Original Article

Generation of An Induced Pluripotent Stem Cell Line from Human Liver Fibroblasts from A Patient with Combined Hepatocellular-Cholangiocarcinoma

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Abstract

Objective: Combined hepatocellular-cholangiocarcinoma (cHCC-CC) is a rare type of primary liver cancer with characteristics of both hepatocellular carcinoma (HCC) and cholangiocarcinoma (CC). The pathogenesis of cHCC-CC is poorly understood due to a shortage of suitable *in vitro* models. Due to scarce availability of human liver tissue, induced pluripotent stem cells (iPSCs) are a useful alternative source to produce renewable liver cells. For use in the development of liver pathology models, here we successfully developed and evaluated iPSCs from liver fibroblasts of a patient with cHCC-CC.

Materials and Methods: In this experimental study, human liver fibroblasts (HLFs) were obtained from the liver biopsy of a 69-year-old male patient with cHCC-CC and transduced with a retroviral cocktail that included four factors - OCT4, SOX2, KLF4, and c-MYC (OSKM). Pluripotency of the iPSCs was determined by alkaline phosphatase (AP) staining, quantitative real-time polymerase chain reaction (PCR), and immunofluorescence. We induced *in vitro* embryoid body (EB) formation and performed an *in vivo* teratoma assay to confirm their differentiation capacity into the three germ layers.

Results: HLF iPSCs derived from the cHCC-CC patient displayed typical iPSC-like morphology and pluripotency marker expression. The proficiency of the iPSCs to differentiate into three germ layers was assessed both *in vitro* and *in vivo*. Compared to normal control iPSCs, differentiated HLF iPSCs showed increased expressions of HCC markers alpha-fetoprotein *(AFP)* and Dickkopf-1 *(DKK1)* and the CC marker cytokeratin 7 *(CK7)*, and a decreased expression of the CC tumour suppressor SRY-related HMG-box 17 *(SOX17)*.

Conclusion: We established HLF iPSCs using liver fibroblasts from a patient with cHCC-CC for the first time. The HLF iPSCs maintained marker expression in the patient when differentiated into EBs. Therefore, HLF iPSCs may be a sustainable cell source for modelling cHCC-CC and beneficial for understanding liver cancer pathology and developing therapies for cHCC-CC treatment.

Keywords: Cholangiocarcinoma, Hepatocellular Carcinoma, Induced Pluripotent Stem Cells

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Introduction

Combined hepatocellular-cholangiocarcinoma (cHCC-CC) is an uncommon primary liver malignancy with characteristics of both hepatocellular carcinoma (HCC) and cholangiocarcinoma (CC). This mixed carcinoma exhibits a more aggressive behaviour and poorer prognosis than HCC or CC (1, 2). However, the pathogenesis of cHCC-CC is poorly understood, partly due to insufficient information and a shortage of proper *in vitro* models (2). With the scarce availability of human liver tissue, induced pluripotent stem cells (iPSCs) may be a valuable source for various models for rare types of liver cancer as they are capable of unlimited self-renewal and can maintain patient specificity (3). HCC is one of the most frequently occurring primary liver cancers worldwide. Despite improvements in prevention, diagnostic techniques, and treatment, the incidence and mortality rate are still increasing (4, 5). CC, in which biliary malignancy arises from the bile duct, is the second most frequent primary liver cancer. Based on anatomical location, CC is classified into three subtypes: intrahepatic, perihilar, or distal (6, 7). Long-term survival of CC is low, ranging from 20–40% for patients after curative resection (8).

