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Fibroblast Growth Factor Receptor 4 and R4-ICD Increased Proliferation, Cell Survival and Metastasis in Cholangiocarcinoma

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By

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ABSTRACT

Fibroblast growth factor receptor 4 (FGFR4) is over expressed in many cancers, including cholangiocarcinoma (CCA). FGFR4 is activated by fibroblast growth factor ligand 19 (FGF19) and plays a critical role in CCA progression. An intracellular cleaved product of FGFR4, referred as R4-ICD (FGFR4 intracellular domain) is also overexpressed in CCA. However, the specific role of R4-ICD in CCA is unknown. In this study, we hypothesized that FGFR4 and R4-ICD play a role in cell proliferation, cell survival and metastasis in CCA. To test this, FGFR4 and R4-ICD were cloned into a cholangiocarcinoma cell line (HuCCT-1) that does not endogenously express FGFR4. Two other CCA cell lines, KMCH and Mz-ChA-1, were also utilized that express endogenous FGFR4. To determine the influence of FGFR4 on CCA proliferation, survival, and migration, FGFR4-selective and pan-FGFR inhibitors were used. Expression of FGFR4 increased CCA proliferation, cell survival and migration. Stable knockdown of FGFR4 using shRNA reduced CCA proliferation, cell survival and migration. FGFR4-selective small molecular inhibitors sensitized cells to TRAIL-induced apoptosis and reduced migration. R4-ICD-expressing HuCCT-1 cells (lacking endogenous FGFR4 expression) were protected against TRAIL-induced apoptosis, indicating an underlying role of R4-ICD. This study suggests that, in addition to FGFR4, R4-ICD can be a potential regulator of CCA.

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LIST OF ABBREVIATIONS

ARDIA	AT-Rich interaction domain 1A
BRCA2	breast cancer type 2 susceptibility protein
CCA	cholangiocarcinoma
CEUS	contrast-enhanced ultrasonography
CO_2	carbon dioxide
CRC	colorectal caner
CT	computed tomography
DAPI	4′,6-diamidino-2-phenylindole
dCCA	distal cholangiocarcinoma
DMEM	Dulbecco's Modified Eagle Medium
DMSO	dimethyl sulfoxide
eCCA	extrahepatic cholangiocarcinoma
EMT	epithelial-mesenchymal transformation
ERCP	Endoscopic Retrograde Cholangiopancreatography
ERK	extracellular signal-regulated kinase
FBS	fetal bovine serum
FGF	fibroblast growth factor
FGFR	fibroblast growth factor receptor
FGFRL1	fibroblast growth factor receptor like-1
GRB2	growth factor bound protein 2
HCC	hepatocellular carcinoma
HER2	Erb-B2 receptor tyrosine kinase

HNSCC	head and neck squamous cell carcinoma
HS	heparan sulfate
iCCA	intrahepatic cholangiocarcinoma
IDH	isocitrate dehydrogenase
Ig	immunoglobulin
KRAS	Kristen rat sarcoma viral oncogene
LB	lysogeny broth
MAPK	mitogen-activated protein kinase
MCL1	Myeloid Cell Leukemia 1
MMP	matrix metalloproteinases
MRI	magnetic resonance imaging
pAKT	phosphorylated protein kinase B
PBRM1	polybromo 1
pCCA	perihilar cholangiocarcinoma
PCR	polymerase chain reaction
pERK	phosphorylated extracellular signal-regulated kinase
PI3K	phosphoinositol 3-kinase
PKB/AKT	protein kinase B
PKC	protein kinase C
PLCγ	phospholipase C-gamma
R4-ICD	fibroblast growth factor receptor 4 intracellular domain
RNAi	ribonucleic acid interference
SBRT	stereotactic body radiation therapy
SOC	super optimal broth with catabolite repression
SOS	son of sevenless
STAT3	signal transducer and activator of transcription 3

TCGA	The Cancer Genome Atlas
TGF-β	transforming growth factor beta
thorotrast	thorium dioxide
TNF	tumor necrosis factor
TP53	tumor protein 53
TRAIL	TNF-related apoptosis-inducing ligand

CHAPTER 1: INTRODUCTION

Significance

Cholangiocarcinoma (CCA) is identified as an aggressive tumor that develops in the biliary tract of the liver. It arises from the bile duct epithelial cells (cholangiocytes) or their progenitor cells [1]. CCA is the second most diagnosed hepatic malignancy after hepatocellular carcinoma (HCC) [2]. CCA accounts for approximately 3% of gastrointestinal tumors [3] and 15% of primary liver tumors [4]. Based on the anatomical location of the tumor, CCA is divided into two major types including intrahepatic CCA (iCCA – within the bile ducts of the liver) and extrahepatic CCA (eCCA – within the bile ducts outside of the liver) [5]. eCCA has been subdivided into perihilar CCA (pCCA) and distal CCA (dCCA) [5]. iCCA is a malignancy known to be located at the periphery of the secondary branch of both right and left hepatic duct. pCCA (also known as Klatskin tumor) is located at the junction of left and right hepatic duct, while dCCA is commonly associated with the common bile duct **(Figure 1)**. CCA is frequently diagnosed in advanced stages due to the asymptotic nature of the tumor in early stages. Due to the late-stage diagnosis, there are limited therapeutic options available to treat the majority of patients with CCA [6, 7]. Recently, it has been observed that the incidence of iCCA is increasing worldwide, in comparison with pCCA and dCCA [6, 8, 9].

CCA has a poor prognosis with the 5-year survival rate between 5-10%. However, the 5-year survival rate is between 25-30% in patients undergoing curative surgery [10, 11]. If the cancer has metastasized to distant parts of the body, the 5-year survival rate reduces to 2%. In the overall cases of pCCA and dCCA, the 5-year survival rate is 10%, while early diagnosis (before tumor advances) of pCCA and dCCA has a 15% survival rate. In case of iCCA, the 5-year survival rate is 9%. If the cancer is diagnosed in early stages (before

tumor advances), survival rate increases to 25%, however, if it spreads to the lymph nodes, the survival rate decreases to 8% [12].

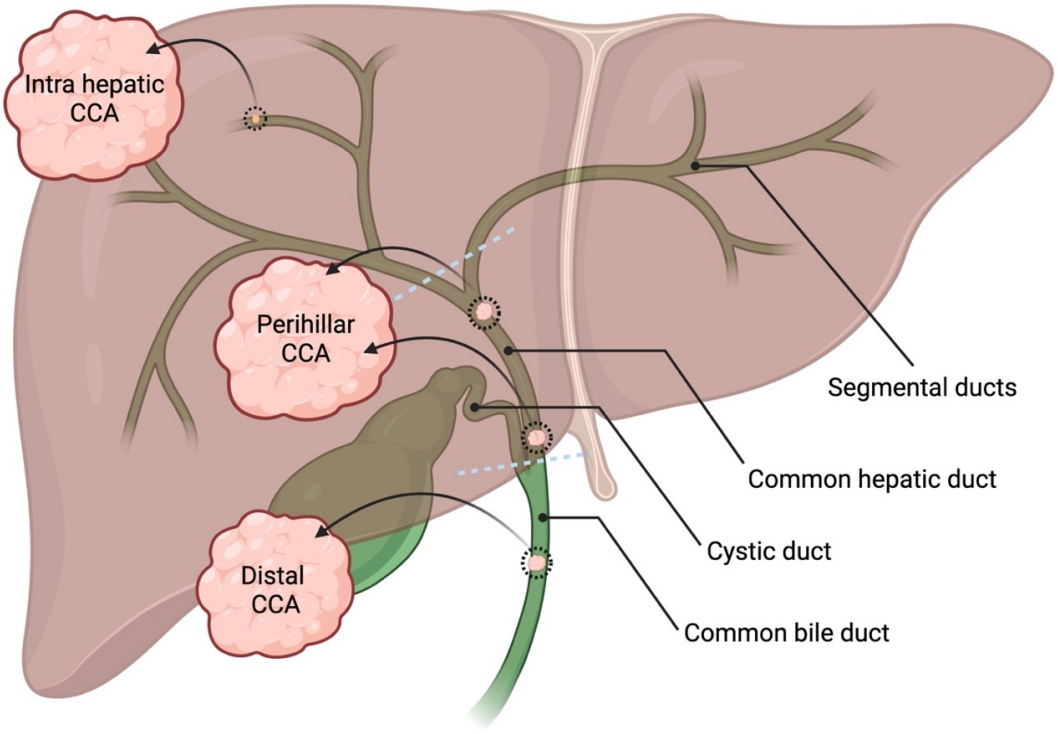


Figure 1: Cholangiocarcinoma (CCA): Anatomical location and classification.

CCA is classified based on tumor location: intrahepatic CCA, perihilar CCA and distal CCA. (Image created with BioRender).

Causes and diagnosis of CCA

The etiology of CCA is not well defined, although a few risk factors and diseases that are associated with CCA have been identified. These include parasitic infection, hepatitis infection, inflammatory bowel disease, liver injury and bile duct disorders. *Clonorchis sinensis* and *Opisthorchis viverrine* are parasites are known to cause liver fluke infection in southeast Asia. These parasites are major risk factors associated with CCA [13]. Also, *Ascaris lumbricoides* (roundworm) infection can lead to CCA [13-15]. All three of these parasites may cause hepatolithiasis, gallstones in the biliary duct that may increase the risk associated with CCA [16].

Besides parasitic infections, a few bacterial infections, including *Helicobacter* species, are also associated risk factors for CCA [14]. Hepatitis infections, including viral hepatitis B and C, are major risk factors of CCA, principally iCCA. Hepatitis C virus is predominantly associated with CCA in USA [17], while hepatitis B-associated CCA is more predominant in Asian countries, including China [18]. Furthermore, patients with primary choledochal cysts, primary sclerosing cholangitis, and ulcerative colitis have a high risk of developing CCA [15, 19, 20]. A well-known association between CCA and genetic aberrations has been identified [21]. In addition, lifestyle and chemical-hazard associated risk factors such as smoking, obesity, alcohol consumption, long term chemical exposure of dichloromethane and 1,2- dichloropropane (printing company workers have exposure) have a higher chance of developing CCA [2, 22, 23]. Long term exposure of radiographic agents such as thorotrast (thorium dioxide) may also lead to CCA [15, 24].

In patients with pCCA and dCCA to the primary symptom is jaundice because of bile blockage between the liver and intestine, while in the case of iCCA jaundice is less frequent. Besides jaundice, other symptoms such as asthenia, abdominal pain, nausea, malaise, weight loss and anorexia also occur in advanced CCA [25]. The most common approaches to diagnose CCA are ultrasonography, contrast-enhanced ultrasonography (CEUS), magnetic resonance imaging (MRI) and computed tomography (CT) scan. Endoscopic Retrograde Cholangiopancreatography (ERCP) imaging methods are considered a gold standard for the detection of biliary disorders and tumors [26]. ERCP has a 74% sensitivity and 70% specificity for the detection of CCA [27]. These techniques are used to determine CCA staging and treatment responses [28]. A CT scan has also been considered a gold standard technique to diagnose CCA as it provides detailed information about the primary tumor and metastasized sites in the abdominal cavity [28]. Moreover, cancer antigen 19-9 (CA 19-9) can also be used as a marker for CCA; the elevated level of CA 19-9 can indicate metastatic disease. However, some patients (Lewis-antigen-

negative) are unable to make CA 19-9, making this marker ineffective in this population, necessitating the need for histopathological assessment for this subgroup [5].

Limitation of current standard of care in CCA

The current standard of care for CCA has many limitations due to limited effectiveness and adverse side effects. The first line chemotherapeutic agents that are used in current clinical practice for CCA management are gemcitabine and platinum-based agents to treat primary as well as advanced CCA [29, 30]. However, these chemotherapeutic agents showed toxicity toward non-cancerous host cells and have severe side effects including nausea, anorexia and vomiting [31]. There are no second line of chemotherapeutic agents or adjuvant therapies presently available for CCA management, due to poor outcomes in the clinical trials [32].

Surgical resection is a potential approach to treat CCA patients but approximately 70% of patients are diagnosed at advanced stages because most patients are not eligible surgical resection [33, 34]. Currently, the advances in the developments of radiotherapy design have made it a potential non-surgical regimen to treat patients with CCA [8]. However, radiation treatment is not a beneficial approach in the case of advanced CCA [33, 34]. Besides radiotherapy, other available treatment regimens include novel systemic therapy, transarterial chemoembolization, ethanol ablation and stereotactic body radiation therapy (SBRT), which are potential non-surgical treatment methods [35, 36]. To manage iCCA, liver transplant is an effective option, which can increase the 5-year survival rates of the patient. In such cases, a combination of chemotherapy and radiation therapy was applied followed by liver transplantation, which increased the 5-year survival rate from 18% to 64% [37-39]. However, a limitation of transplantation is lack of matched donors, and few patients qualify for surgical resection based on tumor staging [40-42].

Another emerging treatment regimen for CCA patients is targeted therapy, where therapeutic target molecules can be used to target isocitrate dehydrogenase (IDH) mutation, Erb-B2 Receptor Tyrosine Kinase 2 (HER2) mutation/amplification, fibroblast growth factor receptor (FGFR) mutation/amplification, and breast cancer type 2 susceptibility protein (BRCA2) mutation in biliary tract cancers [43, 44]. The IDH family is comprised of IDH1 and IDH2, which are the metabolic enzymes participating in the tricarboxylic acid (TCA) cycle. Both IDH1 and IDH2 catalyze the reaction of isocitrate to α-ketoglutarate by $NADP^+$ dependent decarboxylation [45]. Studies showed that a single amino acid substitution (usually arginine) may lead to the gain of function phenotype observed with IDH mutations. The gain of function of IDH works as an oncogenic marker that promotes proliferation and migration of tumor cells. IDH disruptions may further act as stimulators of other pathways such as VEGFR [45 46, 47]. Currently, IDH1 and IDH2 mutations have been associated in 15-20% of iCCA while very few are associated with eCCA [48, 49]. AG-120 (Ivosidenib, Agios) is an IDH-selective inhibitor used to treat patients with CCA [50]. The pharmacokinetic and pharmacodynamic studies of AG-120 in patients with CCA suggested that the drug had good oral response with 500 mg (once per day) with no dose dependent toxicities found in the Phase 1 clinical trial (NCT02073994). The Phase 3 clinical trial study suggested 30% of patients had progression free survival within the first two months of treatment in patients with iCCA [51, 52]. However, an additional clinical trial showed a few side effects including, fatigue, diarrhea, nausea, vomiting and abdominal pain with AG-120 treatment [50, 53]. Another IDH inhibitor, FT-2102 (Olutasidenib, Forma Therapeutics) is currently under clinical trial for iCCA (NCT03684811). The efficacy of other multi-tyrosine kinases that inhibit IDH have been described and showed a positive response against iCCA cells [54]. In addition to IDH, overexpression of FGFR, MET and ERBB were observed in CCA, were related with poor prognosis, and are candidates for targeted therapy [7, 55].

The hybrid capture-based comprehensive genomic profiling (CGP) analysis of 412 patients with iCCA and 57 patients with eCCA was performed. These studies suggested that 1-5 % of patients with iCCA had had genetic aberrations in FGFR1, FGFR2 or FGFR3, with a female predominance. FGFR2-fusions were also observed in 13-50% of patients with CCA [54]. In another study, next generation sequencing (NGS) was performed on CCA samples from 75 patients, finding mutations (66%), loss/deletion (7%), amplifications (20%) and other genetic abnormalities (7%) of genes including tumor protein 53 (TP53), Kristen rat sarcoma viral oncogene homolog (KRAS), IDH1, AT-Rich Interaction Domain 1A (ARID1A), Myeloid Cell Leukemia 1 (MCL1) and polybromo-1 (PBRM1). However, 13% of iCCA and 5% of eCCA had alterations in FGFR family proteins, including mutation, amplification and fusion[56, 57]. In FGFR alterations, 13% of CCA had FGFR2-fusion, 50-60% of CCA had FGFR4 overexpression, rest had shown FGFR1 or FGFR3 mutation [58]. Currently, Phase 3 clinical trials are testing inhibition of FGFR2-fusion proteins through specific targeting molecules to treat CCA [51, 59]. Overall, targeted therapeutics, such as IDH inhibitors and FGFR2-fusion inhibitors are showing beneficial effects in clinical trials with minor side effects, suggesting targeted FGFR therapies are a potential intervention to treat CCA patients.

Fibroblast growth factor receptors

FGFRs are membrane-bound tyrosine kinase receptors [60, 61]. The FGFR family is comprised of highly conserved subtypes FGFR1, FGFR2, FGFR3, and FGFR4, while recently FGFR5/FGFRL1 (fibroblast growth factor receptor like-1) has also been discovered. FGFR5 is known to interact with the family of FGF ligands but it lacks the intracellular tyrosine kinase domain [61]. The structure of FGFR1-4 has been divided into three domains including extracellular immunoglobulin (Ig)-like domains 1-3 (D1-D3), a transmembrane domain and an intracellular tyrosine kinase domain [62]. The FGFR family

of tyrosine kinases differ from other tyrosine kinases as their D1, D2 and D3 domains are rich in hydrophobic amino acid residues and contain an acid box composed of 30 serine amino acid residue that connects D1 and D2 [63, 64].

The FGFR family is activated by FGF ligands in the presence of specific co-receptors and play a distinct role during various stages of embryogenesis such as organogenesis, formation of the limb, nervous system, the lungs and the midbrain [65]. Other than embryonic development, FGFRs play a crucial role in the adult organisms as well. FGFRs regulate tissue homeostasis, angiogenesis, tissue repair and inflammation [65]. Although, the function of FGFR5 is not clearly understood, a recent study indicated that it might be an essential regulator of cell-cell adhesion as it lacks a cytoplasmic intracellular domain [66].

The FGF family includes18 ligands named from FGF1-FGF10 and FGF16-FGF23 [64]. FGF1-10, 16-18 and 20 are known as canonical ligands that require heparan sulfate (HS) proteoglycans as a coreceptor to activate the downstream signaling [67]. However, FGF19, 21 and 23 function as paracrine factors and require Klotho-binding protein as a cofactor to activate downstream signaling [67]. Upon binding of the FGF ligand to the receptor, in the presence of HS/Klotho, the FGFR proteins dimerize with the same or other FGFR proteins on the cell membrane [68]. The dimerized receptors autophosphorylate and/or transphosphorylate the tyrosine residue in the intracellular terminal domain [69]. Further, the phosphorylated residues interact with adaptor proteins such as growth factor receptor bound protein-2 (GRB2), son of sevenless (SOS) and fibroblast receptor substrate 2α (FRS2α). Downstream FGFR signaling is mediated via phosphorylation of FRS2 and phospholipase C-γ (PLC-γ), which subsequently activates protein kinase C (PKC), phosphoinositol 3-kinase (PI3K)/AKT, signal transducer and activator of transcription (STAT) and Ras/mitogen-activated protein kinase (MAPK/ERK) [62, 70]. The dysregulation of FGFR signaling can result in several diseases including cancer [62].

