Anti-migratory Effect of FGFRs Inhibitor on Cholangiocarcinoma Cells

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ABSTRACT

The purpose of this study is to explore the role of FGFRs inhibitor; infigratinib, mediates cytotoxicity and cell migration in CCA cells with highly expressed FGFR2. Functional studies were explored in KKU-100 and KKU-M213 cells. Infigratinib increases cytotoxic effect in dose- and time-dependent manners in both cell lines with the IC50 in a micromolar range. Infigratinib significantly decreased cell migration in KKU-100 and KKU-M213 cells. In the presence of FGF2 ligand, infigratinib significantly suppressed the anti-migratory effect in CCA cells when compared to infigratinib treatment alone, in both CCA cells. These findings indicated the potential use of FGFRs inhibitor to be as a single agent or combination regimen with chemotherapy to suppress CCA cell migration.

บทคัดย่อ

การศึกษานี้มีวัตถุประสงค์ เพื่อศึกษาบทบาทของยายับยั้งตัวรับไฟโบรบลาสโกรทแฟคเตอร์ คือ infigratinib ในการยับยั้งการเคลื่อนที่ของเซลล์มะเร็งท่อน้ำดีที่มีระดับการแสดงออกของโปรตีน FGFR2 สูง ได้แก่เซลล์ KKU-100 และ KKU-M213 ผลของยา infigratinib มีความเป็นพิษต่อเซลล์มะเร็งทั้งคู่ และมีค่า IC50 ในระดับความเข้มข้นไมโครโมลาร์ โดยพบว่าความเป็นพิษของยาดังกล่าวขึ้นกับขนาดยาและระยะเวลาที่ใช้ศึกษาในเซลล์มะเร็ง ยา infigratinib สามารถยับยั้งการเคลื่อนที่ของเซลล์มะเร็งท่อน้ำดีได้อย่างมีนัยสำคัญ โดยฤทธิ์ดังกล่าวจะยิ่งมากขึ้นเมื่อมีการให้ไฟโบรบลาสโกรทแฟคเตอร์ชนิดที่ 2 ร่วมด้วยในเซลล์มะเร็งท่อน้ำดีทั้งคู่ ดังนั้นผลการศึกษานี้ ได้แสดงถึงศักยภาพในการยับยั้งการเคลื่อนที่ในเซลล์มะเร็งท่อน้ำดีซึ่งอาจจะใช้เพียงตัวเดียวหรืออาจให้ร่วมกับยาเคมีบำบัดที่เป็นยามาตรฐานที่ใช้รักษามะเร็งท่อน้ำดี

Keywords: Cholangiocarcinoma, FGFRs, Infigratinib

คำสำคัญ: มะเร็งท่อน้ำดี ตัวรับไฟโบรบลาสโกรทแฟคเตอร์ ยาอินฟิกราทินิป

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Introduction

Cholangiocarcinoma (CCA) is the progressive and deadly malignancy that tumour arises from the biliary epithelial lining cells (Khan et al., 2005). The curative operation is the only chance of better survival. However, the 5-year survival rate after surgical operation has been reported about 27-44 % in all CCA subtypes (Khan et al., 2012). Notably, the vast majority of the CCA patients were diagnosed with the metastatic state and were ineligible for resection. At this stage, the systemic chemotherapy is considered as the palliative treatment option. In the ABC-02 trial, gemcitabine plus cisplatin showed an average of 3.6 months longer than who received monotherapy regimens (Valle et al., 2010). This poor responsiveness was caused by chemo-resistance of CCA tumours and serious side effect of chemotherapy. For instance, the side effects of cisplatin are related to extreme toxicities such as dose-dependent nephrotoxicity and neurotoxicity (Dasari and Tchounwou, 2014). To this point, CCA is a very aggressive tumour that has no curable drugs or any responsive drugs. So, there is really needed to understand the dependent pathway of CCA tumorigenesis, to find the actionable target.

