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Biology of childhood hepatoblastoma and the search for novel treatments

Marjut Pihlajoki [a,*], Katja Eloranta [a], Ruth Nousiainen [a], Ville Väyrynen [a], Tea Soini [a], Antti Kyrönlahti [a], Seppo Parkkila [b,c,d], Jukka Kanerva [a], David B. Wilson [e,f], Mikko P. Pakarinen [a,g], Markku Heikinheimo [a,f,h]

[a] Pediatric Research Center, Children's Hospital, University of Helsinki and Helsinki University Hospital, Helsinki, Finland
[b] Faculty of Medicine and Health Technology, Tampere University, Tampere, Finland
[c] FICAN Mid, Tampere University, Tampere, Finland
[d] Fimlab Ltd, Tampere University Hospital, Tampere, Finland
[e] Department of Developmental Biology, Washington University School of Medicine, St. Louis, MO, United States
[f] Department of Pediatrics, Washington University in St. Louis, St. Louis, United States
[g] Section of Pediatric Surgery, Pediatric Liver and Gut Research Group, Pediatric Research Department of Women's Health, Karolinska Institute, Stockholm, Sweden
[h] Faculty of Medicine and Health Technology, Center for Child, Adolescent, and Maternal Health Research, Tampere University, Tampere, Finland

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ABSTRACT

Our research laboratory has a longstanding interest in developmental disorders and embryonic tumors, and recent efforts have focused on the pathogenesis of pediatric liver tumors. This review focuses on hepatoblastoma (HB), the most common pediatric liver malignancy. Despite advances in treatment, patients with metastatic HB have a poor prognosis, and survivors often have permanent side effects attributable to chemotherapy. In an effort to improve survival and lessen long-term complications of HB, we have searched for novel molecular vulnerabilities using a combination of patient derived cell lines, metabolomics, and RNA sequencing of human samples at diagnosis and follow-up. These studies have shed light on pathogenesis and identified putative targets for future therapies in children with advanced HB.

1. Introduction

Hepatoblastoma (HB) is the most common malignant pediatric liver neoplasm with an annual incidence of 1.9 cases per million (Feng et al., 2019). HB is usually diagnosed before the age of four, the median age of diagnosis being 1.5 years. Most cases of HB are sporadic, but this tumor also arises in the setting of congenital syndromes, such as Beckwith-Wiedemann, Sotos, and familial adenomatous polyposis coli (FAP) syndromes. HB histology resembles embryonal or fetal liver, and low differentiation stage associates with poor prognosis.

* Corresponding author.
E-mail address: marjut.pihlajoki@helsinki.fi (M. Pihlajoki).

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Abbreviations

APC	Adenomatous polyposis coli
AFP	alpha-1-fetoprotein
CTNNB1	beta-catenin
BCL2	B-cell lymphoma 2
BID	BH3 Interacting Domain Death Agonist
CA	Carbonic anhydrase
CQ	chloroquine
CT	contrast-enhanced computed tomography
DEGs	differentially expressed genes
DOX	doxorubicin
ETC	electron transport chain
EMT	epithelial-mesenchymal transition
FAP	familial adenomatous polyposis coli
HB	hepatoblastoma
HCC	hepatocellular carcinoma
NRP	neuropilin
NFE2L2	NFE2 like bZIP transcription factor 2
PHITT	Paediatric Hepatic International Tumour Trial
PARP	Poly(ADP-Ribose) Polymerase
PRETEXT	Pretreatment extent of disease
PPI	protein-protein interaction
TERT	Telomerase reverse transcriptase
UBE2C	Ubiquitin-conjugating enzyme E2 C
WNT	wingless-type MMTV integration site

1.1. Current drug treatment causes severe adverse effects

In Europe, HB patients are treated according to the international PHITT (Paediatric Hepatic International Tumour Trial, ClinicalTrials.gov Identifier: NCT03017326; EudraCT number: 2016-002828-85) treatment protocol. This protocol is based on clinical drug trials conducted by the SIOPEL research group (https://siope.eu) since 1990. Most patients receive doxorubicin and cisplatin- or carboplatin-based chemotherapy before and after surgical treatment. HB drug therapy often leads to long-term side effects, and the intensity of the treatment is tailored to the severity of the disease.