The diagnosis of liver cancer is usually achieved using biomarkers, which can also help in prognosis prediction. CK19 and PRDM5 are specifically increased in the case of cHCC-CC. HCC-related markers such

as alpha-fetoprotein (AFP), GPC3, and APOE are also highly expressed in cHCC-CC (9). AFP is a major serum glycoprotein produced by the foetal liver during liver development and its expression is elevated in the majority of patients with HCC. It is an important diagnostic marker for HCC (10-12). The glycoprotein Dickkopf-1 (DKK1) is known to promote tumour cell proliferation, migration, and invasion via the Wnt/β-catenin signalling pathway dependent (13) and independent (14) mechanisms. Recently, several studies have indicated that elevated expression of DKK1 is closely associated with HCC progression (13, 15, 16). Additionally, cytokeratin 7 (CK7), an intermediated filament protein expressed by epithelial cells, is another prognostic marker that is upregulated in patients with intrahepatic CC (17, 18). During embryonic development, SRY-related HMG-box 17 (SOX17) is considered essential for the formation of gallbladder and bile duct epithelium and its downregulation promotes CC as a tumour suppressor (19). Hypermethylation of the SOX17 promoter has been reported in patients with CC (20).

Reprogramming technology allows the establishment of patient-specific iPSC lines with self-renewal capacity and the ability to differentiate into various somatic cell types (21, 22). Patient-specific induced pluripotent stem cells (iPSCs) may be an effective source for therapeutic development platforms as they retain specific genetic backgrounds and characteristics associated with a particular disease pathology. Several studies have reported the generation of iPSC lines from patient-specific cell sources such as fibroblasts (23), blood samples (24, 25), and urine samples (26, 27).

Importantly, in rare and unique cases such as cHCC-CC, patient-derived iPSCs can be used as a model for understanding the mechanism of disease pathogenesis and for developing individual therapeutic strategies by simulating complex signalling pathways within disease-specific environments (23, 28, 29). Therefore, we hypothesized that iPSCs derived from a patient with cHCC-CC may contain unique biological characteristics of the disease and investigated the expression of several biomarkers associated with disease pathology. To our knowledge, this is the first study to use iPSCs generated using liver fibroblasts from a patient with cHCC-CC.

Materials and Methods

Cell culture

Human skin fibroblasts (HSFs, CRL-2097) were purchased from the American Type Culture Collection (ATCC) for use as non-malignant control cells. In this experimental study, human liver fibroblasts (HLFs) were isolated from the liver specimen of a 69-year-old male patient with cHCC-CC. All experiments were approved by the Institutional Review Board (IRB) at Chungnam National University Hospital (IRB file no. CNUH 2016-03-018) and at Public Institutional Bioethics Committee designated by the South Korea Ministry of Health and Welfare (IRB file no. P01-201703-31-010). The patient provided informed consent. The human liver tissues were washed with cold phosphate-buffered saline (PBS, Thermo Fisher, USA), as previously described (30), and minced in a solution that contained 300 units/ml collagenase type IV (Thermo Fisher, USA). The minced tissue was incubated at 37˚C for 30 minutes until they were digested, then filtered through a 70 μm strainer (SPL Life Science, Korea) and washed with a cold solution of 10% foetal bovine serum (FBS, Thermo Fisher, USA) in PBS. The cells were resuspended in minimal essential medium (MEM, Thermo Fisher, USA) containing 10% FBS and 1% penicillin streptomycin (PS, Thermo Fisher, USA).

Generation of induced pluripotent stem cells

iPSCs were generated using previously described protocols (3, 31). Briefly, HSFs and HLFs (1×10^5 cells/well) were plated in 6-well plates and the cells were transduced with OSKM-retrovirus at a multiplicity of infection of 3 on day 2. Before transduction, the cells were pre-incubated with 8 μg/mL of polybrene (Sigma-Aldrich, USA) for one hour. The medium was replaced with fresh fibroblast culture medium every other day, and then the cells were reseeded onto a γ-irradiated mouse embryonic fibroblast (MEFs) feeder layer in a 6-well plate with a cell density of $1\sim2\times10^5$ cells/well on day 7. The next day, the medium was replaced with iPSC medium (DMEM/F12 (Thermo Fisher, USA), 1X GlutaMAX (Thermo Fisher, USA), 1X MEM-nonessential amino acids (Thermo Fisher, USA), 100 μM β-mercaptoethanol (Thermo Fisher, USA), and 1% PS (Thermo Fisher, USA), 20% knockout serum replacement (Thermo Fisher, USA), and 10 ng/ml basic fibroblast growth factor (bFGF, Peprotech, USA), and then replaced daily. When human embryonic stem cell (hESC)-like iPSC colonies were established, the cells were mechanically picked and expanded on γ-MEF feeder layers.

