyr705) was dramatically reduced in the STAT3 inhibitor treatment (Figure 3c). As a result, we expected that STAT3 may be a downstream signaling of LIN28B and it may be a possible target for against the effect of LIN28B. The result of MTT assay demonstrated that the potent STAT3 inhibitor significantly reduced cell viability of LIN28B-overexpressing MMNK-1 cells harboring constitutively active STAT3 signaling (Figure 3d). Moreover, downregulation of STAT3 targets such as inflammatory gene (IL-6), cell cycleregulatory genes (CDC25A and CDK6), and anti-apoptosis gene (MCL-1) were observed in STAT3 inhibitor treated cells (Figure 3e). Together, our findings indicate that LIN28B can enhance activated STAT3 signaling in cholangiocytes and this possible regulation may contribute to the tumorigenesis of CCA.

**Discussion**

According to the critical role of LIN28B in tumorigenesis, it was reported to be a tumor-initiating factor and sufficient to induce cancer development in multiple contexts in vivo, including lymphoma (Beachy et al., 2012), neuroblastoma (Molenaar et al., 2012), and liver cancer (Nguyen et al., 2014). Nonetheless, the oncogenic
role of LIN28B in CCA is largely unknown. Based on the pathogenesis of CCA, an inflammation process is typically associated with the pathogenesis of bile duct epithelial then allowing the accumulation of proto-oncogenes mutations and impairing of tumor suppressor genes, resulting in CCA formation (Zabron et al., 2013). We have revealed that LIN28B can induce the production of inflammatory cytokines, including IL-6, IL-8, MCP-1, VEGF, IFN-γ, and TNF-α in cholangiocytes. The elevated cytokines were shown to be tumor-promoting inflammatory cytokines and significant role in the carcinogenesis of CCA (Banales et al., 2020). According to the significant role of IL-6/STAT3 pathway in tumorigenesis of CCA (Rizvi and Gores, 2014; Roy et al., 2019), implying that LIN28B may partially involve in CCA initiation by inducing an inflammatory loop IL-6/STAT3 in cholangiocytes. Moreover, our findings also agrees with a previous study by which LIN28B/let-7 axis can sustain the inflammatory feedback loop, LIN28B/let-7/IL-6/STAT3. The effects of inflammatory cytokines on chemoresistance can be applied in LIN28B-overexpressing cholangiocytes by which their effects have been suggested to be involved in the promotion of chemoresistance by inducing pro-survival signals. Moreover, as a function of STAT3 could be applied in our context by which STAT3 primarily confers the chemotherapeutic resistance by inducing expression of several genes that play important functions in anti-apoptosis, cell proliferation, and as well as detoxifying ALDH enzyme.

Figure 4. Propose a Potential Mechanism of LIN28B Induces Reactive Cholangiocytes. The diagram can be drawn by following the molecular cholangiopathy together with our findings. LIN28B would be activated follow by quiescence cholangiocyte exposed oncogenic inducing factors. The let-7 targets, IL-6 was subsequently upregulated in the context of LIN28B expression. The activated STAT3 signaling pathway resulted in transcription of IL-6 and it would turn to activate and create the inflammatory feedback loop, LIN28B/let-7/IL-6/STAT3. The effects of inflammatory cytokines on chemoresistance can be applied in LIN28B-overexpressing cholangiocytes by which their effects have been suggested to be involved in the promotion of chemoresistance by inducing pro-survival signals. Moreover, as a function of STAT3 could be applied in our context by which STAT3 primarily confers the chemotherapeutic resistance by inducing expression of several genes that play important functions in anti-apoptosis, cell proliferation, and as well as detoxifying ALDH enzyme.
mechanism of LIN28B/STAT3 into the tumor-initiation capacity of CCA.

Furthermore, we demonstrated that LIN28B-overexpressing cholangiocytes exhibit low sensitivity to Cisplatin, Etoposide, and Gemcitabine. LIN28B is likely promoted drug resistance by following multifactorial mechanisms of chemoresistance (MOCs) (Marin et al., 2018). The reduction of intracellular drug activity (MOC-2) is proposed by which LIN28B induces the detoxifying ALDH enzyme activity, which has been shown to reduce the effect of gemcitabine and drug resistance in CCA patients (Chen et al., 2016). The expression of ALDH and its activity is specifically found in CSCs sub-populations (Xu et al., 2015) and it has functionally contributed to anti-cancer drugs resistance of CSCs by reducing the cytotoxicity of various harmful aldehydes and alkylating agents (Clark and Palle; Toledo-Guzmán et al., 2019). Considering that LIN28B may potentially promote cancer stem-like properties, chemotherapeutic resistance in cholangiocytes by enhancing ALDH activity. However, the mechanism of LIN28B regulates ALDH expression is unknown. Previous studies have shown that co-expression of LIN28B and ALDH1 isoform was significantly found in cancer stem-like cells and tumor tissues (Chien et al., 2015; Wu et al., 2017). Moreover, the functional studies demonstrated that ALDH expression and its activity in ALDH-positive prostate cancer cells were significantly decreased when inhibited the activation of STAT3 (Han et al., 2014), as well as data shown in CCA cell lines (Beyreis et al., 2019). For that reason, ALDH is likely to be a downstream target of STAT3, hence, implying that LIN28B induces ALDH expression via modulating STAT3 signaling in our context. It will be of interest to next investigate the mechanisms of how LIN28B regulates ALDH in other contexts.

Moreover, LIN28B may implicate in other MOC by which LIN28B induces cholangiocyte secretion of many cytokines associated with ductular reaction which are known to promote apoptosis inhibition (MOC-5a) and cholangiocyte proliferation (MOC-5b) (Sato et al., 2019), those cytokines also play an important role in regulating immune cell activity in the tumor microenvironment (MOC-6) (Pinto et al., 2018). On the other hand, the activity of drug efflux transporter (MOC1b) may not involve in LIN28B declined the inhibition effects of chemotherapeutic drugs in cholangiocytes (Supplementary Figure 4). We also noticed that the viability of cells treated 5-FU was not significantly changed, which is likely due to the normal cholangiocytes are already existed 5-FU metabolizing enzymes, NAD(P)H-quinone oxidoreductase 1 (NQO1) (Zeekpudsa et al., 2014) and rate-limiting enzyme, dihydropyrimidine dehydrogenase (DPD) (Arunsan et al., 2020).

Collectively, we demonstrate that LIN28B enhanced STAT3 signaling causes inflammatory cytokines production and declines chemotherapeutic drugs sensitivity while increasing cancer stem cell markers, ALDH activity. According to such knowledge of STAT3 regulation and our findings, we propose the potential role of LIN28B induces CCA malignant transformation, as shown in Figure 4. This finding provides the evidence that LIN28B/IL-6/STAT3 signaling cascade may be involved in carcinogenesis of CCA and also suggests LIN28B and its downstream signaling may be an interesting therapeutic strategy for patients with CCA.

Author Contribution Statement
Puthdee N; experimental design, drafting original and revision of the manuscript, carrying out the experiments, and data analysis and interpretation. Khramchantuk S; carrying out the experiments and data interpretation. Nuwongsri P; citation of references and preparation of figures. All authors approved the final version of the manuscript.

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Ethical approval
This study was ethically approved from Institutional Review Board of Research Affairs, Faculty of Medicine, Chulalongkorn University, (IRB. No 348/60).

Conflict of interest
The authors declare no potential conflicts of interest

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