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Original Article

CCC-5, a new primary cholangiocellular cell line

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Abstract: Background: Cholangiocellular carcinoma (CCC) is a rare tumor entity and the availability of systemic treatment is limited. Better knowledge of the tumor physiology might lead to an improved treatment. However, basic research is dependent on the availability of cell lines. As there are only few CCC cell lines disposable, we aimed to isolate new CCC cell lines from surgical samples. Material and methods: The cell line was established from a 59-year-old Caucasian male patient who suffered from malignant pleural effusion of a distal cholangiocellular carcinoma (CCC). The cell line was established by explant culture and further characterized with immunohistochemistry and Western blotting. Mutational analysis was performed by means of AmpliSeq Cancer Panel v2. Additionally, we tested sensitivity towards commonly used chemotherapeutics. Results: Immunohistochemistry and Western blotting showed expression of tumor markers typical for CCC cell lines. Analysis of sensitivity towards chemotherapeutics revealed that Irinotecan was the only agent with significant effect on cell proliferation. Mutational analysis identified different mutations in important oncogenes and tumor suppressor genes. Conclusion: The new cell line displays the typical characteristics of a CCC cell line. This new cell line might help to add new insights into this tumor entity and thereby help to find better treatment options for patients with CCC.

Keywords: Cholangiocellular carcinoma, cell lines, chemotherapy resistance, mutational analysis, translational medicine, oncology

Introduction

Cholangiocellular carcinoma (CCC) is a malignant tumor originating from bile duct epithelial cells [1]. In spite of recent advances in diagnostic imaging and therapeutic techniques, prognosis of CCC is still unfavorable [1, 2]. This is also due to late diagnosis of CCC, especially of the peripheral type, and aggressive growth of the tumor [3]. Since effective chemotherapy is still unavailable to date, surgery is the only chance of cure. However, this is only possible in early stages. Prognosis is dependent on the presence of free margins in resected tissues and the absence of lymph node metastases [1].

Since the incidence of CCC is low compared to other malignant entities of the hepatico-pancreatico-biliary spectrum [1], underlying mechanisms of CCC carcinogenesis are poorly characterized and little is known about biochemical markers [4]. However, despite its low incidence compared to hepatocellular carcinoma (HCC), intrahepatic CCC overtook HCC as the most common cause of liver cancer-related death in the mid-1990 [5]. Because the incidence is low, tumor samples are not broadly available and scientific hypotheses cannot be tested easily. Cell lines have the advantage to be an endless source of DNA, RNA and proteins. Additionally, cell lines are a good *in vitro* model of the cancer. CCC cell lines are therefore necessary to

Table 1. Antibody description for IHC and Western blot

Antiboy	Dilution	Antibody Supplier
p53	1:500	Dianova, Hamburg, Germany (DIA 45)
CK7	1:2000	Dako, Hamburg, Germany (*OV-TL* 12/30)
CK8-18	1:50	Dako, Hamburg, Germany (EP17/EP30)
CK20	1:200	Dako, Hamburg, Germany (Ks 20.8)
SOX9	1:1000	Sigma Aldrich, Hamburg, Germany (HPA001758)
CK19	1:1000	Santa Cruz, Heidelberg, Germany (Sc-6278)
p-STAT3	1:1000	Cell Signalling, Frankfurt, Germany (9145)
STAT3	1:1000	Cell Signalling, Frankfurt, Germany (9132)
pERK	1:1000	Santa Cruz, Heidelberg, Germany (Sc-7383)
ERK ½	1:1000	Santa Cruz, Heidelberg, Germany (Sc-135900)
Alpha-Tubulin	1:5000	Abcam, Cambridge, UK (Ab4074)
HNFα	1:1000	Cell Signalling, Frankfurt, Germany (C11F12)

Antibodies and corresponding concentrations used for the purpose of this study.

better understand this tumor entity and to establish successful treatment strategies [6]. About 52 CCC cell lines have so far been reported in literature [7]. However, many of these cell lines were established more than a decade ago and most of the cell lines are not available any more. Therefore, new CCC cell lines need to be established that can be shared with researchers that focus on CCC [8]. The present report describes the establishment and characterization of a new human CCC cell line from malignant pleural effusion of a long time survivor with CCC.

Materials and methods

Patient characteristics

The study was reviewed and approved by the local ethics committee (Medizinische Ethikkomission Mannheim II; http://www.umm.uni-heidelberg.de/inst/ethikkommission) before the study began. All patients gave informed and written consent before operation or intervention. The document of the participant consents are routinely archived together with the patients' notes. The present cell line was established from a 59 year old Caucasian male patient who suffered from malignant pleural effusion of a distal CCC. The primary metastatic Klatskin Bismuth I was diagnosed three years previous to the establishment of the cell line. Initial carcinoembryotic antigen (CEA) was 7.4 g/l and Ca19-9 18510 kU/l. Biliary stents were changed fourteen times. The patient obtained chemotherapy with gemcitabine and cisplatin for seven months initially. After good initial response, the tumor showed progressive growth. Therefore cisplatin was stopped and gemcitabine monotherapy was administered for nine months. After this period, the regimen was changed to capecitabine and oxaliplatin for twelve months. Mitomycin and doxetacel is now the current therapy regime since seven months. The patient was intermittent treated with photodynamic therapy. As the patient was the fifth patient of our study, we named the cell line "CCC-5".

Cell culture condition

The cell line was established by culturing pleural effusion with medium in the ratio of 1:2 as described previously [8]. DME medium (Invitrogen, Karlsruhe, Germany) was used with 20% FCS ("CP-medium") combined with Keratinocyte Serum Free Medium in the ratio 2:1 ("Dresden-Medium") [8]. Medium was supplemented with penicillin (100 U/mL) and gentamicin (2.5 mg/mL) (Invitrogen). The cells were maintained at 37°C in a humidified atmosphere of 5% CO_2 in air, and the medium was replaced every 3 days. The cell line was tested negatively on mycoplasma. All experiments for this study were performed on cell lines between the fourth and ninth passage. As controls, we used hepatocytes and the established HCC cell line HepG2 as well as established CCC cell lines, HCCC-9810 and EGI-1.

