Original Article

Involvement of interleukin-1β in high glucose-activated proliferation of cholangiocarcinoma

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Background: Diabetes mellitus (DM) is associated with the increased risk of development and the advancement of cholangiocarcinoma (CCA). High glucose levels were previously shown for upregulating interleukin-1β (IL-1β) in CCA cells with unclear functions. The present study, thus, aimed to investigate molecular mechanisms linking DM to CCA progression, with IL-1β hypothesized as a communicating cytokine.

Methods: CCA cells were cultured in media with normal (5.6 mM) or high (25 mM) glucose, resembling euglycemia and hyperglycemia, respectively. Expressions of IL-1β and IL-1 receptor (IL-1R) in CCA tissues from patients with and without DM were examined using immunohistochemistry. Functional analyses of IL-1β were performed using siRNA and recombinant human IL-1R antagonist (rhIL-1RA), in which Western blots investigated the knockdown efficacy. BALB/c *Rag-2*$^{-/-}$ *Jak3*$^{-/-}$ (BRJ) mice were implanted with CCA xenografts to investigate hyperglycemia's effects on CCA growth and the anti-tumor effects of IL-1RA.

Results: CCA tumors from patients with hyperglycemia showed significantly higher IL-1β expression than those from non-DM patients, while IL-1β was positively correlated with fasting blood glucose (FBG) levels. CCA cells cultured in high glucose showed increased IL-1β expression, resulting in increased proliferation rates. Suppressing IL-1β signaling by si-IL-1β or rhIL-1RA significantly reduced CCA cell proliferation *in vitro*. Anakinra, a synthetic IL-1RA, also exerted significant anti-tumor effects *in vivo* and significantly reversed the effects of hyperglycemia-induced growth in CCA xenografts.

Conclusions: IL-1β plays a crucial role in CCA progression in a high-glucose environment. Targeting IL-1β might, then, help improve therapeutic outcomes of CCA in patients with DM and hyperglycemia.

Keywords: Cholangiocarcinoma (CCA); diabetes mellitus (DM); glucose; hyperglycemia; interleukin-1β (IL-1β)

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Introduction

Diabetes mellitus (DM) is a metabolic disorder presenting with high blood glucose levels or hyperglycemia (1). Global mortality and prevalence of DM have gradually increased in past years (2,3). The global DM prevalence for the 20–79 age group was estimated to be 10.5% (536.6 million people) in 2021 and is estimated to rise to 12.2% (783.2 million) by 2045. Southeast Asia and South Asia had more than 72 million adults with DM in 2013 and are expected to exceed 123 million in 2035 (4). Additionally, DM is a risk factor for the development of several cancers (5), e.g., cancers of the liver, pancreas, kidney, esophagus, stomach, lung, thyroid, squamous cell carcinoma, and leukemia.

Interleukin-1 (IL-1), a group of inflammatory proteins, is known for its various roles in physiological and pathological functions (6). Evidence suggests that interleukin-1 is crucial in connecting the immunological system with various diseases, not limited to inflammatory conditions. Among the 11 members of the IL-1 family, interleukin-1β (IL-1β) is the most well-known and extensively researched (7). IL-1β is a strong inflammatory cytokine essential for the body's defense against infections and injuries (8). Numerous studies have reported the involvement of IL-1β in carcinogenesis and cancer progression. For instance, in oral squamous cell carcinoma (OSCC) mouse models, elevated IL-1β levels were significantly correlated with higher aggressive stages of OSCC during malignant transformation after receiving carcinogens (9). Patients with high IL-1β levels in the pancreatic ductal adenocarcinoma (PDAC) stroma also exhibited poorer survival rates than those with low IL-1β expression (10). Individuals with hepatitis B infection carrying a high *IL-1B-511C* allele are associated with increased IL-1β production in the liver and are at a higher risk of developing hepatocellular carcinoma (11). These studies suggest important roles for IL-1β in both carcinogenesis and tumor progression.

The highest global incidence of cholangiocarcinoma (CCA), a bile duct malignancy, has been reported in northeastern Thailand, with a high DM mortality rate also reported in the same area (12). Epidemiological studies also indicate an association between DM and CCA in many regions. Meta-analysis of the cancer risks in individuals with DM reveals a positive association between DM and all CCA subtypes (13). However, the mechanistic linkage between DM and CCA development and progression is not fully understood. Insulin, a hormone with dual metabolic and mitogenic functions, is primarily suspected as the associated molecule between DM and CCA (14). The insulin levels are usually high in patients with type 2 DM due to the compensation of the resistance at insulin receptors. This leads to the hypothesis that insulin might have increased the risk of CCA carcinogenesis. Most studies, nevertheless, suggest a null effect of insulin on CCA development (14,15), with only one study suggesting a positive association between insulin and the increased risk of extrahepatic CCA subtype (16). Other medications that increase insulin secretion or its bioavailability, e.g., sulfonylurea and insulin analogs, neither show the effects on CCA development and progression (17). On the other hand, hyperglycemia and other medications are suggested for their involvement in CCA progression or recession (18,19). Later studies are, therefore, mainly focused on the impacts of high glucose levels and anti-diabetic medications on the modifications of risk and progression of CCA.

Our previous studies used transcriptomic analyses to investigate the effects of high glucose levels on global gene expression in CCA cells. Pathway analyses revealed several intracellular signals and molecules linked to hyperglycemia, inflammation, and CCA progression (20).

Highlight box

Key findings

- Interleukin-1β (IL-1β) is underlying the linkage between diabetes mellitus (DM) and cholangiocarcinoma (CCA).

What is known, and what is new?

- DM is associated with poor survival of CCA patients.
- IL-1β is upregulated in CCA cells cultured in high glucose.
- CCA tissues from patients with DM showed high expression of IL-1β compared to those without DM.
- Inhibiting the interleukin-1 (IL-1) signaling pathway significantly reduces the growth of CCA *in vitro* and *in vivo*.

What is the implication, and what should change now?

- IL-1β and IL-1 signaling are promising targets for CCA treatment, especially in patients with DM.