Mycoplasma testing

Mycoplasma contamination of iPSCs was checked via polymerase chain reaction (PCR) using an EZ-PCR Mycoplasma Test Kit (Biological Industries, Israel). Briefly, 1 ml of used iPSC culture supernatant was collected after 24 hours of culture and centrifuged to acquire a pellet. PCR amplification was performed using a primer set provided in the kit.

Alkaline phosphatase staining

Alkaline phosphatase (AP) activity was determined using a commercially available kit (Sigma-Aldrich, USA). Briefly, the iPSCs were treated with a fixation solution for 30 seconds and then incubated with the AP staining solution in the dark for 15 minutes. Images of AP^+ iPSC colonies were obtained using an Olympus microscope.

Short tandem repeat and karyotype analyses

Genomic DNA was isolated from HLFs and the

corresponding HLF iPSCs. Short tandem repeat (STR) analysis was performed by HumanPass, Inc. (Korea). A chromosomal GTG banding karyotype analysis was performed at 550 resolution by GenDix, Inc. (Korea).

Immunofluorescence analysis

For immunostaining, the cells were seeded onto a 4-well plate (Thermo Fisher, USA) or Lab-Tek dish (Ibidi, Germany). Samples were washed with PBS and fixed with 4% paraformaldehyde in PBS for 15 minutes at room temperature (RT). The cells were then permeabilised in 0.25% Triton X-100 (Sigma-Aldrich, USA) in PBS for 15 minutes and incubated in blocking buffer (4% bovine serum albumin/PBS) for one hour at RT. The respective primary antibodies and corresponding Alexa Fluor® conjugated secondary antibodies were incubated with in blocking buffer overnight at 4˚C and for 40 minutes at RT. The samples were washed three times with washing solution (0.05% Tween-20 [Sigma-Aldrich, USA] in PBS) between each incubation step. Staining of the nuclei was performed using 4',6-diamidino-2-phenylindole (DAPI). Florescence images were visualized with an Olympus microscope (32). The list of antibodies used in this study is presented in Table S1 (See Supplementary Online Information at www.celljournal.org).

Total RNA extraction and polymerase chain reaction

Total RNA was extracted using an RNeasy Mini Kit (Qiagen, Germany) and complementary DNA synthesis was performed by a TOPscript™ RT DryMIX kit (Enzynomics, Korea) according to the manufacturer's protocol. Quantitative real-time PCR (qRT-PCR) was carried out using Fast SYBR® Green Master Mix (Applied Biosystems, USA) and a 7500 Fast Real-Time PCR System (Applied Biosystems, USA). The primer sets used in this study are listed in Table S2 (See Supplementary Online Information at www.celljournal.org).

In vitro and *in vivo* differentiation

iPSCs were detached with Dispase (Thermo Fisher, USA) and transferred into 35-mm petri dishes (SPL Life Science, Korea) for *in vitro* differentiation analysis and cultured in iPSC culture medium without bFGF. After four days of suspension culture, the cell aggregates (embryoid bodies, EBs) were attached to Matrigel (Corning)-coated plates and incubated with iPSC basal culture medium supplemented with 10% FBS for one week. *In vivo* differentiation analysis based on teratoma formation was performed as described previously (8). Briefly, 1×10^6 iPSCs were subcutaneously injected into BALB/c nude mice (CAnN.Cg-*Foxn1*^{nu}/CrljOri) (Orient Bio, Inc., Korea). The animal experiments were approved by the Bioethics Committee of KRIBB (KRIBB-AEC-16139).