Cisplatin treatment carries a significant risk of hearing impairment and kidney dysfunction. In the SIOPEL-6 study, which followed the hearing of 101 children and adolescents under 18 years old with standard-risk HB between 2007 and 2014, the risk of hearing impairment was significantly lower among those who received sodium thiosulfate after cisplatin treatment compared to those who did not receive it. At least mild hearing impairment was observed in 63% of children receiving cisplatin treatment, but only in 33% of those who received sodium thiosulfate after cisplatin infusion (Brock et al., 2018).

The development of kidney complications is lessened by hydrating the patient before administering cisplatin. Hyperuricemia and hypoalbuminemia may predispose individuals to cisplatin-induced kidney toxicity. Some individuals with HB also receive anthracycline-based doxorubicin chemotherapy at a relatively high cumulative dose. They are recommended to have lifelong cardiac monitoring. After a liver transplant, patients require lifelong immunosuppressive anti-rejection medication and appropriate follow-up.

Despite advances in HB treatment, new strategies are needed to improve the outcome of the disease. Unraveling the molecular pathways underlying the pathophysiology of HB pathophysiology is paramount. This review summarizes recent developments in the search for druggable targets in HB, especially for children with refractory or relapsed disease still carrying a dismal prognosis.

2. Clinical aspects of hepatoblastoma

2.1. Risk factors and diagnosis of hepatoblastoma

HB is typically diagnosed under the age of five, although it can also occur among adolescents and young adults (Finegold et al., 2008). Several risk factors for HB have been recognized. These include birth weight <1500 g, premature birth, maternal pre-eclampsia, parental smoking, and syndromes such as hemihypertrophy (Beckwith-Wiedemann syndrome), FAP, Li-Fraumeni syndrome, trisomy 18, and Simpson-Golabi-Behmel syndrome (Spector and Birch, 2012; De Fine Licht et al., 2012).

HB diagnosis is based on the clinical and radiological findings and serum alpha-1-fetoprotein (AFP), which is a useful tumor marker. At diagnosis, AFP concentration is typically significantly elevated. However, an exceptionally low AFP concentration (<86 kU/l, 1 kU/L = 2.4 ng/mL) is associated with a poor prognosis (De Ioris et al., 2008). AFP is a useful tumor marker in HB follow-up. Diagnosis is confirmed by histology, and HBs are classified as embryonal, fetal, and mixed subtypes.

2.2. Risk stratification and prognosis

HB treatment is based on the classification of recurrence risk [.e.g. The Children's Hepatic Tumors International Collaboration] (Meyers et al., 2017). Other factors that increase the risk of recurrence include the age [>8 yrs PRETEXT (Pretreatment extent of disease) I–III classes and >3 yrs PRETEXT class IV], serum AFP concentration <86 kU/l, tumor spread in the liver, vascular invasion, tumor growth through the liver capsule, and tumor rupture. The fetal histological subtype is a favorable prognostic factor.

The prognosis of HB patients has significantly improved since the 1990s due to platinum-based chemotherapy and advanced surgical treatment. Internationally, the five-year survival rate for HB ranges from 50 to 100% depending on the extent of the disease and histological subtype. Almost all patients whose tumor can be surgically removed at the diagnosis stage achieve permanent remission. For most patients, chemotherapy is sufficient to treat distant metastases.

2.3. Surgical treatment of hepatoblastoma

The surgical treatment of HB is based on the histopathological diagnosis confirmed from a liver tissue sample and imaging. In addition to abdominal ultrasound, contrast-enhanced computed tomography (CT) and magnetic resonance imaging of the liver and abdominal area, as well as CT with contrast enhancement of the lungs, are performed.

The primary goal of surgical treatment is complete tumor removal, enabling long-term survival without disease recurrence (Lake et al., 2019; Aronson and Meyers, 2016). In most cases, surgical removal of the tumor at diagnosis is not possible, and feasibility of curative liver resection is assessed after two cycles of chemotherapy with repeated imaging (Meyers et al., 2021).