Structure and Function of FGFR4

The structural homology of FGFR4 has a few differences to the other FGFR family proteins. First, the extracellular domain of FGFR4 has only one isoform, while FGFR1-3 have multiple isoforms of loop III [71, 72]. The tyrosine kinase domain of FGFR4 has a distinct amino acid residue, cysteine (C552) instead of tyrosine (Y552), from other FGFR1-3 family proteins [73]. FGFR4 is known to be activated by FGF ligands in both autocrine and paracrine manner. FGFR4 had highest binding activity for ligands FGF1, 2, 4 and 8, which can activate the FGFR4 downstream signaling even in the absence of Klotho co-factor [62, 73]. The role of paracrine ligands FGF19, 21 and 23 was determined, among which, FGF19 showed highest binding affinity to FGFR4 in the presence of Klotho [62, 73]. At a physiological level, FGF19-mediated FGFR4 signaling mainly regulated bile acid synthesis, metabolism, tissue repair and muscle differentiation in growing tissue including liver, bone and lung [73].

FGFR4 signaling in cancer initiation and progression

Recent studies have suggested that alteration in the FGF19-FGFR4 axis plays a crucial role in cancer initiation and advancement [74]. Dysregulation of FGFR4 signaling due to mutation, single nucleotide polymorphism (SNP), overexpression of ligand and receptor has been related with cancer cell proliferation, metastasis, therapy resistance and epithelial to mesenchymal transformation (EMT) [75]. The most common mutation of the extracellular membrane domain of FGFR4 is tyrosine to cysteine at amino acid 367 (Y367C), which causes spontaneous dimerization of the receptors leading to phosphorylation of downstream signaling proteins including ERK and AKT [76]. Mutations in the kinase domain of the intracellular region of the FGFR4 receptor, including valine to glutamic acid (V550E) and asparagine to lysine (N535K), have been identified as markers

of cancer. Both V550E and N535K caused autophosphorylation of FGFR4 and increased the proliferation and migration of tumor cells via STAT3 signaling [77].

The mutations V550E and N535K also lead to the dysregulation of FGFR4 signaling and have been associated with multiple cancers including prostate cancer, breast cancer, rhabdomyosarcoma cancer [75, 78]. The most well-characterized SNP in FGFR4 is present on exon 9. The mutation in the transmembrane domain of FGFR4 from glycine to arginine (G388R) and is associated with multiple cancers and decreased the survival rates of patients [79, 76, 80]. The G388R SNP led to a highly aggressive and metastatic tumor cells that stabilized matrix metalloproteinase (MMP)-14, leading to the degradation of collagen that may increase the risk of tumor metastasis [81]. Besides SNPs and mutations altering the FGFR4 protein signaling cascade, overexpression of FGFR4 and FGF19 also play a crucial role in cancer initiation and progression.

FGFR4 and FGF19 overexpression in cancer progression

The overexpression of FGFR4 and its ligands, including FGF19, accounts for 66% of FGFR4 signaling dysregulation in human cancers including breast, liver, lung squamous, esophageal, bladder, head and neck cancers [73]. FGF19 overexpression has been associated with hepatocellular carcinoma (HCC) [82]. Recombinant human FGF19 activated FGFR4 downstream signaling in Klotho-dependent manner, increased proliferation and inhibited apoptosis of HCC cells. However, RNA interference of FGF19 (siRNA-based silencing) decreased proliferation and increased apoptosis of HCC cell lines, indicating a contributing role of FGF19 in HCC progression [83]. In addition, FGF19 overexpression is also observed in the patients with CCA [84, 85].

Overexpression of FGFR4 has been observed in multiple cancers including liver, colon, breast, rhabdomyosarcoma and pancreatic cancer [86-89]. The overexpression is associated with reduced patient survival rate as well as resistance to radiotherapy and

chemotherapy [88]. The cancer genome atlas (TCGA) analysis data suggested that primary breast cancer had 1.5-fold higher expression of FGFR4 compared to non-cancerous breast cancer tissues[90]. Similarly, TCGA analysis suggested that ovarian cancer and rhabdomyosarcoma showed overexpression of FGFR4 compared to non-cancerous tissues [91, 92]. The overexpression of FGFR4 is linked with the increased in cancer cell proliferation, invasion and survival of colorectal, liver, ovarian and lung cancer [73].

Role of FGFR4 in proliferation, cell survival, and migration in cancer

FGFR4 overexpression was observed in HCC cell lines including Huh7, JHH7, HepG2, PLC/PRF/5. Downregulation of FGFR4 via RNA based interference approach (short hairpin ribonucleic acid-shRNA) highly repressed the viability of both, Huh7 and JHH cells. Similarly, the proliferation of HCC cell line was increased in the presence of recombinant FGF19 in a dose-dependent manner. It indicated that the FGF19/FGFR4 signaling plays a crucial role in cancer cell proliferation. Moreover, it was observed that recombinant FGF19 promoted HCC cell proliferation via p-FRS2α and p-ERK pathway [93]. Similarly, overexpression of FGFR4 was observed in colorectal cancer. Stable knockdown of FGFR4 using shRNA in colorectal cancer cells SW480 and SW48 significantly reduced the cell proliferation via p-ERK signaling compared to FGFR4 expressing parental cells [94].

FGFR4 signaling prevented apoptosis of alveolar rhabdomyosarcoma cells, indicating a role of FGFR4 signaling in cancer cell survival [95]. Furthermore, FGFR4 played a crucial role in the survival of colorectal cancer cells. FGFR4-knockdown cells showed significantly lower cell survival compared to FGFR4 expressing cells, suggesting FGFR4 could be a potential player in the cell survival of colorectal cancer cells. It has been reported that the FGFR4 mainly inhibits apoptosis of cancer cells through downstream signaling via AKT [94]. In addition, shFGFR4 colorectal cancer cells had lower levels of Snail, transforming growth factor beta (TGF-β) and Twist expression and increased expression

of E-cadherin. These downstream effects caused decreased cell adhesion, migration and invasion, supporting the role that FGFR4 plays a vital role in cancer cell migration [94].

FGFR4 overexpression also promoted proliferation of CCA cell lines RBE and QBC939. The knockdown of FGFR4 decreased proliferation, while overexpression of FGFR4 increased proliferation of RBE and QBC939 cells. Similarly, the invasion of CCA cells was increased in the presence of FGFR4, which may promote metastasis to lymph nodes or distal sites. However, a clear underlying mechanism of FGFR4 mediated proliferation, survival and migration in CCA is still unknown to-date [96].

Targeting FGFR4 in cancer

The role of FGFR4 in multiple cancers suggests that FGFR4 could be a potential therapeutic target. Currently, many research groups are developing potential therapeutic components against FGFR4. FGFR4 can be targeted via neutralizing monoclonal antibodies, antisense oligonucleotides, and small molecular inhibitors. Current monoclonal antibodies that target FGFR4 include U3-1784 and LD-1, which significantly reduced tumor cell proliferation and survival in both, *in vitro* and *in vivo* studies [97]. The U3-1784 is human antibody obtained with phage display technology has high affinity for FGFR4 and is known to inhibit the downstream signaling via FRS2 and p-ERK. However, recently published preclinical studies in the nonhuman primate (monkey model) suggested that treatment with U3-1784 antibody increased FGF19 levels, serum bile levels and certain liver enzymes that showed potential damage to the liver indicating side effects of U3-1784 [98]. Furthermore, the antibody therapeutic approach is more expensive compared to other targeted molecules. U3-1784 is currently in a Phase 1 clinical trial to study its efficacy to treat HCC and other advanced solid tumors [99]. Besides monoclonal antibodies, the antisense oligonucleotide ISIS-FGFR4RX has been developed to target FGFR4 mRNA. The efficacy of ISIS-FGFR4RX has not yet been determined in cancers but it is

currently in a Phase 1 clinical trial for obesity [99]. Due to adverse side effects of the FGFR4 antibody in preclinical trails, the small molecule inhibitor approach has gained more attention for targeting FGFR4 in cancer management.

Currently, pan-FGFR and FGFR4-selective inhibitors have been commercialized and their potency against FGFR4 has been determined in many solid cancers. BLU9931 was the first developed potent irreversible covalent FGFR4-selective inhibitor that binds with the Cys552 residue in the ATP binding pocket of intracellular tyrosine kinase residue. Since FGFR1-3 receptors lack a Cys552 residue, BLU9931 cannot bind with other FGFR family members [78]. The efficacy of BLU9931 was determined in HCC, colorectal cancer (CRC), head and neck squamous cell carcinoma (HNSCC) and breast cancer cell lines, in which, BLU9931 has shown efficient antitumor activity [100, 101]. Similarly, BLU554 is an modified version of BLU9931 compound, which showed potential therapeutic potency in solid cancers and is currently being evaluated in a Phase 1 clinical trial for HCC [102]. Furthermore, H3B-6527 is a covalent FGFR4-selective inhibitor that also binds with Cys552 in the ATP binding domain of kinase residues. This compound also showed potential effect against tumorigenesis of HCC cell lines and in *in vivo* models [103]. Another FGFR4 selective inhibitor, Roblitinib (FGF401), is a reversible covalent inhibitor of FGFR4 that interacts with Cys552 residue [104]. FGF401 has shown potent anti-cancer activity in a patient derived xenograft model of HCC [104]. Currently FGF401 is being evaluated under Phase 2 clinical trials for the treatment of HCC and other solid tumors [99].

Furthermore, pan-FGFR4 inhibitors have affinity to block the downstream signaling of FGFR family members including FGFR1-4. PD173074 is a pan-FGFR inhibitor that interacts with the ATP pocket and inhibits FGFR1 [105]. It has been observed that PD173074 increased apoptosis in lung squamous cell carcinoma (SCC) cells within 96 hours [105]. Similarly, PD173074 also inhibited the proliferation of NSCLC cells that overexpress FGFR1 and FGFR2 [106]. BGJ398 is another pan-FGFR inhibitor that has a lower IC50 for

FGFR1-3 but slightly higher IC50 for FGFR4. The efficacy of BGJ398 was determined in MDA-MB-453 breast cancer cells, which exhibited higher expression of FGFR4. BGJ398 showed a dose-dependent effect on the cell proliferation and apoptosis via ERK and AKT inhibition respectively in Hep 3B HCC cells with relatively lower potency compared to the FGFR4-selective inhibitor, BLU9931 [107]. Futibatinib is an irreversible inhibitor of FGFR proteins that binds with the ATP binding pocket of the intracellular kinase domain. Futibatinib inhibited cell proliferation in gastric cancer and bladder cancer cell lines as well as FGFR-driven human tumor xenograft models. The inhibitor has shown dose dependent tumor reduction in the rat xenograft model [108]. Pemigatinib is a pan-FGFR inhibitor that has a high potency for FGFR1-3 while very low potency for FGFR4. Pemigatinib has shown inhibition of FGFR signaling *in vitro* and *in vivo* in various cancer models. Pemigatinib recently recieved FDA approval for the treatment of FGFR2-fusion in CCA [109]. The Pemigatinib mainly inhibited expression of pERK in tumor cell lines that reduced FGFR pathway-dependent cell proliferation [110]. LY2874455 can inhibit FGFR family receptors with high potency. It has dose dependent inhibition of downstream signaling proteins such as ERK and AKT in FGFR4 expressing MDA-MB-453 cells, suggesting inhibition of FGFR4 mediated cancer cell proliferation and apoptosis [78].

Recently, FGFR4 overexpression was observed in CCA patient samples. FGFR4 plays a crucial role in CCA cell proliferation and survival. In addition to FGFR4, a shorter fragment with a lower molecular weight band was also observed on immunoblot. The shorter fragment of FGFR4 was named R4-ICD (FGFR4 intracellular domain) and contains the receptor tyrosine kinase domain. Since R4-ICD contains the ATP binding domain, it may be constitutively active and might play a critical role in cancer initiation and progression, independently from native full-length FGFR4 (Mohr, *et al*, unpublished).

In this study, we showed that overexpression of FGFR4 is associated with CCA progression. The activation of FGFR4 via FGF19 ligand led to the phosphorylation of

intracellular tyrosine kinase domain of FGFR4, which further activated downstream signaling proteins including ERK and AKT. The increased FGFR4 mediated cellular proliferation was dependent upon ERK and the increased cell survival was dependent upon AKT. Besides cellular proliferation and survival, FGFR4 also increased CCA cell migration, although the underlying mechanism of cellular migration is still unknown. The intracellular cleaved product of FGFR4 protein, R4-ICD, increased cell survival independent of AKT. Overall, FGFR4 and R4-ICD play a crucial role in CCA progression **(Figure 2)**.

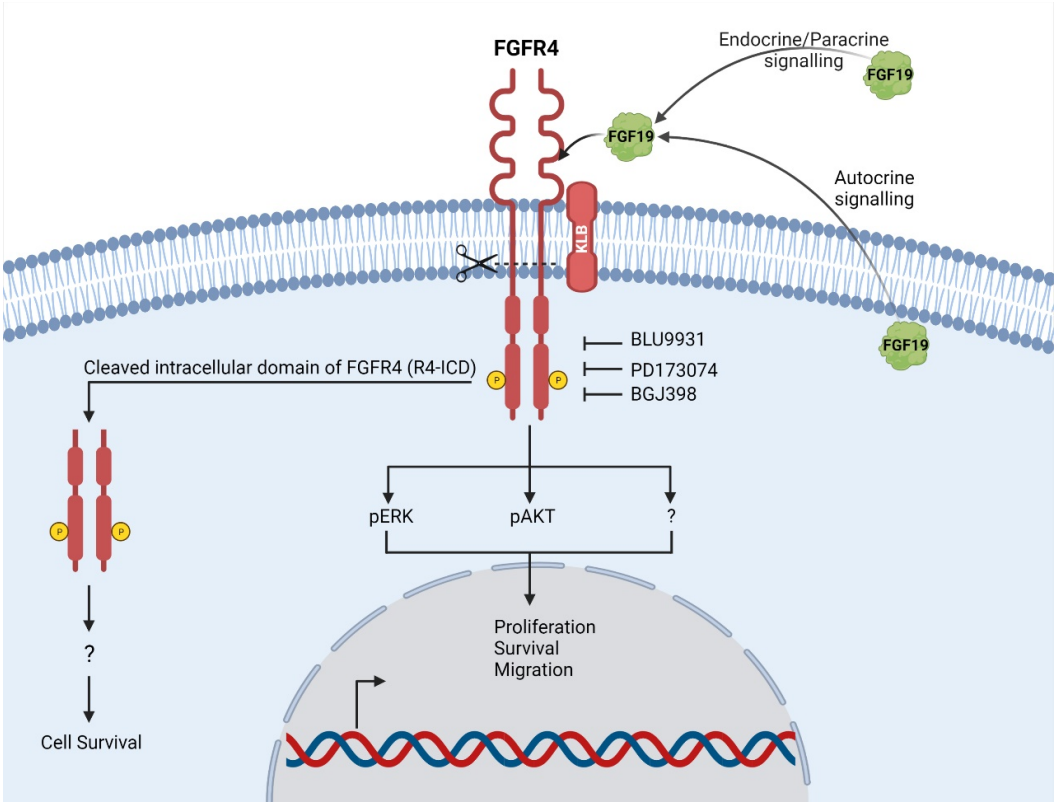


Figure 2: FGFR4 and R4-ICD mediate cancer progression in CCA.

FGFR4 can be activated via FGF19 ligand, leading to activation of downstream signaling pathways including ERK and AKT that regulate cellular proliferation and survival of CCA cells. R4-ICD, an intracellular domain of FGFR4, lead to an increase in CCA cell survival, however the underlying mechanisms is still unknown (Image created with BioRender).

Hypothesis and Objective:

In this study, we hypothesized that FGFR4 and R4-ICD play a role in cell proliferation, cell survival and metastasis in cholangiocarcinoma (CCA).

Specific aims:

Specific aim 1: Determine the efficacy of FGFR4-selective and pan-FGFR inhibitor in CCA cell lines.

To test panel of FGFR4-selective and pan-FGFR inhibitors

- Perform proliferation assay
- Perform caspase assay
- Perform migration assay

Specific aim 2: Generate FGFR4/R4-ICD expressing cells to determine the role of FGFR4/R4-ICD in CCA using *in vitro* and *in vivo* assay.

- Clone R4-ICD into pBABE-zeo vector with mammalian antibiotic resistance gene for zeocin.
- Determine the role of R4-ICD in cell proliferation, cell survival, and cell migration.
- Evaluate the downstream signaling of both FGFR4 and R4-ICD through immunoblotting.
- Determine the efficacy of FGFR4-selective inhibitors in R4-ICD expressing cells.

CHAPTER 2: MATERIALS AND METHODS

Cell culture and reagents

Human cholangiocarcinoma cell lines, HuCCT-1, KMCH, and Mz-ChA-1 were kindly provided from Dr. Gregory Gores. The cells were cultured in complete growth media containing Dulbecco's Modified Eagle Medium (DMEM) (Thermo Fisher, Waltham, MA) medium with 10% fetal bovine serum (FBS) (R&D system, Minneapolis, MN), 50 mg/mL G418, and 0.5 mg/mL insulin. The cells were incubated at 37°C with 5% carbon dioxide (CO_2). Cells were passaged at 80-90% confluency using a 0.25% Trypsin-EDTA (Thermo Fisher) and cultured in tissue culture plates.

Development of R4-ICD-expressing cells

Selection of antibiotic concentration

A kill curve was generated to determine the optimal concentration of zeocin (Invitrogen, #R25001, Carlsbad, CA), a selection marker antibiotic in both, HuCCT-1 parental cells and KMCH shFGFR4 cells. The cells were seeded into a 6-well plate with a seeding density of 3 X 10^3 cells per well. A range (0, 100, 300, 500, 800, 1000 µg/mL) of zeocin concentration was prepared in complete growth media. The selection media was changed after every 2-3 days, and the cells were monitored for 10 days to observe complete cell death.

Standard protocol for restriction digestion

Cloning vector pBABE-Zeo (Addgene, #1766) was used for cloning R4-ICD into HuCCT-1 and KMCH shFGFR4 cells. For restriction digests, 1 µg of plasmid was used using CutSmart buffer (New England Biolabs, Ipswich, MA), 1 µL of restriction enzymes

(New England Biolabs) in a 50 µL reaction. The reaction was incubated at 37°C for 60 minutes. After 30 minutes of incubation additional 1 µL of restriction enzyme was added. Furthermore, the reaction was heat inactivated at 65-80°C temperature for 20 minutes.

