Development of Preclinical Evaluation System for Invasive Papillary Cholangiocarcinoma

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ABSTRACT

Development of Preclinical Evaluation System for Invasive Papillary Cholangiocarcinoma

Invasive papillary cholangiocarcinoma (Intraductal papillary neoplasm with an associated invasive carcinoma) is rare emerging disease entity of the cholangiocarcinoma, it represents about 4% of all malignant epithelial tumors of the extrahepatic duct. However, its biology and characteristics remain unknown because of lack of both *in vitro* and *in vivo* models to elucidate the diagnosis, biological mechanism, and treatment. Establishment of patient-derived xenograft model and cell line of invasive papillary cholangiocarcinoma will be useful for improving the biological progression mechanism, elicit biomarker discovery and knowledge of this disease as an experimental model. Patient-derived xenograft (PDX) was engrafted in NOG mouse from a primary surgically resected tissue (F0) and amplified to third generation (F3). PDX models retained the similarity of genomic expression, histopathological features, and molecular marker expression. Cell line, NCChIPC (National Cancer Center human intrahepatic papillary cholangiocarcinoma), was subsequently generated from F2 tumor tissue of PDX and evaluated their tumorigenicity *in vivo* as

well as drug responses. PDX tumor expanded successfully to develop F1, F2 and F3. PDXs largely retained the genetic and phenotypic features of the original tumor. Histopathological and the molecular features were consistent with the primary tumor of the patient with expression of CK19, MUC1 and MUC5AC while MUC2 and MUC6 were not expressed in patient tissue and through all xenograft tissues. NCChIPC cell line also retained molecular characteristics of the patient tumor and moreover showed tumorigenic ability in mouse with typical histological feature. In addition, the response of anticancer drugs was analyzed and predicted in NCChICP cell line. PDX model and NCChIPC cell line that were developed as a new preclinical system of invasive papillary cholangiocarcinoma will contribute to clarify the biology of invasive papillary cholangiocarcinoma and potentially facilitate translational research. The novel preclinical models here will help to elucidate the IPC etiology and facilitate translational research.

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1. Introduction

1.1. Background of the Study

Cholangiocarcinoma (CCA) is an epithelial cell malignancy arising from varying locations within the biliary tree showing markers of cholangiocyte differentiation. CCA of the intrahepatic large bile ducts and biliary tract including hilar bile ducts usually present with a nodular and/ or sclerosing type, while some of these CCs present predominantly intraductal or papillary growth pattern in the dilated bile ducts(1, 2). The tumor arises from the ductular epithelium of the biliary tree, either within the liver (intrahepatic cholangiocarcinoma) or more commonly from the extrahepatic bile ducts (extrahepatic cholangiocarcinoma) that is further subdivided into Perihilar and distal subtypes(3). This classification has recently been extended to also take into account arterial and venous encasement(4, 5) pCCA is the most common type of CCA. In a large series of patients with bile duct cancer, 8% had iCCA, 50% had pCCA, and 42% had distal CCA. CCA has a poor prognosis; patients have a median survival of 24 months after diagnosis (6).

Recent studies have shown that there are at least two types of pre-invasive neoplasms of the bile ducts preceding cholangiocarcinoma (CCA): biliary intraepithelial neoplasm (BilIN) and intraductal papillary neoplasm of the bile duct (IPNB)(7–9). BilINs are microscopically identifiable intraepithelial epithelial neoplasms and may be the most common precursor of nodular sclerosing, perihilar and distal CCA (p/dCCA) and large-duct intrahepatic CCA (iCCA) .(10–12). In contrast, IPNB has unique clinicopathological features and is defined as an

intraductal growing tumor, developing in the intrahepatic and extrahepatic bile ducts (13–16).

The disease is notoriously difficult to diagnose and is usually fatal because of its late clinical presentation and the lack of effective non-surgical therapeutic modalities. Most patients have unresectable disease at presentation and die within 12 months from the effects of cancer cachexia and a subsequent rapid decline in performance status. Liver failure and recurrent sepsis, secondary to biliary obstruction, can also contribute to the high mortality. Overall survival rate, including resected patients, is poor, with less than 5% of patients surviving to 5 years, a rate which has not changed significantly over the past 30 years (17, 18).

1.2. Epidemiology

Cholangiocarcinoma accounts for 3% of all gastrointestinal tumors. Over the past 3 decades, the overall incidence of CCA appears to have increased. The percentage of patients who survive 5 y after diagnosis has not increased during this time period, remaining at 10%(19, 20)). In the United States, Hispanics and Asians have the highest incidence of CCA (2.8/100,000 and 3.3/100,000 respectively), whereas African Americans have the lowest (2.1/100,000). African Americans also have lower age-adjusted mortality compared with whites (1.4/100,000 vs. 1.7/100,000). Men have a slightly higher incidence of CCA and mortality from the cancer than women.7 With the exception of patients with primary sclerosing cholangitis (PSC), a diagnosis of CCA is uncommon before age 40 y.

Globally, hepatobiliary malignancies account for 13% of cancer-related deaths; 10%–20% of these are attributable to CCA. The mean age of diagnosis of CCA is 50 y. The global incidence of iCCA varies widely, from rates of 113/100,000 in Thailand to 0.1/100,000 in Australia(21). Differences in the prevalence of genetic and other risk factors presumably account for this extensive variation.

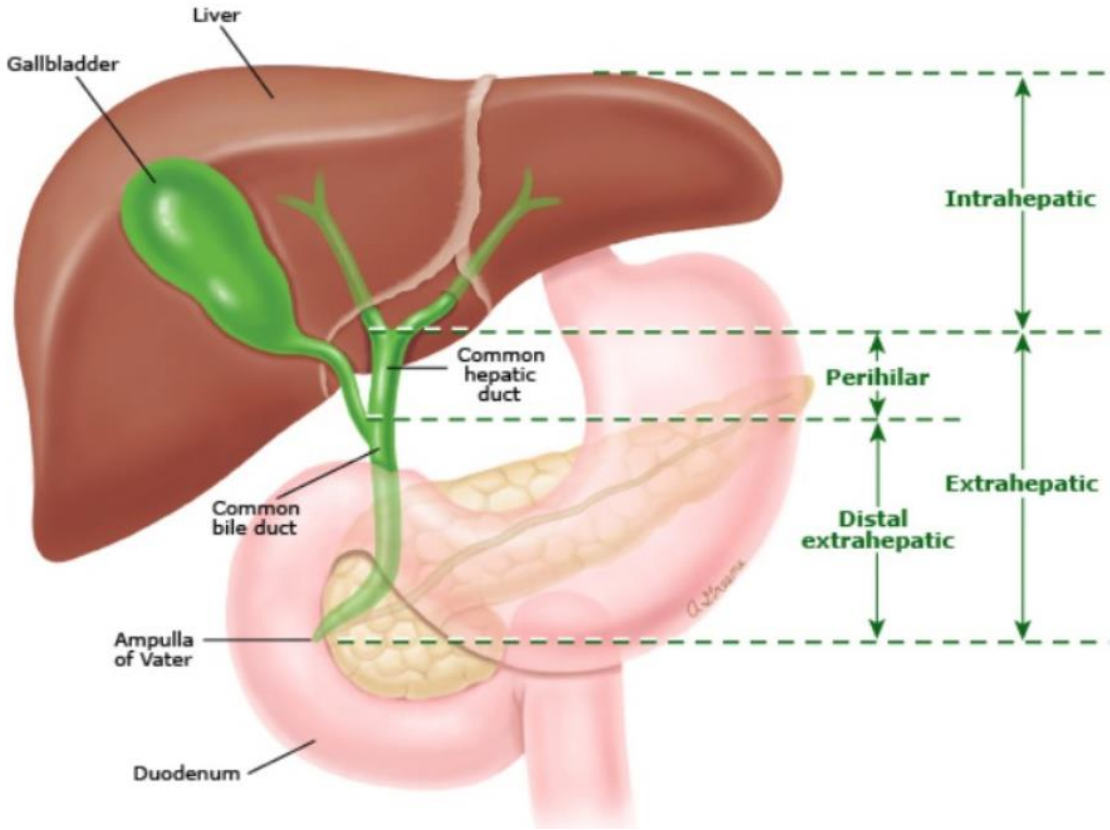

Figure 1. Showing the Biliary tract with Cholangiocarcinoma classification (American Joint committee on cancer staging manual 8th Edition

1.3. Risk Factors and Pathophysiology

CCA in western countries is considered sporadic, (22) however, there are a number of well described risk factors (23). It is proposed that most of these risk factors cause chronic inflammation and cholestasis resulting in a cycle of reactive cell proliferation, genetic and epigenetic mutations and eventually cholangiocarcinogenesis.

An inflammatory Milieu is believed to deregulate or change the expression patterns of growth factors, pro-inflammatory cytokines, and their receptors(24).