If needed, chemotherapy is continued with two additional cycles, although the majority of tumor reduction facilitating surgery occurs during the first two treatment cycles (Meyers et al., 2021; Lake et al., 2019; Aronson and Meyers, 2016). If liver resection is not feasible after completed chemotherapy or if the tumor is classified as PRETEXT IV or has extensively spread to critical blood vessels,

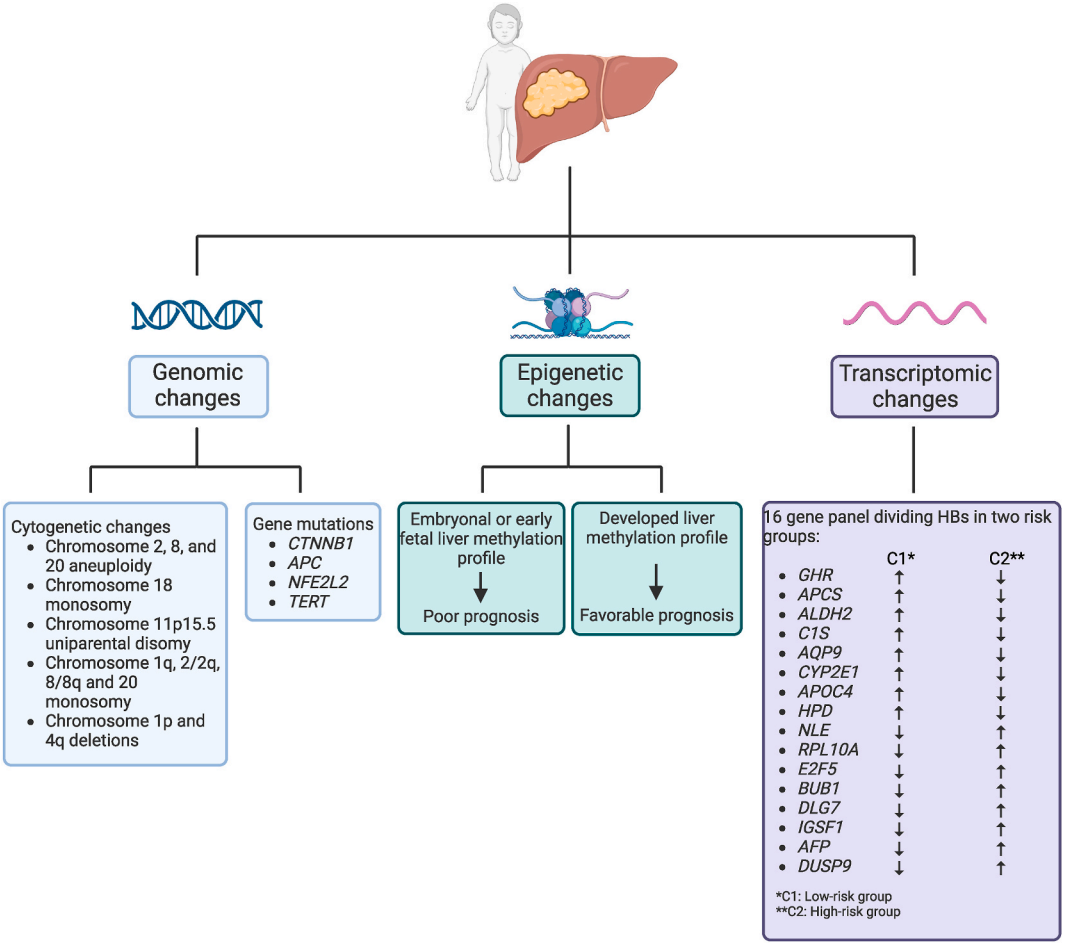

Fig. 1. The cyto- and molecular genetic alterations of hepatoblastoma can be divided into changes in the genome, epigenome, and transcriptome. The arrows represent the quantity of the respective gene product in risk classes in relation to each other, for example, *GHR* is highly expressed in class C1 and less expressed in class C2.

the patient is listed for liver transplantation (Lake et al., 2019; Aronson and Meyers, 2016). Patients are primarily aimed to be treated with partial liver resection due to lower morbidity compared to liver transplantation. This approach also avoids the need for long-term immunosuppressive medication (Tiusanen et al., 2020; Lake et al., 2019).