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A nuclear factor-kappa B (NF-κB) and a signal transducer and activator of transcription 3 (STAT3) are found to cooperate in promoting CCA progression under diabetic glucose conditions. While it is well established that IL-1β can activate the NF-κB signaling cascade, the expression of IL-1β is under transcriptional regulation by both NF-κB and STAT3 pathways and is increased in CCA cells cultured in high glucose. These suggest a cross-talking between the two signaling pathways using IL-1β as a communicative cytokine to promote CCA progression under hyperglycemic conditions. Although IL-1β is well known for its pro-tumorigenic function and is associated with the increased risk of other cancers, this cytokine's role in CCA remains inconclusive. IL-1β levels were increased in patients with CCA compared with benign biliary diseases (21). *IL1B +3954 C/C* gene variant is also associated with shorter overall and disease-free survival of patients with non-liver fluke-associated CCA (22). In contrast, the frequency of a high producer polymorphism *IL1B* -511C/T was not different between the group of benign biliary diseases and liver fluke-associated CCA (21). The discrepancy between liver fluke-associated and non-liver fluke-associated CCA might lead to difficulty developing CCA immunotherapy, especially the treatments based on the effects of cytokine signaling. In addition, CCA generally lacks tumor mutation burden and microsatellite instability, resulting in a relatively lower probability of developing immunotherapy targeting tumor neoantigens and difficulty finding the markers for immunotherapy's response (23). Investigating known potential cytokines in the immune landscape of CCA might provide a better possibility of developing an effective treatment. Targeting IL-1 signaling is thus hypothesized as a promising treatment to improve therapeutic outcomes for patients with CCA and DM (20), who might have higher expression of the IL-1β. We present this article in accordance with the ARRIVE reporting checklist (available at https://tgh.amegroups.com/article/view/10.21037/tgh-24-8/rc).

Methods

Cell lines and cell culture

Human CCA cell lines KKU-213A (RRID: CVCL_M261) and KKU-213B (RRID: CVCL_M264) were established from tumors of Thai patients and obtained from the Japanese Collection of Research Bioresources Cell Bank, Osaka, Japan (#JCRB1557). Both cell lines were cultured in Dulbecco's Modified Eagle Medium (DMEM) containing high glucose (25 mM) (Gibco, Carlsbad, CA, USA) or normal glucose (5.6 mM) (Gibco) supplemented with 10% fetal bovine serum (FBS) (Gibco) and 1% antibiotic-antimycotic (Gibco). Cells were maintained in a 5% CO_2 incubator at 37 ℃ and were subcultured every 3 days. Cells cultured in high glucose medium in at least five passages were assigned as HG cells, while those cultured in normal glucose medium were assigned as NG cells (24).

MTT assay

Cell viability was assessed using an MTT assay. To examine the effect of high glucose on cell proliferation, NG or HG cells of CCA (1.5×10^3 cell/well) in a 96-well plate were incubated for 24, 48, and 72 h. Then, 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) (Invitrogen, Waltham, MA, USA) was added to the final concentration of 0.5 mg/mL, and further incubated for 3 hours. Formazan crystal was dissolved in DMSO (Sigma-Aldrich, St. Louis, MO, USA), and the OD540 nm was determined by a microplate reader (TECAN, Zurich, Switzerland).

To investigate the effect of IL-1β on CCA cell viability, HG cells of CCA (8.0×10^4 cell/well) in a 24-well plate were incubated overnight and transfected with si-IL1-β (Santa Cruz, Dallas, TX, USA) or scramble control (QIAGEN, Hilden, Germany) in serum-free DMEM with high glucose using Lipofectamine 2000 reagent (Invitrogen) according to the previously described protocols (25). After 6 h, transfection complexes were replaced with a complete medium. Cells with knocked down IL-1β and scramble control were then used for the downstream experiments. For functional analysis of IL-1β, HG cells transfected with scramble or si-IL-1β (2.0×10^3 cells/well) were plated in a 96-well-plate, and an MTT assay was performed to examine the effect of suppressing IL-1β on CCA cell growth.

To ensure the requirement of IL-1 signaling on CCA cell proliferation, HG cells (1.5×10^3 cells/well) were incubated in a 96-well plate overnight. Then, cells were incubated with varied concentrations of recombinant human IL-1R antagonist (rhIL-1RA) (ImmunoTools, Lower Saxony, Germany) for 72 h. Cell viability after treatment was then determined by MTT assay.

Immunohistochemistry (IHC)

Formalin-fixed paraffin-embedded CCA tissues (N=51) from patients with and without DM were

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immunohistochemically stained following the standard protocol previously described (24). Briefly, antigen retrieval was performed by heating the samples in 0.1 M citrate buffer, pH 6.0, in a pressure cooker for 5 min. Then, endogenous peroxidases were blocked using 0.5% H_2O_2 in methanol, and non-specific antigens were blocked using 5% bovine serum albumin (BSA) (HIMEDIA, Nashik, India) in PBS. CCA tissues were incubated with anti-IL-1β (1:50) (Proteintech, Rosemont, IL, USA) or anti-IL-1R1 (1:50) (Invitrogen) overnight at room temperature and subsequently incubated with HRP-Labelled polymer anti-mouse antibody (Dako, Santa Clara, CA, USA) or HRP-Labelled polymer anti-rabbit antibody (Dako) at room temperature for an hour. The signals were developed with 3,3'-diaminobenzidine tetrahydrochloride hydrate (DAB) (Sigma Aldrich, St. Louis, MO, USA), then counterstained with Mayer's hematoxylin (Bio-Optica, Milan, Italy) and mounted with Permount (Merck, Darmstadt, Germany). IHC signals were semi-quantitated using H-score systems, as calculated by the formula: Σ (intensity × frequency), and evaluated by two researchers. IL-1β intensity scoring was rated from 0 to 3+ (0= negative staining, 1+= mild intensity, 2+= moderate intensity, 3+= strong intensity). IL-1R1 with membranous pattern expression was scored as 3+ (*Figure 1A*), while cytoplasmic staining was classified as the same system as IL-1β. High or low expressions of IL-1β and IL-1R1 were classified using a median as a cut-off point.

All protocols for using human tissues and human-derived CCA cell lines were reviewed and approved by the Khon Kaen University Ethics Committee for Human Research (approval No. HE661103) based on the Declaration of Helsinki (as revised in 2013) and the ICH Good Clinical Practice. Written informed consents were received from all included patients before the collection of tissue specimens and their clinicopathological data.

Western blot

Antibodies used in this experiment were anti-IL-1β (Proteintech) and anti-GAPDH (Merck).