Cytokines produced by the cholangiocytes and activated microphages can modulate gene expression and lead to activation of carcinogen metabolism. The cytokine can also induce nitric oxide (NO) synthetase expression in cholangiocytes. NO can directly injure DNA. Consumption of cellular detoxification and dysregulation of DNA repair and apoptosis are final steps of biliary carcinogenesis (25, 26).

Bile acids also have been shown to activate inducible cyclo-oxygenase 2 and an anti-apoptotic molecule, myeloid cell leukemia protein 1 in cholangiocytes(27). Thus, an inflammatory milieu and toxic bile constituents act together to promote carcinogenesis in the biliary tree.

Table 1: Risk factors

1. Cholestatic liver disease
    - Primary sclerosing cholangitis
    - Fibro-polycystic liver disease
    - Congenital hepatic liver disease
    - Caroli's disease
    - Choledochal cystic
    - Biliary hematomas

2. Liver cirrhosis (any etiology)

a) Biliary stone Disease
- Cholecystolithiasis
- Hepatolithiasis
- Choledocholithiasis

b) Infections
- Liver flukes
- Hepatitis B and C
- Chronic typhoid disease
- Recurrent pyogenic Cholangitis
- Human immunodeficiency virus (HIV)

c) Inflammatory disorders
- Inflammatory bowel disease
- Chronic pancreatitis
- Gout
- Thyrotoxicosis

d) Toxins
- Alcohol
- Tobacco
- Thorotrast (contrast agent)
- Chemical toxins (e.g Vinyl Chloride, Nitrosamines)

e) metabolic conditions
- Diabetes
- Obesity
- Non-alcoholic fatty liver disease (NAFLD)

f) Genetic disorders

- Lynch syndrome (hereditary Nonpolyposis Colorectal Cancer

g) Others

- Bile salt transporter protein gene defect
- Intraductal papillary Neoplasm of the bile duct (IPNB).

1.4. Laboratory Tests and Diagnosis

Extrahepatic CC presents with classic signs of cholestasis including jaundice, dark urine, pale stools, pruritus, malaise, and weight loss. Laboratory investigations reveal increased alkaline phosphatase, gamma-glutamyl trans-peptidase and bilirubin. Prolonged obstruction of the main bile ducts can cause increased prothrombin time and a reduction in fat soluble vitamins. As the disease advances further, albumin, hemoglobin and lactate dehydrogenase can decrease. A glycoprotein tumor marker, CA 19-9, can be found elevated in 85% of such cases. A value of > 100 U/mL in PSC patients has a sensitivity of 89% and specificity of 86% for the diagnosis of CC. CC should not be diagnosed only on the basis of elevated CA 19-9. However, in patients without PSC, the sensitivity of CA 19-9 > 100 U/mL is 53% (28–30).

There is still need for better tumor markers for early diagnosis. The most widely used circulating marker for CCA is carbohydrate antigen (CA) 19-9(30). However, (CA) 19-9 is also elevated in pancreatic cancer, gastric cancer, and

primary biliary cirrhosis and has been shown it gives false positive results (31). Carcinoembryonic antigen (CEA) is the other common tumor marker used for detecting CCA. CEA is not specific, being mainly used for colorectal cancers, and can be elevated in other types of cancer, such as gastrointestinal or gynecologic malignancies(31). Differences in expression profiles between normal liver and CCA tissues were studied, because CCA is contained in liver tissue and is suggested to arise from the same stem cells as HCC(25, 32). We have previously compared CCA and HCC cell lines using proteomic techniques in order to investigate potential CCA markers for early diagnosis (33).

1.5. Intraductal Papillary Neoplasm of the Bile duct (IPNB)

The concept of epithelial tumors arising from non-invasive intraepithelial dysplasia or neoplasm is well-established in various human cancers(34). Recent studies have shown that there are at least two types of pre-invasive neoplasms of the bile ducts preceding cholangiocarcinoma (CCA): biliary intraepithelial neoplasm (BilIN) and intraductal papillary neoplasm of the bile duct (IPNB)(7, 8, 13, 35–37). BilINs are microscopically identifiable intraepithelial epithelial neoplasms and may be the most common precursor of nodular sclerosing, perihilar and distal CCA (p/dCCA) and large-duct intrahepatic CCA (iCCA) (10, 36, 38). In contrast, IPNB has unique clinicopathological features and is defined as an intraductal growing tumor, developing in the intrahepatic and extrahepatic bile ducts (13, 15, 16). About half of IPNBs show stromal invasion at the time of

surgical resection. Mucinous cystic neoplasm (MCN) is another pre-invasive intraepithelial neoplasm associated with ovarian-like stroma and lacks communication with the bile duct lumen (13).

Historically, IPNBs have been studied with reference to intraductal papillary mucinous neoplasm of the pancreas (IPMN), as the biliary tree and pancreas are located closely anatomically, and at least some biliary diseases show similarities to pancreatic diseases(7, 39–43). Through these comparative studies, the main pathological characteristics of IPNB have been recognized, including the presence of four subtypes, slow progression with intraepithelial mucosal spreading around the main tumor and mucus hypersecretion. The radiological comparison of biliary diseases, including IPNB, with their pancreatic counterparts has also been attempted(44–46) . Approximately half of IPNBs reportedly showed histopathological features similar to those of IPMNs (38, 47–49). However, IPNB differed from IPMN in its higher histological grade, more advanced stage, higher frequency of associated invasive cancer, worse prognosis and some differences in the oncogenic signal pathways and genetic changes(42, 43, 50). According to recent studies including such comparative processes, IPNB is now being established as an independent disease along the biliary tree. While IPNBs have been given several different names reflecting their characteristic features, the World Health Organization (WHO) published the Classification of Digestive System Tumors 5th edition (2019), in which the only term IPNB was proposed using one chapter.

1.6. Clinical Features, Epidemiology, and Imaging

IPNB is a recently defined pathologic entity and premalignant disease characterized by a low incidence, high risk of malignant transformation and an uncertain prognosis Its clinical characteristics and classification as well as radiological features have yet to be established (13, 51). IPNBs typically affect middle-aged to elderly adults and show a slight male predominance(52–56) . Intermittent or recurrent, right-upper-quadrant abdominal pain, fever and acute cholangitis or jaundice are the most common clinical manifestations, but a certain percentage of patients (about 12%) have no symptoms at the diagnosis (13, 51). Elevated levels of alkaline phosphatase, carcinoembryonic antigen (CEA) and carbohydrate antigen 19-9 (CA19-9) have been reported, although they are unlikely to have high sensitivity or specificity for the diagnosis of IPNB. The serum levels of CA19-9 may reflect the tumor burden and level of invasiveness.

IPNB is a rare disease entity with a prevalence of 4% to 15% among bile duct tumors(7, 51) .IPNB was mainly reported in East Asia, and the incidence is regarded to be higher in these countries than in others (10) examined the ratio of IPNB/mucinous cystic neoplasm of liver (MCN-L) and showed this ratio to be 5.7:1 in Seoul but 1:3.0 in Seattle (WA, USA) and 1:6.3 in London (UK). This difference was mainly attributable to the considerably greater number of IPNB patients in Seoul than in Seattle and London.

The most common abnormal preoperative imaging findings for IPNB are intraductal masses and the involvement of bile duct dilation. The most important

morphological changes are the presence of (a) intraductal mass(es) and surrounding intraepithelial neoplastic biliary mucosa, (b) diffuse or segmental bile duct dilatation with or without cystic changes (maximum 126 mm) and (c) ductal and periductal invasion including macro-invasion of the liver (57).In ultrasound sonography (US), IPNB was recognizable by variable features, including hyperechoic nodules (37.5%), focal bile duct dilatation (37.5%) and diffuse bile duct dilatation with intraductal nodules (25%) (58). Magnetic resonance imaging (MRI) reveals IPNB as isointense to hypointense masses on T1-weighted images and hyperintense masses on T2-weighted images.

1.7. Classification Based on Radio-pathological Appearance

Several classifications have been proposed based on the gross and radiological appearance. Recently, (59) proposed a modified anatomical classification of IPNB: extrahepatic type, wherein the main lesions are confined to the extrahepatic hepatic duct; intrahepatic type, wherein the main lesions are located at the intrahepatic bile ducts; and diffuse type, wherein the main lesions are located over a wide are of the intrahepatic and extrahepatic bile ducts.

1.8. Histology of Intraductal Papillary Neoplasm of the bile duct (IPNB)

IPNBs are a pre-invasive, papillary/villous biliary neoplasm with variable tubular components, covering fine fibrovascular stalks or with fibrous stroma in

dilated bile ducts. Some cases of IPNB, particularly oncocytic subtype, show mildly widened stroma due to edema and inflammatory cell infiltration (48). The histology of IPNB is heterogeneous, depending on the subtypes, mucin production, grade of cytoarchitectural atypia, invasion, and tumor location along the biliary tree.