Morphology and immunocytochemistry

Growth patterns and cell morphologies were determined *in vitro* using a Zeiss phase-contrast microscope (Zeiss, Jena, Germany). Cells were stained with hematoxylin-eosin, Masson goldner and periodic acid Schiff according to standard protocols. For immunocytochemistry, a cell suspension of approximately 6×10^7 cells/ml was prepared and cytocentrifuged. The cell pellet was fixed in methanol for 10 min. The pellet was then paraffin embedded. After the blocking procedure, cells were incubated in antibody buffer with primary antibody for 60 minutes at room temperature. **Table 1** shows the details of the antibodies used.

After three washing steps (5 minutes each) with TBS-buffer (DAKO, Hamburg, Germany), secondary antibody diluted in antibody buffer was applied for 15 minutes at room temperature. After washing three times (at least 5 minutes each) with TBS-buffer, protein antibody binding was visualized using EnVision™ System-HRP-3,3'-diaminobenzidine chromogen solution system (Dako, Hamburg, Germany). Cells were counterstained with hemalum for 5 minutes and slides were coverslipped with an aqueous-based mounting medium.

Cell doubling time

Cell doubling time was determined by counting the number of viable cells derived from freshly trypsinized monolayers in duplicate. 6-well plates with 50,000 cells per well with 3 ml medium were used. Cells were counted at 24 h intervals for 7 days. The culture medium was changed after two days. The doubling time of the cell population was calculated from the logarithmic growth curve by the following formula:

v = lgN-lgN0/lg2 (t-t0), with doubling time = 1/v.

Chemosensitivity

For viability assays 15,000 cells were seeded into 96-well plates in triplicates. Untreated CCC-5 cells were used as control. CCC-5 cells were treated with irinotecan, gemcitabine and oxaliplatin. Concentrations of irinotecan test series were 13, 130 and 1300 ng/ml. Concentrations of gemcitabine test series were 0.45, 4.5 and 45 µg/ml. Concentrations of oxaliplatin test series were 1, 10 and 100 µg/ml. Concentrations were according to those used in current literature [9-11]. Treatment with 1% Triton X-100 was used as positive control. Mitochondrial activity was quantified by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) reduction assay. Viability was measured at 570 nm and expressed as relative values compared to untreated control cells.

Western blotting

Thawed cells were prepared in loading buffer with dithiothreitol 50 mM (Sigma-Aldrich, Germany) 1:4. Proteins were electrophoresed in NuPAGE MOPS Running Buffer (Invitrogen, Carlsbad, USA) with a 12% SDS-PAGE-gel, NuPAGE® Novex Bis-Tris gels 1.0 m (Invitrogen, Carslbad, USA) and transferred with NuPAGE Transfer Buffer (Invitrogen) to a nitrocellulose membrane (MF Protran BA 83 0, 2 µm, Schleicher & Schuell BioScience, München, Germany). To block non-specific binding, the membrane was incubated with 5% Top Block (Sigma-Aldrich, Munich, Germany) for phosphorylated proteins, milk powder for unphosphorylated proteins and BSA for sensitive proteins. Primary antibodies were used as recommended by the producer: SOX9 (Sigma-Aldrich, Munich, Germany), Cytokeratin 19, pERK and ERK (all Santa Cruz Biotechnology, Dallas, USA), Stat3, pStat3 and HNF4 (all Cell Signaling, Frankfurt, Germany). A-Tubulin served as control (Abcam, Cambridge, UK). After washing with TBST, goat anti-rabbit or anti-mouse secondary antibody (Santa Cruz Biotechnolody, Dallas, USA) was incubated in 1:3,000 dilution (**Table 1**). After washing in TBS, the protein was visualized using Luminol/Enhancer solution and peroxidase buffer 2:1 (Thermo Fisher, Rockford, USA).

AmpliSeq™ cancer panel v2

The 50-gene Ion AmpliSeq Cancer Hotspot panel v2 (Life Technologies) was used for mutational analysis. The Hotspot panel explores regions of 50 cancer-genes: ABL1, AKT1, ALK, APC, ATM, BRAF, CDH1, CDKN2A, CSF1R, CTNNB1, EGFR, ERBB2, ERBB4, EZH2, FBXW7, FGFR1, FGFR2, FGFR3, FLT3, GNA11, GNAS, GNAQ, HNF1A, HRAS, IDH1, IDH2, JAK2, JAK3, KDR/VEGFR2, KIT, KRAS, MET, MLH1, MPL, NOTCH1, NPM1, NRAS, PDGFRA, PIK3CA, PTEN, PTPN11, RB1, RET, SMAD4, SMARCB1, SMO, SRC, STK11, TP53, VHL. Details on target regions of the panel are at http://www.lifetechnologies.com. [12]. Twenty ng of DNA were used for each multiplex PCR amplification. Emulsion PCR was performed with the One Touch2 system (Life Technologies). The quality of the obtained libraries was evaluated by the Agilent 2100 Bioanalyzer on-chip electrophoresis (Agilent Technologies). Sequencing was run on the Ion Torrent Personal Genome Machine (PGM, Life Technologies) loaded with 316 chips. Data analysis, including alignment to the hg19 human reference genome and variant calling, was done using the Torrent Suite Software v.3.6 (Life Technologies). Filtered variants were annotated using the SnpEff software v.3.1. Alignments were visually verified with the Integrative Genomics Viewer; IGV v.2.2, Broad Institute [13].