Cells were lysed by radioimmunoprecipitation assay (RIPA) lysis buffer, and the Bradford assay (Bio-Rads, Hercules, CA, USA) was used to determine the total protein amount. Total proteins (20 µg/well) were resolved in 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred onto polyvinylidene difluoride (PVDF) membrane (Merck). The membranes were blocked with 3% BSA in TBST before applying the primary antibody at 4 ℃ overnight, followed by secondary antibodies for another 1 h at room temperature. The signals were detected using Enhanced Chemiluminescence reagent (Merck) by Amersham Imager 600 (GE-Healthcare Bio-Science AB, Uppsala, Sweden).

Immunocytofluorescence

Normal glucose (NG) and high glucose (HG) cells of CCA (1×10^4 cells/well) were seeded in a 48-well plate and incubated for 24 h. Cells were fixed using 4% paraformaldehyde for 30 min, and non-specific antigens were blocked with 5% FBS in PBS. Anti-IL-1R1 primary antibody (1:100) (Invitrogen) was applied and incubated at 4 ℃ overnight. The secondary antibody (Invitrogen) was then incubated for a further 1 h, and the nuclei were stained with Hoechst (Invitrogen) for 10 min. The fluorescent signals were visualized and photographed by a fluorescence microscope (Nikon, Tokyo, Japan).

CCA xenograft and effects of IL-1RA in vivo

A 4-week-old male BALB/c *Rag*-$2^{-/-}$ *Jak3*$^{-/-}$ (BRJ) mice (26) (5 mice/group) were bred and housed in the husbandry of the Northeast Laboratory Animal Center, Khon Kaen University. After acclimatization, mice were randomly allocated into non-diabetic control, diabetic, and diabetic-receiving IL-1RA groups. Mice were induced to have DM using a single intraperitoneal injection with a high-dose streptozotocin (200 mg/kg) (Sigma) in 0.1 M citrate buffer pH 4.0, while a control group received an equal amount of citrate buffer. DM and hyperglycemia in the diabetic group were considered when fasting blood glucose (FBG) was ≥250 mg/dL by a digital glucometer test (EasyMax, Hsinchu). After the mice developed DM, KKU-213A cells (1×10^5 cells/site) in a 50% Matrigel solution were subcutaneously injected into the left flank of each mouse. A week after xenograft implantation, anakinra, a synthetic IL1-R1A (Med Chem Express, Monmouth Junction, NJ), was given via intraperitoneal injection (1 mg/kg/day), 5 days/week. The tumor sizes were measured twice a week using a digital Vernier caliper by a researcher who was blinded for the allocating process. Tumor volumes were calculated using the formula: tumor volume = $(L \times W^2)/2$, where L is the longest diameter, and W is the shortest diameter of the tumor. All mice were euthanized on day 11 after treatment and included in the analysis, as the tumor volumes were significantly different between the control

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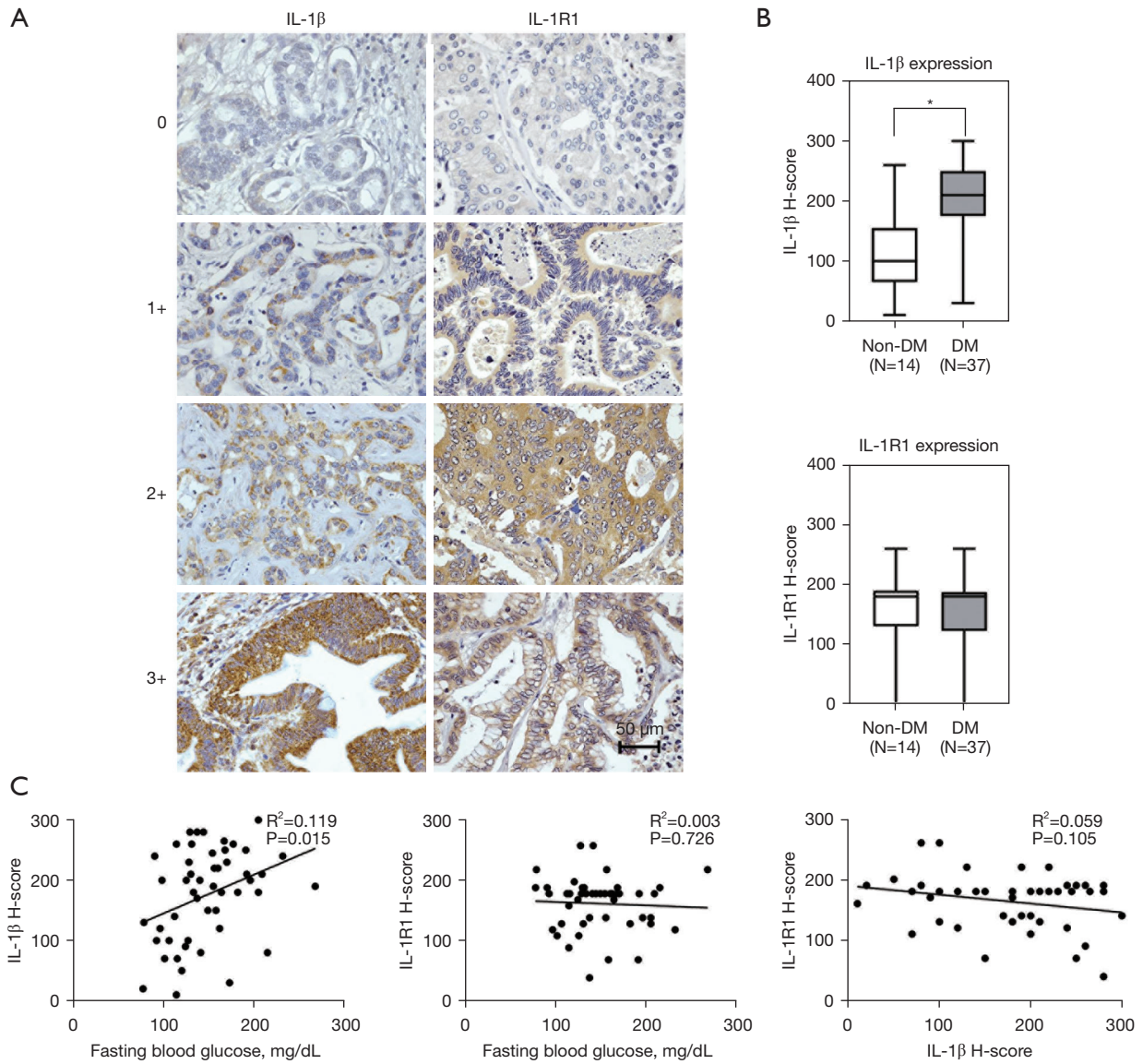