Statistical analysis

The graphs represent the mean ± SEM relative to mRNA expression levels of triplicate samples used for the PCR analysis. All data analyses were performed on Microsoft Office Excel (version 2019). The student's t test was conducted to evaluate inter-group comparisons, and $P<0.05$ indicated statistical significance.

Results

Generation of human liver fibroblast induced pluripotent stem cells from a patient with combined hepatocellular-cholangiocarcinoma

The HLFs were isolated from the patient with cHCC-CC, who showed mixed histopathology of both HCC and CC and were mainly charged by CC in the liver biopsy sample. The HLFs were reprogrammed into iPSCs (HLF iPSCs) using retroviral transduction of OSKM (Fig.1A). HSFs were also reprogrammed into iPSCs for use as a normal control (Con iPSCs). Both Con iPSC and HLF iPSC colonies expanded well with a typical hESC-like morphology (Fig.1B). The HLF iPSCs were negative for mycoplasma contamination (Fig.1C). Both types of iPSCs retained the undifferentiated characteristics of strong AP activity (Fig.1D). The established HLF iPSCs STR profile matched that of the original fibroblasts (Fig.1E).

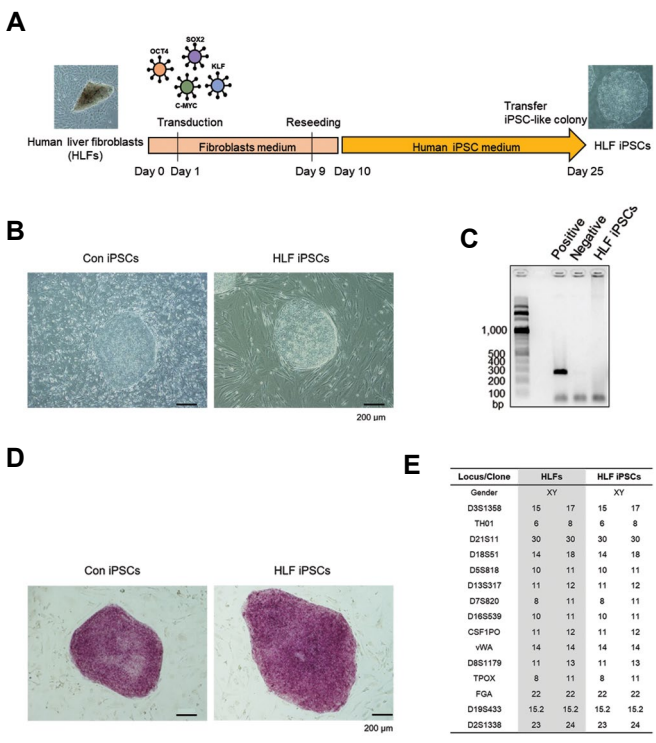


Fig.1: Generation of human liver fibroblast induced pluripotent stem cells (HLF iPSCs) from a patient with combined hepatocellular-cholangiocarcinoma (cHCC-CC). **A.** Experimental schematic for the generation of human iPSCs. HLFs derived from a patient with cHCC-CC and human skin fibroblasts (HSFs) from a healthy individual were reprogrammed into iPSCs using retroviral transduction of OSKM. **B.** Representative morphology of HSFs-derived control iPSCs (Con iPSCs) and cHCC-CC patient-derived iPSCs (HLF iPSCs). **C.** Confirmation of lack of mycoplasma contamination of the HLF iPSCs. **D.** Representative alkaline phosphatase (AP) activity of Con iPSCs and HLF iPSCs. **E.** Short tandem repeat (STR) profiles of HLFs and HLF-derived iPSCs (scale bar: 200 µm).