The diagnostic criteria for low- and high-grade dysplasia of IPNB may not be the same among global regions, institutions and pathologists, and sampling error may also be a challenging issue for this two-tiered system, particularly in small specimens from IPNB, a grossly visible tumor with non-homogenous histology. Recently, Japan–Korea expert pathologists discussed the possibility of subclassification of IPNB based on the structural changes of IPNB combined with a two-tiered grading system (low-grade and high high-grade-dysplasia), and proposed type 1 and type 2 subclassification(50, 59).

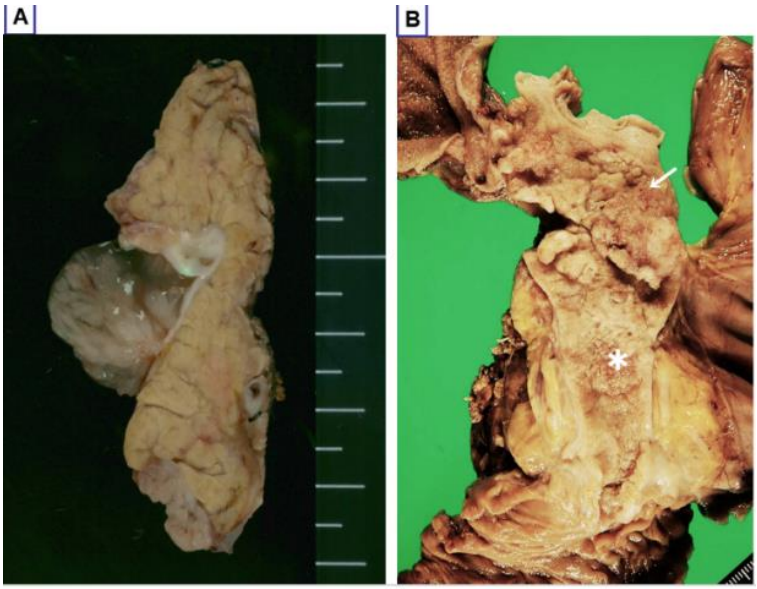

Figure 2. Gross features of intraductal papillary neoplasm of the bile duct (IPNB). A. Single papillary Neoplasm in the extrahepatic bile duct is covered by is covered by mucin layer. B. Polyploid lesions → and surrounding granular or rough mucosa (*) are regionally distributed in the perihilar.

1.9. Morphological Features Characterizing IPNB type 1 and type 2.

a) Type 1 IPNB

This type of IPNB shows regular papillary, villous, or tubular structures and a relatively homogeneous appearance. Papillary fibrovascular stalks are generally thin (depending on the subtype), while fibrovascular stalks are variably widened at the basal side in some cases. The structures are regular and homogeneous in appearance. IPNBs with low-grade dysplasia (about 10% of all IPNBs) and those with high grade dysplasia with regular structures (30%) belong to type 1. Type 1 IPNB is histologically classified into four classifiable subtypes on the lining epithelial cells and architecture, including fibrovascular stroma: pancreatobiliary, Intestinal, Gastric and oncocytic. While many cases are predominantly composed of individual subtype, a mixture of other subtypes are frequently observed as is the case in IPMN (Albores-Saavedra et al 2010. WHO Classification of digestive system.)

b) Type 2 IPNB

This type shows irregular structures and a non-homogeneous appearance and is composed of high-grade dysplasia and irregular structures (60% of all IPNBs). In addition, this type commonly shows foci of complicated lesions or structures, such as cribriform, compact tubular and solid components or patterns, as well as

relatively large cystic changes within the tumor and foci of bizarre cells and nuclear changes appearing as overt malignancy.

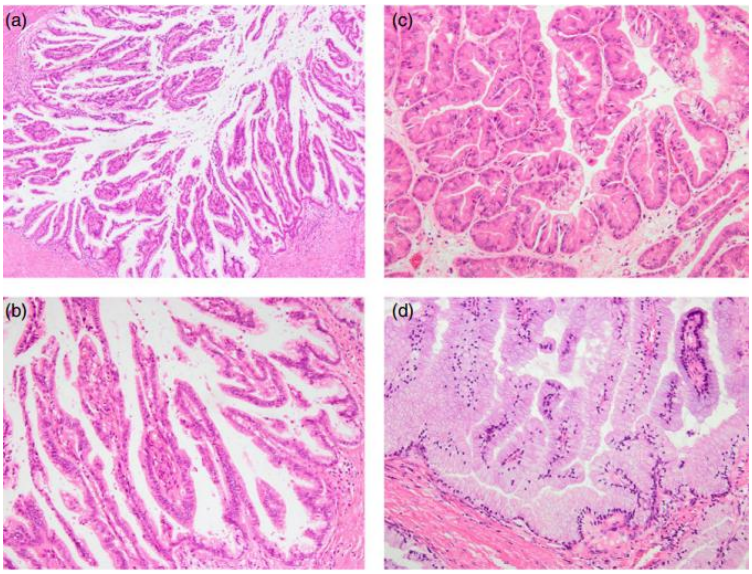

Figure 3. Histopathological features of classical intraductal papillary neoplasm of the bile duct (IPNB) type 1. (a, b) Tumor cells are arranged in an overall well-organized, high-papillary architecture. (. a) Pancreatobiliary type. b) Intestinal c) Oncocytic type with complex architecture d) Gastric type

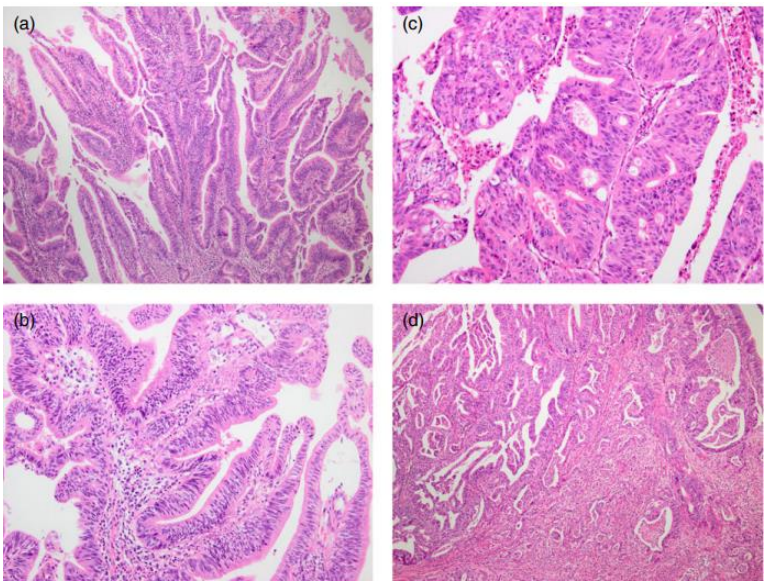

Figure 4. Histopathological features of type 2 IPNBs (papillary cholangiocarcinoma). (a, b) The papillary architecture is complex with irregular branching and thick fibrovascular stalks. (c) Neoplastic cells show a tubular growth with intraluminal necrosis. (d) The tumor is associated with invasive cancer

Table 2. Characteristics of the four subtypes of Intraductal papillary Neoplasm of the bile duct (IPNB)

Four subtypes	Description	Immunohistochemistry
Intestinal subtypes	➢ Neoplastic epithelial lining with columnar cells showing cigar-shaped nuclei and basophilic or amphophilic cytoplasm with variable amount of mucin. ➢ Presenting mainly with tubular patten	➢ Positive for CD20/CDX2 in their cytoplasm ➢ Positive for MUC2 in the goblet cells
Gastric type	➢ Neoplastic lining of tall columnar cells with basally oriented nuclei and abundant pale mucinous in the cytoplasm ➢ High grade dysplasia showing columnar epithelial cells with more complicated structures	➢ Positive for MUC5AC in foveolar areas and MUC6 in the pyloric gland.
Pancreatobiliary type	➢ Ramifying fine and think branches and papillae covered by cuboidal to low columnar epithelial cells ➢ Round hyperchromatic Nucleoli ➢ Irregular papillary architectures.	➢ Positive for S100P and MUC 1, and negative for MUC5AC

Oncocytic subtype	➢ Complex and arborizing papillae with a stroma lined with by several layers of cuboidal columnar cells ➢ Hyperchromatic, round, large, and uniform nuclei ➢ Frequent in intraepithelial lumina	➢ Positive for MUC5AC

Table 3. Pathological features of two types of biliary papillary neoplasm

Features	Type 1 IPNB(Classical)	Type 2 IPNB Papillary Carcinoma
Location	Commonly intrahepatic bile duct	Intrahepatic and extrahepatic bile duct including perihilar
Gross Mucin	Common (approximately 80%)	Rare (approximately 10%)
Histological architecture	Well organized papillary growth with thin fibrovascular stalks. Relatively uniform growth in the tumor. Gastric or oncocytic types show a tubular architecture	Complex papillary growth with thick papillae or irregular branching with fine fibrovascular cores. Different growth patten in the same tumor. Tubular or solid components with necrosis are observed**.**
Histological types	Intestinal or oncocytic types. More than one histological type co-exists.	Pancreatobiliary or intestinal types. More than one histological type may co-exist.
Associated with invasive cancer	Approximately 50%	Approximately over 90%

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1.10. Diagnosis, Treatment and Post -operative Outcomes of IPNB

A high potential for high-grade dysplasia (or carcinoma in situ) and frequently invasive nature but usually confined to the duct(13) at the diagnosis are hallmarks of IPNB. Furthermore, the recurrence rate of IPNB is high, being found in up to 29% of cases, potentially impairing the long-term outcomes (15).