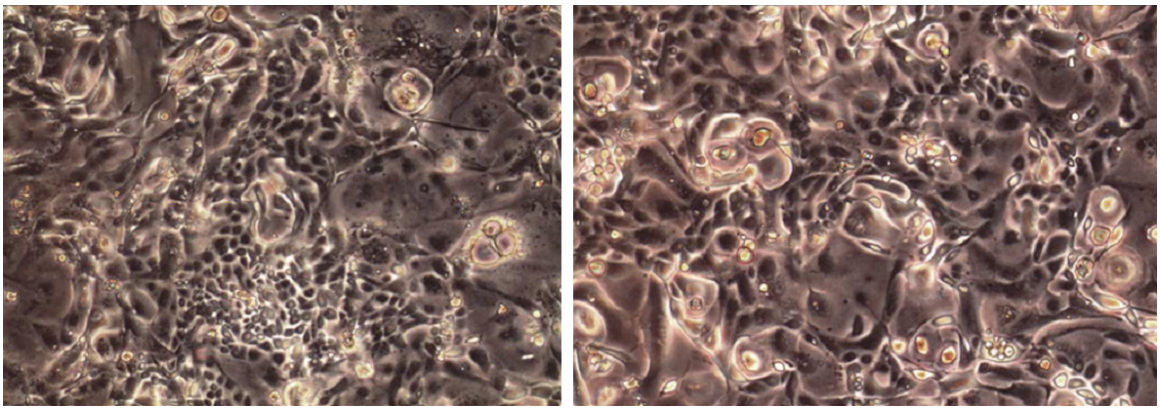

Figure 1. Morphology of CCC-5 by light microscopy (40×).

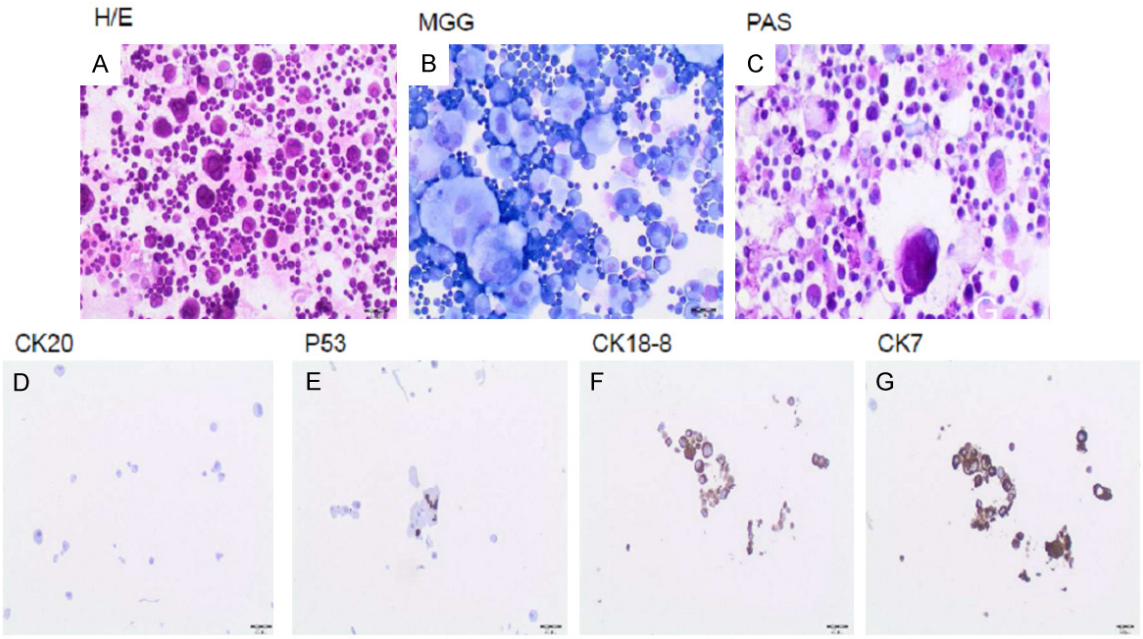

Figure 2. Histological staining of CCC-5 with hematoxylin and eosin staining (A), May-Grünwald-Giemsa staining (B) and Periodic acid-Schiff staining (C) (40×). Immunocytochemical staining of CCC-5 for CK20 (D), p53 (E), CK18-8 (F), and CK7 (G) (40×).

Statistical analysis

For statistical analysis, we used Excel 2003 (Microsoft) and SPSS 13.0 for Windows. A p-value<0.05 was defined as significant in Student's t-test.

Results

Morphology and growth characteristics

In order to assess morphology we used phase contrast microscopy. CCC-5 grows as typical epithelial cell culture. The colonies grow as epithelial monolayer in a cobblestone pattern. The cell line exhibited spindle- to polygonal-shaped morphology of the cells. Especially the bigger cells have a prominent nucleus (**Figure 1**). The H/E and May-Grünwald-Giemsa staining revealed anaplastic, multinucleated giant cells as wells as small cuboidal cells with eosinophilic cytoplasm and round central nuclei. The high number of small cells indicates a high rate of cell divisions (**Figure 2A**, **2B**). Periodic acid-Schiff staining revealed mucin production in CCC-5 cells (**Figure 2C**).

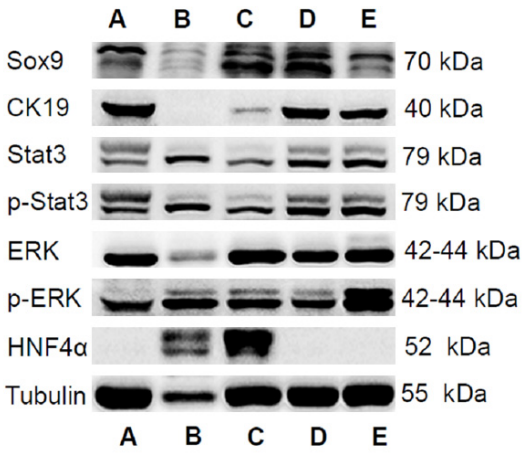

Figure 3. Western blot analysis of CCC-5 (A), human hepatocytes (B), HepG2 (C), HCCC-9810 (D) and EGI-1 (E) (*CK19 = cytokeratin-19; STAT-3 = Signal transducer and activator of transcription 3; HNF4α = Hepatocyte nuclear factor 4 alpha; ERK = Extracellular-signal Regulated Kinase*).