Genomic research has been using to refine the molecular taxonomy of a spectrum in cancers and discover novel genetic alterations contributing to tumorigenesis. Significant proportion of CCA has potentially targetable genetic alterations including aberrations of fibroblast growth factor receptors (FGFRs) pathway which is involved in cancer cell proliferation, differentiation, migration, and angiogenesis (Kongpetch et al., 2020). Inhibition of FGFRs signalling has been showed a promising effect in cancers harbouring FGFRs genetic alterations. For example, CCA cells harbouring *FGFR2* fusion gene, showed the responsiveness to FGFRs inhibitor by decreasing the proliferation, migration as well as invasiveness (Arai et al., 2014). However, the majority in subset of Thai CCA were found to harbour FGFR2 overexpression without FGFR genetic alterations. So, this could raise the questions whether this subset of non-harbouring FGFR alterations, may gain benefit from the targeted FGFR inhibition as the advent of personalized therapy.

The FGFRs is a member of tyrosine kinase receptor family and consists of FGFR1, FGFR2, FGFR3 and FGFR4 subtypes. When FGFs (fibroblast growth factor) bind to FGFRs, as results in activation of downstream signalling pathway to promote cell differentiation, proliferation, survival, migration, angiogenesis, and resistance to anticancer agents. Stimulating of downstream signalling appears via intracellular receptor substrates FGFR substrate 2 (FRS2) and phospholipase C_γ (PLC_γ), subsequently triggers upregulation of RAS/mitogen-activated protein kinase (MAPK), phosphoinostitide-3-kinase (PI3)/AKT, and STAT-dependent signalling pathways (Touat et al., 2015).The role of FGF/FGFRs signalling contributed to oncogenesis, has been reported in various cancers. Aberrant FGFRs signalling can stimulate tumour growth by driving cancer cell proliferation as well as promoting tumour angiogenesis. Raising data reveals that this pathway plays a key role in oncogenesis via gene amplification, activating mutations and chromosomal translocation (gene fusion) (Turner and Gores, 2010).

Recently, the data of FGFR gene expression with clinicopathological data from CCA patients, showed a good correlation between high FGFR2 expression and reduction of tumour. This result indicated that FGFR2 might be an important regulator in CCA tumorigenesis. Moreover, overexpression FGFR2 has been found in various cancers including lung, pancreatic, and breast cancers (Bane et al., 2009; Behrens et al., 2008; Nomura et al., 2008). Huang *et al* reported that overexpressed FGFR2 is a tumour specific event in gastric cancer. The overexpressed levels of FGFR2 only found in cancer area compared to adjacent normal tissues. Moreover, the overexpressed FGFR2 was associated with stage III and IV patients (Huang et al., 2017). The pharmacological drugs to target FGFRs signalling are now available in small-molecule TKIs competitively inhibit ATP binding to the cytoplasmic kinase domain or inhibit the autophosphorylation of tyrosine residue (Chae et al., 2017). Infigratinib is a potent, orally bioavailable, highly selective pan-FGFR inhibitor with main action against FGFR1-3. This drug has been testing in clinical trial phase III (PROOF-301) in advanced or metastatic CCA patients with FGFR2 fusion/translocation (Makawita et al., 2020). Of note, a subset of wild type (WT)-FGFR overexpression (without alterations; mutation, amplification and fusion gene), has not yet elucidated the treatment outcome of FGFR inhibitors.

As alluded to above, we then aim to determine anti-oncogenic effect of the FGFRs inhibitors focusing on the cancer cells migration in CCA cells with highly expressed FGFR2. Our preclinical study will provide novel insights into a subgroup of CCA that may benefit from FGFR-targeted therapies.

Objective of the study

This aim of this study was to investigate the role FGFRs inhibitor (infigratinib) on the anti-migratory effect in CCA cells with highly expressed FGFR2.

Materials and methods

Cell lines and cell cultures

The human cholangiocarcinoma (CCA) cell lines, KKU-100, KKU-M213, KKU-M156 and KKU-452 were employed in this study. KKU-100, KKU-M213 and KKU-M156 cells were generously provided by Prof. Banchob Sripa, Department of Pathology, Faculty of Medicine, Khon Kaen University. The KKU-452 cells were established from CCA tumour tissue according to previous report (Saensa-Ard et al., 2017). CCA cells were cultured in Ham's F12 media and supplemented with 10 mM N-2-hydroxyethylpiperazine-N´-2-ethanesulfonic acid (HEPES), pH 7.3, gentamicin sulfate (50 µg/ml), penicillin (100 unit/ml), and 10 % (v/v) fetal bovine serum (FBS). CCA cells were maintained under an atmosphere of 5% CO_2 and 37 °C. The CCA cells were subcultured every 3 days using 0.25 % trypsin-EDTA.