Figure 1 Expression profiles for IL-1β and IL-1R1 in cholangiocarcinoma. (A,B) Immunohistochemistry reveals that IL-1β levels are significantly increased in CCA tissues from patients with DM, while IL-1R1 expressions are not different between DM and non-DM groups. (C) The expressions of IL-1β are significantly correlated with preoperative fasting blood glucose of patients with CCA. (B) Student's *t*-test. (C) Pearson's correlation coefficient test. *, P<0.05. IL-1β, interleukin-1β; IL-1R1, interleukin-1 receptor-1; DM, diabetes mellitus; CCA, cholangiocarcinoma.

and treatment groups.

The protocol for the *in vivo* study was reviewed and approved by the Institutional Animal Care and Use Committee of Khon Kaen University (approval No. IACUC-KKU 139/64) based on the National Guidelines of the National Research Council of Thailand and the ARRIVE guidelines (27). All mice were maintained in individual ventilation cages with a restricted environment of 12 h dark-light cycle, 23±2 ℃, and 30–60% relative humidity, and were allowed access to food and drink ad libitum. The mice's body weights were measured once a week, and health inspections were performed daily by the veterinarian to assess the early humane endpoints. The euthanasia was performed using overdose isoflurane

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inhalation, followed by thoracotomy, which was used as a secondary euthanasia.

Statistical analysis

The clinicopathological associations of IL-1β and IL-1R1 expressions were determined by Pearson's Chi-squared test. The correlations between IL-1β, IL-1R1, and FBG were analyzed by Pearson's correlation coefficient. All quantitative data were compared using the Student's *t*-test, one-way ANOVA, or two-way ANOVA with Tukey's multiple comparisons when appropriate using SPSS 17.0 software (IBM, Chicago, IL, USA). Statistical significance was considered when $P<0.05$.

Results

IL-1β levels were upregulated in CCA tissues from patients with DM

Immunohistochemistry was performed to assess IL-1β and IL-1R1 expressions in tissues from Thai CCA patients with and without DM. Patients with pre-operative FBG ≥126 mg/dL were classified as having DM and hyperglycemia, and those with FBG <100 mg/dL were classified as having euglycemia. IL-1β expressions were significantly upregulated in the CCA tumors from patients with DM, compared with the non-DM group ($P<0.01$) (*Figure 1A,1B*). On the other hand, IL-1R1 expressions were not different between the groups (*Figure 1B*). In addition, IL-1β levels showed a significantly positive correlation with FBG ($P<0.05$) (*Figure 1C*). DM was also associated with increased levels of IL-1β expression in CCA tissues [odd ratio (OR) =7.03, 95% confidence interval (CI): 1.65–30.11, P=0.009] (*Tables 1,2*). However, there was no association between the expression of IL-1R1 and any clinicopathological characteristics of CCA patients (*Table 3*).

High glucose increased IL-1β expression and CCA cell proliferation

Consistent with CCA tissues from patients with DM, CCA cells cultured in high glucose medium (HG cells) also expressed higher levels of IL-1β compared with those cultured in normal glucose medium (NG cells) (*Figure 2A,2B*). HG cells also showed a significantly higher proliferation rate compared with NG cells (*Figure 2C*). In contrast, IL-1R1 expressions were not different between HG and NG cells (*Figure 2D,2E*). It is, thereby, hypothesized that high glucose promotes CCA cell proliferation via the upregulation of IL-1β.

Silencing IL-1β expression and IL-1R antagonist attenuated cell proliferation

To affirm the roles of IL-1β in CCA cell proliferation, siRNA-mediated knockdown of IL-1β was performed in HG cells of CCA. IL-1β expressions were significantly decreased at 24 and 48 h after knocking down (*Figure 3A,3B*), resulting in significantly decreased proliferation of CCA cells (*Figure 3C*). The administration of IL-1R antagonist (IL-1RA) also attenuated the proliferation of CCA cells, suggesting the requirement of IL-1 signaling in CCA cell proliferation (*Figure 3D*).

IL-1R antagonist suppressed CCA tumor growth in vivo

The protocol and timeline of *in vivo* experiments are summarized in *Figure 4A*. After 1 week of streptozotocin injection, all induced mice developed DM and had significantly increased levels of FBG compared with the control group (*Figure 4B*), while the body weights of the mice in each group were not different (*Figure 4C*). The tumor volumes and tumor weights in mice with hyperglycemia were significantly higher than those in the mice with euglycemia, whereas the IL-1R antagonist significantly reduced both tumor volumes and tumor weights in diabetic mice to similar levels of the non-diabetic group (*Figure 4D-4F*).

Discussion

IL-1β is a well-known cytokine playing a central role in the inflammatory process and innate immunity. Several studies have shown that IL-1β has significant roles in carcinogenesis and cancer progression (28). Pro- and anti-tumorigenic roles of IL-1β have been both reported in various cancers and found to be cancer-type specific. Notably, our study is the first to demonstrate the promotive effects of IL-1β in CCA. In addition, the present study also demonstrated that hyperglycemia in patients with DM was associated with increased levels of IL-1β in CCA cells, affirmed by *in vitro* studies showing that high glucose conditions increased the expression of IL-1β in CCA, resulting in growth promotion. Further affirmation was evidenced when the suppression of IL-1β expression or administration of IL-1R antagonist reversed the effects of high glucose-induced aggressiveness of CCA, as depicted in the schematic summary (*Figure 5*).