Immunocytochemistry and protein analysis

To proof origin of the cell line we used immunocytochemistry. The analysis of CCC-5 cells revealed expression of CK18-8 and CK7 but not expression of CK20 (**Figure 2D**, **2F**, **2G**). Expression of p53 was strong in a subpopulation of tumor cells (**Figure 2E**).

Expression of different other genes that might be of importance in CCC were analysed in CCC-5 by means of Western blot analysis. As control served primary human hepatocytes, the HCC cell line HepG2 and the CCC cell lines HCCC-9810 and EGI-1. CCC-5 showed expression of Sox9 that could also be seen in the other tumor cell lines but not in normal hepatocytes. The same holds true for CK19. STAT-3 and phospho-STAT-3 were expressed in all cell lines. Unphosphorylated ERK was only seen in tumor cell lines but not in normal hepatocytes. HNF4α was specifically expressed in normal hepatocytes and the HCC cell line (**Figure 3**).

Chemosensitivity

The doubling time of CCC-5 cells was 60.5 hours (**Figure 4**). Different agents that are routinely used for the treatment of CCC were tested by means of MTT assay for their chemotherapeutic effect on CCC-5. Irinotecan was the only agent with significant effect on metabolic activity at concentrations of 1300 ng/ml compared to untreated controls after 48 hours ($P<0.01$) but also 72 hours ($P<0.01$) indicative for a dose-dependent cytotoxicity. CCC-5 cells cultured with medium containing gemcitabine or oxaliplatin showed no significant cytotoxicity within the applied concentrations neither after 48 h nor after 72 h (**Figure 5**).

Mutational analysis

DNA was successfully amplified in multiplex PCR for the 56 genes and an adequate library for deep sequencing was obtained. The mean read length was 78 base pairs and a mean coverage of 1800× was achieved, with 87.1% target bases covered more than 100×. A minimum coverage of 20× was obtained in all cases. CCC-5 showed mutations in 30 different genes (**Table 2**).

Among these genes there are several receptors like epidermal growth factor receptor (EGFR) platelet-derived growth factor receptor (PDGFR) and fibroblast growth factor receptor (FGFR). Different kinases that mediate cellular activation were also mutated. We also found mutations within classical oncogenes like p53, the APC gene, RB1, ATM and VHL. Interestingly, there were also mutations frequently found in pancreatic cancer like BRAF, SMAD4 and NOTCH1 (see also Supplementary Table 1).

Discussion

Cell lines are an economic and uncomplicated two dimensional tumor model. Such models are a prerequisite for basic research. Most experimental studies in cancer use cancer cell lines for testing hypothesis and getting insight into tumor pathology. Different established cell lines are available for this purpose. One problem in rare tumor entities is the limited number of cell lines for research. As a limited number of cell lines cannot mirror the genetic heterogeneity of the native tumors the isolation of more cell lines is necessary.

Another problem is that many cell lines were established many years ago and these cell lines accumulate new mutations that were not part of the initial set of mutations. This is called genetic drift [7, 8]. For these reasons, it is important to establish new carcinoma cell lines [7, 8]. The cell culture lab of the Surgical Department of the University Medicine Mannheim establishes cell lines from surgical samples in a routinely fashion. The aim of the present

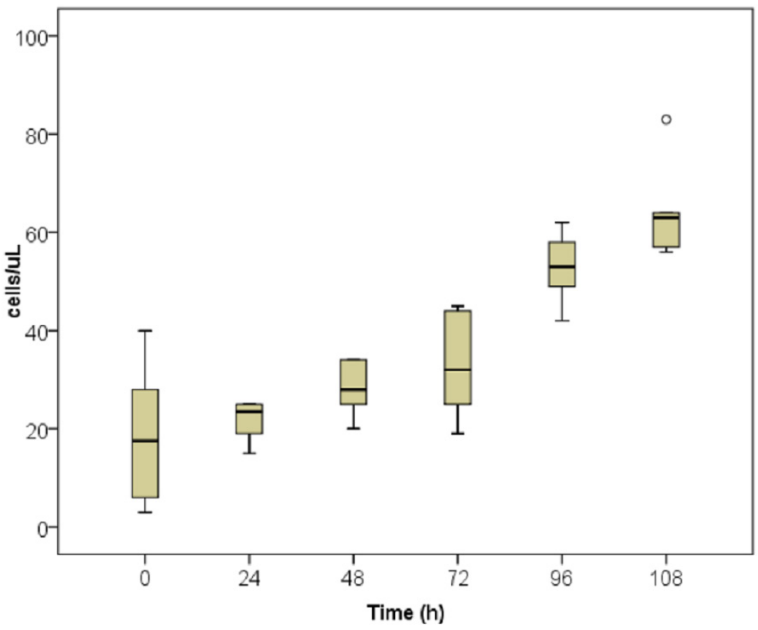

Figure 4. Growth curve of CCC-5 cells.

study was to characterize a newly established cell line, CCC-5. This cell line was established out of pleural effusion of a long term survivor with primary metastatic extra-hepatic CCC. To proof the cholangiocellular origin of the cell line we performed different analyses. Microscopically, we found the typical morphology of a malignancy of epithelial origin. Cells had tight cell-cell contacts and grew in colonies; this is typical for carcinoma cell lines. CCC-5 synthesizes mucin as seen in other CCC cell lines [7].