Western blot analysis

The protein samples were separated by SDS-PAGE, then transferred to PVDF membranes by semi-dry electroblotting and blocked with 5% (w/v) bovine serum albumin (BSA) in TBST. The blotting membranes were washed with TBST and incubated overnight at 4 ºC with primary antibody: rabbit polyclonal antibody against FGFR2 (Abcam, Cambridge, UK) and goat polyclonal antibody against β-actin (Santa Cruze, Texas, USA). The membranes were washed with TBST and incubated at room temperature for 3 h with the applicable horseradish peroxidase-conjugated secondary antibody. Protein bands were detected using Luminata™ Forte Western HRP substrate (Millipore Corporation, Billerica, MO, USA). The intensity of specific protein band was detected by the ChemiDoc™ MP imaging system (Bio-Rad) and the Image Lab software. Protein expression was normalized using β-actin as loading control.

Sulforhodamine B (SRB) assay

To determine the cytotoxic effect of infigratinib (MedChemExpress, NJ, USA), KKU-100 and KKU-M213 cells were seeded into 96-well plate with a density of 3000 and 4000 cells/well, respectively. For treatment procedure, the culture medium was replenished with various concentrations of infigratinib and incubated for 48 and 72 hours. After an incubation period, the culture medium was removed and the cells were washed with sterilized PBS buffer and fixed with 10 % trichloroacetic acid (TCA) (Sigma-Aldrich, MO, USA) for 1 hour, and stained with 0.4 % SRB (Sigma-Aldrich, MO, USA) in 1 % acetic acid solution for 30 minutes. Excess dye was removed by washing several times with 1 % acetic acid solution. After that, 10 mM Tris base pH 10.5 was added to solubilize the protein-bound dye and measured the absorbance at a wavelength of 540 nm using a microplate reader. The percentage of viable CCA cell in triplicate experiments will be calculated as, % cell viability = (absorbance of the treated group/absorbance of the control group) x100.

Wound healing assay

The wound healing assay was determined using the scratch wound method. Briefly, KKU-100 cells were seeded into 24-well plate at density of 300,000 cells/well and allowed to growth nearly 90 % cell-confluence. A scratch wound was created by using a sterile 200 µl-pipette-tip, followed by washing with PBS to eliminate detached cells. After that, the cells were incubated in complete media consisting of the various concentrations of infigratinib with or without FGF2 (ProSpec, NJ, USA). The images of the scratched wound in two different areas from each well, were taken from 0 to 48 h. The closing of scratched wound represented the phenotypic cell migration and measured by Image-Pro Plus software (Media cybernetic, LP, USA).

The transwell cell migration assay

This assay was performed using a 24-well transwell plate. In brief, the lower chamber of transwell insert was filled with complete media. Next step, KKU-M213 cells were seeded into the upper insert-chamber at a density of 20,000 cells in 100 µl of infigratinib with or without FGF2 in HamF12 serum

free medium. After an incubation period of 48 hours, cells on the upper surface were gently removed with a cotton swab and the migrated cells on a lower surface were fixed with cold absolute methanol for 20 minutes then stained with 0.5 % crystal violet in 2 % ethanol solution for 15 minutes. The staining of migrated cells on the inserted membrane was captured under a microscope and measured by Image-Pro Plus software (Media cybernetic, LP, USA).

Statistical analysis

Data were presented as the mean ± SEM of three independent experiments. Comparison among each treatment groups was performed by Sigma Stat software using one-way ANOVA. Results considered to be significant at p-value < 0.05.

Results

Basal level of FGFR2 protein expression in CCA cells

To analyse the constitutive levels of FGFR2, the protein expression of FGFR2 in KKU-100, KKU-M156, KKU-M213 and KKU-452 cell lines, were measured by Western blot analysis. The highest expression level of FGFR2 was in KKU-M213, subsequent levels were in KKU-M156 and KKU-100. The lowest expression level was in KKU-452 cells (Figure 1A and B). Due to the migratory activity, the KKU-M213 and KKU-100 cells were employed to use as a representative cell lines with highly expressed FGFR2 for further functional studies.