Pluripotency characterisation of human liver fibroblast induced pluripotent stem cells

Based on real-time PCR analysis, we observed higher mRNA expressions of pluripotent stem cell markers such as *OCT4*, *SOX2*, *NANOG*, and *REX1* in the Con iPSCs and HLF iPSCs than in the corresponding fibroblasts (Fig.2A). High expression of pluripotent markers such as OCT4, TRA-1-60, NANOG, and SSEA4 proteins were observed in both Con iPSCs and HLF iPSCs, as determined by immunostaining (Fig.2B). Total and endogenous pluripotency markers were well-expressed, but exogenous expressions similar to those of an hESC line, H9, were not detected by PCR (Fig.S1A, See Supplementary Online Information at www.celljournal.org). Karyotype analysis showed that no genetic abnormality had occurred during the reprogramming process of the iPSCs (Fig.S1B, See Supplementary Online Information at www.celljournal.org).

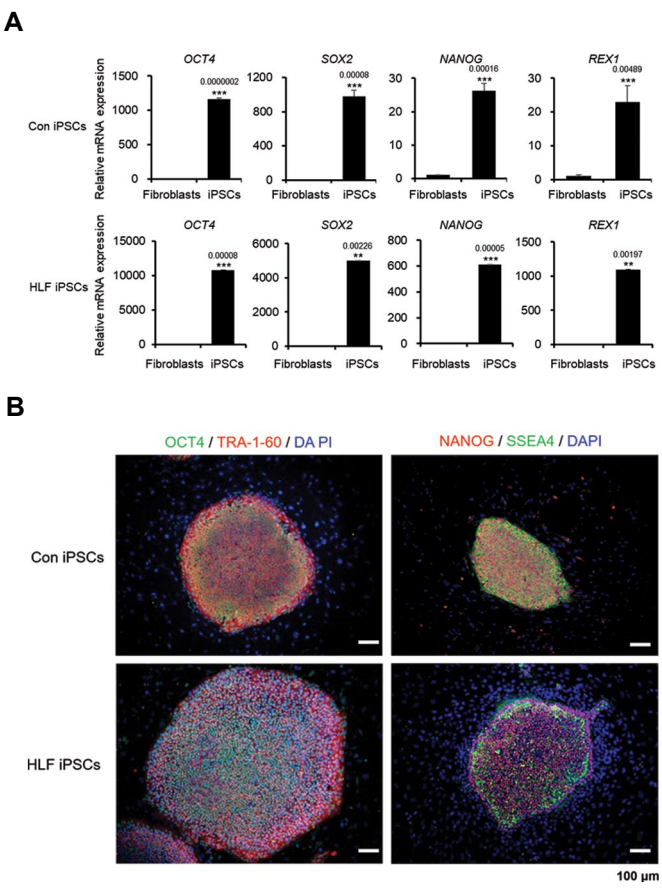


Fig.2: Pluripotency marker expressions in combined hepatocellular-cholangiocarcinoma (cHCC-CC)-derived human liver fibroblast induced pluripotent stem cells (HLF iPSCs). **A.** mRNA expression levels of pluripotency markers *OCT4*, *SOX2*, *NANOG*, and *REX-1* in fibroblasts and iPSCs from normal control (upper) and cHCC-CC patient (lower). **B.** Representative immunostaining images of HSFs-derived control iPSCs (Con iPSCs) (upper) and cHCC-CC patient-derived iPSCs (HLF iPSCs) (lower) stained for pluripotency markers using the indicated antibodies. Data are expressed as the mean ± SEM (n=3) and were analysed using the student's t test, **; P<0.01 and ***; P<0.001 (scale bar: 100 µm).

To further determine the full pluripotency potential of the iPSCs, *in vitro* and *in vivo* differentiation analyses were carried out (Fig.3). The formation of EBs allowed the spontaneous differentiation of iPSCs into the embryonic three germ layers *in vitro* (Fig.3A). Both Con iPSCs and HLF iPSCs, which differentiated *in vitro*, expressed representative markers for ectoderm (TUJ1), mesoderm (alpha smooth muscle actin, α-SMA), and endoderm (FOXA2), as shown in Figure 3A. Teratoma induced *in vivo* by subcutaneous injection of iPSCs contained representative tissues of each particular layer, including ectoderm (pigment epithelium containing melanocytes or neural rosettes), mesoderm (cartilage), and endoderm (gut-like epithelium), as revealed by haematoxylin and eosin staining (Fig.3B). These results demonstrated that the established HLF iPSC line retained pluripotency *in vitro* and *in vivo*, which was indistinguishable from that of Con iPSCs.