Standard ligation protocol

Sticky and blunt-end DNA ligation was performed using 1:3 ratio of Vector:Insert. The vector and insert were incubated with 10X T4 DNA ligase buffer and 1 µL of T4 DNA ligase enzyme (New England Biolabs, #M0202S). The ligation reaction (20 µL) mix was incubated at room temperature for 30 minutes and the enzyme was heat inactivated at 65°C for 15 minutes.

Standard transformation protocol

After the completion of ligation, One Shot™ TOP10 Chemically Competent *E. coli* (ThermoFisher,Scientific # 404003, Waltham, MA) were thawed on ice, 3.33 µL of ligation reaction was added, and the tubes were incubated on ice for 30 minutes. After incubation, the competent cells were subjected to heat shock at 42°C for 30 seconds. Next, the cells were placed on ice for a minute and 250 µL of super optimal broth (SOC) media (Invitrogen, #15544-034) was added. The competent cells in SOC media were incubated at 37°C while shaking for 1 hour. Agar plates containing 50 of µg/mL ampicillin were prewarmed at 37°C. The SOC media containing competent cells (75 µL) was plated on the agar plates and incubated overnight at 37°C. After incubation, isolated single colonies were picked using sterile p10 tips and incubated overnight with 3 mL of ampicillin (1:1000) containing lysogeny broth (LB broth). Lastly, the plasmid was isolated using Monarch Plasmid Miniprep Kit (NEW ENGLAND Biolabs, #T1010S) according to manufacturer's protocol.

Cloning strategy

The construct containing FGFR4 (pmscvFL-FGFR4-deltaPA) was generously provided by Dr. Javed Khan (National Cancer Institute) and previously used to generate an R4-ICD construct pcDNA.FLAG.HA.R4.ICD.WT. However, the selection marker needed to be altered to zeocin (pBABE-zeo). To generate a construct with R4-ICD, we first cloned in a polyA signal into the backbone plasmid (pBABE-zeo). To amplify the bGH polyA tail and to introduce the NotI restriction enzyme site (needed for subsequent cloning of R4-ICD), polymerase chain reaction (PCR) was performed. The PCR product was run on a 1% agarose gel to ensure a single band of the appropriate size measured against a 100 bp ladder. Later, the bGH insert was gel purified using QIAquick Gel Extraction (QIAGEN, #28704, Hilden Germany). The bGH polyA tail was inserted into the TOPO TA PCR 2.1, and the vector was transformed into chemical competent E. coli cells using the standard transformation protocol as mentioned above. The competent E. coli cell suspension was seeded into the agar plate containing ampicillin and grown overnight at 37 °C. After incubation, the isolated colonies were picked and cultured overnight into LB broth medium containing 50 of µg/mL ampicillin. The plasmid was isolated using the Monarch Plasmid Miniprep Kit. The diagnostic digest was performed with the TOPO 2.1 TA bGH vector using EcoRI restriction enzyme and run on a 1.5% of agarose gel. The positive vector had a band at 3913 bp and 269 bp. The 269 bp band was cut from the gel and gel purified. Later, the recipient vector (pBABE-Zeo) was cut with EcoRI restriction enzyme and treated with shrimp alkaline phosphatase (rSAP). The gel purified bGH poly A from TOPO TA PCR 2.1 vector was introduced into linearized pBABE-Zeo vector. The standard transformation protocol was followed to grow the transformed E. coli cells and to isolate plasmids. Lastly, pBABE-Zeo.bGH was digested with SnaBI and XhoI and rSAP. The R4-ICD fragment was cut from pcDNA.FLAG.HA.R4.ICD.WT using XhoI and NruI (4683 bp and 2050 bp) and the 2050 bp DNA inserted into pBABE-Zeo.bGH using T4

ligase and plasmid was isolated via standard transformation. Positive clones were identified using restriction digestion analysis with SacII and SnaB1. The plasmid size was tested for expected bands at 5963 bp and 1147 bp on 1% agarose gel. The positive clones were identified using the restriction digestion analysis and sequencing with primers flanking the insert and one within the insert (pBABE 5', pBABE 3' and pCDNA FLAG HA Fwd).

Stable transfection protocol

Stable transfection was performed in HuCCT-1 parental and KMCH shFGFR4 cells. Cells were seeded into a 6-well plate with a seeding density of 3 X 10^5 cells/well. After 24 hours, the linearized plasmid (pBABE-Zeo.bGH.R4-ICD) was induced using lipofectamine 3000 (Invitrogen, #100022050). The plasmid was linearized using ScaI at 37°C for 1 hour and heat inactivated at 80 °C for 20 minutes. To prepare the lipofectamine reagents, first, 125 µL of Opti-MEM media was mixed with 3.75 µL of lipofectamine reagent. Secondly, 125 µL of Opti-MEM media, 10 µL of P300 and 1 µg linearized DNA (50 µL) were mixed. Both of these reagents were mixed and incubated at room temperature for 15 minutes. After the incubation, the DNA: lipofectamine mixture was added into each well. Cells without DNA (lipofectamine only), empty vector (pBABE-Zeo) and R4-ICD plasmid (pBABE-Zeo.bGH.R4-ICD) were considered for all of the transfection experiments. Transfection media was removed after 24 hours, and cells were subjected to zeocin antibiotic containing media (800 µg/mL zeocin concentration for HuCCT-1 cells and 500 µg/mL zeocin concentration for KMCH shFGFR4 cells). Once cells reached 85-90% confluency, they were transferred into a 10 cm dish using complete growth media (without antibiotic), which was replaced with the selection media after 24 hours. Individual colonies were picked and seeded into a 96 well plate. Positive clones were determined

via immunoblotting using FGFR4 antibody (Cell Signaling TECHNOLOGY, #8562S, Danvers, MA).

Cell Proliferation Assay

Cell proliferation was determined by methylene blue stained based assay [111]. Cells were seeded at 2 X 10^3 density per well into a 96 well plate overnight. Cells were treated with the required treatment (FGFR4-selective inhibitor or pan-FGFR inhibitor) and maintained at 37°C with 5% CO_2. Each inhibitor was used at a specific concentration as follow: FGFR4 selective inhibitors including (i) BLU9931 (3 μM), (MiliporeSigma, #L1003) (ii) Roblitinib (3 μM), (Selleck Chemicals, #S8548, Houston, TX) (iii) H3B-6527 (1 μM), (Selleck Chemicals, #S8675) and (iv) Blu554 (5 μM) (Selleck Chemicals, #8503) as well as pan-FGFR inhibitors including (i) PD173074 (3 μM) (Selleck Chemicals, #S1264) and (ii) BGJ398 (5 μM) (Selleck Chemicals, #S2183). For each treatment (+/- inhibitor), eight technical replicates were considered. In control group, cells were treated with the vehicle (DMSO) without any inhibitors. After the treatment, cells were washed with 1X PBS (pH 7.4) and fixed with 10% formalin at 0, 24, 48,72 and 96 hours. Next, the fixed cells were stained using 80 μM 1% methylene solution prepared in 0.01 M borate buffer for 2 hours. After staining, each well was washed with 150 μM of 0.01 M of borate buffer (pH 8.5), for four times. Once excess dye was washed off, the remining stained was eluted for 30 minutes using 100 μM of elution buffer containing 95% v/v ethanol and 0.1 M HCL. The absorbance was measured at 650 nm.

Cell Survival Assays

Caspase assay

Apo-ONE Homogeneous Caspase-Glo 3/7 assay kit (Promega Corporation, Madison, WI) was used to determine caspase 3 and caspase 7 activity. The caspase 3/7 assay kit is based on the detection cleaved substrate rate by monitoring the rate of rhodamine 110 using a specific excitation (475 nm) and emission wavelength (525 nm). HuCCT-1, HuCCT-1 clones, KMCH and KMCH shFGFR4 cells were seeded in 96 well plates with a seeding density of 1 X 10^5 cells/well at 37°C overnight. The next day, cells were treated with vehicle and/or specific inhibitors such as 0.003 μM to 30 μM concentration of BLU9931 and 1 μM concentration of Akt Inhibitor VIII, (Santa Cruz, sc-202048) for 24 hours. For the final 6 hours of treatment, TNF-related apoptosis-inducing ligand (TRAIL) (R&D system #375-7L) was added (positive cell death control). For each treatment four technical replicates were considered. Following treatment, the Apo-ONE Caspase assay was performed according to manufacturer's protocol. Briefly, the Caspase Substrate Z-DEVD-R110 was prepared using a 1:100 ratio in the Apo-ONE® Homogeneous Caspase-3/7 substrate and Buffer (Promega, #G777A and #G778A). After completion of the treatment, 170 μL of media was removed from each well and 30 μL of prepared caspase reagent was added. The plate was wrapped in an aluminum foil and stored in dark for 1.5 hours at room temperature. Once the incubation was over, the fluorescence was measured at the optimal excitation wavelength of 475 nm and emission wavelength of 525 nm.

Apoptotic nuclei assay

The detection of dead cells using 4′,6-diamidino-2-phenylindole (DAPI) was performed as follows. Cells were seeded in a 24 well plate with a seeding density of 5 X 10^5 cells per well. The cells were incubated overnight at 37°C with 5% CO_2. Cell death

was indued using TRAIL for 6 hours. After incubation, the cells were stained by adding 1 µL of a 5 µg/mL stock of DAPI, incubated for 5 minutes at 37°C and imaged using fluorescence microscope. Positive apoptotic nuclei showed condensed or fragmented nuclear staining and reported as a percentage of total cells per field.

Migration assay

Transwell inserts with 6.5 mm diameter of transwell with 8.0 µm pore size (MilliPORE SiGMa, #CLS3422, Burlington, MA) was used to determine cell migration. In the upper chamber, cells with a seeding density of 1 X 10^4 cells / transwell were seeded using 100 µL of serum free media. The inhibitor treatment was given in the upper chamber with serum free media. The bottom chamber was filled with 600 µL serum containing media (10% FBS), which touched the bottom of the transwell membrane. For each treatment, three technical replicates were considered. The plate was incubated at 37°C with 5% CO_2 for 8-24 hours (HuCCT-1 cells) and 24 hours (KMCH cells). After the incubation, the inserts were washed with PBS and excess media was removed using cotton tipped applicator. The insert was fixed and stained using KWIK-DIFF stain kit (Thermo Fisher Scientific, catalog 9990701). The inserts were dipped into each solution for 20 times and washed with DI water in between. The inserts were air-dried at room temperature and counted under an inverted microscope.

Immunoblotting

Cells were collected at 70-80% confluency and washed using PBS. After removing PBS, the cells were lysed with lysis buffer containing 50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 1 mM α-phenylmethylsulfonyl fluoride, 1 mM Na3VO4, 100 mM NaF, protease inhibitor (Roche) and 1% Triton X-100 (pH 7.4). The cells were scraped

in lysis buffer and transferred into 1.5 mL tubes and kept on ice. The cell lysates were centrifuged at 13,000 rpm for 15 minutes at 4°C and the supernatant was collected into sterile 1.5 mL tubes. The protein was quantified using Bradford reagent (200 µL of Bradford reagent with 6 µL of protein). The protein quantification was performed against the standard curve using Nanodrop. After quantification, 50 µg of protein (for KMCH and KMCH sHFGFR4 cell lysate) and 25 µg of protein (for HuCCT-1 and HuCCT-1 clone lysate) was aliquoted. The samples were diluted using lysis buffer and 5X laemmili with Dithiothreitol (DTT) to make total volume of 35 µL. Next, the samples were boiled at 95°C for five minutes. The samples were loaded into 10% SAD-PAGE gel (ThermoFisher Scientific, #XP00100BOX) and run at 110 V for 1.5 hours. Next the protein was transferred to nitrocellulose membrane, blocked using 5% non-fat dried milk mixed in 1X TBST, and incubated overnight with primary antibody (1:1000). Next, the blot was washed with 1X TBST for three times, 20 minutes each followed by addition of secondary antibody. The secondary antibody was washed by 1X TBST for three times, 20 minutes each. The blot was developed with SuperSignal West Pico PLUS Chemiluminescent substrate (1:1 ratio) (Thermo SCIENTIFIC, #34580), for 1 minute and developed with the film. The specific protein was verified against the molecular weight marker (BIO-RAD, #161-0374, Hercules, CA). The FGFR4 antibody (Cell Signaling TECHNOLOGY, #8562S, Danvers, MA) recognizes the intracellular domain of the receptor protein. Other antibodies used were pAKT, Thr-308 (Cell Signaling TECHNOLOGY, #2965S, tAKT (Cell Signaling TECHNOLOGY, #4691S), pERK (Cell Signaling TECHNOLOGY, #9101S), tERK (Cell Signaling TECHNOLOGY, #4695S), pSTAT3 (Cell Signaling TECHNOLOGY, #9134S), tSTAT3 (Cell Signaling TECHNOLOGY, #12640).

CHAPTER 3: RESULTS

FGFR4 is overexpressed in many cancer types including breast cancer, HCC, and colorectal cancer, and previous studies have shown that the overexpression of FGFR4 plays a crucial role in cancer progression [73]. This project focused on determining the expression of FGFR4 in cholangiocarcinoma. Previously, a panel of CCA tumor samples were analyzed for FGFR4 expression (Phillips, *et al., in preparation)*. This analysis showed that the majority of the CCA patient samples over-expressed FGFR4. In addition to the FGFR4, all of the tumor samples had higher expression of fibroblast growth factor receptor 4 intracellular domain (R4-ICD), a lower molecular weight (45-50 kDa) cleaved product of FGFR4. To investigate the role of FGFR4, the expression of FGFR4 in CCA cell lines was first evaluated. Immunoblot analysis of the panel of CCA cell lines showed that KMCH had highest expression of FGFR4 while Mz-ChA-1 had moderate level of FGFR4 **(Figure 3A)**. However, HuCCT-1 had no detectable amount of FGFR4 on immunoblot analysis **(Figure 3A)**. The KMCH cells are human cell lines that are derived from a patient with a tumor showing histologic features of combined HCC and CCA (in culture, KMCH cells consistently display a CCA-type phenotype). The KMCH cells showed highest expression of FGFR4. The Mz-ChA-1 is a malignant biliary epithelial cell line and showed moderate amount of FGFR4 expression on immunoblot. Lastly, HuCCT-1, derived from a patient with intrahepatic CCA (iCCA), did not show any expression of endogenous FGFR4.

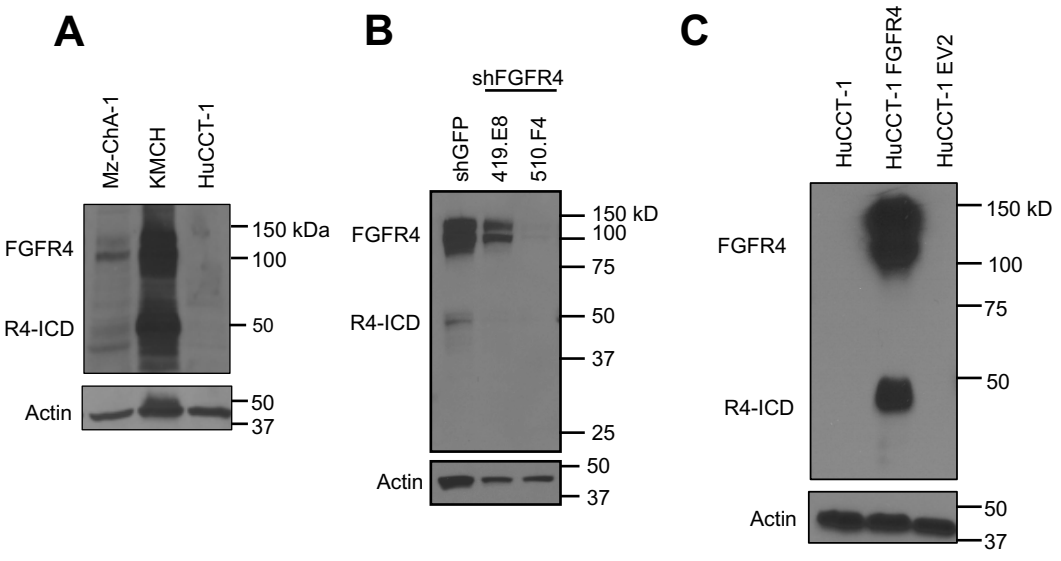


Figure 3: FGFR4 expression in CCA cells

(A) FGFR4 protein expression in three CCA parental cell lines KMCH, Mz-ChA-1, and HuCCT-1. **(B)** Two shRNAs targeting FGFR4 were tested (shRNA 419 and 510) in KMCH cells, compared to shRNA against GFP (knockdown control). **(C)** Parental HuCCT-1 cells were modified to stably express FGFR4, HuCCT-1 FGFR4 (stable clone of FGFR4), or HuCCT-1 EV2 (empty vector control).

Role of FGFR4 in CCA cell proliferation, survival and migration

To understand the role of FGFR4 in KMCH cells, RNA interference (short hairpin RNA) based silencing was used to knockdown FGFR4 expression. Immunoblot analysis results indicated the absence of FGFR4 as well as its cleaved product R4-ICD in KMCH shFGFR4 510.F4 cells **(Figure 3B)** (the FGFR4 antibody recognizes the c-terminus of the protein). The shFGFR4 510.F4 cells were used throughout this study as they show strong knockdown of FGFR4. Next, FGFR4 was stably cloned in HuCCT-1 cells, which inherently do not express FGFR4 **(Figure 3C)**. Stable expression of FGFR4 in the cloned HuCCT-1 FGFR4 cells was detected via western blot analysis **(Figure 3C).** Moreover, the empty

vector cells HuCCT-1 EV2 were transfected with an empty puromycin plasmid, which did not show expression of FGFR4 or R4-ICD, as predicted, and were used as control **(Figure 3C)**. To determine the role of FGFR4 in cell proliferation, a methylene blue proliferation assay was performed. HuCCT-1 FGFR4 cells showed similar cellular proliferation as HuCCT-1 parental cells (control) at 24 hours. However, HuCCT-1 FGFR4 cells showed significantly increased cell proliferation at 48, 72 and 96 hours compared to HuCCT-1 parental cells (control) ($p < 0.001$) **(Figure 4A)**. Similarly, HuCCT-1 FGFR4 cells showed significantly higher proliferation compared to HuCCT-1 EV2 cells at 72 and 96 hours ($p < 0.001$) **(Figure 4B)**. Overall, the HuCCT-1 cell line provides a useful cell model to modulate FGFR4 levels and increased FGFR4 promoted increased proliferation.