3. Molecular genetic background of hepatoblastoma

Modern molecular profiling techniques have increased our understanding of the genetic and epigenetic changes related to the development and progression of HBs. Whole exome sequencing studies have shown that the mutation frequency in HBs is relatively low. On average, only a few functional genetic changes are found per tumor, which, according to a study comparing mutation frequencies across various childhood tumors, is the lowest among 24 cancer types (Gröbner et al., 2018).

The key molecular pathways involved in HB pathophysiology are depicted in Fig. 1. The most frequent mutation found in HB is in the *CTNNB1* gene, which encodes for beta-catenin, and is detected in the majority (over 80%) of tumors (Sumazin et al., 2017). These mutations increase the activity of beta-catenin, leading to enhanced signaling through the wingless-type MMTV integration site (WNT) pathway. The WNT pathway has been shown to play a significant role in regulating organ development, maintaining stem cells, and contributing to the formation of various types of cancers. Other mutations that enhance WNT pathway activity are also present in HB, including mutations in the Adenomatous polyposis coli (*APC*) gene, which are most notably associated with FAP (Yang et al., 2018). FAP is characterized by adenoma polyps in the colon, but it has also been linked to an increased susceptibility to HB formation, among other conditions. Less frequently occurring mutations in HB include changes in the NFE2 like bZIP transcription factor 2 (*NFE2L2*) gene that encodes the NRF2 transcription factor, which have been associated with poorer survival rates, and mutations in the Telomerase reverse transcriptase (*TERT*) gene promoter, primarily found in atypical late-onset HBs (patients over ten years old) (Fig. 1) (Eichenmüller et al., 2014).

Among cytogenetic findings, the most common ones are aneuploidies in chromosomes 2, 8, and 20, as well as monosomy of chromosome 18 (Ferreira et al., 2017). About one-third of HBs show uniparental disomy on the 11p15.5 chromosomal region (Carrillo-Reixach et al., 2020). Copy number changes frequently involve duplications of chromosomes 1q, 2/2q, 8/8q, and 20, as well as deletions of chromosomes 1p and 4q (Fig. 1). In a recent study, HBs lacking copy number changes were histologically characterized as embryonal-fetal subtype and classified as small or medium risk tumors. Tumors with abundant copy number changes were histologically epithelial-embryonal subtype and high risk tumors (De Ioris et al., 2008).

In addition to the WNT-beta-catenin pathway, activation of signaling pathways related to liver development, such as hedgehog, Hippo/YAP, and insulin-like growth factor signaling, is commonly observed in connection with these tumors (Zhang et al., 2021). Both genetic and epigenetic changes play a role in activating these pathways.

The epigenetic profile of HBs differs from that of normal liver tissue. Methylation analyses indicate that HBs resemble the profile of embryonal or fetal liver tissue, explaining the activity of these developmental signaling pathways (Carrillo-Reixach et al., 2020). This approach can also complement conventional clinical risk assessment. Patients whose tumors exhibited methylation profiles resembling embryonal or early fetal liver tissue have shown poorer prognosis compared to those whose HB profile represents more developed liver tissue (Fig. 1) (Murawski et al., 2020). In a recent preclinical publication Clavería-Cabello et al. performed an integrative transcriptional analysis of 180 epigenetic modifiers and found significant alterations in the expression of epigenetic genes. Among these genes, the histone-lysine methyltransferase G9a was identified as a candidate involved in HB growth and pharmacological targeting of G9a significantly inhibited growth of HB cells, organoids, and patient-derived xenografts (Clavería-Cabello et al., 2023). Currently, methylation analysis is not routinely utilized in HB diagnostics.

Changes at the transcriptome level can also be utilized for molecular subtyping of HB. By assessing the expression of 16 genes, HBs can be divided into two risk groups (C1 and C2) (Cairo et al., 2008). The high-risk group C2 associated with poor prognosis displays increased expression of genes related to stem cell-like features and cell cycle regulation (such as BUB1 and AFP), compared to the low-risk group C1 (Fig. 1) (Cairo et al., 2020). Risk class C2 tumors also commonly exhibit activity in the MYC signaling pathway.