The diagnosis of IPNB can be challenging due to its varying clinico-radiological presentations (15, 60). Imaging plays a major role in not only the diagnosis of IPNB but also the management strategy employed, and with improvements in imaging equipment and diagnostic technology, including cholangioscopy, the early diagnosis rate of IPNB is increasing (45, 49, 51).

CT and MRI are frequently used in the diagnosis of IPNB, with typical findings being biliary tract dilatation and an intraductal mass. A preoperative tissue diagnosis provides important information, particularly when a villous or papillary neoplasm is obtained However, its practical application remains limited at present. A preoperative misdiagnosis of IPNB can occur in clinical practice due to its low incidence, lack of specific tumor markers and unclear pathogenesis(16).

Early surgical resection is strongly advisable for radiologically suspected IPNB to prevent disease progression(13, 54), and surgery is performed in the same manner as surgical resection for conventional p/dCCA and large duct iCCA(13, 16, 41, 47, 61–64). Regional lymphadenectomy should also be performed.

Extrahepatic IPNBs tend to be removed by bile duct resection or pancreato-duodenectomy (13, 60), while IPNBs of the intrahepatic bile duct and perihilar bile ducts tend to be removed by hepatobiliary resection (60). Local excision of the biliary tract is applicable for lesions of the middle part of the extrahepatic bile duct, and pancreato-duodenectomy is suitable for distal bile duct tumor [18]. The type 1 and 2 subclassification of IPNB may be helpful for making decisions concerning the surgical approach, as type 1 IPNB usually shows less aggressive behavior than type 2 IPNB and develop preferentially in the intrahepatic bile duct(13, 65) . Therefore, a significant difference in the surgical procedures used has been found between these two types (13). Hepatic resection is mainly performed for patients with type 1 IPNB, whereas patients with type 2 IPNB undergo hepatic resection, pancreato-duodenectomy or bile duct resection.

1.11. Histological Grades of IPNB

IPNB can be classified into 1) Low grade IPNB, II) High grade IPNB and III) invasive IPNB. Low grade IPNB presents so called low grade biliary epithelial dysplasia or borderline lesion and cellular and nuclear atypia are mild, and so-called papillary adenoma or biliary papilloma or papillomatosis were included in this category. High grade IPNB is non-invasive or in situ papillary adenocarcinoma with fine fibro-vascular connective tissue and show cellular/nuclear and structural atypia enough for malignancy. Invasive IPNB is IPNB with evident invasion of carcinoma cells to the bile duct wall and/or the surrounding structures including

liver parenchyma and pancreas and show basically papillary adenocarcinoma and show cellular/nuclear and structural atypia within the intraductal papillary tumor, similar to high grade IPNB. When the invasion into the duct wall and periductal tissue was suspicious and not evident, such cases were included in high grade IPNB. Some morphologic features of IPNB have been identified, such as diffuse or segmental ductal dilatation and the appearance of an intraductal growing mass. Nearly one-third of IPNB cases are associated with macroscopic mucin hypersecretion, therefore, bile duct dilation is often observed (60, 66).

Invasive IPNB was further divided into minimal (microscopically identifiable) invasive type and grossly visible invasion. The former was confirmed mainly at the invasion to the bile duct wall and periductal tissue, and the latter showed grossly visible invasion to the periductal tissue, including the surrounding structures such as the periductal connective tissue, liver parenchymal and pancreas. Some of the latter could be grossly described as mass or nodule formation or thickening of affected bile duct.

1.12. Patients-Derived Xenograft Models (PDX)

Patient-derived xenograft (PDX) mouse models are used preclinically in cancer drug development and the molecular profiling of tumors. They have been shown to recapitulate the histologic and genetic features of human primary tumors and to be useful in assessing treatment response(67). Evidence has shown that PDXs retain the genome-wide exomic nucleotide variants, gene copy number

alterations, and DNA methylation patterns of their corresponding tumors(68, 69)irrespective of the number of passages, although clonal selection during initial engraftment and passaging of PDXs has been observed(70). The development of novel therapeutics has been hampered in part through high clinical and biologic heterogeneity and the lack of distinguishable histologic subtypes. However, the age of next-generation sequencing and integrated genomics is providing increasing evidence for molecularly defined subtypes. Although strict correlation with clinical outcome remains elusive, tumors can now be classified by their genome copy number, fusion gene profiles, mutational landscapes, and even mRNA splicing patterns(71–73).

In vivo propagation of a patient's tumor tissue in immunodeficient mice can enable the simultaneous evaluation of the tumor response to several drugs and treatment regimens, leading to the identification of an effective therapy for the patient. Nowadays, pathogenesis and drug response are usually studied on preclinical models represented by cell lines, primary cultures, and xenografts. Xenografts and orthotopic models obtained by CCA cell lines, carcinogen-induced and genetically engineered mouse model for CCA has been created (74).

However, the development of molecular targeted drugs, which target specific molecules in diverse and complex tumors, requires a model that permits the appropriate expression and function of the target molecule in tumor cells(75, 76) . In this context, the xenograft model, which involves the implantation of cultured tumor cell lines established from tumor tissue into immunodeficient mice, has been often used as an in vivo model for cancer research (75). As is the case for in vitro

models, constant tumor cell proliferation is maintained in xenograft models, and the validity of anti-cancer drugs based on the results of non-clinical studies has been assured to some extent. Furthermore, the correlation of the outcomes of preclinical studies using xenograft models of cell lines possessing certain driver mutations with clinical efficacy is known(77). However, because cultured cell lines consist only of specific tumor cells adapted to culture conditions that differ markedly from the in vivo environment, xenograft models of cultured cells are not considered, at present, to reflect the diversity and complexity of tumors(77).

In the last years, patient-derived cancer xenograft (PDX) models have been established by directly engrafting surgically resected human tumor tissues into immune compromised mice. Molecular and genetic analysis demonstrated that PDXs rely primary tumor characteristics, making them suitable models to study pathogenesis and to test anti-cancer drugs activity.

PDXs are established from different cancer types, including gastric, breast, ovarian, colon, lung, prostate, and pancreatic cancers (78–82).

The development of novel therapeutics has been hampered in part through high clinical and biologic heterogeneity and the lack of distinguishable histologic subtypes. However, the age of next-generation sequencing and integrated genomics is providing increasing evidence for molecularly defined subtypes. Although strict correlation with clinical outcome remains elusive, tumors can now be classified by their genome copy number, fusion gene profiles, mutational landscapes, and even mRNA splicing patterns(72, 83).

To exploit emergent discoveries for mechanistic understanding and therapeutic advances, focus must now turn to the development of a new generation of preclinical models that capture the "omic" diversity of cancer. Preclinical cancer models for *in vivo* drug tests are commonly based on immune-deficient mice carrying subcutaneous cancer cell line xenografts. Unfortunately, these models fail to reproduce the diverse heterogeneity observed in the clinic, partly due to the increased homogeneity of established cell lines after long-term in vitro culturing. Furthermore, cell line xenografts rarely possess the tissue architecture of the original cancer specimens from which the cell lines were derived and, consequently, do not accurately represent the complex biochemical and physical interactions between the cancer cells and various components of their microenvironment as found in the original malignancies. Unsurprisingly, therefore, cell line xenografts frequently fail to adequately predict the efficacy of anticancer agents in the clinic(84).

In theory, patient-derived cancer tissue xenograft models, based on direct implantation of fresh cancer tissue specimens into immunodeficient mice [NOG] (severe combined immunodeficient) mice], provide the needed clinical relevance. In other cancers, these xenografts retain the cellular heterogeneity, architectural and molecular characteristics of the original cancer, and its microenvironment.(85).

1.13. IPC Study Justification

IPC, a phenotype of papillary Cholangiocarcinoma is treated by surgical resection and palliatively with radiotherapy and chemotherapy. Recurrence rate after surgery is more than 60% in less than 4 months. Pathological description is still very confusing despite the realization that IPC is a unique pathology from all IPNBs. Clinical manifestation is not known because the tumor biology is not known.

The lack of proper CCA experimental model is a major limitation even though the therapeutic outcome of CCA differs significantly depending on the degree of invasion and the choice of anticancer agent according to the tumor subtype. Currently, 15 different CCA cell lines are commercially available and several kinds of *in vivo* animal models(81-83).