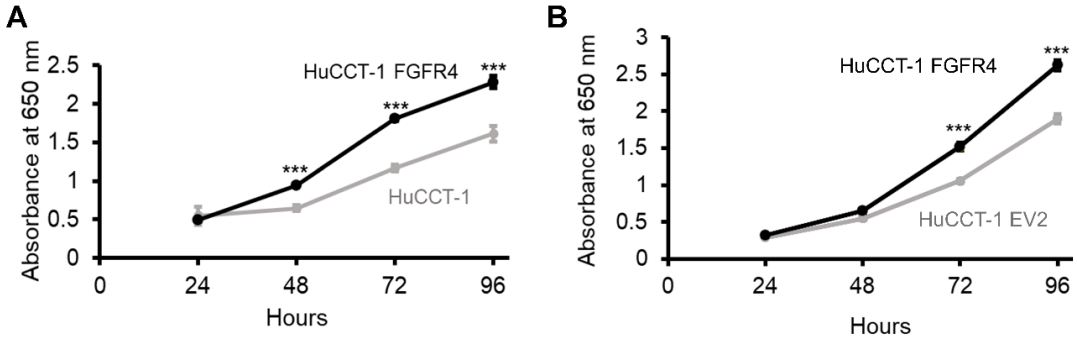


Figure 4: FGFR4 increases CCA cell proliferation

Comparison of cellular proliferation between **(A)** HuCCT-1 FGFR4 clones and HuCCT-1 parental cells, and **(B)** HuCCT-1 FGFR4 clones with empty vector HuCCT-1 EV2 cells from 0 to 96 hours (*** $p < 0.001$). Each experiment was performed two times, n=8.

The role of FGFR4 in cell survival was investigated by determining caspase 3/7 activity in HuCCT-1 EV2 and HUCCT-1 FGFR4 cells with or without TRAIL sensitization **(Figure 5A)**. Caspase activity was used as a marker for apoptosis. It was observed that HuCCT-1 EV2 cells were more sensitive to TRAIL-induced apoptosis compared to HuCCT-1 cells expressing FGFR4. TRAIL-treated HuCCT-1 EV2 cells showed significantly higher caspase 3/7 activity ($p < 0.01$) compared to the HUCCT-1 FGFR4 cells

(Figure 5A). The magnitude of protection was small, so we extended our investigation to test the effect of knockdown of FGFR4 from KMCH cells. To determine cell survival in KMCH cells, DAPI staining (for apoptotic nuclei) was performed following 6 hours of TRAIL sensitization. Cells which lack FGFR4, KMCH shFGFR4, were sensitive to TRAIL induced apoptosis ($p < 0.001$) while the KMCH shGFP cells were resistant **(Figure 5B)**. In general, HuCCT-1 cells (which endogenously lack FGFR4) are sensitive to TRAIL-induced apoptosis while the KMCH cells (FGFR4 expressing) are more resitant to TRAIL-induced cell death. These data showed that FGFR4 expression protected HuCCT-1 and KMCH cells from TRAIL induced apoptosis.

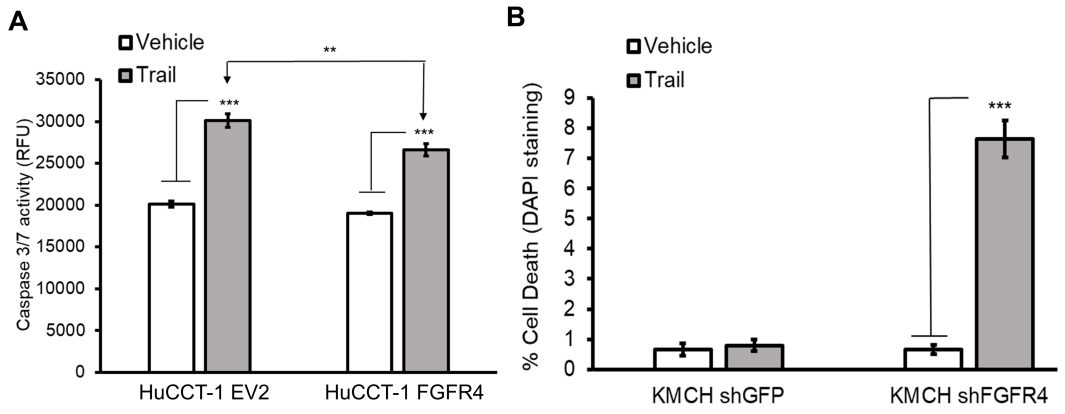


Figure 5: FGFR4 protects against TRAIL-induced cell death.

(A) Caspase 3/7 activity in HuCCT-1 EV2 (lacking FGFR4), and HuCCT-1 FGFR4 cells with or without 6 hours of TRAIL treatment (vehicle: white bar, TRAIL: gray bar) (**$p < 0.01$, ***$p < 0.001$). **(B)** DAPI staining for apoptotic nuclei was performed with KMCH shGFP (expressing FGFR4) and KMCH shFGFR4 cells with 6 hours of TRAIL treatment (***$p < 0.001$). **A** was performed three times, n=4, and **B** was performed once, n=3.

Lastly, a transwell cell migration assay was performed to examine the role of FGFR4 in cell migration. HuCCT-1 FGFR4 cells showed significantly increased cell migration compared to HuCCT-1 parental cells ($p < 0.001$) after 8 hours **(Figure 6A)**. However, surprisingly HuCCT-1 FGFR4 cells showed no difference in cell migration

compared to HuCCT-1 EV2 cells after 24 hours **(Figure 6B)**. In each replicate, we found that the number of migrated cells could vary substantially, so results were always compared within the same experiment (e.g., more HuCCT-1 parental cells migrated in the experiment depicted in panel B compared to the experiment in panel A, despite using the same HuCCT-1 parental cell line in both). We discuss below the potential effect of clonal variability, on the migration phenotype (and others) and sought additional experimental evidence that FGFR4 regulated migration. FGFR4-knockdown KMCH cells (KMCH shFGFR4) cells showed significantly reduced cell migration compared to parental KMCH cells ($p < 0.01$) **(Figure 6C)**. Overall, FGFR4 appears to increase cell migration in KMCH and HuCCT-1 cells.

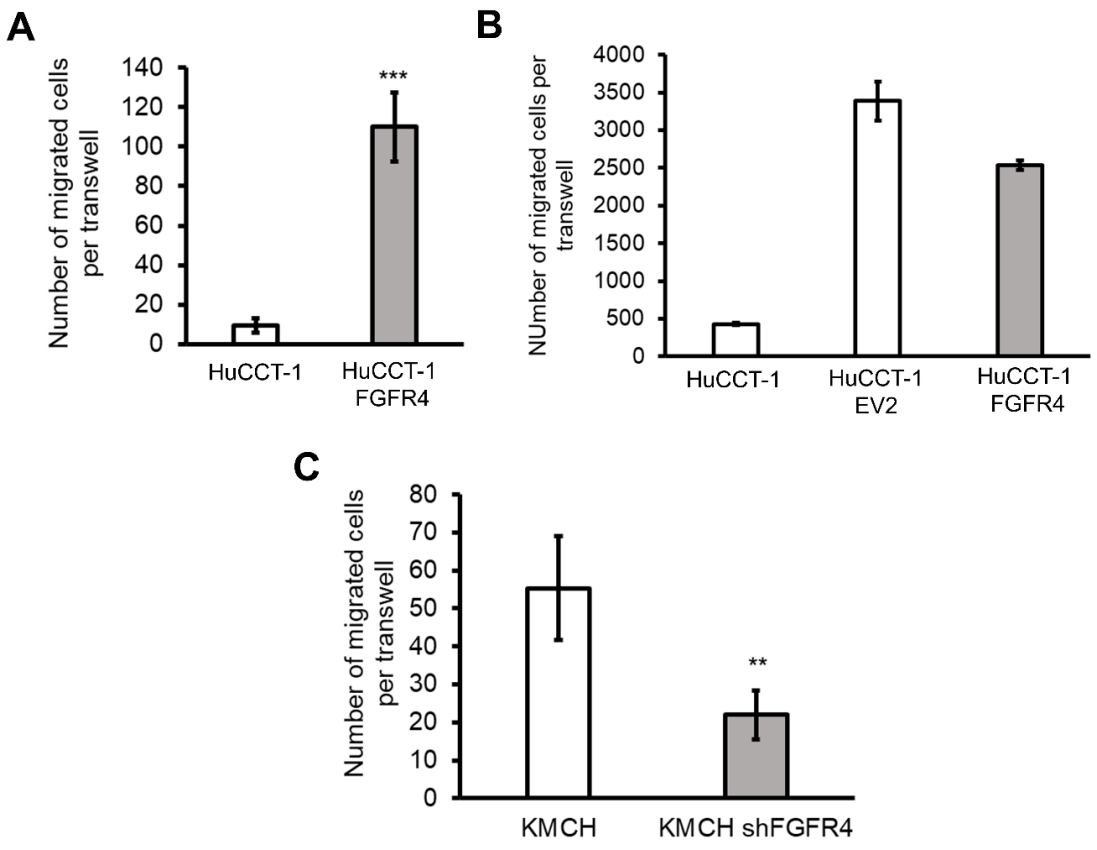


Figure 6: FGFR4 expression alters CCA cell migration in vitro.

(A) Transwell cell migration assay indicates migration of parental HuCCT-1 and HuCCT-1 FGFR4 cells toward serum containing media after 8 hours. (**B**) Transwell cell migration assay indicates migration of parental HuCCT-1, HuCCT-1 EV2 and HuCCT-1 FGFR4 cells toward serum containing media after 24 hours. **(C)** Transwell migration assay indicating the role of FGFR4 in KMCH cell migration after 24 hours of treatment (**$p < 0.01$, ***$p < 0.001$). **A**, **B** and **C** were performed two times, n=3.

Determining the efficacy of small molecule inhibitors in CCA cell proliferation

To target FGFR4 in CCA cell lines, small molecule inhibitors that selectively target FGFR4 (BLU9931, Roblitinib, and H3B6527) or pan-FGFR inhibitors that target all FGFR family members (BGJ398, PD173074) were used. The efficacy of the FGFR4-selective and pan-FGFR inhibitors was determined using methylene blue cell proliferation assay. KMCH cell proliferation was significantly reduced with treatment of 3 μM of BLU9931 at 24 ($p < 0.05$), 48 ($p < 0.05$), 72 ($p < 0.001$) and 96 hours ($p < 0.001$) **(Figure 7A)**. However, two other FGFR4-selective inhibitors, Roblitinib (1 μM and 3 μM) and H3B6527 (1 μM), did not reduce KMCH cell proliferation compared to control **(Figure 7B-D)**. Pan-FGFR inhibitors BGJ398 and PD173074 significantly reduced KMCH cell proliferation at 72 and 96 hours ($p < 0.001$) **(Figure 7E-F)**. These results indicated that among above mentioned inhibitors, FGFR4-selective inhibitor BLU9931 as well as the two pan-FGFR4 inhibitors, BGJ398 and PD173074, can effectively inhibit KMCH cell proliferation and therefore were selected for use in further experiments.

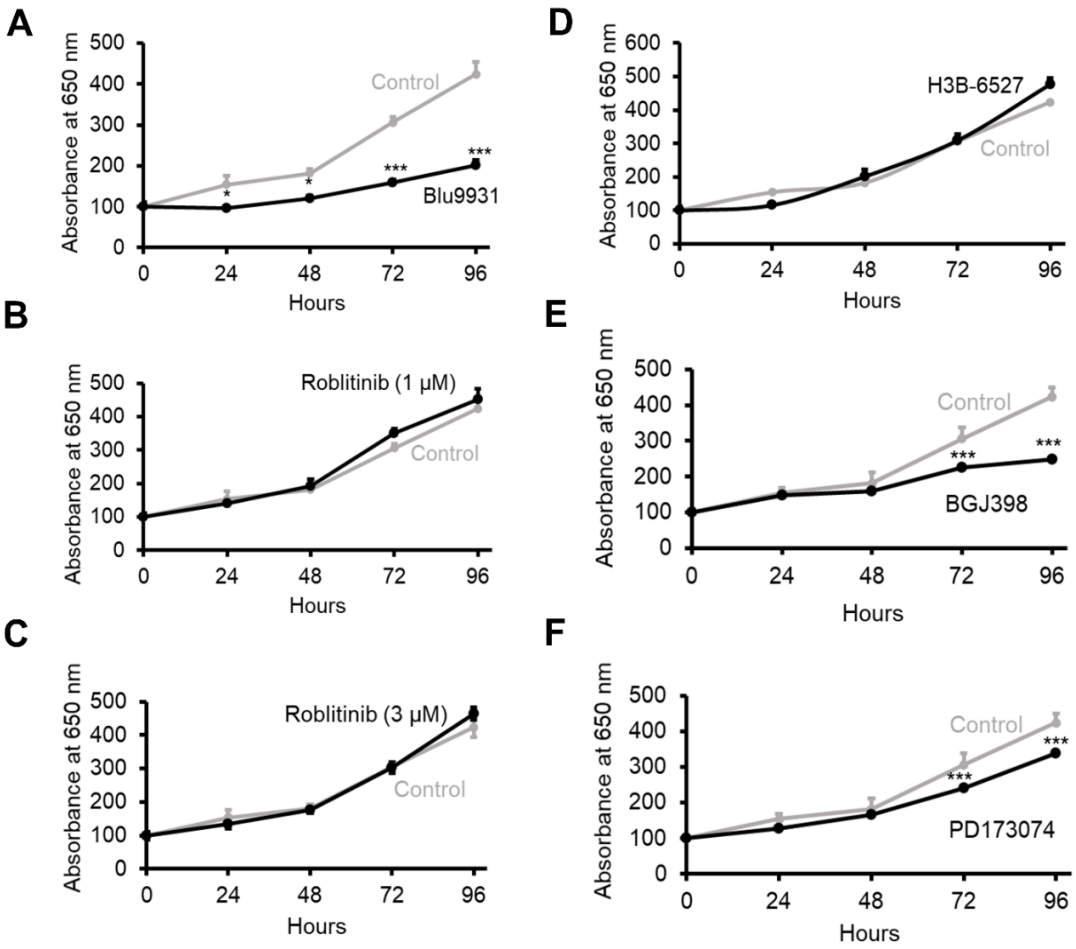


Figure 7: Pan-FGFR4 inhibitors and FGFR4-selective inhibitor BLU9931 reduced KMCH cell proliferation.

Comparison of cellular proliferation between control (DMSO) and FGFR4-selective inhibitors **(A)** BLU9931 (3 μM), **(B)** Roblinitib (1 μM) **(C)** Roblinitib (3 μM) (**D)** H3B-6527 (1 μM) from 0 to 96 hours. Comparison of cellular proliferation between control (DMSO) and pan-FGFR inhibitors **(E)** BGJ398 (5 μM), and **(F)** PD173074 (5 μM) from 0 to 96 hours (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). Each experiment was performed three times, n=8.

HuCCT-1 parental and HuCCT-1 FGFR4 cells treated with BLU9931 showed significantly reduced cell proliferation at 72 ($p < 0.001$) and 96 hours ($p < 0.001$) **(Figure 8A & 8D)**. Pan-FGFR inhibitors, PD173074 and BGJ398, significantly reduced HuCCT-1 and HuCCT-1 FGFR4 proliferation at 48 ($p < 0.001$ for HuCCT-1), 72 ($p < 0.001$) and 96 hours ($p < 0.001$) **(Figure 8B, 8C, 8E, 8F)**. These results indicate that the FGFR4-selective inhibitor, BLU9931, may be exhibiting off-target effects as the FGFR4 non-expressing HuCCT-1 cells had a reduction in proliferation.

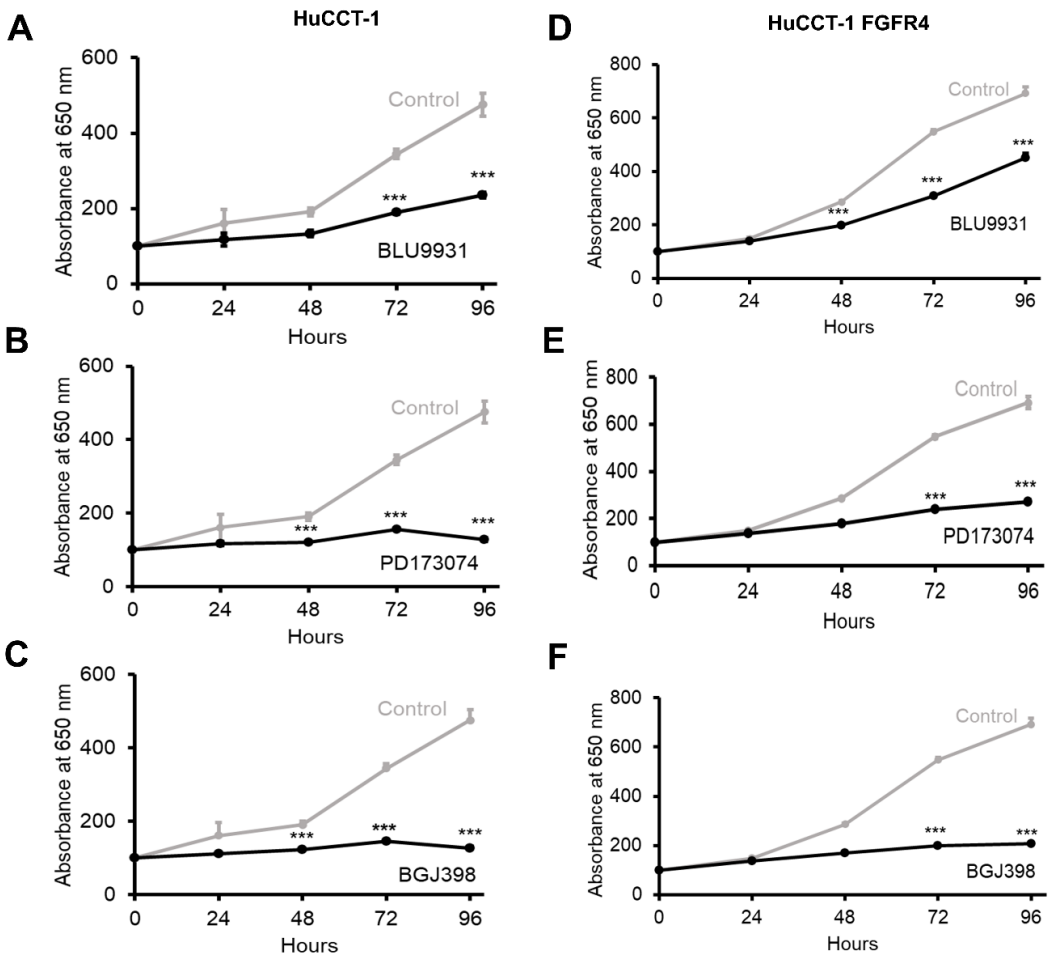


Figure 8: FGFR4-selective and pan-FGFR inhibitors reduced proliferation in parental HuCCT-1 and HuCCT-1 FGFR4 cells.