4. New avenues to tackle HB: from GATA transcription factors to screening novel therapeutic compounds

4.1. Transcription factor GATA4 in HB pathophysiology

GATA transcription factors are a family of six evolutionarily highly conserved zinc finger DNA-binding proteins that serve important physiological and pathological functions in various organs. GATA1/2/3 are mainly expressed in hematopoietic cells, whereas GATA4/5/6 are present in mesoderm- and endoderm-derived tissues (Viger et al., 2008).

GATA factors have been implicated in a variety of human developmental diseases. They are also connected to several human cancers including leukemia and solid tumors of many tissues affecting the metabolism, proliferation, cell death signaling, and invasiveness of tumor cells (Zheng and Blobel, 2010). GATA1/2/3 are especially important for normal and aberrant hematopoiesis, and accordingly GATA2 is involved in myeloid neoplasias and immunodeficiencies. GATA4/6 are expressed in developing heart, gonads, lung, gastrointestinal tract, and yolk sac, and their aberrant expression has been linked to congenital heart disease, diaphragmatic hernia, gonadal abnormalities and tumors, and malignancies of the gastrointestinal tract (Molkentin, 2000; Pihlajoki et al., 2016).

GATA4 is thought to be among the first transcription factors promoting endodermal differentiation, and it is abundantly expressed in early fetal liver hepatoblasts (Dame et al., 2004). GATA4 is crucial for normal liver development, but its expression declines in developing hepatocytes later in gestation. In postnatal liver, GATA4 expression is mainly restricted to endothelial cells, hepatic stellate cells, and liver macrophages. GATA4 has been connected to liver fibrosis regression and inhibition of nonalcoholic fatty liver disease

(Arroyo et al., 2021; He et al., 2023). In adult hepatocellular carcinoma (HCC), GATA4 has been suggested to act as a tumor suppressor (Enane et al., 2017; Lu et al., 2020).

A well-known side effect of DOX therapy is cardiomyopathy. Downregulation of GATA4, abundantly expressed in normal cardiomyocytes and essential for their function, has been implicated in pathogenesis of DOX-induced cardiomyopathy (Kim et al., 2003). DOX is shown to suppress GATA4 expression and its DNA-binding activity in cardiomyocytes. Furthermore, silencing GATA4 in these cells enhances the DOX-induced autophagy and apoptosis by affecting BCL2 (B-cell lymphoma-2) mediated apoptosis (Aries et al., 2004). Since DOX is effective for treatment of HB, we hypothesized it may act partially through GATA4 mediated apoptotic mechanisms also in HB cells.

Our studies revealed that GATA4 is highly expressed in the majority of HBs whereas its expression is absent in normal hepatocytes (Soini et al., 2012, 2018). Especially tumor areas with a distinct embryonal, undifferentiated histology were highly positive for GATA4. To assess the role of GATA4 in DOX-induced apoptosis of HB cells further, we used siRNA to silence *GATA4* from these cells. We treated the *GATA4* silenced cells with DOX and measured cell viability and apoptosis. We found that *GATA4* silencing sensitizes HB cells to the apoptotic effect of DOX. We also noticed that expression level of anti-apoptotic B-cell lymphoma 2 (BCL2) was decreased whereas pro-apoptotic BH3 Interacting Domain Death Agonist (BID) was increased after *GATA4* silencing and DOX treatment shifting the balance of the intrinsic apoptotic pathway towards a proapoptotic direction (Soini et al., 2017).