Immunocytochemical characteristics were that of a CCC cell line, too. CCC-5 revealed no expression of CK20. CK20 is a typical marker for colorectal cancer metastasis [14]. While expression of p53 was strong in a subpopulation of cells, expression of CK18-8 and CK7 was very strong. Although p53 and CK7 are markers in CCC diagnosis, they are also sensitive for pancreatic and lung cancer. CK18 is a typical marker for gastrointestinal and hepatopancreatico-biliary malignancies [14]. The expression of markers seems to be that of a CCC cell line. By means of a Western Blot we further analysed expression of different markers and cell messagers with a known pathophysiological role in CCC: Sox9, CK19, STAT-3, phospho-STAT-3 and ERK. Sox9 was expressed in CCC-5. Sox9 is a biliary marker regulated by Notch and therefore typically expressed in CCC. CK19 as a typical marker of CCC was also expressed, while this was not the case in HCC [15]. STAT-3 was also expressed by CCC-5. STAT-3 is characteristically associated with poor differentiated CCC [16] and is believed to play a role in microenvironment and cancer development of hepato-biliary tumors [17]. The reason for high STAT-3 expression might be that the cell line was isolated from pleural effusion and therefore from an advanced tumor. HNF4α is not expressed by CCC-5. This is in accordance to our hypothesis because HNF4α is typically expressed in liver and intestinal epithelial cells, but is not present in biliary tract epithelial cells [18]. In conclusion, Western Blot showed that our cell line expresses typical markers of CCC.

To further analyse clinic-pathological characteristics of our new cell lines we tested the effect of standard systemic therapies. Usually Gemcitabine and platinic derivate are used first line for the treatment of advanced CCC, Irinotecan is used as second line agent [19]. The effect of the agents on cell survival was tested by MTT assay. Surprisingly, Irinotecan was the only agent with significant effect on cell proliferation. This might be due to previous treatment of the tumor host. Our patient was treated with Oxaliplatin and Gemcitabine initially and showed good response. However, after 7 months progression was observed and the systemic therapy was changed. Maybe the initial treatment selected subpopulations within the tumor with resistance towards Gemcitabine and Oxaliplatin.

To learn which frequent mutation within tumor-associated genes might be present in CCC-5, we performed mutational analysis. By this, we could identify 30 missense mutations in important tumor-associated genes. Among these genes were several cell membrane receptors like epidermal growth factor receptor (EGFR), platelet-derived growth factor receptor (PDGFR) and fibroblast growth factor receptor (FGFR). These genes are known oncogenes and especially mutations of EGFR [20], PDGFR [21] and FGFR1, -2, -3 [22] seem to play a role in tumor progression in CCC. We also found mutations

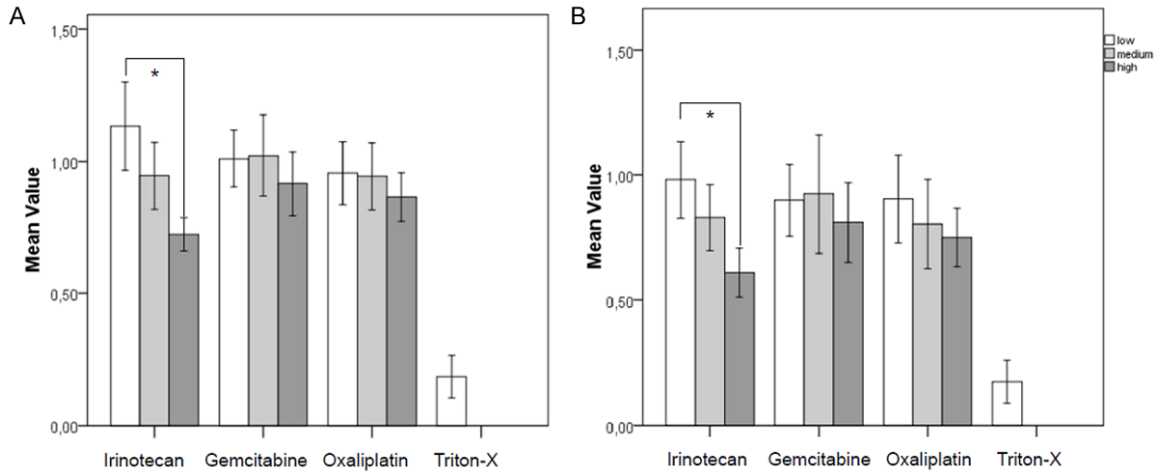

Figure 5. Chemosensitivity assay of CCC5 for Irinotecan, Gemcitabine and Oxaliplatin after 48 (A) and 72 (B) hours. All sets were compared with untreated controls (mean value = 1). White bars indicate low concentration, light grey bars indicate medium concentration and dark grey bars indicate high concentration of the corresponding agent. Error bars show 95% confidence interval.

within classical oncogenes like p53, the APC gene and RB1.

Mutations of p53 are found in up to 37% of intrahepatic CCC and seem to be an prognostic factor for survival [23]. APC mutations in CCC have also been described in different series before [24]. In one other study, RB1 seemed not to be of prognostic value [25]. Interestingly, there we found also mutation of genes in CCC-5 that are frequently found in pancreatic cancer like BRAF, SMAD4 and NOTCH1 [26].

BRAF encodes a protein of the raf/mil family of serine/threonine protein kinases. This protein plays a role in downregulation of MAP kinase/ERKs signaling pathway thereby affecting cell division, differentiation and secretion. BRAF mutation has been associated with various cancers and is therefore point of interest in current research. Dysfunctional BRAF mutation has been associated with better prognosis in CCC. Mutations of SMAD4 were already described in CCC [22]. SMAD4 encodes a member of the SMAD family of signal transduction proteins. SMAD proteins are phosphorylated and activated by transmembrane serine-threonine receptor kinases in response to TGF-beta signaling. The function of the TGF-beta pathway is pleiotropic and mutations or deletions in SMAD4 are consequently correlated with many cancers and syndromes [27]. Notch signaling network is an evolutionarily conserved intercellular signaling pathway which regulates interactions between physically adjacent cells [28]. The gene plays a key role in development. This protein functions as a receptor for membrane bound ligands, and has pleiotropic function during development. Notch1 promoted tumor survival and cancerogenesis in mice and rat models of CCC [14].