Hyperglycemia has been recognized as a diabetogenic

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Table 1 Univariate analysis of the association between diabetes mellitus and clinicopathological characteristics

Clinicopathological data	Diabetic status		Crude OR (95% CI)	P
	DM	Non-DM		
Sex (N=49)			1.7 (0.47–6.11)	0.42
Male	18	9		
Female	17	5		
Age (N=49)			0.79 (0.18–3.48)	0.75
≥56 years	26	11		
<56 years	9	3		
Tumor size (N=49)			0.89 (0.25–3.12)	0.85
≤4 cm	14	6		
<4 cm	21	8		
Histological grading (N=32)			–	0.45
Well-differentiated	15	9		
Moderately differentiated	3	3		
Poorly differentiated	2	0		
Histotype (N=49)			1.06 (0.29–3.86)	0.93
Papillary	13	5		
Non-papillary	22	9		
Perineural invasion (N=45)			0.39 (0.10–1.46)	0.16
Present	10	7		
Absent	22	6		
Tumor involved margin (N=46)			0.43 (0.12–1.59)	0.20
Present	11	7		
Absent	22	6		
Lymphovascular invasion (N=48)			0.56 (0.15–2.00)	0.37
Present	17	9		
Absent	17	5		
IL-1β expression (N=49)			7.03 (1.65–30.11)	0.009
High	23	3		
Low	12	11		
IL-1R1 expression (N=46)			1.32 (0.36–4.81)	0.68
High	20	7		
Low	13	6		

IL-1β, interleukin-1beta; IL-1R1, interleukin-1 receptor type 1; DM, diabetes mellitus; OR, odds ratio; 95% CI, 95% confidence interval.

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Table 2 Univariate analysis of the association between IL-1β expression and clinicopathological characteristics

Clinicopathological data	IL-1β expression		Crude OR (95% CI)	P
	High	Low		
Sex (N=49)			1.11 (0.36–3.45)	0.85
Male	14	13		
Female	12	10		
Age (N=49)			0.28 (0.07–1.22)	0.66
≥56 years	17	20		
<56 years	9	3		
Tumor size (N=49)			1.61 (0.51–5.09)	0.42
≤4 cm	12	8		
>4 cm	14	15		
Histotype (N=32)			–	0.26
Well-differentiated	12	12		
Moderately differentiated	2	4		
Poorly differentiated	2	0		
Papillary neoplasm (N=49)			0.58 (0.18–1.87)	0.36
Papillary	8	10		
Non-papillary	18	13		
Neural invasion (N=45)			2.12 (0.61–7.32)	0.24
Invaded	11	6		
Not invaded	13	15		
Tumor involved margin (N=46)			2.31 (0.68–7.89)	0.18
Involved	12	6		
Not involved	13	15		
Lymphovascular invasion (N=48)			1.36 (0.44–4.27)	0.59
Invaded	15	11		
Not invaded	11	11		
Diabetes (N=49)			7.03 (1.65–30.11)	0.009
Diabetes	23	12		
Non-diabetes	3	11		
IL-1R1 expression (N=46)			1.39 (0.43–4.51)	0.59
High	15	12		
Low	9	10		

IL-1β, interleukin-1beta; IL-1R1, interleukin-1 receptor type 1; OR, odds ratio; 95% CI, 95% confidence interval.

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Table 3 Univariate analysis of the association between IL-1R1 expression and clinicopathological characteristics

Clinicopathological data	IL-1R1 expression		Crude OR (95% CI)	P
	High	Low		
Sex (N=46)			0.89 (0.27–2.89)	0.85
Male	15	10		
Female	12	9		
Age (N=46)			0.28 (0.052–1.50)	0.14
≥56 years	19	17		
<56 years	8	2		
Tumor size (N=46)			0.95 (0.29–3.11)	0.93
≤4 cm	11	8		
>4 cm	16	11		
Histotype (N=29)			–	0.61
Well-differentiated	14	8		
Moderately differentiated	3	3		
Poorly differentiated	0	1		
Papillary neoplasm (N=46)			0.81 (0.244–2.69)	0.73
Papillary	10	8		
Non-papillary	17	11		
Neural invasion (N=43)			2.06 (0.56–7.53)	0.28
Invaded	12	5		
Not invaded	14	12		
Tumor involved margin (N=43)			1.05 (0.30–3.62)	0.94
Involved	10	7		
Not involved	15	11		
Lymphovascular invasion (N=45)			1.00 (0.30–3.32)	>0.99
Invaded	15	10		
Not invaded	12	8		
Diabetes (N=46)			1.32 (0.36–4.81)	0.68
Diabetes	20	13		
Non-diabetes	7	6		
IL-1β expression (N=46)			1.39 (0.43–4.51)	0.59
High	15	9		
Low	12	10		

IL-1R1, interleukin-1 receptor type 1; IL-1β, interleukin-1beta; OR, odds ratio; 95% CI, 95% confidence interval.