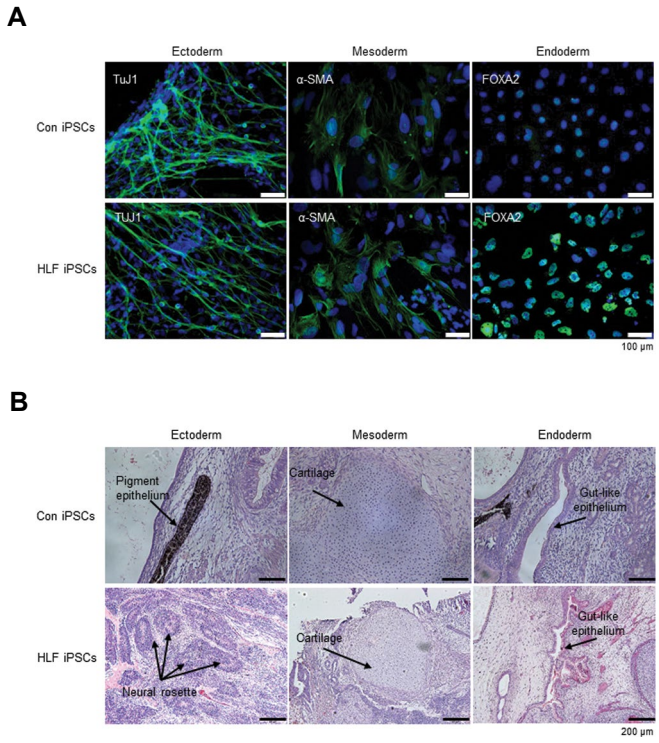
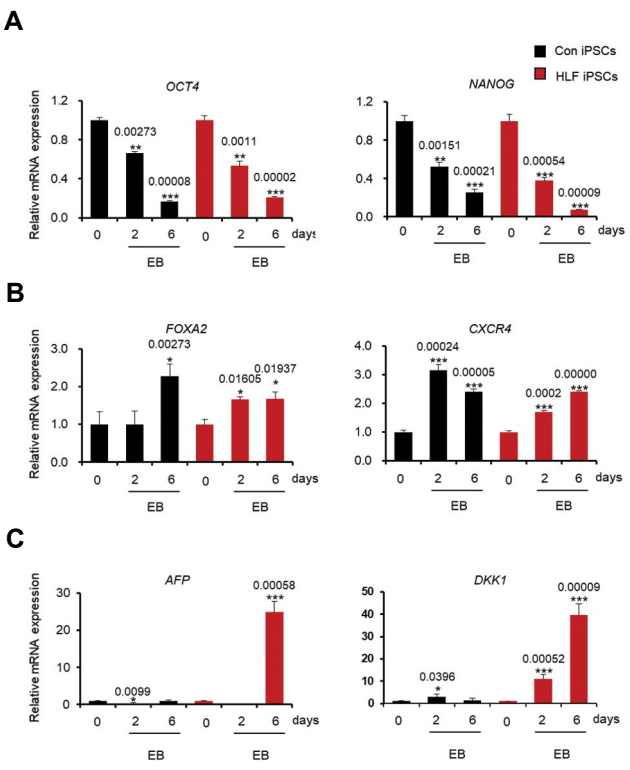


Fig.3: *In vitro* and *in vivo* differentiation potential of combined hepatocellular-cholangiocarcinoma (cHCC-CC)-derived human liver fibroblast induced pluripotent stem cells (HLF iPSCs). **A.** *In vitro* differentiation of the iPSCs determined through embryoid body (EB) formation. Representative immunofluorescence images of ectodermal, mesodermal, and endodermal markers in differentiated cells from HSFs-derived control iPSCs (Con iPSCs) (upper) and cHCC-CC patient-derived iPSCs (HLF iPSCs) (lower) (scale bar: 100 µm) **B.** *In vivo* differentiation of Con iPSCs (upper) and HLF iPSCs (lower) determined through teratoma formation. Representative histology of teratomas, including the three germ layers, stained with haematoxylin and eosin (scale bar: 200 µm).