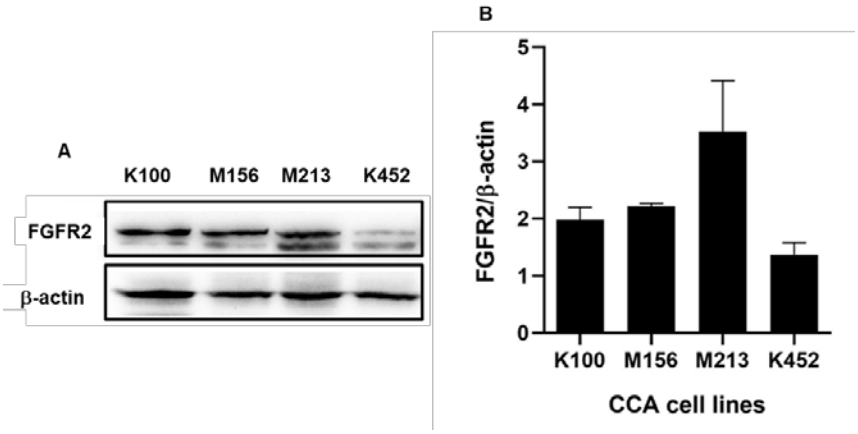

Figure 1 Protein expression of FGFR2 in CCA cell lines. (A and B) Protein expression levels of FGFR2 in KKU-100, KKU-M156, KKU-M213 and KKU-452. Western blot analysis was performed to measure the expression of FGFR2. The relative intensity of target proteins was normalized with β-actin as a loading control and was calculated as a ratio to the control group. Each bar represents the mean ± SEM from three independent experiments. The representative bands from one experiment.

Infigratinib mediates cytotoxic effect in CCA cells

Since the FGFRs play a significant function in promoting cancer cell survival, we then evaluated the cytotoxic effect of infigratinib on CCA cells. After both CCA cells were exposed with various concentrations of infigratinib, the results showed that the drug significantly suppressed CCA cell viability in KKU-100 and KKU-M213 cells in the concentration- and time-dependent manners (figure 2A and 2B). In KKU-100 cells, the IC50 values of infigratinib were in a range of 4.23 to 4.98 μM. In addition, the IC50 values in KKU-M213 cells, were in a range of 4.53 to 5.28 μM (Table1). To compare the responsiveness between two CCA cells, the IC50 values of KKU-100 cells showed more sensitive to infigratinib than in KKU-M213 cells.

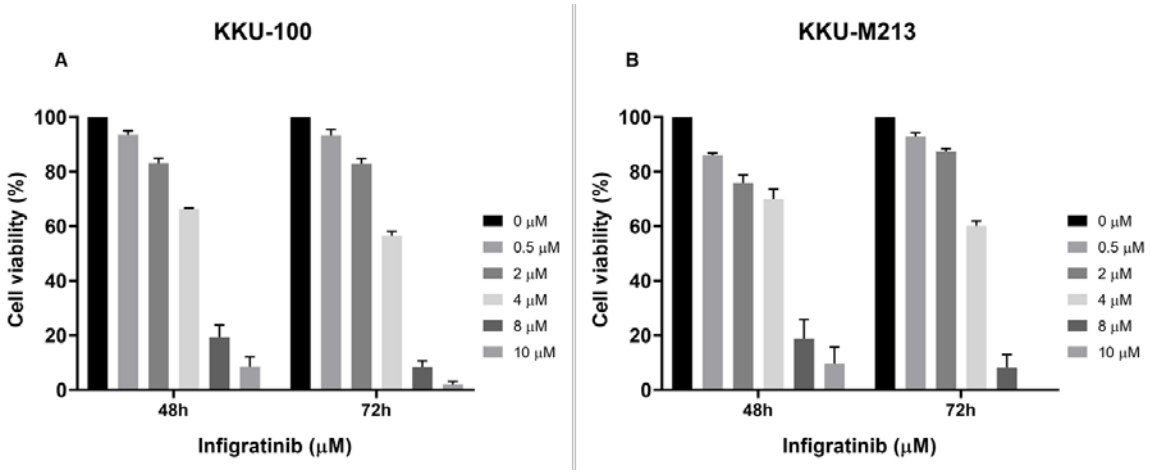

Figure 2 Infigratinib mediates cytotoxic effect in CCA cells. KKU-100 (A) and KKU-M213 (B) were treated with infigratinib with 0, 0.5, 2, 4, 8, 10 μM, incubated 48 h and 72 h. The cytotoxicity was analysed by SRB assay and calculated in % cell viability. Each bar represents the mean ± SEM from three independent experiments.