To further investigate the role of GATA4 in HB pathophysiology we performed an mRNA microarray hybridization on *GATA4* silenced and control HB cells (Soini et al., 2018). Gene Ontology (GO) analysis on the differentially expressed genes (DEGs) revealed that a substantial proportion of the altered genes and biological processes were connected to epithelial-mesenchymal transition (EMT), a gradual process, in which the polarized epithelial cell detaches from the basement membrane and adjacent cells, obtains enhanced migratory capacity, and produces increased amounts of extracellular matrix components. To validate our microarray results we performed qPCR analysis on genes related to epithelial-mesenchymal-balance, adhesion, migration, and invasion from the list of DEGs. We verified altered expression of several genes known to promote cell motility and EMT in other cell types. Phalloidin staining of *GATA4* silenced cells revealed a change in the organization of the filamentous actin fibers. In control HB cells actin stress fiber formation was evident, indicating a motile, mesenchymal-like phenotype, while in *GATA4* silenced cells filamentous actin was re-localized mainly in the peripheral parts of the cell, adjacent to cell membrane and stress fiber formation was reduced. Cadherin switch, downregulation of epithelial E-cadherins and upregulation of mesenchymal N-cadherins, is a hallmark molecular phenomenon in the EMT process. In metastatic HCCs cadherin switch is connected to poor prognosis. Our results indicated a reverse cadherin switch in *GATA4* silenced HB cells. We also assessed the effects of GATA4 on HB cell motility by wound healing and transwell assays. Our results showed that *GATA4* silencing significantly impairs migration of HB cells suggesting that GATA4 is a key factor required for maintaining the migratory capacity of HB cells. Finally, we overexpressed GATA4 in human primary hepatocytes to see whether GATA4 overexpression is able to induce the migratory and mesenchymal-like gene expression gene expression profile in normal hepatocytes. We found that majority of the EMT-associated genes altered in *GATA4* silenced HB cells were significantly altered in primary hepatocytes after GATA4 overexpression implying that GATA4 can change the gene expression profile of hepatocytes to a mesenchymal direction.

Our results demonstrate that transcription factor GATA4 is frequently overexpressed in HB tumors compared to normal liver and silencing *GATA4* enhances DOX-induced apoptosis, decreases migration, and shifts HB cell gene expression towards a more epithelial phenotype (main findings summarized in Fig. 2). Small molecular combounds inhibiting GATA4 function by interacting with the

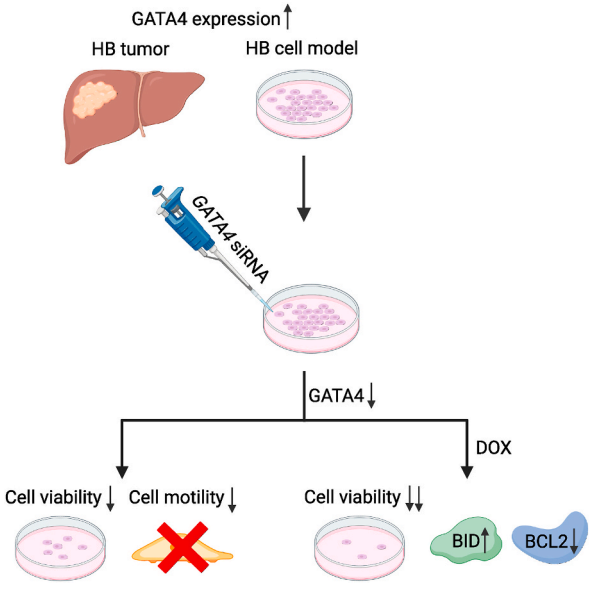

Fig. 2. Schematic view of the role of GATA4 in HB pathobiology.

interaction of GATA4 and its cofactors offer an intriguing possibility to affect GATA4 function also in HB. These combounds have been used successfully *in vitro* and *in vivo* (Kinnunen et al., 2018). Our unpublished results reveal that these combouds can also be used in human HB PDX cell lines to interfere with GATA4 function (Kinnunen et al., unpublished).

4.2. Neuropilin-2 is associated with increased HB cell viability and motility

Neuropilins (NRP1 and -2) are multifunctional glycoproteins and co-receptors with multiple different functions during development of several tissues and are associated with numerous signalling pathways including those activated by Epidermal Growth Factor, Fibroblast Growth Factor, Hepatocyte Growth Factor, Insulin-like Growth Factor, Platelet Derived Growth Factor, and Transforming Growth Factor beta (Ball et al., 2010; Gluzman-Poltorak et al., 2000; Grandclement et al., 2011; Matsushita et al., 2007). NRPs were originally documented as regulators for neurogenesis, angiogenesis, and lymphangiogenesis, but abundant expression of NRPs has also been associated with tumor progression, aggressive phenotype, and resistance to chemotherapy in many cancers, including another liver malignancy HCC (Dong et al., 2017).