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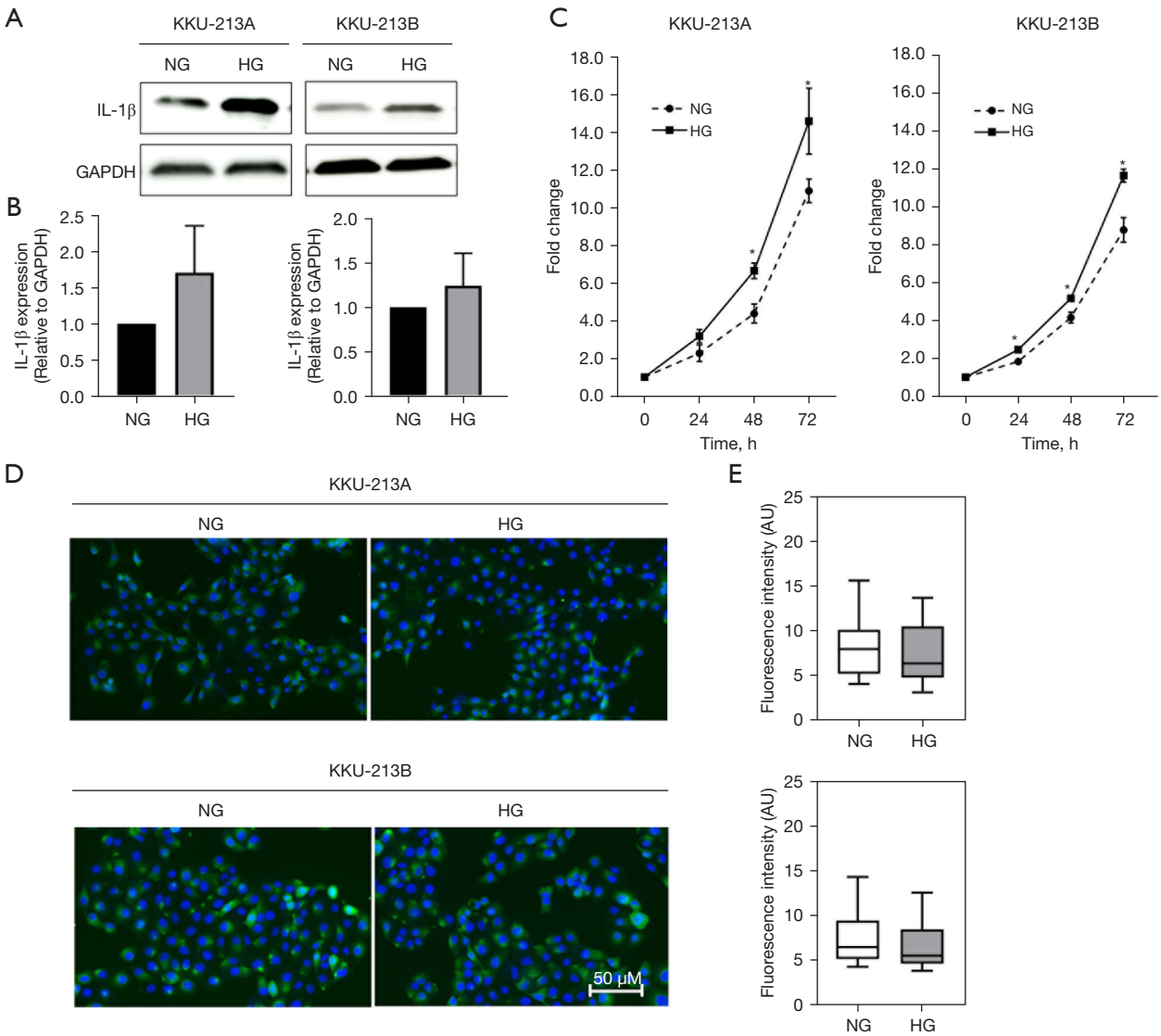

Figure 2 Effects of glucose on IL-1β expression and proliferation of CCA cells. (A,B) CCA cells cultured in high glucose (HG cells) expressed higher IL-1β compared with cells cultured in normal glucose (NG cells). (C) HG cells of both CCA cell lines showed significantly increased proliferation compared with NG cells. (D,E) The expressions of IL1R1 by immunocytofluorescent staining are not different between HG and NG cells of CCA. (B,E) Student's *t*-test. (C) Two-way ANOVA with multiple comparisons. *, P<0.05. IL-1β, interleukin-1β; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; NG, normal glucose; HG, high glucose; AU, arbitrary unit; CCA, cholangiocarcinoma; IL-1R1, interleukin-1 receptor-1; ANOVA, analysis of variance.

factor that is involved in diabetic complications. One emerging mechanism of high glucose-induced complications is promoting the inflammatory process. Our studies showed that high glucose stimulates inflammatory pathways in CCA cells, namely STAT3 and NF-κB (20,24). Furthermore, high glucose can promote CCA progression via the activation of glycogen synthase kinase-3β (GSK3β)/β-catenin pathways (18), epidermal growth factor receptors (29), increased expression of cell cycle machinery (30), and increased reactive oxygen species (31).

IL-1β, an inflammatory cytokine, was also upregulated by high glucose conditions. Nevertheless, mechanisms of action and how IL-1β is regulated are not fully understood since IL-1β occupies a dual position for both the upstream activator

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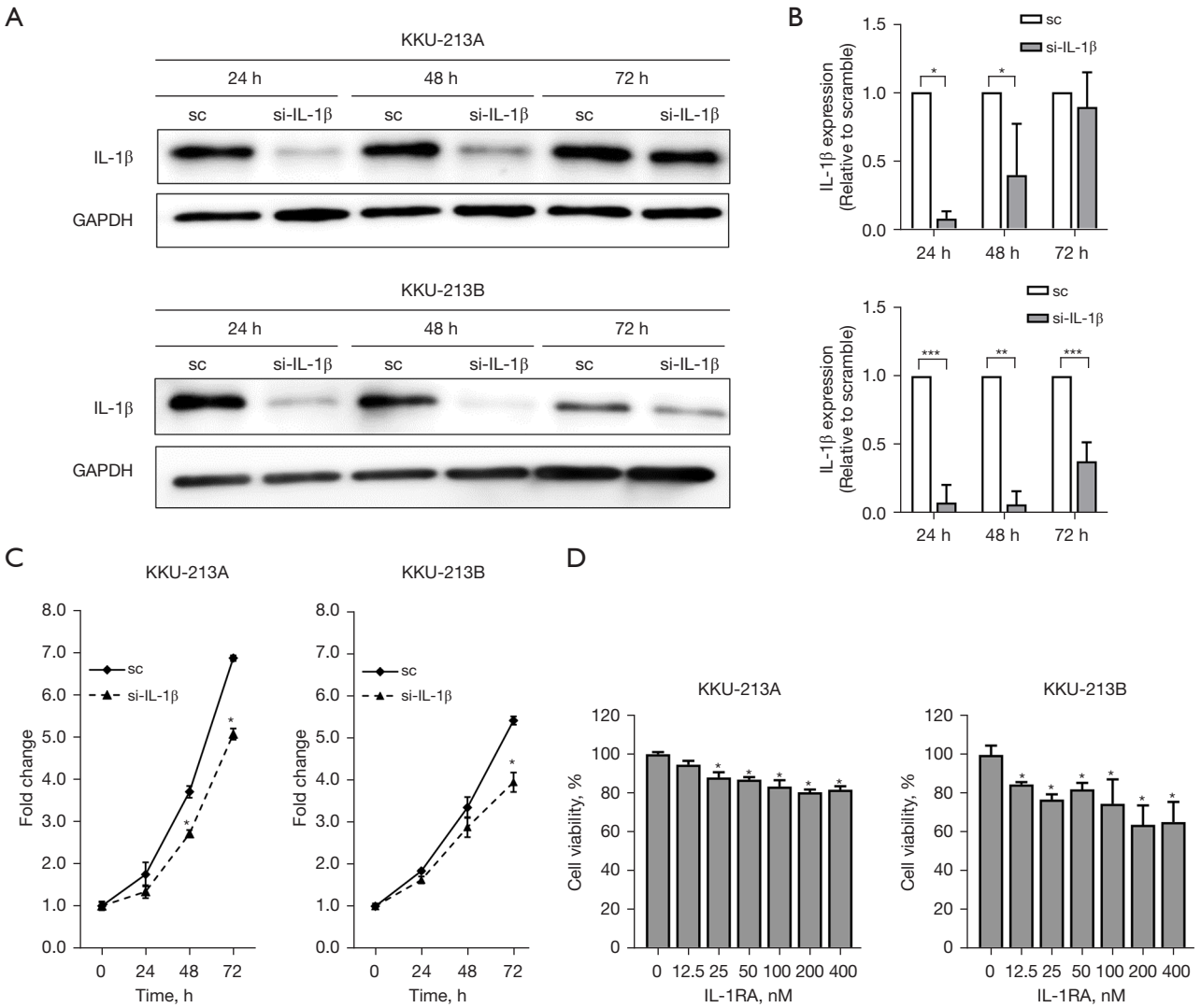