Hepatocellular carcinoma and cholangiocarcinoma marker expression in human liver fibroblast induced pluripotent stem cells during differentiation

We evaluated the expressions of various makers during *in vitro* differentiation to determine if HLF iPSCs could maintain the characteristics of the patient (Fig.4). The expressions of pluripotency markers *OCT4* and *NANOG*

gradually decreased upon differentiation and the levels did not differ significantly between Con iPSCs and HLF iPSCs (Fig.4A). In contrast, the expressions of definitive endoderm markers *FOXA2* and *CXCR4* (Fig.4B), ectoderm markers *NESTIN* and *OTX2*, and mesoderm markers *VIMENTIN* and *BRACHYURY* (Fig.S1C, See Supplementary Online Information at www.celljournal.org) increased during differentiation in both Con iPSCs and HLF iPSCs. These results were consistent with the data shown in Figure 3A and confirmed that the *in vitro* differentiation potential of the HLF iPSCs was not different from that of the normal Con iPSCs. However, expression levels of HCC markers *AFP* and *DKK1* substantially increased by 25-fold and 40-fold, respectively, compared to undifferentiated HLF iPSCs. In contrast, the expression levels of *AFP* and *DKK1* were similar in undifferentiated and differentiated cells from Con iPSCs (Fig.4C). We also examined the expressions of CC markers, *CK7* and *SOX17*. *CK7* expression increased aberrantly in the differentiated EBs from HLF iPSCs compared to the undifferentiated HLF iPSCs (by 56.2-fold); it was more than 10 times higher than the difference between undifferentiated and differentiated normal Con iPSCs. Furthermore, *SOX17*, which is known to have tumour suppressive roles in CC patients (21, 22), did not increase during differentiation of HLF iPSCs. However, its expression continually increased in Con iPSCs during differentiation (Fig.4D). Overall, these results demonstrated that patient-derived HLF iPSCs exhibited the expression of pluripotency markers, general differentiation potential, and HCC and CC patient-specific marker expression. Therefore, HLF iPSCs may provide a renewable cell source for modelling cHCC-CC.



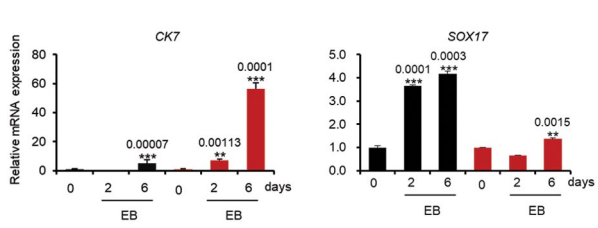


Fig.4: Expression of markers for hepatocellular carcinoma (HCC) and cholangiocarcinoma (CC) in combined hepatocellular-cholangiocarcinoma (cHCC-CC)-derived human liver fibroblast induced pluripotent stem cells (HLF iPSCs) during differentiation. **A.** mRNA expression levels of stem cell markers *OCT4* and *NANOG* on days 0, 2, and 6 after embryoid body (EB) formation of HSF-derived control iPSCs (Con iPSCs) and HCC-CC patient-derived iPSCs (HLF iPSCs). **B.** mRNA expression levels of definitive endoderm markers *FOXA2* and *CXCR4* on days 0, 2, and 6 after EB formation of Con iPSCs and HLF iPSCs. **C.** mRNA expression levels of HCC markers *AFP* and *DKK1* on days 0, 2, and 6 after EB formation of Con iPSCs and HLF iPSCs. **D.** mRNA expression levels of CC marker *CK7* and CC tumour suppressor marker *SOX17* on days 0, 2, and 6 after EB formation of Con iPSCs and HLF iPSCs. Data are shown as the mean ± SEM (n=3) and were analysed by the student's t test, *; P<0.05, **; P<0.01, and ***; P<0.001.