Some of the identified mutations might be an interesting target for new therapeutic approaches. E.g. in case reports effective treatment of three CCC patients with multikinase inhibitor Sorafenib targeting FLT3 has been described [29]. Therefore our new cell line might be interesting for this kind of research.

Mutations of some of the tested genes were firstly described for CCC in the present study like ATM, GNA11, KDR, PTPN11, RET, SMARCB1 and STK11. Further investigation regarding these genes might provide new insights. For example, RET could already be correlated with other tumor entities [30]. Altogether, CCC-5 shows an interesting genetic profile. Besides several typical oncogenes like AKT1 and TP53 several important cellular signalling pathways are affected. This primary cell line may be an interesting *in vitro* model for investigating effects of major oncological pathways in cholangiocarcinoma.

Conclusions

In conclusion, we successfully established a new primary CCC cell line. CCC-5 originates of a

Table 2. Mutations found in CCC-5 cell line

	Gene	Official full name	Function
1	AKT1	V-akt murine thymoma viral oncogene homolog 1	Critical mediator of growth factor-induced neuronal survival
2	APC	Adenomatous polyposis coli	Tumor suppressor protein that acts as an antagonist of the Wnt signaling pathway
3	ATM	ATM serine/threonine kinase	Cell cycle checkpoint kinase as a regulator of a wide variety of downstream proteins; associated to various cancers
4	BRAF	B-Raf proto-oncogene, serine/threonine kinase	Regulating the MAP kinase/ERKs signaling pathway, affecting cell division, differentiation and secretion; associated to various cancers
5	CDH1	Cadherin 1, type 1, E-cadherin (epithelial)	Cell-cell adhesion glycoprotein decreasing proliferation, invasion, and/or metastasis
6	CDKN2A	Cyclin-dependent kinase inhibitor 2A	Stabilizer of the tumor suppressor protein p53
7	EGFR	Epidermal growth factor receptor	Role in cell proliferation
8	ERBB4	v-erb-b2 avian erythroblastic leukemia viral oncogene homolog 4	Induces cellular responses including mitogenesis and differentiation
9	FBXW7	F-box and WD repeat domain containing 7, E3 ubiquitin protein ligase	Subunit SCFs function in phosphorylation-dependent ubiquitination
10	FGFR1	Fibroblast growth factor receptor 1	Interacts with fibroblast growth factors
11	FGFR2	Fibroblast growth factor receptor 2	Interacts with fibroblast growth factors
12	FGFR3	Fibroblast growth factor receptor 3	Interacts with fibroblast growth factors
13	FLT3	Fms-related tyrosine kinase 3	Regulates hematopoesis
14	GNA11	Guanine nucleotide binding protein (G protein), alpha 11 (Gq class)	Modulator resp. transducer of various transmembrane signaling systems
15	GNAS	GNAS complex locus	key component of many signal transduction pathways
16	IDH2	Isocitrate dehydrogenase 2 (NADP+), mitochondrial	Intermediary metabolism and energy production
17	KDR	Kinase insert domain receptor (a type III receptor tyrosine kinase)	Role in VEGF receptor mediated endothelial proliferation, survival and migration
18	MET	MET proto-oncogene, receptor tyrosine kinase	Protein possesses tyrosine kinase activity, importance for invasive growth of tumors
19	NOTCH1	Notch 1	Role in cell fate decisions
20	PDGFRA	Platelet-derived growth factor receptor, alpha polypeptide	Receptor for mitogens for cells of mesenchymal origin
21	PIK3CA	Phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha	Catalytic subunit using ATP to phosphorylate PtdIns, PtdIns4P and PtdIns(4,5)P2
22	PTPN11	Protein tyrosine phosphatase, non-receptor type 11	Signaling molecule involved in cell growth, differentiation, mitotic cycle, and oncogenic transformation
23	RB1	Retinoblastoma 1	Negative regulator of the cell cycle
24	RET	Ret proto-oncogene	Involved in regulation of cell growth and differentiation
25	SMAD4	SMAD family member 4	Complexes with other SMAD proteins to regulate transcription of target genes
26	SMARCB1	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily b, member 1	Part of a complex that relieves repressive chromatin structures, allowing the transcriptional machinery to access its targets more effectively
27	SMO	Smoothened, frizzled class receptor	Component of the hedgehog signaling pathway, can act as oncogene
28	STK11	Serine/threonine kinase 11	Member of the serine/threonine kinase family, regulates cell polarity and functions as a tumour suppressor
29	TP53	Tumor protein p53	Tumor suppressor protein inducing cell cycle arrest, apoptosis, senescence, DNA repair, or changes in metabolism
30	VHL	Von Hippel-Lindau tumor suppressor, E3 ubiquitin protein ligase	Component of the protein complex involved in ubiquitination and degradation of hypoxia-inducible-factor

long time survivor with good response to different oncological regimens. The present study gives relevant information on pathophysiology of the cell line. Hopefully, this cell lines will help other researchers to better understand and treat CCC.

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Disclosure of conflict of interest

None.