Comparison of HuCCT-1 proliferation between control (DMSO) and FGFR4-selective inhibitors **(A)** BLU9931 (3 μM), as well as pan-FGFR4 inhibitors **(B)** PD173074 (5 μM) and **(C)** BGJ398 (5 μM) from 0 to 96 hours. Comparison of HuCCT-1 FGFR4 proliferation between control (DMSO) and FGFR4-selective inhibitors **(D)** BLU9931 (3 μM), as well as pan-FGFR4 inhibitors **(E)** PD173074 (5 μM) and **(F)** BGJ398 (5 μM) from 0 to 96 hours (***$p < 0.001$). Each experiment was performed twice, n=8.

Moreover, BLU9931, PD173074 and BGJ398 showed significantly reduced proliferation of Mz-ChA-1 cells at 48, 72 ($p < 0.001$) and 96 hours ($p < 0.001$), which expressed moderate levels of FGFR4 **(Figure 9A-C)**. Overall, FGFR4-selective and pan-FGFR small molecule inhibitors reduced proliferation, even in cells lacking FGFR4. This

effect in HuCCT-1 cells was unexpected, but consistent among the three inhibitors used (BLU9931, BGJ398, and PD173074). These data indicate that the FGFR4-selective inhibitor and pan-FGFR inhibitors may regulate pathways other than FGFR4. Previous data (not shown) reveal that KMCH cells express FGFR1, FGFR3 and FGFR4 and HuCCT-1 cells express FGFR1 (expression of FGFR2 and FGFR3 were not tested). Combined with data from Figure 4, these experiments suggest that FGFR4, and potentially an additional unidentified kinase or target, contribute to cholangiocarcinoma proliferation.

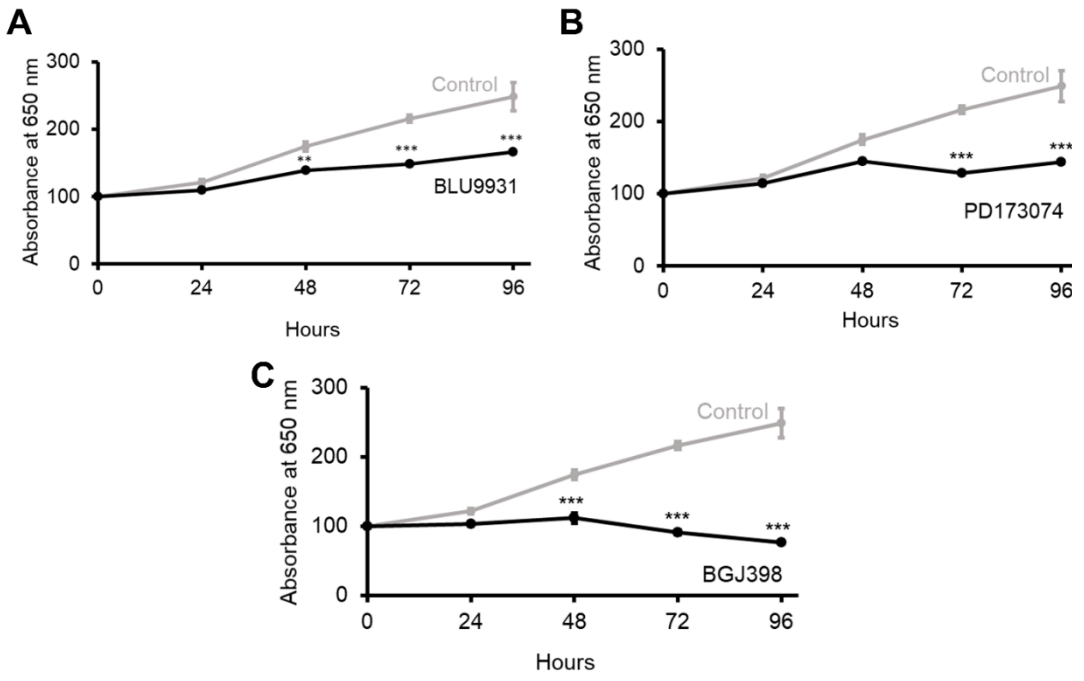


Figure 9: FGFR4-selective and pan-FGFR inhibitors reduced the proliferation of Mz-ChA-1 cells.

Comparison of Mz-ChA-1 proliferation between control (DMSO) and FGFR4-selective inhibitors **(A)** BLU9931 (3 μM), as well as pan-FGFR4 inhibitors **(B)** PD173074 (5 μM) and **(C)** BGJ398 (5 μM) from 0 to 96 hours (**$p < 0.01$, ***$p < 0.001$). Each experiment was performed twice, n=8.

Effect of small molecule inhibitors in CCA cell apoptosis

To determine the effect of FGFR4-selective inhibitor BLU9931 in KMCH cell survival, caspase 3/7 activity was used as a marker of cell death (apoptosis). The caspase 3/7 activity in KMCH cells treated with BLU9931 at 0.003 μM, 0.03 μM, 0.3 μM, 3 μM and 30 μM was measured and compared with vehicle (DMSO). The FGFR4-selective inhibitor BLU9931 significantly sensitized KMCH cells at 3-30 μM in the presence of TRAIL (p < 0.001) **(Figure 10)**. BLU9931 at 30 μM concentration also caused higher caspase 3/7 activity without TRAIL, indicating cytotoxic effects of BLU9931 at 30 μM **(Figure 10)**.

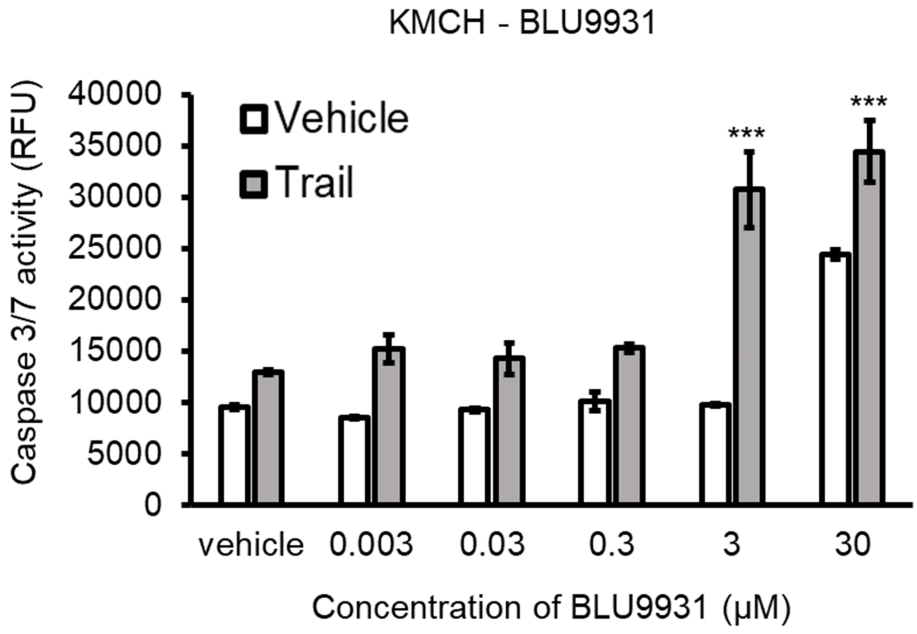


Figure 10: FGFR4-selective inhibitor sensitizes cells to TRAIL-induced cell death parental KMCH cells.

Caspase 3/7 activity was measured in KMCH cells treated with FGFR4-selective inhibitor BLU9931 at 0.003 μM, 0.03 μM, 0.3 μM, 3 μM and 30 μM concentration (24 hours), with or without 6 hours of TRAIL treatment (***p < 0.001). This experiment was performed twice, n=4.

Additional pan-FGFR inhibitors were tested for their efficacy on KMCH cell apoptosis

To determine the effect of pan-FGFR inhibitors Futibatinib, Pemigatinib and LY2874455 in KMCH cell survival, caspase 3/7 activity was measured. The inhibitors were considered at 0.1 μM, 1 μM and 10 μM concentration and caspase activity was measured after 6 hours **(Figure 11)** and 24 hours **(Figure 12)** of treatment with and without TRAIL. After 6 Hours of treatment, Futibatinib showed slightly increased caspase 3/7 activity at 10 μM ($p < 0.001$) in KMCH cells **(Figure 11A)**. Pemigatinib (at 0.1 μM, 1 μM and 10 μM concentrations) showed no difference in caspase activity compared to vehicle, with or without TRAIL treatment **(Figure 11B)**. LY2874455 had significantly increased ($p < 0.001$) caspase 3/7 activity in KMCH cells after 6 hours of TRAIL sensitization **(Figure 11C).**

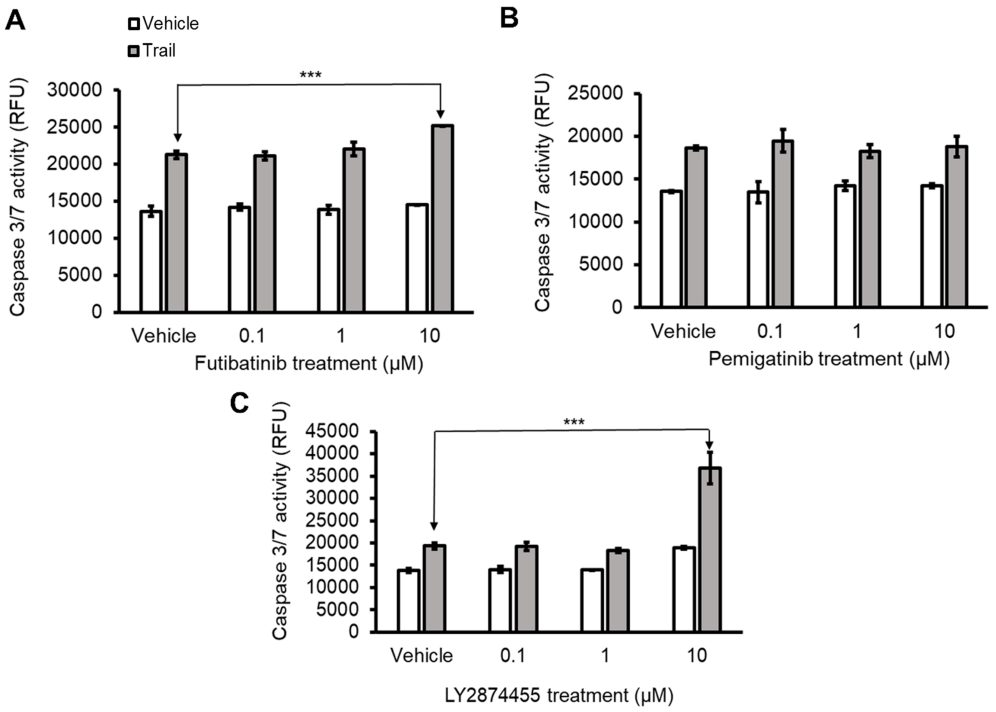


Figure 11: Additional pan-FGFR inhibitors were tested for efficacy using caspase 3/7 activity assay in parental KMCH cells after 6 hours of treatment.

The efficacy of pan-FGFR inhibitors Futibatinib (A), Pemigatinib (B), and LY2874455 (C) to promote caspase 3/7 activity at 0.1 μM, 1 μM and 10 μM concentration during 6 hours of treatment, with or without 6 hours of TRAIL treatment in KMCH parental cells (***$p < 0.001$) n=4.

After 24 hours of treatment, Futibatinib showed no apoptosis sensitization at 0.1 and 1 μM of concentration in the presence of TRAIL while 10 μM of Futibatinib with or without TRAIL increased the caspase 3/7 activity after 24 hours, indicating toxicity **(Figure 12A)**. Pemigatinib had no effect on the caspase 3/7 activity of KMCH cells at 0.1 and 1 μM of concentration. However, Pemigatinib at 10 μM concentration with TRAIL significantly increased caspase 3/7 activity compared to vehicle **(Figure 12B)**. The LY2874455 at 0.1 μM, 1 μM and 10 μM concentration had dose dependent increase in caspase 3/7 activity compared to the vehicle without TRAIL sensitization **(Figure 12C)**.

Similarly, LY2874455 at 0.1 μM, 1 μM and 10 μM concentration also showed dose dependent caspase 3/7 activation after 6 hours to TRAIL sensitization **(Figure 12C)**.

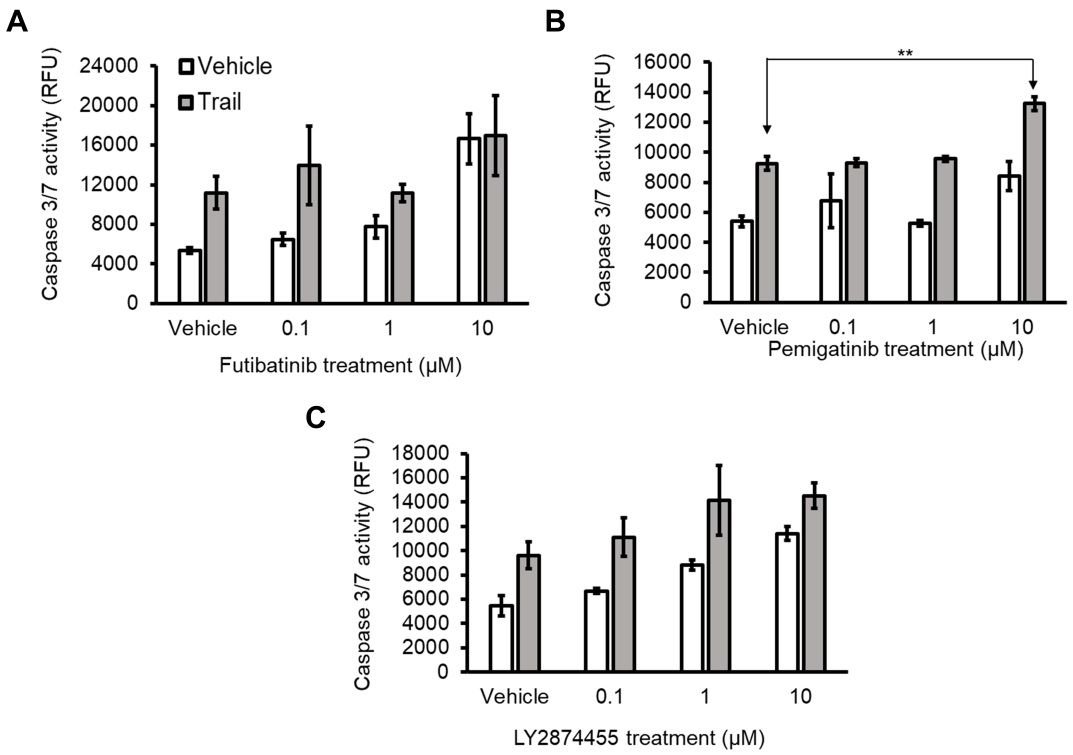


Figure 12: Additional pan-FGFR inhibitors were tested for their efficacy using caspase 3/7 activity in parental KMCH cells after 24 hours of treatment.

The efficacy of pan-FGFR inhibitor, Futibatinib **(A)**, Pemigatinib **(B)**, and LY2874455 **(C)** were measured at 0.1 μM, 1 μM and 10 μM concentration (24 hours of treatment) with or without 6 hours of TRAIL treatment (**$p < 0.01$). n=4.

Effect of FGFR small molecule inhibitors in CCA cell migration

Previously it was observed that FGFR4-expressing parental KMCH cells showed increased cell migration compared to FGFR4-knockdown KMCH shFGFR4 **(Figure 6C)**. Similarly, HuCCT-1 FGFR4 cells showed significantly increased cell migration compared to HuCCT-1 cells ($p < 0.001$) **(Figure 6A)**. Based on these observations, 3 μM of BLU9931 and 5 μM of pan-FGFR inhibitors PD173074 and BGJ398 were used to determine if small-molecule FGFR4 inhibition reduced cell migration in both KMCH and HuCCT-1 FGFR4 cells. It was observed that all of the inhibitors, BLU9931, PD173074 and BGJ398 significantly reduced the number of migrated HuCCT-1 FGFR4 cells compared to the vehicle after 8 hours of treatment ($p < 0.01$) **(Figure 13A)**. Next, treatment with PD173074 ($p < 0.01$) and BGJ398 ($p < 0.001$) significantly reduced KMCH cell migration compared to vehicle (DMSO) after 24 hours of treatment **(Figure 13B)**. BLU9931 treatment showed variable results in the inhibition of KMCH cell migration. BLU9931 at 3 μM concentration had no significant reduction in KMCH cell migration at 24 hours **(Figure 13B)** while the same treatment significantly reduced the KMCH cell migration at 24 hours ($p < 0.1$) **(Figure 13C)**. Overall, the data suggests that BLU9931, PD173074 and BGJ398 significantly inhibited cell migration of KMCH and HuCCT-1 FGFR4 cells, however further investigation into the specific pathway of BLU9931 is needed.

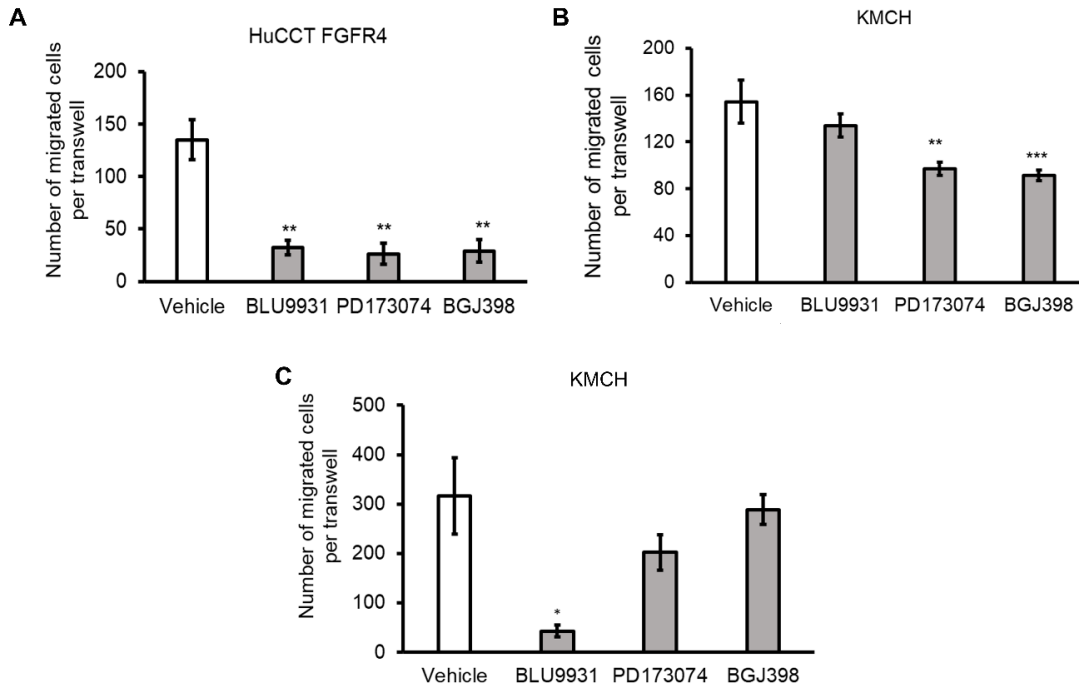


Figure 13: FGFR4-selective inhibitor, BLU9931 and pan-FGFR inhibitors (PD173074 and BGJ398) reduced cell migration.