Table 1 The IC50 value of infigratinib in KKU-100 and KKU-M213 cells

CCA cells	IC50 for 48 h (Mean ± SD)	IC50 for 72 h (Mean ± SD)
KKU-100	4.98 ± 0.52 μM	4.23 ± 0.20 μM
KKU-M213	5.28 ± 0.89 μM	4.53 ± 0.19 μM

Infigratinib mediates anti-migratory effect in CCA cells

Metastatic stage is a state for cancer progression that contributing to the poor prognosis and shorter survival rate of patients, including CCA patients. It is necessary to investigate the anti-oncogenic effect focusing on suppression of cancer cell-migration. The wound healing assay was performed in KKU-100 cells, to evaluate the suppressive effect of infigratinib on the cell-migration by measured the wound closure area. The concentration of 2.5 and 5 μM of infigratinib, significantly suppressed KKU-100 cell-

migration at 18 hours. However, only infigratinib at the concentration of 5 μM, significantly inhibited KKU-100 cell-migration at 24 and 48 hours (Figure 3A and 3B). The FGF2 is a ligand of FGFR2 which has been documented to stimulate cancer invasiveness in many cancers. The FGF2 alone or in combination with 5 μM of infigratinib, were employed to explore whether the FGF2 stimulate CCA cell migration and this effect could be blocked by FGFRs inhibitor. The FGF2 treatment alone did not increase KKU-100 cell-migration as showed by the wound closure area did not change compare to the control group. However, in the presence of FGF2, infigratinib significantly potentiated the anti-migratory effect in KKU-100 cells when compared to infigratinib treatment alone (Figure 3C and 3D).

To confirm the anti-migratory effect of infigratinib in CCA, KKU-M213 cells were employed to perform a transwell cell migration assay. The infigratinib alone was significantly suppressed KKU-M213 cell-migration at both concentrations of 2.5 and 5 μM. The cells were treated with FGF2 alone or with the combination of 2.5 and 5 μM of infigratinib. Consistency to KKU-100 cells, FGF2 did not alter KKU-M213 cell-migration as compare to control group. Moreover, the combination of infigratinib (2.5 μM) and FGF2 were significantly inhibited KKU-M213 cell-migration when compare to infigratinib (2.5 μM) or FGFR2 treatment alone (figure 4A and 4B). Altogether, these data demonstrated that FGFRs inhibitor potentially mediated anti-migratory effect in highly expressed FGFR2 CCA cells.

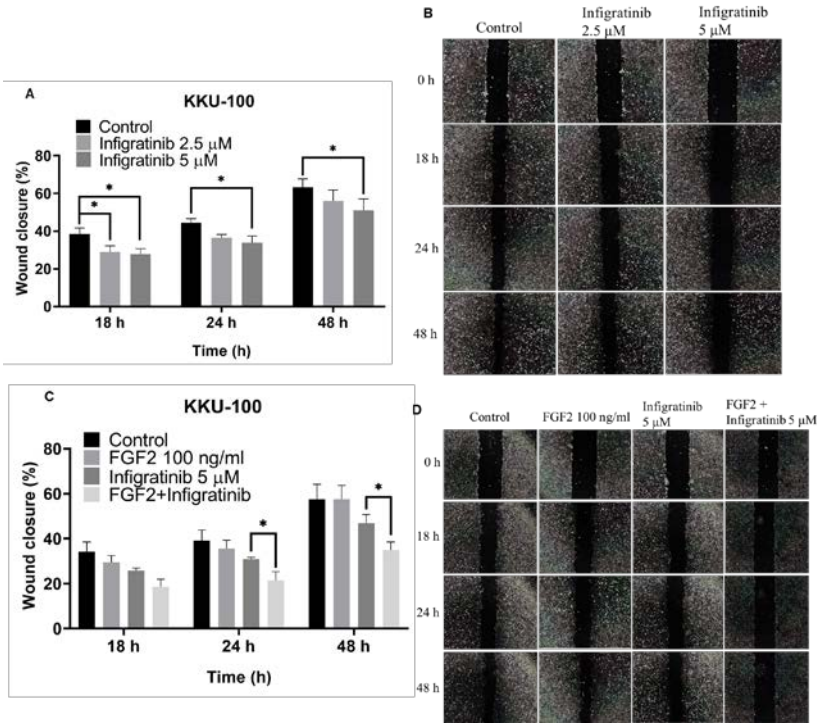

Figure 3 Infigratinib mediated anti-migration in KKU-100 cells. (A) A wound healing assay performed after treatment with infigratinib at the 2.5 and 5 μM for 48 h. (C) The combination effect between 100 ng/mL of FGF2 and 5 μM of infigratinib, on wound closure. Each bar represents the mean ± SEM of three experiments. *P < 0.05 vs control. (B & D) The representative figures from one experiment.