Figure 3 Inhibiting IL-1 signaling suppresses the growth of CCA. (A,B) siRNA significantly inhibits the expression of IL-1β in CCA cells in the first 48 h, and the expression levels rebound at 72 after siRNA administration. (C) CCA cells with silenced IL-1β expressions show a decreased proliferation at 48 and 72 h. (D) rhIL-1RA significantly inhibits the growth of CCA cells. Western blot is representative of at least 3 biological replications, and the graphs represent the average band intensities from at least 3 biological replications. (B) Student's *t*-test. (C) Two-way ANOVA with multiple comparisons. (D) One-way ANOVA. *, P<0.05; **, P<0.01; ***, P<0.001. IL-1β, interleukin-1β; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; sc, scramble control; IL-1RA, interleukin-1 receptor antagonist; si-IL-1β, siRNA targeting *IL1B* (interleukin-1β) gene; CCA, cholangiocarcinoma; rhIL-1RA, recombinant human interleukin-1 receptor antagonist; ANOVA, analysis of variance.

and downstream target of the NF-κB pathway. Other studies have also demonstrated that high glucose conditions can upregulate IL-1β via several mechanisms. High glucose-activated PKC-α and ERK1/2 in human monocytes lead to NF-κB activation, resulting in increased IL-1β expressions (32). In diabetic retinopathy, hyperglycemia-enhanced IL-1β expression leads to PKC activation and vascular dysfunction (33). IL-1β has also been implicated in pro-tumorigenic effects on several types of cancer. In glioblastoma, cells with CD133 expression can modulate the tumor microenvironment through IL-1β pathway (34). IL-1β can also promote colon cancer progression and

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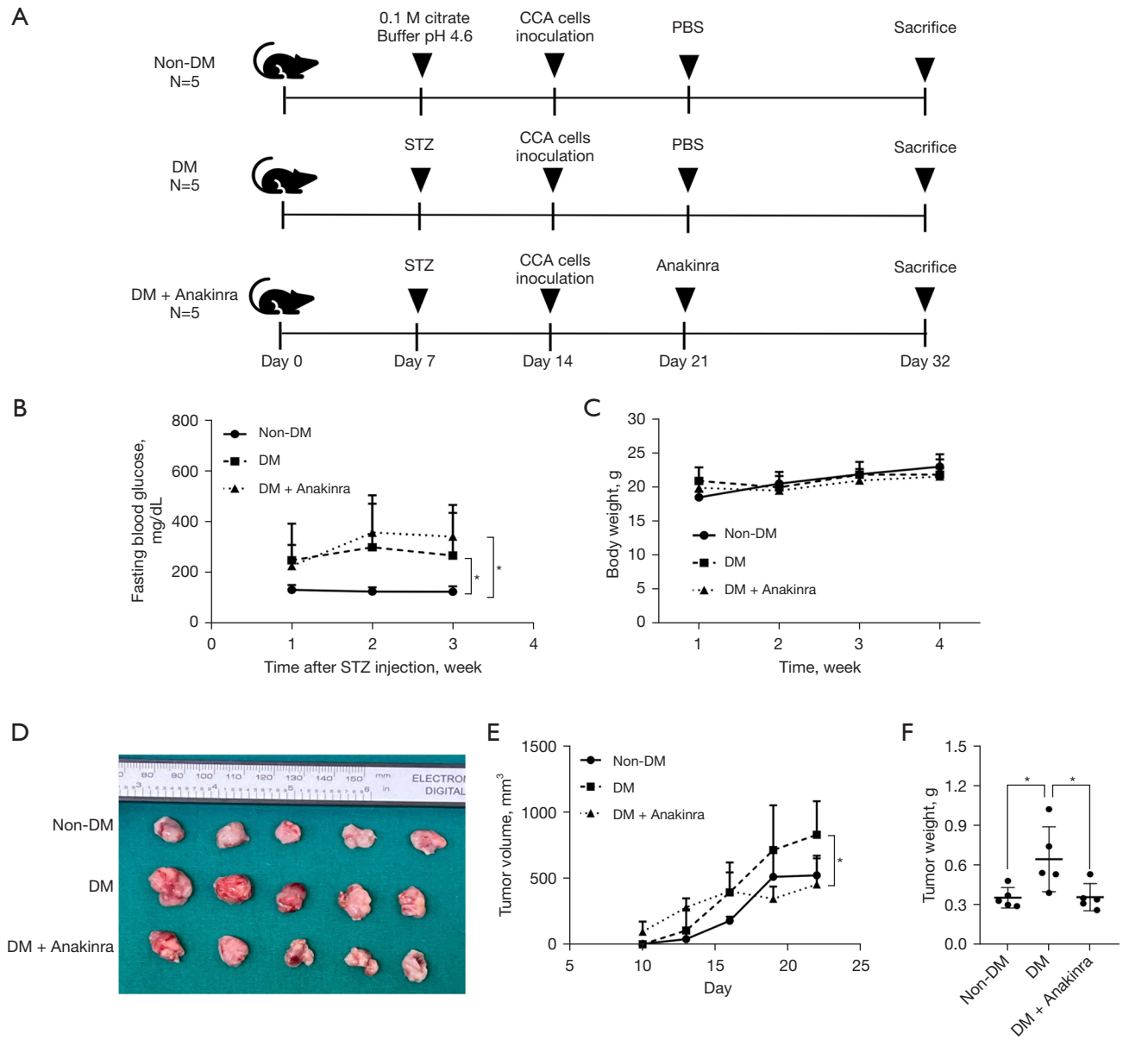