The efficacy of BLU9931 (3 μM), PD173074 (5 μM), and BGJ398 (5 μM), was compared with vehicle (DMSO) to inhibit cell migration after 8 hours of treatment in **(A)** HuCCT-1 FGFR4 clone and **(B)** and **(C)** parental KMCH cells after 24 hours. (*$p < 0.1$, **$p < 0.01$, ***$p < 0.001$) Each experiment was performed twice, n=3.

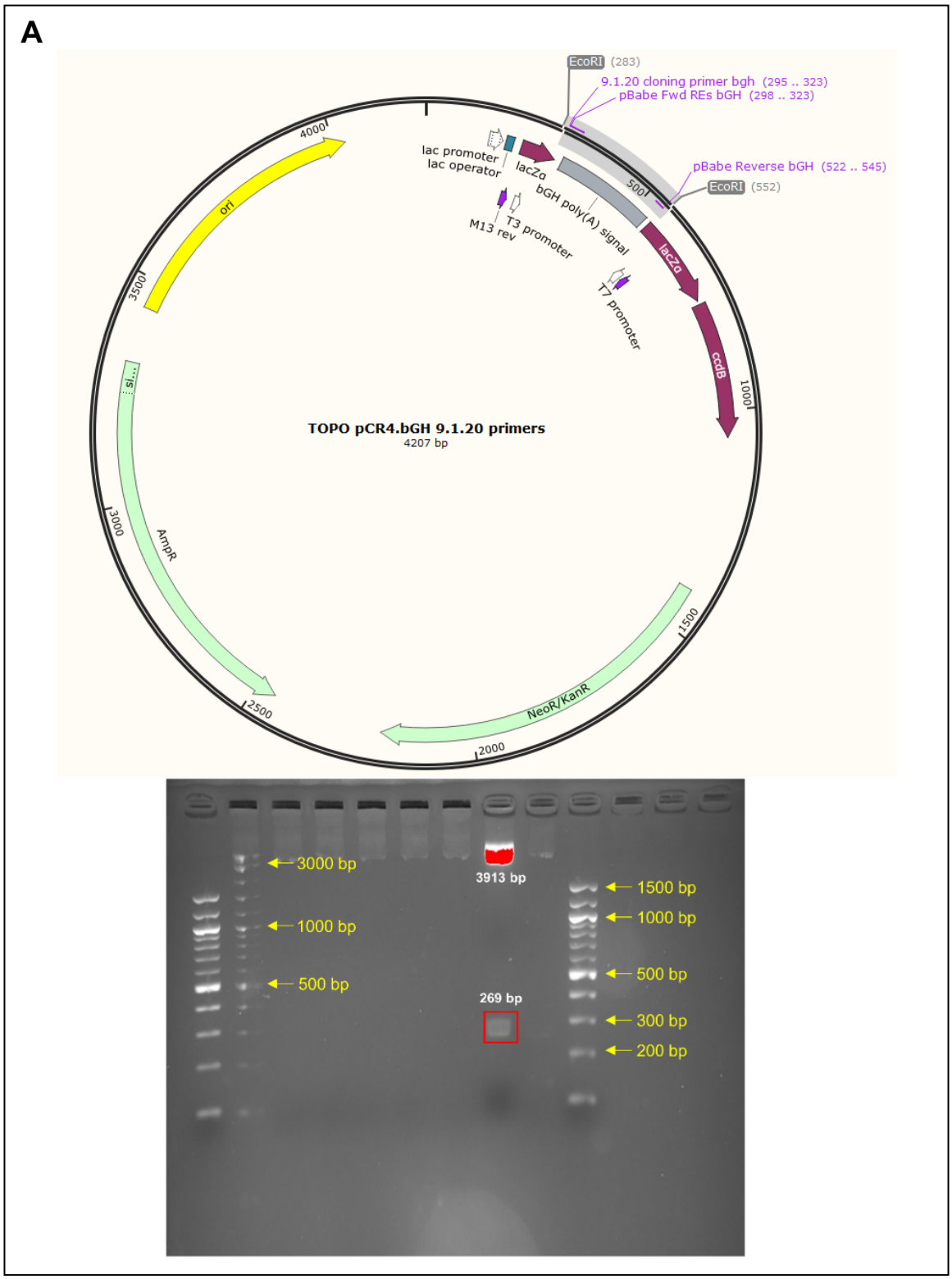
Cloning strategy for the stable expression of R4-ICD in HuCCT-1 and KMCH shFGFR4 cells

To investigate the role of R4-ICD in CCA, R4-ICD was stably cloned in HuCCT-1 cells, which endogenously do not express either FGFR4 or R4-ICD. We included the selectable antibiotic resistance gene for zeocin to allow selection of transfected cells by growth in antibiotic-containing media. The optimal antibiotic concentration for selection of resistant clones was determined using kill curve assay. HuCCT-1 and KMCH parental

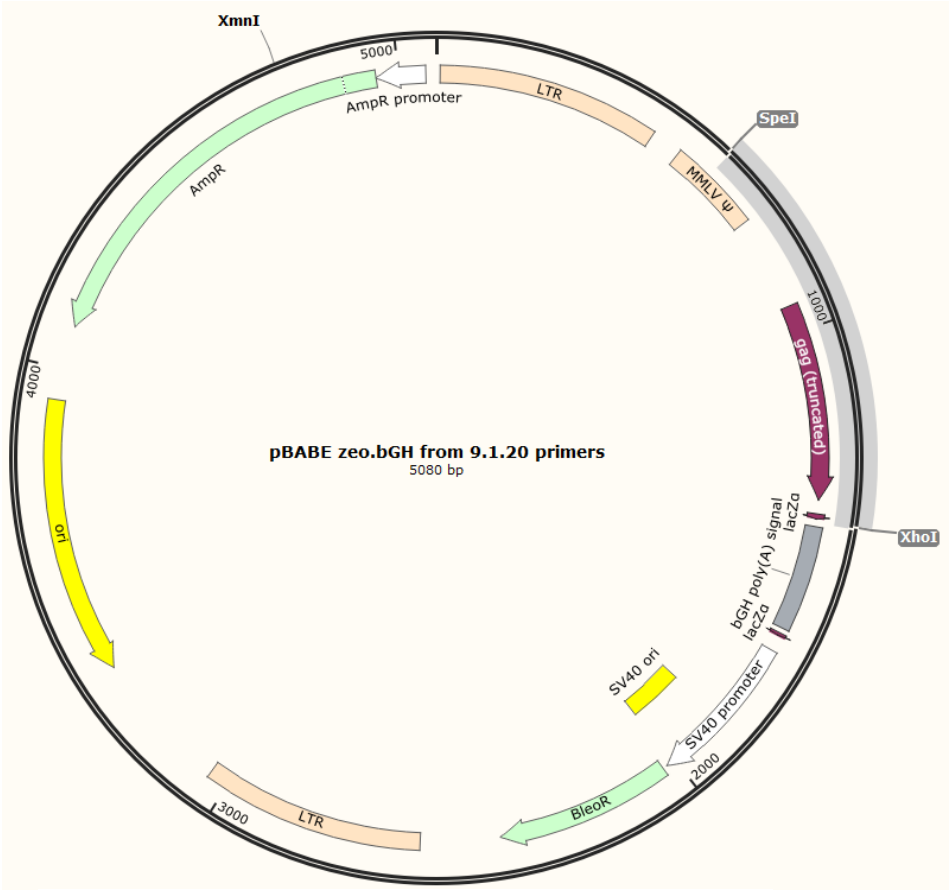
cells showed loss of viability at 800 mg/mL and 500 mg/mL of zeocin antibiotic respectively.

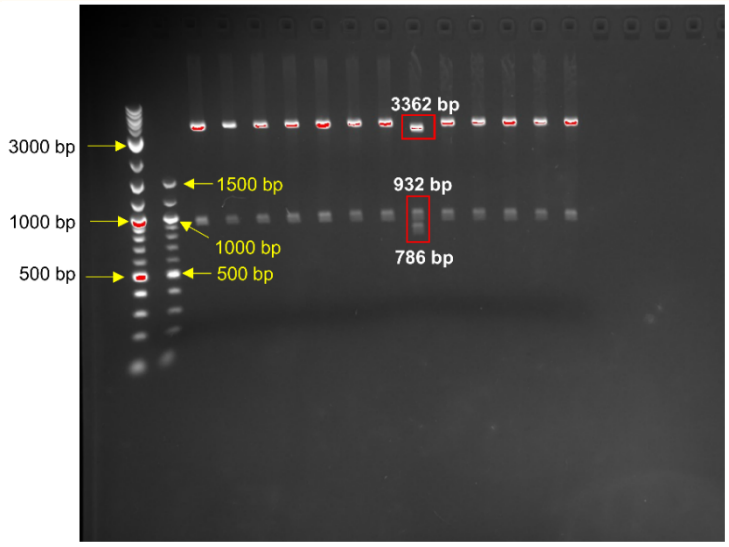
The overall strategy to incorporate R4-ICD into a plasmid with a new selection marker (zeocin) is described below. First, the neither the zeocin vector nor our plasmid containing R4-ICD contained a polyA signal. To start the cloning process, the bGH polyA signal from pcDNA.FLAG.HA.R4.ICD.WT. was inserted into TOPO TA 2.1. The TOPO TA 2.1 vector was digested with EcoRI and run on 1% agarose gel **(Figure 14A)** and the 269 bp band was gel purified. Next, the pBABE-zeo was digested with EcoRI, rSAP treated to remove phosphate groups from the 5' end and run on 1% gel to confirm the single band at 4811 bp. The gel extracted bGH polyA tail was ligated with EcoRI treated pBABE-zeo. The resulting plasmid, pBABE-zeo.bGH was confirmed by diagnostic digest with XmnI, XhoI, and SpeI for both size and orientation of the insert. The positive clone of pBABE-zeo.bGH was expected to have bands at 3362, 932 and 786 bp on 1% agarose gel **(Figure 14B)**. One positive clone with the bGH polyA insert was identified by diagnostic digest and was digested with SnaBI, XhoI and rSAP treated. The pcDNA.FLAG.HA.R4.ICD.WT. plasmid (previously generated to include amino acids 391-802 of FGFR4 after an N-terminal HA/FLAG epitope tag) was digested with XhoI and NruI. A 2050 bp (R4-ICD) band was gel extracted. The R4-ICD fragment was ligated into pBABE-zeo.bGH. Colonies were digested with SnaBI and SacII **(Figure 14C)**. The positive pBABE-zeo.bGH.R4-ICD clone was identified by diagnostic digest and sequencing was done to confirm the sequence of R4-ICD using pBABE 5', pBABE 3' and pcDNA FLAG HA Fwd primers **(Figure 14D)**. HuCCT-1 cells were transfected with pBABE-zeo.bGH.R4-ICD plasmid or an empty vector plasmid (pBABE-zeo). Subsequent clonal selection and immunoblotting was performed to validate the expression of R4-ICD. R4-ICD transfected HuCCT-1 cells (R4-ICD.C3 and R4-ICD.C6) showed single band at 50-60 kDa against FGFR4 antibody

on immunoblot **(Figure 14E)**, consistent with the epitope-tagged R4-ICD. The level of exogenous R4-ICD is comparable to that of cleaved R4-ICD in the HuCCT-1 FGFR4 cells.

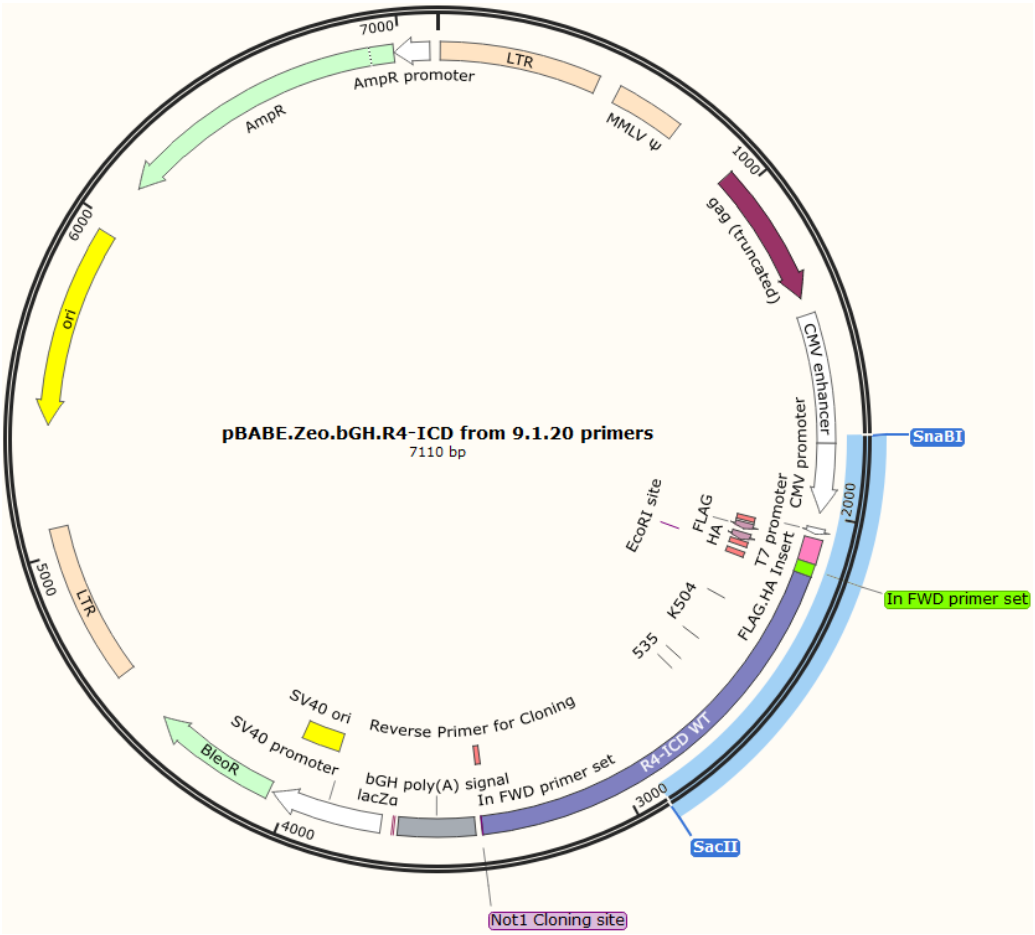


B


XmnI
5000
AmpR promoter
LTR
SpeI
MMLV Ψ
AmpR
1000
gag (truncated)
4000
pBABE zeo.bGH from 9.1.20 primers
5080 bp
ori
signal lacZα
XhoI
bGH poly(A)
lacZα
SV40 ori
SV40 promoter
2000
3000
LTR
BleoR


3362 bp
3000 bp
1500 bp
932 bp
1000 bp
1000 bp
786 bp
500 bp
500 bp

C


pBABE.Zeo.bGH.R4-ICD from 9.1.20 primers
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7000
AmpR promoter
LTR
MMLV Ψ
1000
gag (truncated)
CMV enhancer
CMV promoter
SnaBI
2000
T7 promoter
FLAG.HA Insert
In FWD primer set
EcoRI site
FLAG
HA
K504
535
R4-ICD WT
3000
SacII
Not1 Cloning site
In FWD primer set
bGH poly(A) signal
lacZα
Reverse Primer for Cloning
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SV40 promoter
BleoR
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5000
LTR
ori
6000
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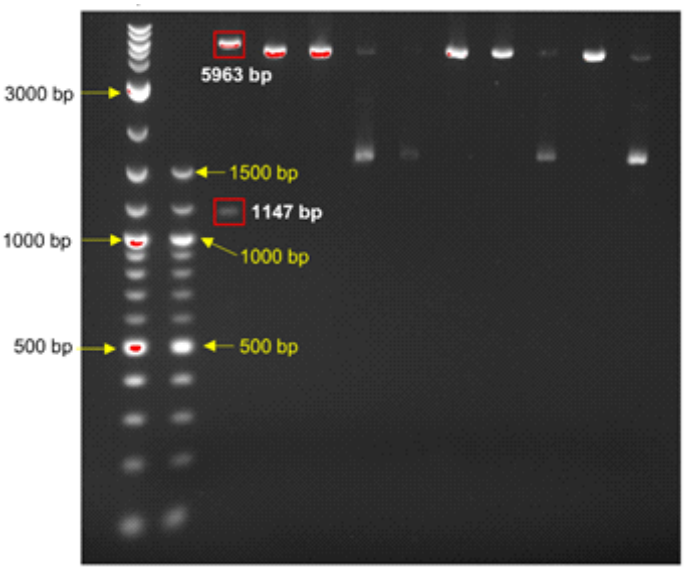
3000 bp
1000 bp
500 bp
5963 bp
1500 bp
1147 bp
1000 bp
500 bp

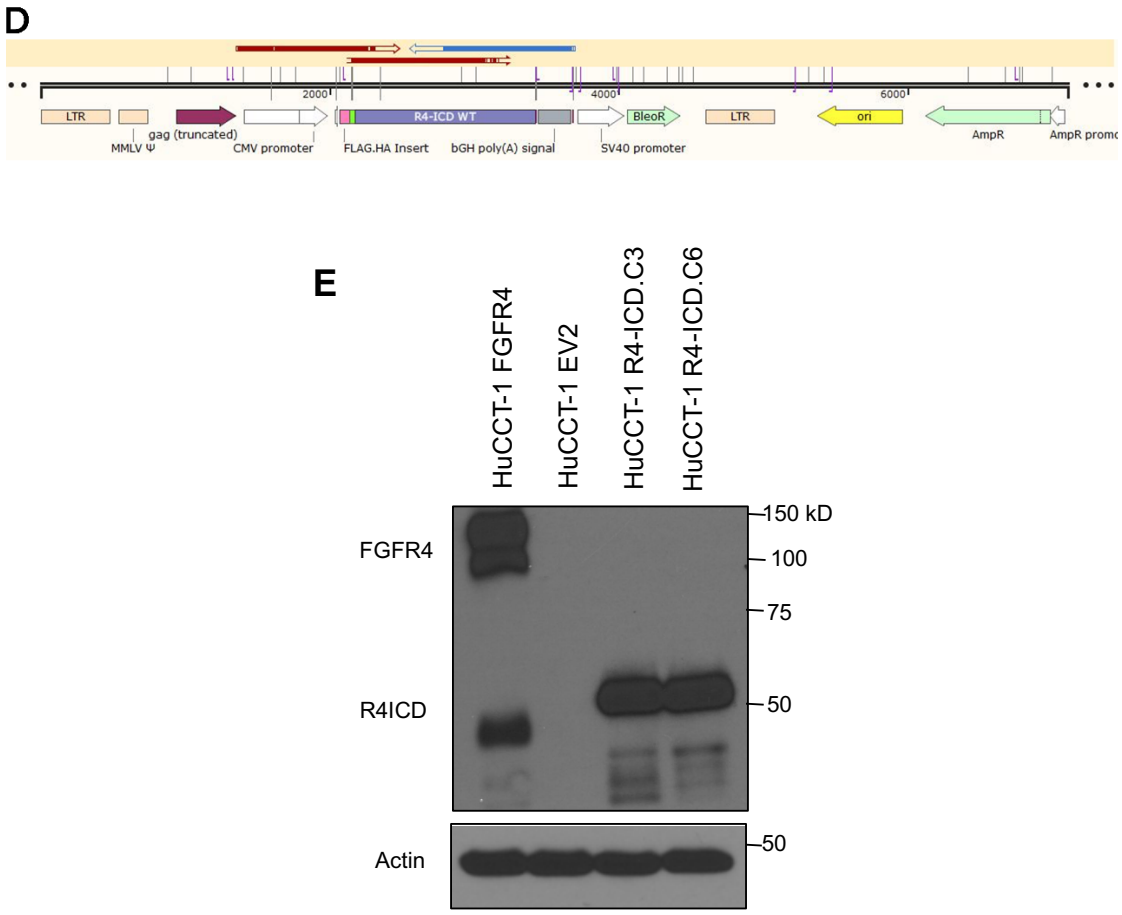

Figure 14: Cloning strategy to generate pBABE-zeo.bGH.R4-ICD.