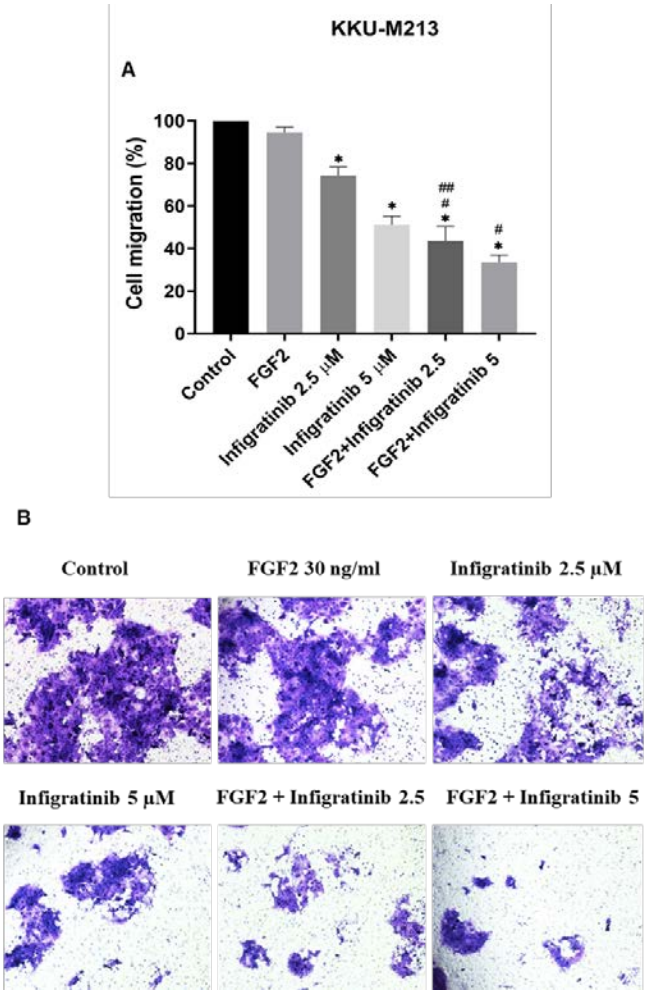

Figure 4 Infigratinib mediated anti-migration in KKU-M213 cells. A transwell cell migration assay was performed to measure the capability of cell migration. (A) The % migration of infigratinib (2.5 and 5 µM) treatment alone or combined with FGF2 (30 ng/mL) in KKU-M213 cells. Each bar represents the mean ± SEM of three experiments. *P < 0.05 vs control, #P <0.001 vs FGF2, ##P <0.05 vs infigratinib 2.5 µM. (B) The representative figures from one experiment.

Discussion

Many FGFR inhibitors have been clinically investigated in the cancer patients harbouring FGFR alteration with promising outcomes such as the tumour shrinkage and decrease in the lymph node metastasis. For example, a multicenter, phase II study on infigratinib (BGJ398), in advanced or metastatic CCA with FGFR alterations. Notably, this oral agent with an ATP-competitive pan-FGFR kinase inhibitor showed the most responsive cases in the group harbored FGFR2 fusions. The overall response rate was 14.8% and this response was even higher in the patients harbouring FGFR2 fusion only (18.8%) (Javle et al., 2018). In another study, the derazantinib (ARQ 087) were further investigated the treatment outcome in inoperable intrahepatic CCA harbouring *FGFR2* gene fusions. This FGFRs inhibitor with potent pan-FGFR suppression showed an overall response rate of 20.7% and disease control rate of 82.8%

(Mazzaferro et al., 2019). Altogether, these clinical responses towards FGFR inhibitors suggest that certain FGFR alterations, particularly *FGFR2* fusions, may serve as biomarkers for personalized CCA therapy. Other FGFR alterations such as point mutations and amplifications or FGFRs overexpression have not shown obvious correlation, although larger cohort studies are necessary required. Recently, pemigatinib is the first FGFRs inhibitor that was approved by US FDA in April 2020, in patients with advanced or metastatic CCA harbouring *FGFR2*-fusion genes.