An ideal preclinical cancer model is the one that recapitulates both human tumor heterogeneity as well as the TME, considering the complexity of cancer (88, 89). Currently *in vivo* and *in vitro* models rely on the reconstruction of the original human tumors and in some cases, subsequent deconstruction with combinations of selected cell types (clonal cells) (90)

We aimed to establish *in vivo* and *in vitro* models that retains the intrinsic characteristics of the tumor and increase the success rate of model establishment. In addition to evaluate the derived model for biomarker identification for invasive papillary cholangiocarcinoma. Patient-derived xenograft (PDX) models are an important missing component in the

development of IPC diagnostic and therapeutic system, that would enable the examination of tumor tissue without affecting the heterogeneity, proteomic, genomics, and architecture of IPC. Furthermore, the strategy was to establish a cell line from PDX tissue to increase the success rate of cell line establishment.

To the best of our knowledge, this is the first study to report the successful establishment of PDX and PDX-derived cell line for IPC using tissue obtained from a patient. Our results will help develop new suitable models for the translational and preclinical studies of IPC.

Establishing tumor models for IPC will help:

a) To gain a better understanding of IPC tumor biology
b) Be able to investigate novel anticancer combinations that can be used in IPC
c) Be able to monitor treatment response and resistance, thus design models that can overcome this.
d) Be able to design personalized medicine.

1.14. Purpose of the Study of Invasive Papillary Cholangiocarcinoma (IPC)

Establishment of preclinical evaluation systems that can contribute to the clarification of IPNB (with invasive) [IPC] biology and facilitate translational research.

2. Material and Methods

2.1. Ethics statement

The study was approved by the institutional review board (IRB) of the National Cancer Institute (NCC), and the patient provided written informed consent. All processes complied with the Declaration of Helsinki (IRB approval No: NCC-2015-0245).

All animal studies were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of the National Cancer Centre Research Institute (NCCRI) (NCC-16-313, NCC-21-313G). The NCCRI is a facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC) and abides by the guidelines of the Institute of Laboratory Animal Resources (ILAR) (Accredited unit-NCCRI: unit number 1392).

2.2. Patient Details

Our study participant was a 71-year-old woman diagnosed with intraductal papillary neoplasm with an associated invasive carcinoma by pathological examination and imaging. The patient was admitted to the hospital for surgical resection in November 2016 with ECOG 1 and underwent surgery of the liver, extended right hemihepatectomy with bile duct resection, at the NCC. There was metastasis in one lymph nodes (1/10), the pathological stage was pT3N1, and the tumor was moderately differentiated (Table 4).

Table 4. Characteristics of the patient with invasive papillary cholangiocarcinoma reported in this study.

Characteristics	
Gender	Female
Age	71 years
Primary tumor origin	Common bile duct
	Common bile duct involved, cystic duct obstruction. 1 lymph node involved
Tumor histology	Intraductal papillary neoplasm with an associated invasive carcinoma
Tumor grade classification	pT3N1
CA19-9	43.3 U/ml
CEA Baseline	45.2 ng/ml

2.3. Establishment of PDX from Patient Tissue

Female immunodeficient NOG mice aged 5–8 weeks (Harlan Laboratories, Inc. Indianapolis, IN, USA) were housed in a specific pathogen-free environment under controlled light and humidity conditions and were allowed food and water. The NOG mouse was anesthetized using 2% isoflurane in 100% oxygen. The patient tissue (F0) was removed from the medium and cut into approximately 3 mm3 pieces in a sterile petri dish with fresh medium and maintained on ice. To establish an F1 generation of PDX, a 5 mm horizontal incision was made on the flank of the mouse to create a subcutaneous pocket. a tumor piece mixed with growth medium and Matrigel was inserted in the incision and sealed with a black silk suture. Povidone iodine was applied at the incision site. After completion of the procedure, the mouse was returned to the storage box and observed for complications. Tumor growth rate was monitored twice per week by measuring the tumor size using a caliper (Mitutoyo, Japan) and calculated using the formula: (Width2 x Length)/2. The mouse was euthanized with CO2 once the tumor size reached approximately 1500 mm3. The tumor was then removed and triaged for cryopreservation and expansion in the secondary recipient mouse for establishing the F2 and F3 generations of PDX and for performing histological and molecular analyses.

2.4. H&E and Immunohistochemical Analysis

The tumor was fixed in formalin, implanted (embedded) in paraffin, sectioned, and stained for histopathological assessment using hematoxylin and eosin (H&E). Immunohistochemical detection of CK19, MUC1, MUC2 and MUC5AC was performed using the rabbit anti-human MUC1 mAb clone: EPR 1025,1:3000 dilution, rabbit anti-human MUC2 mAb clone EPR 6145 1:5000 dilution, rabbit anti-human MUC5AC mAb clone EPR 16904 1:500 dilution, ant-CD74 [EPR4064] ab 108393,1:100 dilution, and rabbit amt-human CK19 mAb clone Ep1580Y 1:400 dilution. Incubation with the respective antibodies was performed overnight at 4 °C. Immunodetection was performed using an Envision Plus system (Dako Carpinteria, CA, USA) with 3,3-diaminobenzidine (DAB/H2O2) chromogen. The immunostained sections were then counterstained with hematoxylin and coverslipped for microscopic assessment. All images were captured by Vectra® Polaris™ imaging system (PerkinElmer, USA).

2.5. Establishment of the Human Invasive Papillary CCA Cell line, NCChIPC

The cell line was established from the PDX F2. The tumor mass from F2 was minced and dissociated mechanically and chemically by GentleMACS dissociator (Milteny Biotec, Germany) for 45 min. The cell suspension was washed by centrifugation and resuspended in Dulbecco's modified Eagle's medium (DMEM)/F-12 supplemented with epidermal growth factor (EGF) and the

antibiotics zellshields™ (Minerva Biolabs, Berlin Germany). The cell suspension was filtered through a strainer with a mesh size of 70 µm, suspended in a growth medium and incubated at 37 ºC and 5% CO2. The cells were seeded and cultured until the doubling time could be estimated. The cells were trypsinized, fresh medium added and counted. Primary cultured cells were observed periodically, and contamination with fibroblasts was aseptically removed by trypsinization until they were free of fibroblasts. The cultured cells were initially subcultured every week until they grew at a stable rate. When they reached 70% confluence using trypsin-EDTA (Invitrogen), the cells were cultured in growth media after a few passages of primary culture. Contamination with mycoplasma was monitored periodically using an e-mycoTM Mycoplasma PCR detection kit [ver.2.0]. The culture remained free of mycoplasma during the experiments.

2.6. Cell Proliferation Assay

NCChIPC cells were seeded at a density of 5,000 cells/well (100 µL of suspended cells in enriched DMEM) in a 96-well plate. Cells suspended in growth medium were incubated in Incucyte Zoom (Sartorius, Essen Bioscience, USA) at 37 ºC and 5% CO_2. The cells were then monitored for 6 days and assayed for proliferation.

2.7. NCChIPC Cell line-derived Xenograft

To detect *in vivo* tumorigenicity, a mixture of *NCChIPC* cells (5.0×10^6) and 1:1 ratio of growth factor to Matrigel was injected subcutaneously in an NOG mouse subcutaneously. The tumor growth was monitored twice per week. When the tumor size reached 1500 mm3, the mouse was sacrificed, and the tissue samples were obtained and fixed in 10% phosphate-buffered formalin overnight. The tissue sample was then embedded in paraffin for histopathological evaluation.

2.8. Cytotoxicity Assays for Anticancer Drugs

Gemcit® (Gemcitabine-HCl) and cisplatin were obtained from Dong-A ST Co., Ltd., Seoul, Korea; albumin-bound paclitaxel (Abraxane®) was obtained from Celgene Corporation, NJ, USA; and onivyde (Irinotecan liposome injection) was obtained from Servier, France. 5-FU, Oxaliplatin, erlotinib, epirubicin, carboplatin, and devimistat were purchased from MedChem Express. For analyzing the drug responses, NCChIPC cells (4 × 103 cells/well) were seeded in 384-well plates and stabilized for 24 h, followed by incubation with the following drugs for 72 h: 0.001 μM to 10 μM of Gemcit®, albumin-binding paclitaxel (Abraxane), and 0.01 μM to 100 μM of 5-FU, onivyde, oxaliplatin, cisplatin, erlotinib, epirubicin, carboplatin, and devimistat. Cell cytotoxicity was measured using the Cell Titer-Glo® Viability assay kit (Promega Corporation, WI, USA). Plates were read using luminescence infinite 200 Pro (Life Sciences). Dose-dependent response was determined using the GraphPad Prism5 software.