(A) Diagnostic digest indicating one positive clone with 269 bp band (bGH polyA) on 1% agarose gel**. (B)** Diagnostic digest of pBABE-zeo.bGH with bands at 3362, 932 and 786 bp on 1% agarose gel. **(C)** Diagnostic digest of pBABE-zeo.bGH.R4-ICD with bands at 5963 and 1147b bp on 1% agarose gel. **(D)** The sequencing result of pBABE-zeo R4-ICD bGH with pBABE 5, pBABE 3' and pcDNA FLAG HA Fwd primers. **(E)** Immunoblot analysis showing the expression of R4-ICD in HuCCT-1 cells.

Role of R4-ICD in cell proliferation

To determine the role of R4-ICD on CCA proliferation, methylene blue cell proliferation assay was performed using HuCCT-1 R4-ICD-expressing stable positive clones R4-ICD.C3 and R4-ICD.C6. In addition, previously generated HuCCT-1 R4-ICD.PB1 cells transfected with pcDNA.FLAG.HA.R4.ICD.WT were included, which showed R4-ICD expression on immunoblot (see below for R4-ICD expression). Methylene blue proliferation assay results indicated that the HuCCT-1 FGFR4 and HuCCT-1 R4-ICD.PB1 cells had significantly higher proliferation compared to empty vector EV2 cells ($p < 0.001$) **(Figure 15A)**. However, R4-ICD.C3 showed no difference in cell proliferation, while R4-ICD.C6 showed reduced cell proliferation compared to the empty vector cells (EV2) **(Figure 15A)**. Currently, results are mixed whether R4-ICD can support cell proliferation.

Role of R4-ICD in cell survival

To investigate the role of R4-ICD in cell apoptosis, caspase 3/7 activity was measured in HuCCT-1 FGFR4 cells, R4-ICD-expressing HuCCT-1 clones (R4-ICD.C3, R4-ICD.C6 and R4-ICD.PB1) with or without TRAIL sensitization. Parental HuCCT-1 cells are sensitive to TRAIL-induced apoptosis, while HuCCT-1 FGFR4 significantly protected CCA cells from apoptosis by reducing caspase3/7 activity ($p < 0.01$) **(Figure 15B)**. Importantly, R4-ICD-expressing clones R4-ICD.PB1, R4-ICD.C3 and R4-ICD.C6 showed significantly reduced caspase 3/7 activity compared to HuCCT-1 EV2 cells ($p < 0.001$) **(Figure 15B)**. Thus, both full-length FGFR4 and R4-ICD could protect from TRAIL-induced apoptosis.

Role of R4-ICD in cell migration

The HuCCT-1 R4-ICD cell migration was determined using a transwell migration assay. HuCCT-1 parental cells, empty vector HuCCT-1 EV2 cells, HuCCT-1 FGFR4, R4-ICD.C3, R4-ICD.C6 and R4-ICD.PB1 cells were each evaluated for their migratory potential. HuCCT-1 EV2 cells unexpectedly showed the highest cell migration at 24 hours while HuCCT-1 FGFR4, R4-ICD.C3, R4-ICD.C6 and R4-ICD.PB1 cells showed reduced cell migration compared to empty vector (HuCCT-1 EV2) cells, but increased migration compared to HuCCT-1 parental cells **(Figure 15C)**. Thus, R4-ICD likely supports cholangiocarcinoma cell migration, though effects of clonal variation may complicate this interpretation.

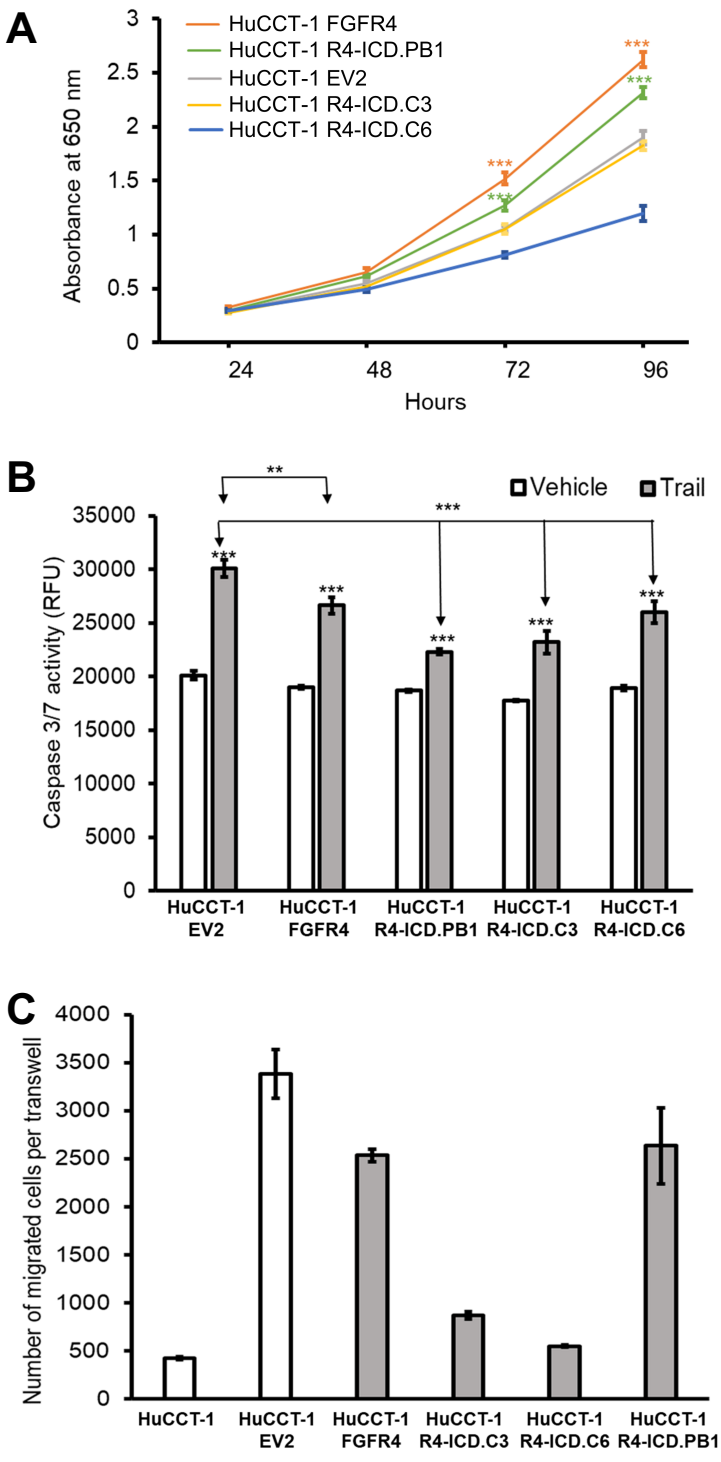
A
HuCCT-1 FGFR4
HuCCT-1 R4-ICD.PB1
HuCCT-1 EV2
HuCCT-1 R4-ICD.C3
HuCCT-1 R4-ICD.C6
Absorbance at 650 nm
Hours
B
Vehicle
Trail
Caspase 3/7 activity (RFU)
HuCCT-1 EV2
HuCCT-1 FGFR4
HuCCT-1 R4-ICD.PB1
HuCCT-1 R4-ICD.C3
HuCCT-1 R4-ICD.C6
C
Number of migrated cells per transwell
HuCCT-1
HuCCT-1 EV2
HuCCT-1 FGFR4
HuCCT-1 R4-ICD.C3
HuCCT-1 R4-ICD.C6
HuCCT-1 R4-ICD.PB1

Figure 15: The role of R4-ICD in cellular proliferation, apoptosis and migration.

(A) Methylene blue cell proliferation assay compares proliferation of HuCCT-1 EV2, HuCCT-1 FGFR4 and HuCCT-1 R4I-CD clones from 0 to 96 hours. n=8. (**B)** Caspase 3/7 activity in HuCCT-1 EV2, HuCCT-1 FGFR4, and HuCCT-1 R4-ICD clones after 24 hours with or without 6 hours of TRAIL treatment. n=4. **(C)** Transwell cell migration assay indicates migration of HuCCT-1 parental, EV2, HuCCT-1 FGFR4, HuCCT-1 R4-ICD clones toward serum containing media after 24 hours (n.s.). n=3. (**$p < 0.01$, ***$p < 0.001$). Each experiment was performed twice.

Determine the role of AKT downstream of FGFR4/R4-ICD signaling

To determine the role R4-ICD mediated cell survival of CCA via downstream AKT mediated signaling, an Akt Inhibitor at 1 μM (AKTi) was used in the presence and absence of TRAIL (6 hours). The caspase 3/7 activity of HuCCT-1 EV2, HuCCT-1 FGFR4, R4-ICD.PB1, R4-ICD.C3 and R4-ICD.C6 cells were evaluated. After 24 hours of AKTi treatment, HuCCT-1 EV2 and HuCCT-1 FGFR4 cells increased their sensitivity to TRAIL-induced cell death (***$p < 0.001$) compared to only TRAIL. However, R4-ICD-expressing cells (R4-ICD.PB1, R4-ICD.C3 and R4-ICD.C6) did not show an increase in caspase 3/7 activity with AKT inhibition compared to TRAIL alone, indicating the R4-ICD mediated cell survival is independent of pAKT mediated downstream signaling **(Figure 16)**.

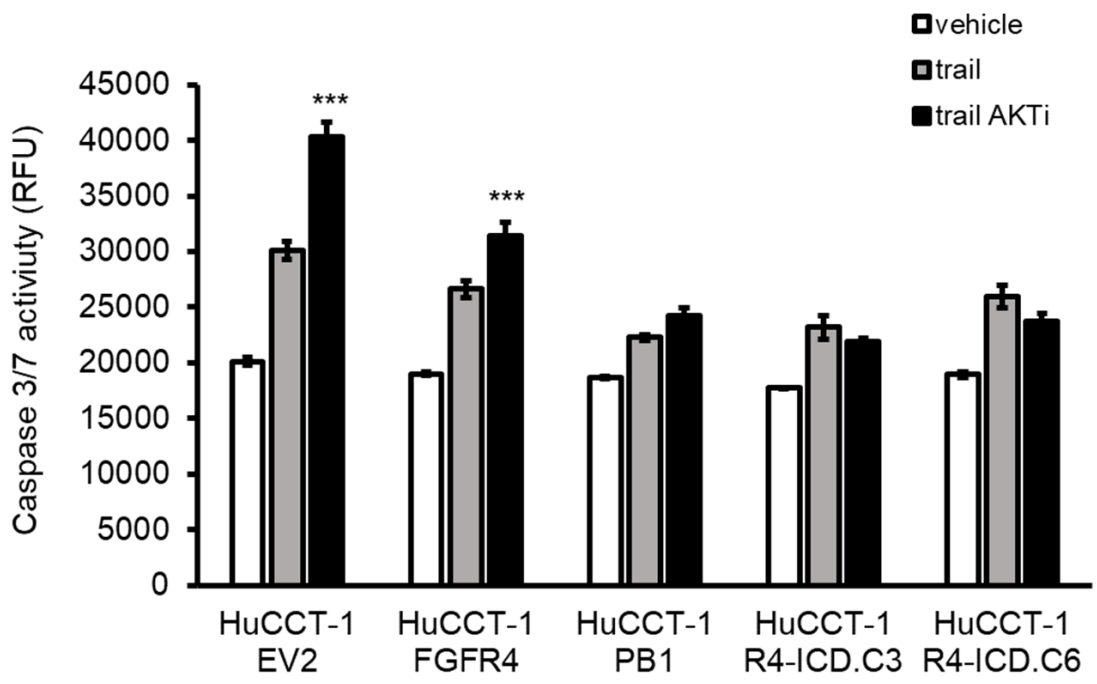

Figure 16: R4-ICD mediated CCA cell survival is independent of AKT.

HuCCT-1 EV2, HuCCT-1 FGFR4, R4-ICD.PB1, R4-ICD.C3 and R4-ICD.C6 cells were treated with AKT inhibitor at 1 μM concentration in the presence or absence of TRAIL (***$p < 0.001$) n=4.

Determining downstream signaling of FGFR4 and R4-ICD

It is known that FGFR4 mediates downstream phosphorylation of ERK to regulate cell proliferation. Also, FGFR4 downstream signaling phosphorylates AKT to regulate cell survival. Based on such observations in other studies, the level of phosphorylated proteins in the presence and absence of FGFR4 were determined using immunoblot. To examine the effect of FGFR4 to activate downstream signaling, phosphorylation of various downstream proteins including phospho- and total-ERK (regulates cell proliferation) and phospho- and total-AKT (regulates cell proliferation and cell survival) were determined using immunoblotting. The expression of FGFR4 was evaluated in KMCH shGFP and

KMCH shFGFR4 cells (**Figure 17A**), in which KMCH shFGFR4 have mild reduction in FGFR4 using the 419 shRNA and greater reduction of FGFR4 using 510 **(Figure 3B).** The amount of phosphorylated AKT was higher in FGFR4-expressing KMCH shGFP cells, while the shFGFR4 cells did not show any signal against anti-pAKT. However, the total-AKT was similar in both cells **(Figure 17A)**. Such observation indicates that FGFR4 plays a crucial role in the activation of pAKT. Secondly, the expression of phosphorylated ERK in both cell lines was measured using anti-pERK antibody. KMCH shGFP had enhanced level of pERK compared to FGFR4-knockdown cells, although the level of total ERK did not change in both cell lysate **(Figure 17A)**. Thus, ERK is not activated in parental cells, but was active in cells with shRNA to GFP or to FGFR4, indicating the activation is not necessarily FGFR4-mediated. Lastly, the KMCH shGFP and KMCH shFGFR4 cells had same signal for actin, indicating same protein loading on the gel **(Figure 17A)**.

Similarly, the downstream signaling of both FGFR4 and R4-ICD was examined in HuCCT-1 EV2, HuCCT-1 FGFR4, R4-ICD.C3, R4-ICD.C6, and R4-ICD.PB1 cells. The HuCCT-1 FGFR4 cells had lower level of phosphorylated AKT, STAT and ERK than parental, while the total level of AKT, STAT and ERK did not change **(Figure 17B)**. Next, R4-ICD-expressing cells R4-ICD.C3, R4-ICD.C6, and R4-ICD.PB1 showed similar levels of pAKT and pERK compared to HuCCT-1 EV2 **(Figure 17B)**. However, HuCCT-1 FGFR4 showed reduced levels of pAKT and pERK compared to HuCCT-1 EV2 **(Figure 17B)**. The level of pAKT was similar in stable R4-ICD clones, R4-ICD.C3 and R4-ICD.C6, as was total AKT **(Figure 17B)**. The level of pERK in R4-ICD.C3 was slightly higher compared to HuCCT-1 and HuCCT-1 EV2 cells with equal level of total AKT in each of them **(Figure 17B)**. Lastly, we compared aliquots of R4-ICD.PB1 cells (arbitrarily called A, B, C, and D) and showed an increased level of pAKT in a R4-ICD dependent manner while the total AKT was equal in all R4-ICD.PB1 on immunoblot **(Figure 17C)**. This immunoblot indicates

that R4-ICD may increase pAKT. We note however, our previous studies suggested that the R4-ICD mediated cellular survival was independent of pAKT **(Figure 16**).