In our recently published study, we characterized the expression patterns of NRP1 and NRP2 in human HB specimens and cell models and examined the functional consequences of *NRP2* gene silencing in HB cells (Eloranta et al., 2021). Our results indicated that both NRPs are expressed in majority of HB tissues and cell models, whereas normal hepatocytes remained negative. RNA interference mediated inhibition of *NRP2* significantly suppresses HB cell viability in two cell models measured with clonogenic assay. Furthermore, stress fiber formation and cell motility are impaired in *NRP2* silenced HB cells.

These findings suggest that NRP2 contributes to the malignant phenotype of HB and NRP2 targeted interventions have potential in the management of aggressive HB (findings summarized in Fig. 3).

4.3. UBE2C expression is elevated in HB and correlates with inferior patient survival

Highly proliferating tumor cells require metabolic reprogramming to boost their survival in nutrient-deprived and hypoxic environment. A metabolic switch is also associated with initiation of tumorigenesis, and it has been demonstrated to promote all hallmarks of cancer (Nong et al., 2023). Many oncogenes and tumor suppressors participate in dysregulation of metabolic pathways in cancer,

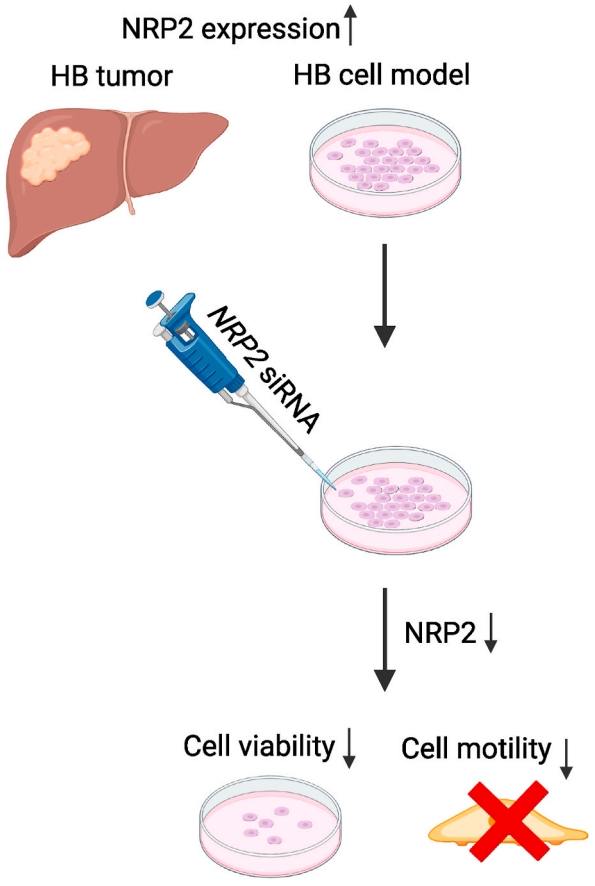

Fig. 3. Schematic view of the effects of *NRP2* silencing on HB cells.

and genes coding for metabolic enzymes have been described to be aberrantly expressed in various tumor types (Sreedhar and Zhao, 2018). Alterations in genes regulating ubiquitination and deubiquitination and their role as modulators of the metabolic changes of tumor cells are also known to be essential in cancer progression (Sun et al., 2020).