2.9. Short Tandem Repeats

Short tandem repeat (STR) analysis was performed at 10 loci on different chromosomes to verify that the PDX, PDX-derived cells, and xenograft tissue F1, F2, and F3 samples were derived from the patient (F0). STR loci (*TH01, D21S11, D5S818, D13S317, D7S820, D16S539, CSF1PO, AMEL, vWA, TPOX*) amplification was performed using a Gene Print R10 system kit (Promega, Madison, WI, USA) according to the manufacturer's instructions. Samples were run on an ABI 3730 DNA Analyzer (Thermo Fisher Scientific) and analyzed using Gene Mapper v4.0.

3. Results

3.1. Establishment of PDX Model for Invasive Papillary CCA

The Overall strategy of the establishment of preclinical model for IPC is summarized in Figure 5. The features of primary IPC patient tumors are shown in Figure 6A and B. To generate the PDX model, small pieces of tumor were engrafted on the left flank of an NOG mouse and tumor growth was monitored by palpating the site of the implant. When the engrafted tumor reached 1500 mm3, the mouse was sacrificed, and the tumor was removed by sharp dissection. Tissue from the first mouse was considered the first generation of PDX or F1 tissue. A second NOG mouse was then implanted with tissue from F1 to form the next generation in serial order. Tissue-derived xenograft morphology was analyzed using H&E staining. The retention of histopathological characteristics of xenografts derived from F1, F2, and F3 was determined by immunohistochemistry for IPC markers such as CK19, MUC1, 2, 5AC .CK19 is well expressed in all xenograft tissues, similar to the parent tumor. MUC1 and MUC5AC are also expressed in all tissues; in contrast, MUC2 was not expressed in IPC, which was expected. Additionally, STR analysis was performed to confirm the paternity authentication for the presence of chromosomal aberrations in the PDX model compare with that in the original tumor. STR analysis at 10 loci demonstrated that PDX-derived models were unique and matched with the original patient tumor (Table 5). This PDX model is a promising first report of IPC PDX.

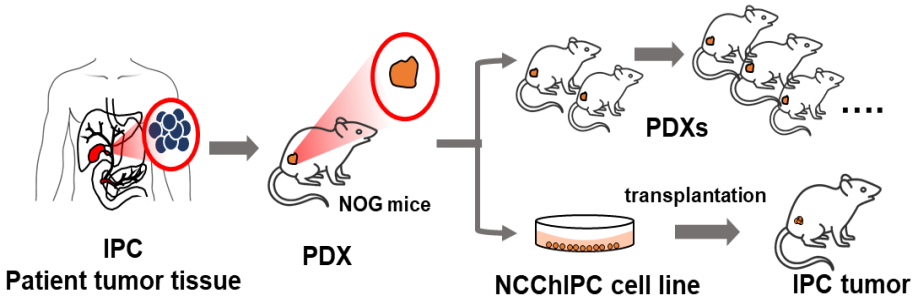

Figure 5. Schematic diagram of the establishment of a preclinical model of invasive papillary cholangiocarcinoma (IPC) both in vitro & in vivo. The first step is the generation of a patient-derived xenograft (PDX) from the surgical tissue of IPC. Next, the primary patient tumor sample is engrafted in immunocompromised (NOG) mice. Then, the xenograft tumor was expanded in successive mice to develop the F2 and F3 passages. Thereafter, PDX tumor tissue-derived cells can be isolated (temporarily termed the NCChIPC cell line) and form tumor nodules with IPC characteristics by engraftment in immunodeficient mice, which can then be used as an *in vivo* model of IPC.

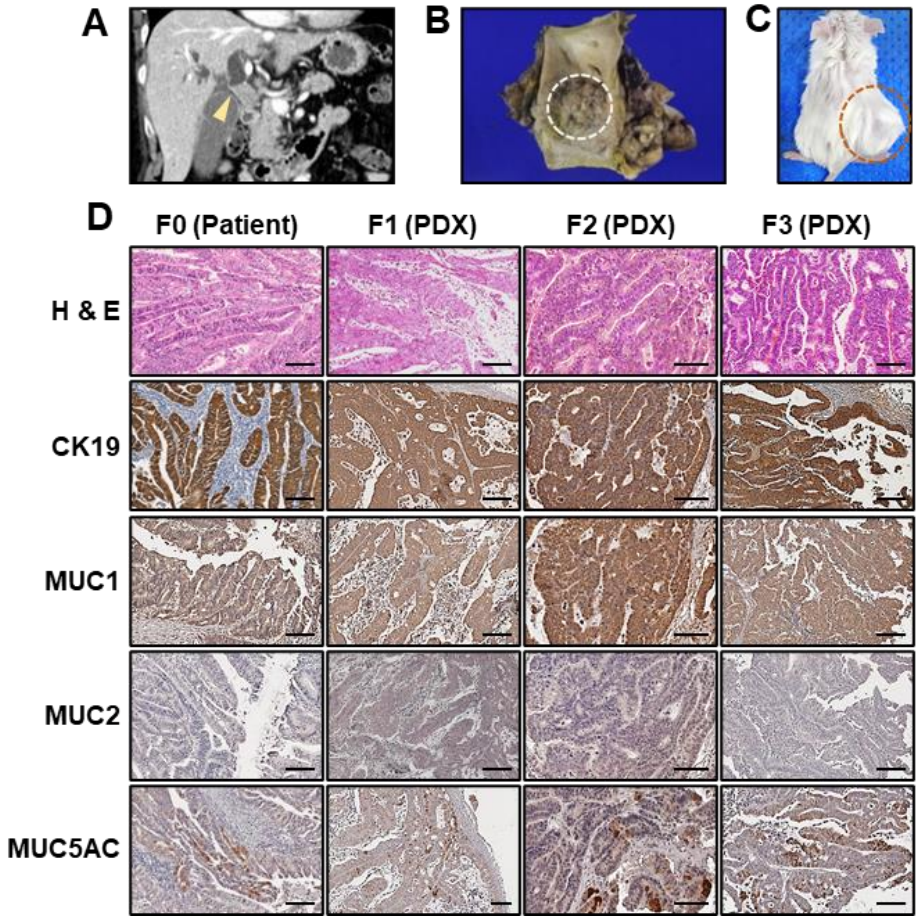
A
B
C
D
F0 (Patient)
F1 (PDX)
F2 (PDX)
F3 (PDX)
H & E
CK19
MUC1
MUC2
MUC5AC

Figure 6. Establishment of IPC PDX model and retention of histopathological features of primary tumors by PDX tumors. (A) The patient abdominal CT image showed a bile duct defect as indicated by yellow arrowhead and (B) papillary tumor region of bile duct in patient surgical resected specimen is indicated by white circle. (C) The patient's surgical tumor sample was subcutaneously engrafted in the flank of an immunocompromised NOG mouse for the generation of PDX F1. The tumor nodule is indicated by the yellow arrow. (D) Morphological and histological features between the patient (F0) and PDXs (F1–F3) are validated based on H&E staining and immunohistochemical analysis of CK19 and mucin subtypes. Histological and morphological features resembled the parent tumor. CK19 was expressed in all xenograft tissues similar to the parent tumor. MUC1 and MUC5AC were highly expressed in all the tissues. MUC2 were not expressed in IPC tissues. Scale bars = 100 μm.

Table 5. Short tandem repeat analysis performed at 10 loci on different chromosomes to verify that the PDX F1, F2, F3 and cell-derived xenografts were derived from the primary patient sample F0. PDX were consistent with F0 (patient).

Locus	*TH01*	*D21S11*	*D5S818*	*D13S317*	*D7S820*	*D16S539*	*CSF1PO*	*AMEL*	*vWA*	*TPOX*
F0	7,9	29,32.2	11	9,12	8	12	12	X	17,18	8
F1	7,9	32.2	11	9,12	8	12	12	X	17,18	8
F2	9	32.2	11	9,12	8	12	12	X	17,18	8
F3	7,9	32.2	11	9,12	8	12	12	X	17,18	8
NCChIPC	9	32.2	11	9,12	8	12	12	X	17,18	8

3.2. Establishment of a New Cell line from Invasive Papillary CCA

Cancer cells were isolated from PDX F2 tissue and showed an adherent epithelial-like morphology. We termed these cell lines NCChIPC, human IPC at NCC (Figure 7A). Proliferation monitoring using the Incucyte machine revealed that the population doubling time was approximately 28-36h (Figure 7B). We inoculated different anticancer drugs available for CAA and observed that Abraxane was the most toxic, followed by epirubicin. Other drugs including gemcit, erlotinib, carboplatin, and 5-FU were less toxic (Figure 7C). These results suggest that the NCChIPC cell line can be used as an in vitro model for evaluating the drug response in IPC treatment.