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Supplementary Table 1. Comprehensive presentation of mutations (*chrom. = chromosomes*)

Position	Type	Ref	Length	Gene	Transcript	Chrom.	Location	Exon	Function	Genotype	Coding	*P* value
105246515	SNV	G	1	AKT1	NM_005163,2	14	Exonic	3	Missense	G/A	c,85C>T	0.033
105246553	SNV	C	1	AKT1	NM_005163,2	14	Exonic	3	Missense	C/T	c,47G>A	0.000
112174577	SNV	C	1	APC	NM_000038,5	5	Exonic	16	Nonsense	C/T	c,3286C>T	0.013
112175343	SNV	C	1	APC	NM_000038,5	5	Exonic	16	Missense	C/T	c,4052C>T	0.014
112175385	SNV	G	1	APC	NM_000038,5	5	Exonic	16	Missense	G/A	c,4094G>A	0.037
112175440	SNV	G	1	APC	NM_000038,5	5	Exonic	16	Missense	G/A	c,4149G>A	0.021
112175610	SNV	C	1	APC	NM_000038,5	5	Exonic	16	Missense	C/T	c,4319C>T	0.011
112175820	SNV	G	1	APC	NM_000038,5	5	Exonic	16	Missense	G/A	c,4529G>A	0.071
108206608	SNV	C	1	ATM	NM_000051,3	11	exonic	56	Nonsense	C/T	c,8188C>T	0.063
108155132	SNV	G	1	ATM	NM_000051,3	11	Exonic	26	Missense	G/A	c,3925G>A	0.002
108205766	SNV	G	1	ATM	NM_000051,3	11	Exonic	55	Missense	G/A	c,8081G>A	0.129
108206597	SNV	C	1	ATM	NM_000051,3	11	Exonic	56	Missense	C/T	c,8177C>T	0.068
108218077	SNV	G	1	ATM	NM_000051,3	11	Exonic	59	Missense	G/A	c,8656G>A	0.006
140453123	SNV	CCAT	1	BRAF	NM_004333,4	7	Exonic	15	Nonsense	CCAT/TCAT	c,1812G>A	0.004
140453115	SNV	G	1	BRAF	NM_004333,4	7	Exonic	15	Missense	G/A	c,1820C>T	0.131
140453134	SNV	T	1	BRAF	NM_004333,4	7	Exonic	15	Missense	T/C	c,1801A>G	0.063
68846077	SNV	C	1	CDH1	NM_004360,3	16	Exonic	8	Missense	C/T	c,1048C>T	0.000
21971028	SNV	C	1	CDKN2A	NM_000077,4	9	Exonic	2	Nonsense	C/T	c,330G>A	0.083
21970966	SNV	C	1	CDKN2A	NM_000077,4	9	Exonic	2	Missense	C/T	c,392G>A	0.078
21970985	SNV	C	1	CDKN2A	NM_000077,4	9	Exonic	2	Missense	C/T	c,373G>A	0.040
21971177	SNV	C	1	CDKN2A	NM_000077,4	9	Exonic	2	Missense	C/T	c,181G>A	0.027
21971188	SNV	G	1	CDKN2A	NM_000077,4	9	Exonic	2	Missense	G/A	c,170C>T	0.001
55241707	SNV	G	1	EGFR	NM_005228,3	7	Exonic	18	Missense	G/A	c,2155G>A	0.004
55242428	SNV	C	1	EGFR	NM_005228,3	7	Exonic	19	Missense	C/T	c,2198C>T	0.001
55242511	SNV	G	1	EGFR	NM_005228,3	7	Exonic	19	Missense	G/A	c,2281G>A	0.155
212530063	SNV	G	1	ERBB4	NM_005235,2	2	Exonic	15	Missense	G/A	c,1856C>T	0.006
212530073	SNV	G	1	ERBB4	NM_005235,2	2	Exonic	15	Missense	G/A	c,1846C>T	0.000
212576865	SNV	G	1	ERBB4	NM_005235,2	2	Exonic	9	Missense	G/A	c,1034C>T	0.058
212576901	SNV	G	1	ERBB4	NM_005235,2	2	Exonic	9	Missense	G/A	c,998C>T	0.004
212587202	SNV	C	1	ERBB4	NM_005235,2	2	Exonic	7	Missense	C/T	c,799G>A	0.011
212587235	SNV	C	1	ERBB4	NM_005235,2	2	Exonic	7	Missense	C/T	c,766G>A	0.029
153249433	SNV	C	1	FBXW7	NM_018315,4	4	Exonic	8	Missense	C/T	c,1105G>A	0.037
38285938	SNV	G	1	FGFR1	NM_015850,3	8	Exonic	4	Missense	G/A	c,374C>T	0.003
123274749	SNV	C	1	FGFR2	NM_000141,4	10	Exonic	9	Missense	C/T	c,1169G>A	0.000
123274768	SNV	C	1	FGFR2	NM_000141,4	10	Eexonic	9	Missense	C/T	c,1150G>A	0.000
123274833	SNV	G	1	FGFR2	NM_000141,4	10	Exonic	9	Missense	G/A	c,1085C>T	0.000
123279498	SNV	G	1	FGFR2	NM_000141,4	10	Exonic	7	Missense	G/A	c,934C>T	0.024
1806170	SNV	C	1	FGFR3	NM_000142,4	4	Exonic	9	Missense	C/T	c,1189C>T	0.000
1808904	SNV	G	1	FGFR3	NM_000142,4	4	Exonic	18	Missense	G/A	c,2336G>A	0.000
28592611	SNV	C	1	FLT3	NM_004119,2	13	Exonic	20	Missense	C/T	c,2534G>A	0.003
28608300	SNV	C	1	FLT3	NM_004119,2	13	Exonic	14	Missense	C/T	c,1756G>A	0.013
28608315	SNV	C	1	FLT3	NM_004119,2	13	Exonic	14	Missense	C/T	c,1741G>A	0.044
3118929	SNV	G	1	GNA11	NM_002067,2	19	Exonic	5	Missense	G/A	c,613G>A	0.000
57484421	SNV	G	1	GNAS	NM_000516,4	20	Exonic	8	Missense	G/A	c,602G>A	0.005
90631935	SNV	G	1	IDH2	NM_002168,2	15	Exonic	4	Missense	G/A	c,418C>T	0.001
55946319	SNV	G	1	KDR	NM_002253,2	4	Exonic	30	Missense	G/A	c,3860C>T	0.000
55955132	SNV	G	1	KDR	NM_002253,2	4	Exonic	26	Missense	G/A	c,3413C>T	0.001
55962492	SNV	C	1	KDR	NM_002253,2	4	Exonic	19	Missense	C/T	c,2632G>A	0.072
55980327	SNV	C	1	KDR	NM_002253,2	4	Exonic	6	Missense	C/T	c,764G>A	0.009
55980331	SNV	C	1	KDR	NM_002253,2	4	Exonic	6	Missense	C/T	c,760G>A	0.008
116339629	SNV	C	1	MET	NM_000245,2	7	Exonic	2	Missense	C/T	c,491C>T	0.003
116403176	SNV	C	1	MET	NM_000245,2	7	Exonic	11	Missense	C/T	c,2437C>T	0.022
116403236	SNV	C	1	MET	NM_000245,2	7	Exonic	11	Missense	C/T	c,2497C>T	0.013