Discussion

While cHCC-CC is a rare type of primary hepatic cancer, there are limited therapeutic options due to an incomplete understanding of its pathogenesis. One of the biggest challenges in developing therapeutics for liver diseases is the lack of accessibility to human liver tissue, which results in difficulty for *in vitro* modelling of disease progression (2, 33). For uncommon cases of liver cancer such as cHCC-CC, patient-specific models are crucial to understand novel mechanisms of disease pathology and to develop personalized therapies. Patient-derived iPSCs that retain individual characteristics and exhibit unlimited self-renewal and differentiation potential into various cell types (34) can be a valuable source for modelling rare diseases, including liver cancer.

In this study, we isolated HLFs from a cHCC-CC specimen and reprogrammed them into indefinitely proliferative iPSCs. These patient-derived HLF iPSCs demonstrated prominent pluripotency and differentiation potential similar to that of normal Con iPSCs. Notably, when the HLF iPSCs were differentiated *in vitro* via EB formation, unique marker expression of the patient was clearly observed. For example, AFP is considered a gold standard in liver cancer diagnosis and DKK1 has been implicated in tumourigenesis in many tissues, including HCC. Both are involved in embryonic liver development (13, 15, 16). The combined expression of AFP and DKK1 demonstrates a more precise diagnosis of HCC than AFP or DKK1 alone (35). The biliary marker CK7 is highly expressed in intrahepatic CC and thus has been studied as a prognostic marker in this type of cancer (17, 36). SOX17 is also necessary for the normal formation of the biliary epithelium, and its epigenetic downregulation by aberrant hypermethylation of the *SOX17* promoter is observed in CC patients (19). The expressions of these markers was clearly distinguishable in the cHCC-CC-derived HLF iPSCs.

Here, we demonstrated the spontaneous differentiation of the HLF iPSCs into three germ layers via EB formation; however, further direct differentiation of iPSCs into specific endodermal cell types such as hepatocytes (37) or cholangiocytes (38) is also possible. Recent developments in organoid technology (39) make it possible to differentiate iPSCs to recapitulate three-dimensional (3D) miniature livers that maintain cell composition and organ function (40). Our group has also generated expandable and functional 3D human liver organoids from iPSCs and demonstrated that iPSC-derived liver organoids can be used for toxicity prediction and drug screening in conditions with long-term maintenance of individual characteristics (30, 32) . Therefore, we aim to generate liver organoids in future using the cHCC-CC -derived HLF iPSCs established in the current study. Patient-derived liver organoids may provide a personalized disease modelling platform for revealing the molecular mechanisms of individual pathogenesis, developing therapeutics, and identifying hepatotoxic responses against targeted anti-cancer drugs. Moreover, patient-derived iPSCs may also provide various types of organoid models for the brain, lung, kidney, and gut in addition to the liver and may prove to be a valuable resource for modelling rare diseases.

Conclusion

We generated iPSCs using liver tissue-derived fibroblasts from a patient with rare cHCC-CC. The HLF iPSCs exhibited prognostic marker expression of cHCC-CC upon differentiation. Therefore, the HLF iPSC line may be used as a practical and renewable cell source for personalized disease modelling, uncovering the molecular mechanisms of individual pathogenesis, and developing therapeutics.

Acknowledgments

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Authors' Contributions

H.-S.A., J-S.R, S.J.M., K.-S.C., M.J.S.; Study conception and design. H.-S.A., J-S.R, J.L., S.J.M., Y.-H.H.; Acquisition of data. H.-S.A., J-S.R, J.L., S.J.M., K.-S.C., M.J.S.; Analysis and interpretation of data. H.-S.A., J-S.R, J.L., Y.S., K.-S.C., M.J.S.; Drafting of manuscript. All authors read and approved the final manuscript.

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