3.3. Recapitulation of Parental Molecular Characteristics in PDXs and NCChIPC Cell line

To evaluate whether the established patient-derived preclinical models have retained the characteristics of the original tumor, we performed RNA sequencing to determine the similarity between the gene expression of the three generations of PDX (F1, F2, and F3) and NCChIPC cell lines with those of the F0 tissue. Overall, the correlation of PDXs with F0 tissues showed very high similarity (94%–98%). Moreover, the NCChIPC cell line reflected about 81% of the patient's transcriptomics characteristics. Next, we determined the RNA levels of 94 genes expressed in our library among 100 target genes corresponding to the proliferative

or metabolic subclasses of eCCA, which was reported. While only a few genes related to the metabolic class were overexpressed, most proliferation class-related genes were highly expressed, and the level was retained not only in several passages of PDX generations but also in NCChIPC cells. These results suggest that our preclinical models for IPC reflect the genomic characteristics of the patient.

3.4. Tumor Formation by Xenograft of NCChIPC Cells and Histological evaluation

Subcutaneous xenografts of NCChIPC cells successfully formed tumor nodules and exhibited a prominent papillary growing pattern of CCA morphologically by H&E staining despite of cell line implantation model. Immunohistochemical analysis of the tissues showed that they exhibited positive expression of CK19 and MUC5AC, which are the typical markers of IPC. These results strongly suggest that NCChIPC cell-derived *in vivo* models can be easily used in IPC research.

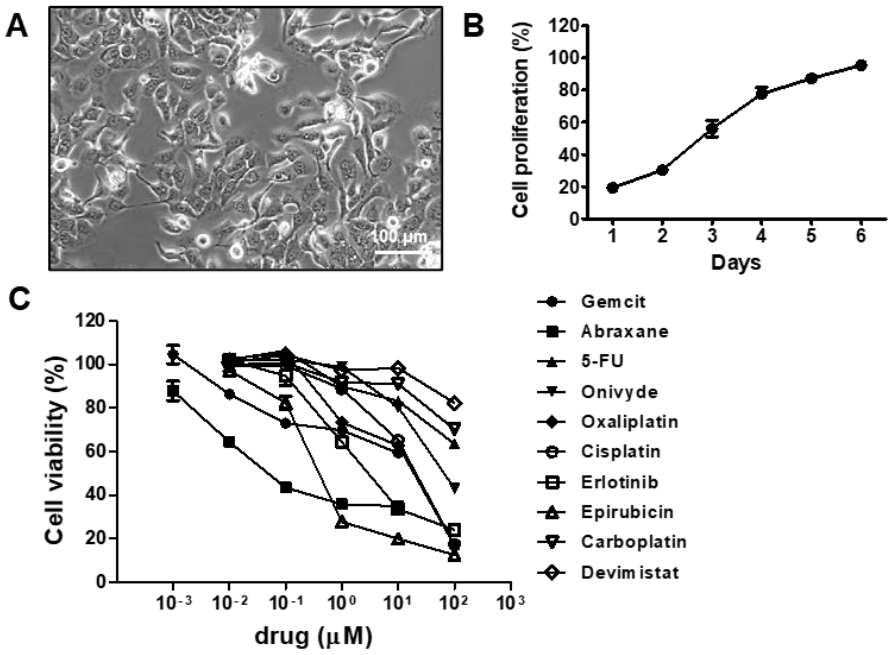
A
100 µm
B
Cell proliferation (%)
120
100
80
60
40
20
0
1
2
3
4
5
6
Days
C
Cell viability (%)
120
100
80
60
40
20
0
10-3
10-2
10-1
100
101
102
103
drug (µM)
Gemcit
Abraxane
5-FU
Onivyde
Oxaliplatin
Cisplatin
Erlotinib
Epirubicin
Carboplatin
Devimistat

Figure 7. Establishment of the NCChIPC cell line for IPC. NCChIPC cells were isolated from the PDX tumor tissue. (A) The NCChIPC cell morphology of passage 10 in monolayer culture. (B) Proliferation curve of NCChIPC cells in culture medium through Incucyte. (C) Dose-response curve in NCChIPC according to different drugs. NCChIPC cells were seeded in a 384-well plate and incubated overnight. The cells were then exposed to drugs according to the indicated concentrations, there were different responses (toxicities) to different cancer medicines, with stronger effects of epirubicin, Abraxane®, Gemcit and weaker effects with erlotinib. Error bars represent the standard deviation of three independent experiments.

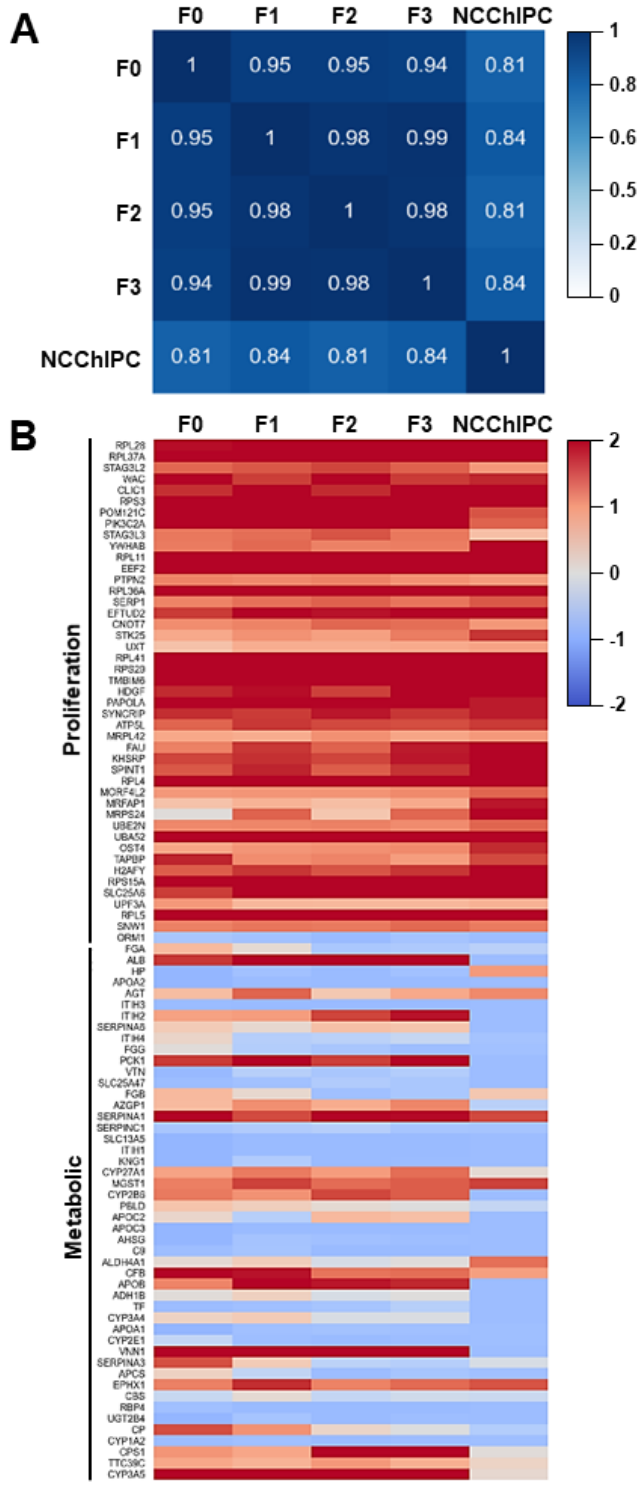
A
F0
F1
F2
F3
NCChlPC
1
0.95
0.95
0.94
0.81
0.95
1
0.98
0.99
0.84
0.95
0.98
1
0.98
0.81
0.94
0.99
0.98
1
0.84
0.81
0.84
0.81
0.84
1
1
0.8
0.6
0.5
0.2
0
B
F0
F1
F2
F3
NCChlPC
2
1
0
-1
-2
Proliferation
RPL28
RPL37A
STAG3L2
WAC
CLIC1
RPS3
POM121C
PIK3C2A
STAG3L3
YWHAB
RPL11
EEF2
PTPN2
RPL36A
SERP1
EFTUD2
CNOT7
STK25
UXT
RPL41
RPS23
TMBIM6
HDGF
PAPOLA
SYNCRIP
ATP5L
MRPL42
FAU
KHSRP
SPINT1
RPL4
MORF4L2
MRFAP1
MRPS24
UBE2N
UBA52
CST4
TAPBP
H2AFY
RPS15A
SLC25A6
UPF3A
RPL5
SNW1
ORM1
Metabolic
FGA
ALB
HP
APOA2
AGT
ITIH3
ITIH2
SERPINA5
ITIH4
FGG
PCK1
VTN
SLC25A47
FGB
AZGP1
SERPINA1
SERPINC1
SLC13A5
ITIH1
KNG1
CYP27A1
MOGAT1
CYP2B6
PBLD
APOC2
APOC3
AHSG
C9
ALDH4A1
CFB
APOB
ADH1B
TF
CYP3A4
APOA1
CYP2E1
VNN1
SERPINA3
APCS
EPHX1
CBS
RBP4
UGT2B4
CP
CYP1A2
CPS1
TTC39C
CYP3A5

Figure 8. High correlation between patient (F0) and PDX (F1-F3) & NCChIPC cell line from Transcriptome. (A) A correlation matrix showing the Pearson correlation coefficient calculated from the expression of 27686 genes (FPKM). (B) A heat-map showing the expression of genes related to two extrahepatic cholangiocarcinoma (eCCA) subclasses (proliferative and metabolic classes). The molecular subtype of patients with IPC was successfully engrafted as PDX and NCChIPC. Gene expression was analyzed by calculating the Z-score by using the normalized data.