139399408	INDEL	GCAC	3	NOTCH1	NM_017617,3	9	Exonic	26	Non frame-shift Deletion	GCAC/G	c,4732_4734delGTG	0.000
139390785	SNV	G	1	NOTCH1	NM_017617,3	9	Exonic	34	Missense	G/A	c,7406C>T	0.087
139390834	SNV	C	1	NOTCH1	NM_017617,3	9	Exonic	34	Missense	C/T	c,7357G>A	0.000
55144549	SNV	G	1	PDGFRA	NM_006206,4	4	Exonic	15	Missense	G/A	c,2023G>A	0.011
55144565	SNV	G	1	PDGFRA	NM_006206,4	4	Exonic	15	Missense	G/A	c,2039G>A	0.001
55152129	SNV	G	1	PDGFRA	NM_006206,4	4	Exonic	18	Missense	G/A	c,2561G>A	0.000
178927478	SNV	G	1	PIK3CA	NM_006218,2	3	Exonic	7	Missense	G/A	c,1241G>A	0.002
178927481	SNV	C	1	PIK3CA	NM_006218,2	3	Exonic	7	Missense	C/T	c,1244C>T	0.028
178938844	SNV	G	1	PIK3CA	NM_006218,2	3	Exonic	14	Missense	G/A	c,2086G>A	0.000
178952081	SNV	G	1	PIK3CA	NM_006218,2	3	Exonic	21	Missense	G/A	c,3136G>A	0.000
178952090	SNV	G	1	PIK3CA	NM_006218,2	3	Exonic	21	Missense	G/A	c,3145G>A	0.000
112888160	SNV	C	1	PTPN11	NM_002834,3	12	Exonic	3	Missense	C/T	c,176C>T	0.044
112888187	SNV	G	1	PTPN11	NM_002834,3	12	Exonic	3	Missense	G/A	c,203G>A	0.000
48953760	SNV	C	1	RB1	NM_000321,2	13	Exonic	14	Monsense	C/T	c,1363C>T	0.118
48941639	SNV	C	1	RB1	NM_000321,2	13	Exonic	10	Missense	C/T	c,949C>T	0.003
49037877	SNV	G	1	RB1	NM_000321,2	13	Exonic	21	Missense	G/A	c,2117G>A	0.207
43609969	SNV	G	1	RET	NM_020630,4	10	Exonic	11	Missense	G/A	c,1921G>A	0.006
43615601	SNV	G	1	RET	NM_020630,4	10	Exonic	15	Missense	G/A	c,2680G>A	0.006
43617424	SNV	G	1	RET	NM_020630,4	10	Exonic	16	Missense	G/A	c,2761G>A	0.001
48584560	SNV	C	1	SMAD4	NM_005359,5	18	Exonic	6	Nonsense	C/T	c,733C>T	0.000
48593427	SNV	G	1	SMAD4	NM_005359,5	18	Exonic	10	Missense	G/A	c,1178G>A	0.038
24143236	SNV	G	1	SMARCB1	NM_003073,3	22	Exonic	4	Missense	G/A	c,468G>A	0.081
128845180	SNV	C	1	SMO	NM_005631,4	7	Exonic	3	Missense	C/T	c,674C>T	0.017
128846049	SNV	G	1	SMO	NM_005631,4	7	Exonic	5	Missense	G/A	c,979G>A	0.024
128846383	SNV	C	1	SMO	NM_005631,4	7	Exonic	6	Missense	C/T	c,1219C>T	0.068
128846407	SNV	G	1	SMO	NM_005631,4	7	Exonic	6	Missense	G/A	c,1243G>A	0.015
128851558	SNV	G	1	SMO	NM_005631,4	7	Exonic	11	Missense	G/A	c,1883G>A	0.044
1223100	SNV	G	1	STK11	NM_000455,4	19	Exonic	8	Missense	G/A	c,1037G>A	0.000
7578212	SNV	GAA	1	TP53	NM_000546,5	17	Exonic	6	Nonsense	GAA/AAA	c,637C>T	0.032
7577118	SNV	C	1	TP53	NM_000546,5	17	Exonic	8	Missense	C/T	c,820G>A	0.090
7577538	SNV	CG	1	TP53	NM_000546,5	17	Exonic	7	Missense	CG/TG	c,743G>A	0.001
7578401	SNV	G	1	TP53	NM_000546,5	17	Exonic	5	Missense	G/A	c,529C>T	0.014
7578406	SNV	C	1	TP53	NM_000546,5	17	Exonic	5	Missense	C/T	c,524G>A	0.006
7578472	SNV	G	1	TP53	NM_000546,5	17	Exonic	5	Missense	G/A	c,458C>T	0.029
7578544	SNV	G	1	TP53	NM_000546,5	17	Exonic	5	Missense	G/A	c,386C>T	0.005
7579485	SNV	C	1	TP53	NM_000546,5	17	Exonic	4	Missense	C/T	c,202G>A	0.000
10188208	SNV	G	1	VHL	NM_000551,3	3	Exonic	2	Nonsense	G/A	c,351G>A	0.015
10188275	SNV	C	1	VHL	NM_000551,3	3	Exonic	2	Missense	C/T	c,418C>T	0.004
10188288	SNV	G	1	VHL	NM_000551,3	3	Exonic	2	Missense	G/A	c,431G>A	0.005