To our hypothesis, whether the CCA patients with WT-FGFR2 overexpression could obtain the beneficial effect from these FGFRs inhibitors, so we have designed the preclinical study using CCA cell lines with highly expressed FGFR2 levels. In our study, we have demonstrated the anti-oncogenic action of infigratinib, focusing on anti-migratory effect. The infigratinib is the representative of FGFRs inhibitor used in this study. The IC50 of infigratinib was in a range of 4-5 μM in two CCA cell lines. Moreover, our results showed infigratinib mediated anti-migratory in both KKU-100 and KKU-M213. The suppressive effect of this agent to CCA cell migration, was even higher with the present of FGF2.

Considerably, *in vitro* cancer models, the FGFRs inhibitor were demonstrated to suppress oncogenic activities in various cancer cells. For example, AZD4547 showed the suppression to the growth of FGFR-amplified or overexpressed FGFRs in breast cancer cells (Kang et al., 2019). Furthermore, monoclonal antibody PRO-007 that targets FGFR2 amplification, showed the reduction of cell viability and cell invasiveness in KATO III gastric cell line carried FGFR2 amplification (Kim et al., 2019). Consistency to our finding that infigratinib also mediated the cytotoxicity in both CCA cells (KKU-100 and KKU-M213).

The aggressive cancer phenotype has been determined in CCA cells including migratory activity. Both CCA cells were employed to analyze the migration using wound healing and transwell cell migration assays. The results from wound healing assay, the dose of infigratinib of 2.5 and 5 μM showed the dose-dependent effect to inhibit the cell migration in KKU-100 cells in an early treatment time of 18-hour. The role of FGFRs inhibitors in cell migration were explored in many cancer models. For example, TKI-258 (Dovitinib) was treated in various concentrations in SCC-4 oral squamous cell carcinoma and the effect significantly decreased cell migration in a concentration-dependent manner (Carneiro et al., 2017). The ligand of FGFR is FGF which plays crucial roles in tissue growth and homeostasis. The FGF ligand has been investigated its role in cancer progression including migration and invasion. FGF2 was reported to stimulate the transition of primary prostate carcinomas to be metastatic phenotype. In addition, the switching of FGFR isoforms IIIb to be IIIc-isoform, was caused by FGF2 secreting by cancer cells (Nakamoto et al., 1992). In another study, FGF2 stimulated the migration of HOC313 and TSU oral squamous cell carcinoma (OSCC) by inducing the increase in expression of p-ERK1/2 which is a downstream effector of FGFRs. In addition, the use of selective FGFR1 inhibitor, SU5402, significantly suppressed cell migration via downregulation of p-ERK1/2 (Osada et al., 2019). However, our results showed that FGF2 did not stimulate the CCA cell migration. Even though FGF2 did not show the correlation to the migration of KKU-100 and KKU-M213, it potentiated the anti-migratory

activity of FGFRs inhibitor when used in a combination of infigratinib. This controversial finding could be explained by the effect of ligand-independent stimulation. This mechanism is not needed the specific ligand to bind and to activate the downstream signalling. For example, an extremely high activity of FGFRs due to *FGFR2* fusion gene, can initiate cancer cell migration via the oligomerization and autophosphorylation without its ligand binding (Wu et al., 2013). However, our findings were required further investigations including the expression level of phosphorylation form of the downstream effectors of FGFR2. This could be confirmed whether the overexpressed FGFR2 lead to lack of responsiveness to its specific ligands (Hallinan et al., 2016).

Conclusion

In summary, the present study demonstrated that infigratinib inhibited cell migration in CCA cell lines with highly expressed FGFR2. This preclinical study provided the results on potential use of FGFRs inhibitor in CCA with overexpressed FGFR2, especially in metastatic CCA tumours. Further study on FGFRs inhibitors with molecular mechanisms and beneficial effect to inhibit CCA tumorigenesis, might be warranted to explore in preclinical study as well as in clinical trials.

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