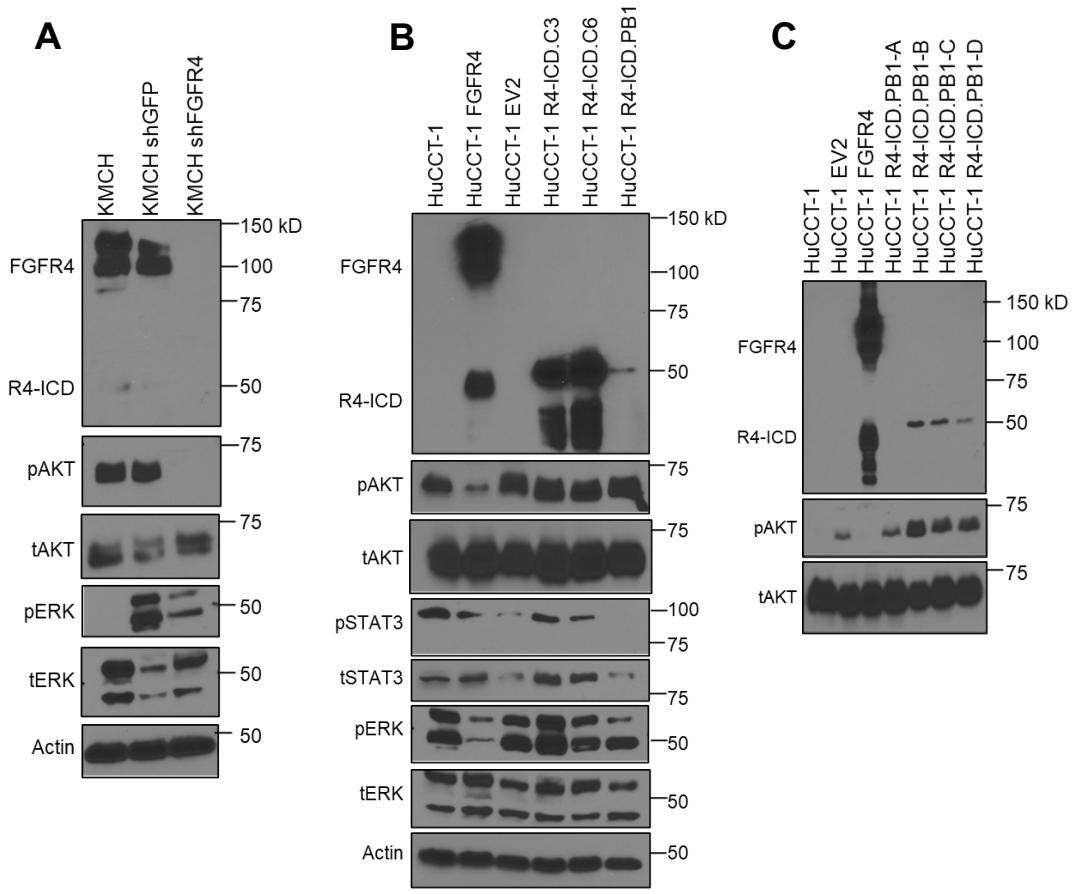

Figure 17: Evaluation of FGFR4 dependent downstream signaling using immunoblot analysis with anti-FGFR4, anti-pERK, anti-tERK, anti-pAKT anti-tAKT and anti-actin (loading control) antibodies.

(A) Immunoblot analysis of FGFR4-expressing parental KMCH and KMCH shFGFR4 cell lysate in comparison with FGFR4-knockdown (KMCH shFGFR4) cells. **(B)** Determining the role of FGFR4 and R4-ICD using HuCCT-1 FGFR4 and R4-ICD.C3, R4-CID.C6 and R4-ICD.PB1 with comparison of HuCCT-1 EV2 cell lysate. **(C)** Immunoblot showing the correlation of R4-ICD and phosphorylation of AKT in R4-ICD.PB1 clones. **A** and **B** were performed three times and **C** was performed one time.

CHAPTER 4: DISCUSSION

To investigate the role of fibroblast growth factor receptor 4 in cholangiocarcinoma, cell line-based functional assays were performed. In this thesis, I provided evidence that FGFR4 and R4-ICD promoted cell proliferation, protected cancer cells from apoptosis, and increased cell migration. We employed both genetic FGFR4 depletion and small molecule kinase inhibitors. Finally, we characterized some of the functional effects mediated by the R4-ICD form of FGFR4. Unexpectedly, the R4-ICD signal in cells may differ from the role of full-length receptor. Each of these cancer phenotypes and signaling pathways are discussed below.

HuCCT-1 cells exogenously expressing FGFR4 were more proliferative compared to HuCCT-1 parental and HuCCT-1 EV2 cells (both lacking FGFR4), indicating FGFR4 plays a role in cellular proliferation of CCA cells. FGFR4 is a known regulator for cellular proliferation via activation of ERK1/2 signaling in colorectal cancer and HNSCC [94], [112]. The level of phosphorylated ERK (pERK) protein was examined in this study using immunoblotting and we found lower levels of pERK in HuCCT-1 FGFR4-expressing cells compared to HuCCT-1 parental and HuCCT-1 EV2 cells. This immunoblot data suggested that FGFR4-mediated cellular proliferation may not be dependent upon pERK-mediated downstream signaling in HuCCT-1 cells. However, the level of pERK was also examined in KMCH shGFP and shFGFR4 cells. In the KMCH cell lines, knockdown of FGFR4 reduced levels of pERK, indicating the downstream FGFR4 signaling in KMCH cells may be via ERK. FGFR4 can also regulate cellular proliferation via STAT3 signaling, although in our study, FGFR4-expressing cells showed no increase in STAT signaling compared to their FGFR4 negative control cells [113].

In addition to cellular proliferation, the role of FGFR4 in cell death was determined via caspase 3/7 activity. HuCCT-1 cells showed sensitivity against TRAIL-induced

apoptosis. Based on these findings, FGFR4 was cloned into HuCCT-1 cells to study the role of FGFR4 induced cell survival. HuCCT-1 FGFR4 cells showed lower caspase 3/7 activity compared to HuCCT-1 EV2 cells in the presence of TRAIL, suggesting FGFR4 protects CCA cells from TRAIL-induced apoptosis. FGFR4 is known to regulate the cell survival via phosphorylated AKT (pAKT) in multiple cancers including colorectal cancer, breast cancer and HNSCC [94, 112, 114]. The level of pAKT was lower in HuCCT-1 FGFR4 cells compared to the control HuCCT-1 FGFR4 EV2 cells. However, knockdown of FGFR4 in KMCH reduced levels of pAKT, indicating downstream FGFR4 mediated pAKT signaling that could be a marker of cellular survival. These data suggested that FGFR4-mediated cell survival pathway may be cell line specific.

To further gain insight into FGFR4 mediated cell migration, transwell migration assays were performed. HuCCT-1 FGFR4 cells had significantly higher numbers of migrated cells compared to HuCCT-1 parental cells, although the HuCCT-1 FGFR4 cells had lower migration compared to control HuCCT-1 EV2 cells. From this observation, it was not clear that the increased cellular migration resulted from the overexpression of FGFR4 or the presence of empty (puromycin) vector. To further clarify these results, KMCH and KMCH shFGFR4 cells were used in the transwell migration assay. The FGFR4-knockdown cells (KMCH shFGFR4) showed significantly reduced cell migration compared to parental KMCH cells, indicating the important role of FGFR4 in CCA cell migration. Previous studies from our lab indicated that the KMCH cells expressed higher levels of FGF19 ligand compared to HuCCT-1 cells. Since HuCCT-1 FGFR4 cells may have expressed lower levels of FGF19, FGF19-FGFR4 mediated cell migration may play less of a role in HuCCT-1 FGFR4 cells than in KMCH cells. In accordance with our observations, similar results were reported in a previous article that showed the importance of FGF19 in cell migration. In that study, HCC cell migration increased in the presence of recombinant FGF19 [82]. This study indicated that higher levels FGF19 may be

required to activate cell migration, therefore further experiments are needed to determine if higher levels of FGF19 can mediate HuCCT-1 FGFR4 cell migration. Overall, our findings suggest that FGFR4 inhibition via RNA inference results in decreased pERK and pAKT. Similarly, HuCCT-1 cells that endogenously lack FGFR4 expression, when subjected to FGFR4 overexpression (HuCCT-1 FGFR4), showed increased cellular proliferation and survival. However, the cellular proliferation and survival was not perfectly correlated with ERK- and AKT-mediated downstream signaling in HuCCT-1 FGFR4 cells.

To determine the efficacy of pan-FGFR inhibitors and FGFR4-selective inhibitors in CCA cell lines, cell proliferation assays were performed. Two FGFR4-selective inhibitors, including H3B-6527 and Roblitinib, did not show any effect on cellular proliferation in cholangiocarcinoma cells. One FGFR4-selective inhibitor, BLU9931, significantly reduced proliferation of the FGFR4-expressing CCA cells KMCH, HuCCT-1 FGFR4 and Mz-ChA-1 cells. Similar observations are reported in past where BLU9931 significantly reduced the proliferation of FGFR4 overexpressing pancreatic ductal adenocarcinoma (PDAC) at 2 μM and HCC at 3 μM concentration [115, 116]. Pan-FGFR inhibitors BGJ398 and PD173074 reduced cellular proliferation of KMCH, HuCCT-1 FGFR4 and Mz-ChA-1 cells. FGFR4-selective inhibitor (BLU9931) and pan-FGFR4 inhibitors (PD173074 and BGJ398) also reduced the cellular proliferation of HuCCT-1 cells that lack FGFR4, suggesting the inhibition of other FGFR family protein in HuCCT-1 cells or an off-target effect of an inhibitor. KMCH cells express FGFR1, FGFR3 and FGFR4, while HuCCT-1 cells are known to express FGFR1, while the expression of FGFR2 and FGFR3 is unknown.

In cells treated with FGFR4-selective inhibitor BLU9931, the cells were sensitized to TRAIL-induced apoptosis. At 3 μM BLU9931 treatment alone did not increase cell death, however, at 30 μM BLU9931 has toxic effects on the cells in the absence of TRAIL. Similarly, 3 μM BLU9931 treatment reduced cell proliferation in FGFR4-expressing cells.

In another study, BLU9931 increased the caspase 3/7 activity of FGFR4 overexpressing HCC cells in a dose dependent manner, suggesting the induction of caspase cascade in HCC cell line [116].

The caspase activity induced by three additional pan-FGFR inhibitors was determined in the presence or absence of the death ligand TRAIL. However, apoptosis sensitization was not universally noted. LY2874455 at 10 μM sensitized KMCH cells to apoptosis in the presence of TRAIL. When the inhibitors were given for 24 hours, Pemigatinib was the only inhibitor to significantly sensitize KMCH cells for TRAIL induced apoptosis. These data suggest that the Pemigatinib has a potential to reduce FGFR4-expressing cell survival. Pemigatinib is an FDA approved drug (a front-line chemotherapy) used to target FGFR2-fusion in an advanced unresectable and metastatic CCA [109].

The FGFR4-selective inhibitor BLU9931 and two pan-FGFR inhibitors, PD173074 and BGJ398, significantly reduced cell migration in FGFR4-expressing cells, suggesting their role in inhibiting cell migration. Related to this, BLU9931 significantly reduced cell migration in PDAC cells that had FGFR4 overexpression [115]. Overall, our results indicated that BLU9931, PD173074 and BGJ398 reduced proliferation, increased apoptosis, and reduced migration in CCA cells that express FGFR4. However, the FGFR4-selective inhibitor and pan-FGFR inhibitors reduced proliferation in cells that lack FGFR4 expression, calling into question its selectivity for FGFR4.

R4-ICD is a cleaved product of FGFR4 that is highly expressed in CCA patient samples and CCA cell lines. Similar proteolysis of other tyrosine kinase receptors generated by gamma secretase has been reported and may facilitate kinase relocalization in the cell [117]. Another study showed that the FGFR1 also has a cleaved product of intracellular domain that may plays a role in cancer progression. The subcellular localization of intracellular domain of cleaved FGFR1 receptor increased levels of pERK compared to cells that lacked the FGFR1 intracellular domain, indicating the involvement

of this cleaved product in pERK mediated signaling [118]. Similarly, patients exhibiting the cleaved product of ErbB4 in breast cancer had lower survival compared to cell membrane bound ErbB4, and correlated with worse clinical outcomes in ER-positive breast cancer patients [119]. To determine the role of R4-ICD in CCA, HuCCT-1 cells (lacking endogenous FGFR4 or R4-ICD) were stably transfected with a plasmid encoding only the intracellular domain of FGFR4 (R4-ICD). Cell proliferation, survival and migration assays were performed on R4-ICD-expressing R4-ICD.C3, R4-ICD.C6, and R4-ICD.PB1 cell lines compared to empty vector control cells (HuCCT-1 EV2) to determine the phenotype of R4-ICD in CCA. We hypothesized that R4-ICD-mediated effects would mimic those of FGFR4 in CCA progression. HuCCT-1 FGFR4 and R4-ICD.PB1 cells had higher cellular proliferation while the R4-ICD.C3 and R4-ICD.C6 cells had reduced cell proliferation. Similar to our findings, epidermal growth factor receptor 4 (ErbB4) is known to be cleaved into a lower molecular weight product, an intracellular domain (ICD) in breast cancer cells. The cleaved ICD of ErbB4 promoted the cellular proliferation of breast cancer cells [119]. The data indicates that R4-ICD may be a regulator of CCA cell proliferation as R4-ICD.PB1 had higher cellular proliferation compared to empty vector (HuCCT-1 EV2). However, R4-ICD.C3 and R4-ICD.C6 had reduced cell proliferation. ERK signaling is a known mechanism of FGFR4 mediated cellular proliferation, therefore ERK signaling was evaluated in R4-ICD-expressing cells. Our immunoblot data suggested that the HuCCT-1 FGFR4 cells and R4-ICD.PB1 cells did not express higher level of pERK compared to HuCCT-1 EV2, suggesting FGFR4 and R4-ICD mediated cellular proliferation may not be dependent upon pERK-mediated cellular signaling. Therefore, further investigation is required to determine the degree to which FGFR4, and R4-ICD mediate cellular proliferation and the mechanism of this signaling.

Caspase 3/7 activity was evaluated in R4-ICD and FGFR4-expressing cells. HuCCT-1 FGFR4 cells were protected from TRAIL-induced apoptosis compared to the

empty vector (HuCCT-1 EV2) cells. Similarly, R4-ICD-expressing cells including R4-ICD.C3, R4-ICD.C6 and R4-ICD.PB1 had more protection against TRAIL-induced cell death compared to both EV2 and FGFR4. FGFR4 may regulate cancer cell survival via AKT-mediated signaling. Therefore, the level of pAKT was determined under steady-state conditions for HuCCT-1 FGFR4 and HuCCT-1 R4-ICD clones. FGFR4-expressing cells had lower levels of pAKT compared to EV2. Similarly, the R4-ICD cells have no significant difference in the expression of pAKT signal. These data indicate R4-ICD might not regulate cell survival via pAKT. To further evaluate whether the FGFR4 and R4-ICD mediated survival was dependent upon pAKT, AKT inhibitors were used in the cell apoptosis assay. The caspase 3/7 activity of HuCCT-1 FGFR4 and HuCCT-1 R4-ICD cells were determined in the presence and absence of TRAIL. AKT inhibition in the presence of TRAIL sensitized control HuCCT-1 EV2 and HuCCT-1 FGFR4 cells to death. However, the R4-ICD-expressing clones (R4-ICD.PB1, R4-ICD.C3 and R4-ICD.C6) did not show significant increase in TRAIL-induced cell sensitization in the presence of AKT inhibitor suggesting R4-ICD mediated cell survival independently of pAKT signaling. This challenges our hypothesis that R4-ICD could directly substitute for full length FGFR4 and suggests that R4-ICD signaling may be qualitatively different than FGFR4 signaling. In previous studies, the intracellular cleaved product of FGFR1 receptor has been translocated to the nucleus and identified as a transcriptional regulator of different target gene [118]. Similarly, it is possible that R4-ICD might translocate to the nucleus and act as transcription factor to mediate CCA cell survival or have other means to activate signaling not available for the full-length receptor. Therefore, further investigation is required to determine if R4-ICD translocates and mediates non-canonical FGFR4 signaling.

In this study FGFR4 and R4-ICD both were cloned into HuCCT-1 cells. For control (HuCCT-1 EV2), HuCCT-1 cells were transfected with empty puromycin plasmid that lack both FGFR4 and R4-ICD. The plasmid containing the gene of interest (FGFR4 or R4-ICD)

was transfected into HuCCT-1 cells using lipofectamine transfection reagent. Stable clones were selected by resistance to puromyocin or zeocin in the media. Stable clones have random insertion at different genomic sites. Random insertion may lead to alteration in the amino acid sequence (i.e., via frameshift), dysregulation of a specific gene or its expression, and can cause phenotypic heterogeneity in different selective clones. During single cell screening, clones are selected based on resistance to antibiotic alone. This included clones that have genetic alterations that allowed them to expand more rapidly and have better survival, independent of the transgene. Besides clonal variation and selection of single clonal population, our recently grown HuCCT-1 EV2 cells exhibited very unusual phenotypic changes compared to our past experiments. Our previous studies have suggested that the HuCCT-1 EV2 cells behaved very similar to HuCCT-1 parental cells. However, our recent experiments have shown that the HuCCT-1 EV2 have developed a distinct phenotype compared to HuCCT-1 parental cells, which might have resulted from clonal drift. Overall, further investigation is required to confirm whether HuCCT-1 EV2 cells have a stable phenotype and are similar to HuCCT-1 parental cells.

Regarding the role of R4-ICD in cell migration, FGFR4 and R4-ICD-expressing cells had higher cell migration compared to FGFR4-lacking HuCCT-1. However, the highest cell migration was observed in empty vector (EV2) cells. Previously, KMCH cells had shown higher cell migration compared to the FGFR4-knockdown cells. To further understand the role of R4-ICD in CCA cell proliferation, future studies will test cell survival and migration in KMCH shFGFR4 cells with restored R4-ICD expression. Overall, R4-ICD protected cells from cell apoptosis and R4-ICD mediated cell survival was independent of pAKT. However, the role of R4-ICD in cellular proliferation and migration is still not clear and further investigation is required to understand the R4-ICD-mediated signaling pathways that regulate cell proliferation, survival and migration.

Conclusion

Fibroblast growth factor receptor 4 overexpression was associated with 50-60% cholangiocarcinoma progression and therefore it is considered as a potential target in CCA management. In our study, it was observed that FGFR4 increased proliferation, survival and migration of CCA cells. The inhibition of FGFR4 expression using shRNA, lead to the reduction of cellular proliferation, cell survival and migration. Moreover, shRNA mediated FGFR4 inhibition resulted in reduced downstream signaling by way of pERK and pAKT, which are markers of cellular proliferation and survival, respectively. Furthermore, inhibition of FGFR4 via pan-FGFR (PD173074 and BGJ398) and FGFR4-selective (BLU9931) small molecular inhibitors resulted in reduced survival and migration of FGFR4-expressing CCA cells. In the case of FGFR4-selective inhibitor, BLU9931, further study is required to rule out off target effects on proliferation. An intracellular cleaved product of FGFR4, named as R4-ICD was successfully cloned into HuCCT-1 cells that endogenously lack FGFR4. These cells were used to determine the role of R4-ICD in CCA progression. It was observed that R4-ICD protected cells from TRAIL induced apoptosis independently of FGFR4, indicating its important role CCA cell survival. It was observed that the R4-ICD mediated cell survival was independent of AKT-mediated downstream signaling. Furthermore, the role of R4-ICD in cellular proliferation and migration is still not clear to-date and further investigation is required to understand R4-ICD mediated CCA progression. In conclusion, this study strongly suggests that FGFR4 and R4-ICD promote tumor features and are candidate targets for CCA management.

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