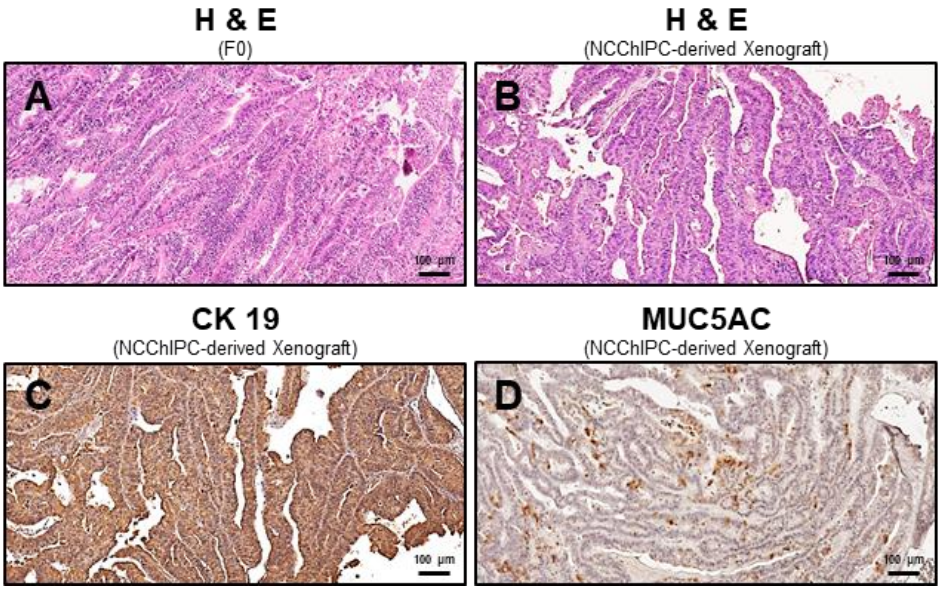

Figure 9. Tumor formation in vivo from subcutaneous implantation of the NCChIPC cell line in NOG mouse. (A-B) The morphology of the NCChIPC cell-derived xenograft tumor (B) was similar to the IPC patient tumor tissue (A) from H&E staining, showing the papillary shape. (C-D) CK19 and MUC5AC were expressed in NCChIPC cell-derived xenograft tumor tissues. Scale bars = 100 μm.

4. Discussion

IPC is a minor phenotype of CAA, for which preclinical models are difficult to establish. Nevertheless, it has been shown that preclinical models are key to basic and translational research, with the potential for indispensable to assess human tumor biology, identification of therapeutic targets, biomarker and preclinical testing and evaluation of drugs for various cancers (76). Previously, cell line xenografts were used as standards in preclinical research; however, generally cell lines did not precisely mirror the true picture and behavior of the host tumor and were able to adapt to in vitro growth, losing the native properties of the host tumor (91).

PDXs are platforms that can represent the complexity and diversity of cancer and are known to preserve major important biological and morphological properties of tumors from which they were obtained, and maintain the same stability across passages(92, 93). These models can predict clinical outcomes and are useful for precision medicine. PDX models are established by engrafting patient tumor tissues in immunocompromised NOG mice and subsequently observing the passage of tumor cells from human tissues to the animal(76). Xenografts derived directly from patients' surgical tissue or biopsy samples with minimal in vitro manipulation retain the morphological and molecular markers of the source tumors despite serial passaging across several generations of mice(92, 94).

In the present study, we established a PDX and cell line for IPC with an invasive phenotype using patient-derived tumor tissues. The PDX was established by engrafting surgically resected tumor tissues in NOG mice. The PDX expanded successfully to generate subsequent tumors at F1, F2, and F3 generations, with the retention of the histological and molecular features of the original tumor. Histology of original papillary carcinoma patient (F0) showed typical phenotypes of fibrovascular core and mitotic figure. This papillary growth pattern was retained throughout PDX passage. Meanwhile, at F3, a slightly complex papillary with a distinct shape was observed. However, overall, the invasive papillary type was well-maintained. CK19 and mucin expression in F1, F2, and F3 tumors were consistent with that in the patient's tumor tissue. CK19 as a positive marker of CCA showed strong positive expression in all PDX and original tumor. In addition, we observed the expression of MUC1 and MUC5AC and no expression of MUC2 in all the tissues (Figure 6D). MUC1 and MUC5AC were significantly expressed in both PDX and patient tissue.

A papillary growth pattern was retained throughout the passage. CK19 and mucin expression in F1, F2, and F3 tumors were consistent with that in the patient's tumor tissue. MUC1 is a surface membrane bound type of mucin (glycoprotein) detected in most epithelial cells(Guillen P et al 2000) .Mucin gene changes in esophageal adenocarcinoma showing a down regulation of MUC2, MUC5AC, and MUC6 and upregulation of MUC1 as reported seen in dysplasia ,adenocarcinoma and squamous cell carcinoma as reported (94, 96). We also observed the expression of MUC1 and MUC5AC and no expression of MUC2 in

all the tissues (Figure 6). MUC1 is membrane-bound mucoprotein that is highly expressed in invasive adenocarcinoma. The tumor biological characteristic is shown by their mucin (MUC) expression pattern. MUC1 expression and/or absence of MUC2 expression correlated with the aggressive and invasive features of IPC. MUC5AC is consistently expressed in all types of xenografts across generations. Meanwhile, at F3, a slightly complex papillary with a distinct shape was observed. However, overall, the invasive papillary type was well-maintained. The NCChIPC cell line established from the PDX F2 generation showed a typical epithelial monolayer with polygonal to spindle shapes and regular dimensions and grew exponentially as a monolayer in the growth medium (Figure 7).

Transcriptome analysis revealed that the gene expression in the NCChIPC cell line was very similar to that in the patient's tissue and the PDX. The similarity in correlation coefficient values is higher when it is closer to 1; it was observed to be almost 0.8 or higher (Figure 8A), thus reflecting the high reliability of our cell line(97). Moreover, in the heat-map, we observed that the patient's tumor belonged to the proliferative subclass (Figure 8B), and hence, anticancer drugs that target cell cycle or proliferation can be administered to this patient. Our cell line showed a very high response to Abraxane® which is known to have inhibitory effect of cell cycle progression. Therefore, these result support that our model can be used to predict drug response. The tumorigenic ability of the NCChIPC cells was also very good. In addition, a distinctly shaped, invasive papillary CCA was formed despite changes in the stromal components that occur during engraftment, whereas a homogenous tumor is formed with a cell line in general (Figure 9).

In addition, STR profiling with 10 loci performed for validation of the PDX with the tumor demonstrated that all PDX-derived models were unique and matched the original patient tissue, as observed in the DNA fingerprinting results (Table 5). These results further proved that all xenografts were derived from the primary tumor of the IPC. STR validation using the corresponding results suggests that our model is reliable. This further supports that PDX, and the established cell line are reliable tools for pre-clinical use.

Despite the many advantages of PDX for cancer modeling, there are constraints to use of PDX, such as loss of the tumor microenvironment, immune response (98, 99), selection of clonal subpopulations different from the original tumor (100) and cost effectiveness (76). However, despite these challenges, the value and use of PDX in oncology for addressing preclinical models are improving, as they continually reflect the complexity, heterogeneity, and diversity of clinical tumors. Here also used PDX for cell line establishment retaining the original heterogeneity and enhancing the success rate. NCChIPC and PDX-derived cell lines showed in vitro and in vivo heterogeneity to some extent with the characteristics of tissue architecture.

Therefore, this study is the first to establish PDX and cell lines as tools for detecting and understanding IPC.

The PDX models have been valuable tool in preclinical drugs testing in many types of cancers, as was the case while testing the effectiveness of nab-paclitaxel with gemcitabine in pancreatic cancers(100), they are also advantageous in preclinical studies of targeted agents. After genomic characterization of PDX

models, a subset of PDX model with the same genomic characteristic can be useful in target therapeutics. Furthermore, for systematic drugs testing preclinical PDX models have been found useful in testing the drugs sensitivity (Nardella C Lunard et al 2011)). PDX can also be used for generating drug resistant and sensitive tumor models(102). Additionally, the predictive power of PDX models has been applied to conduct co-clinical trials for novel cancer therapeutics (103). Collectively, the PDX model is of such a great importance in both preclinical and clinical setting to facilitate cancer research.

In conclusion, our study generated a PDX, the NCChIPC cell line, and NCChIPC-derived xenograft as preclinical models. These novel preclinical models could help in improving our understanding of the etiology of IPC and potentially facilitate translational research.

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