Figure 4 IL-1RA reverses hyperglycemia-induced growth of CCA *in vivo*. (A) BRJ mice were induced to have DM using streptozotocin, and the control groups were given a vehicle solution. After DM and hyperglycemia were confirmed, KKU-213A were subcutaneously inoculated, and anakinra, a synthetic IL-1RA, was given. (B) Diabetic mice had hyperglycemia, and the high blood glucose levels were maintained until the end of the experiments. (C) The body weights of mice in each group were not different. (D-F) Xenografted tumors in DM mice had significantly increased volumes and weights compared with non-DM mice, whereas anakinra exerted significant anti-tumor effects on CCA xenografts and reversed the effects of hyperglycemia-induced CCA growth. (B,C,E) Two-way ANOVA with multiple comparisons. (F) One-way ANOVA. *, P<0.05. DM, diabetes mellitus; CCA, cholangiocarcinoma; PBS, phosphate buffered saline; STZ, streptozotocin; IL-1RA, interleukin-1 receptor antagonist; BRJ, BALB/c *Rag-2*$^{-/-}$ *Jak3*$^{-/-}$; ANOVA, analysis of variance.

invasion through the activation of cells' self-renewal and endothelial-mesenchymal transition (EMT) via Zinc-finger E-box-binding homeobox 1 (Zeb-1) (35). Our study was therefore designed to investigate the association between DM, IL-1β, and the progression of CCA, a cancer that is highly prevalent in the same areas as DM in Thailand.

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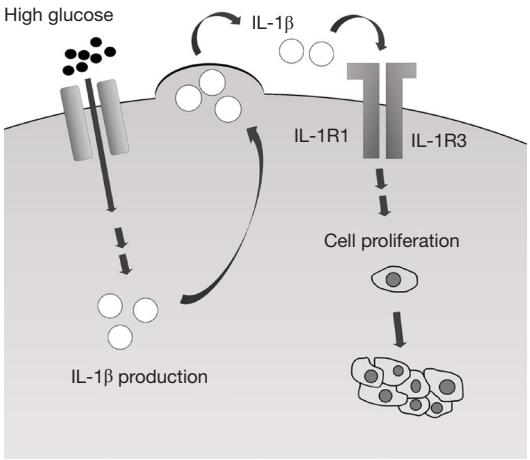

Figure 5 Schematic summary. High glucose increases IL-1β expression in cholangiocarcinoma cells, which then secrete and act as autocrine regulators of CCA cell growth under hyperglycemic conditions. IL-1β, interleukin-1beta; IL-1R1, interleukin-1 receptor type 1; IL-1R3, interleukin-1 receptor type 3.

IL-1β can be expressed and secreted from cancer and other cells in the tumor microenvironment (28). Our results confirmed and emphasized the findings that IL-1β proteins were expressed in CCA cells both *in vitro* and in the tumor tissues of patients and that the expression levels were significantly correlated with preoperative FBG levels of the patients. IL-1β, as a key pro-inflammatory cytokine, might then play an important role in liver fluke-associated CCA in which DM and hyperglycemia provided the additive effects for carcinogenesis and tumor progression. In line with this, the increased progression of CCA by IL-1β signaling and function in a present study requires IL-1R1 (36). Upon IL-1β/IL-1R1 complex formation, the downstream signaling cascades are activated (37). Our results showed that the expression of IL-1R1 was, however, not different between NG and HG cells of CCA, consistent with indifferent expressions of IL-1R1 in CCA tissues from patients with and without DM. Suppression of IL-1β expression using siRNA significantly reduced the growth of CCA *in vitro* while blocking IL-1β/IL-1R1 complex formation by using IL-1RA also suppressed CCA growth both *in vitro* and *in vivo*. Notably, IL-1RA significantly reversed the growth of CCA in diabetic mice, affirming that the promotive effects of DM and hyperglycemia on CCA growth are partly via IL-1β signaling. These suggest that controlling blood glucose or inhibiting molecules induced by high glucose might complement the standard treatment of CCA patients with DM. Our report then not only shows the linkage between hyperglycemia and IL-1β for cancer progression, but it also emphasizes the pathogenic roles of simple nutrients like glucose when its levels are beyond physiological needs. Moreover, the development of targeted therapy on IL-1β/IL-1R is then promising for application in CCA treatment. Repurposing available drugs, namely IL-1RA (38) or monoclonal antibodies against IL-1β (39,40) that have been studied in other cancers, is thus also promising for the development of CCA treatment.

This study, however, has some limitations. Firstly, CCA cell lines and tissues used in this study were derived from liver fluke-associated patients, for which chronic inflammation is a major pathogenesis. The roles of IL-1β in non-liver fluke-associated CCA with and without DM remain to be clarified. Secondly, the major effects of IL-1β induced by high glucose in the present study are from autocrine modulation. The effects of IL-1β via paracrine and endocrine routes from circulating leukocytes and in tumor microenvironments exposed to hyperglycemia should also be further investigated. Finally, fuller clarification is required to see if controlling glucose levels to slow CCA progression via inhibiting IL-1β signaling benefits CCA patients who have DM and hyperglycemia.

Conclusions

Hyperglycemia increased the expression of IL-1β in CCA cells and promotes CCA growth *in vitro* and *in vivo*. Suppression of IL-1β expression or inhibition of the complex formation between IL-1β and IL-1R significantly reduced the growth of CCA both *in vitro* and *in vivo*, suggesting IL-1β signaling as a potential target for improving the outcome of CCA treatment, especially in patients with hyperglycemia.

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passed away in January 2024 while serving as an overseas advisor and one of the principal investigators.
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Footnote

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Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013) and the ICH Good Clinical Practice. The study was approved by the Khon Kaen University Ethics Committee for Human Research (approval No. HE661103), and informed consent was obtained from all individual participants. Animal experiments were performed under a project license granted by the Institutional Animal Care and Use Committee of Khon Kaen University (approval No. IACUC-KKU 139/64) based on the National Guidelines of the National Research Council of Thailand and the ARRIVE guidelines.

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