In our recent publication we characterized the landscape of metabolic genes in HB using RNA sequencing data and bioinformatics analyses (Nousiainen et al., 2023). RNAseq data of six HB cell models and healthy primary hepatocytes were analyzed, and approximately 9000 DEGs in each cell line were identified compared to primary hepatocytes of which about 3000 were shared among all six cell lines. We used a list of known human metabolic genes to identify the metabolic genes in our HB cell lines. Approximately 1400 DEGs in each cell line were classified as metabolic genes. 490 of these DEGs were shared among all six HB cell lines. Next, the online tool of STRING database was utilized to construct protein-protein interaction (PPI) networks for each cell line using the lists of differentially expressed metabolic genes as input. Highly interconnected clusters in each PPI network were identified, scored, and ranked on size and density. Next, to identify enriched pathways and gene sets, the list of protein-coding genes in each cell line's highest-scoring PPI cluster was uploaded to the online tool Enrichr and the results were ranked by p-value. Ubiquitination-related pathways were the topmost statistically significant GO terms in the highest-ranking clusters. Four genes that were observed in every HB cell line's highest-scoring clusters (*RNF130*, *UBE2C*, *HERC3*, and *RNF144B*) were also found to be significantly altered in the publicly available microarray dataset containing 53 HB tissue samples and 14 noncancerous liver tissue samples. In this patient cohort, higher Ubiquitin-conjugating enzyme E2 C (*UBE2C*) mRNA expression correlated with distant metastasis, occurrence of events, and death. Next, we assessed the protein expression of UBE2C in HB patient samples by immunohistochemistry. 20/25 HBs showed positive staining for UBE2C while 5/6 normal liver samples remained negative. Finally, to examine UBE2C function *in vitro*, *UBE2C* was silenced in two HB cell models by siRNA. Cell viability measurements with *UBE2C* silenced cells showed significant decrease in cell viability. RNA sequencing of *UBE2C* silenced HB cells revealed that *UBE2C* knockdown is linked with alterations in RNA expression of genes connected to cell cycle regulation and p53 signaling pathway.

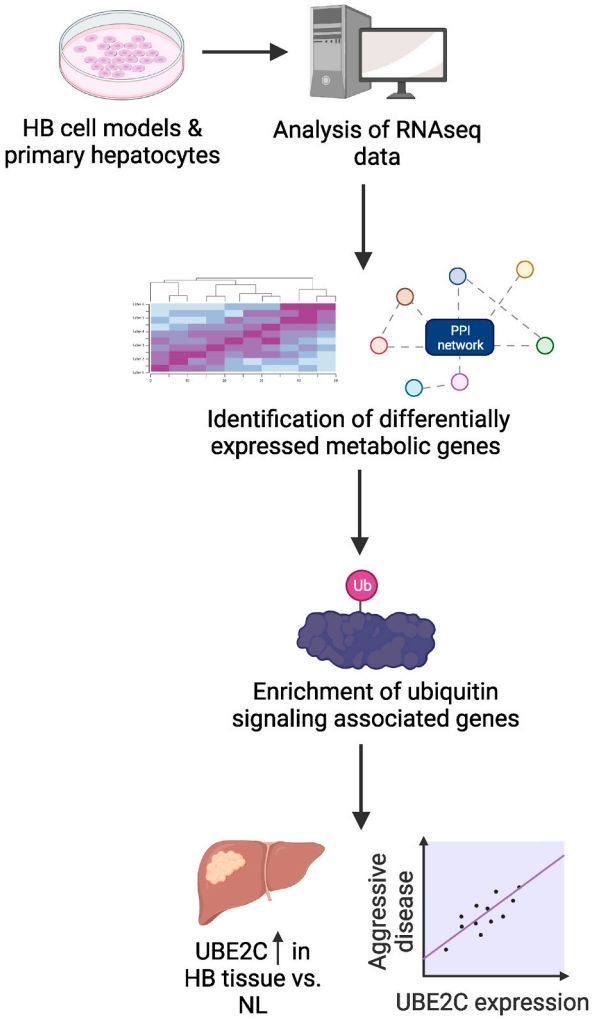

Fig. 4. Schematic view of the metabolic gene analyses in HB cells.

These findings indicate that metabolic alterations in HB tumors are diverse and that ubiquitination-related factors may have a significant role in HB progression. Furthermore, UBE2C may hold prognostic utility in HB and the ubiquitin pathway is a potential therapeutic target in this tumor. The findings are summarized in Fig. 4.

4.4. SLC-0111, an inhibitor of carbonic anhydrase IX, attenuates HB cell viability and migration

Carbonic anhydrases (CAs) are evolutionarily conserved metalloenzymes catalyzing reversible hydration of CO_2 to HCO_3^- and H^+. Carbonic anhydrase 9 (CAIX) is a transmembrane metalloenzyme maintaining both physiological intracellular and slightly acidic extracellular pH in hypoxic environment typical for solid tumors (Pastorekova et al., 2006). CAIX is a tumor associated isoform that promotes an aggressive cancer phenotype. Its expression in healthy liver is restricted to bile duct cells (Pastorekova et al., 1997). Overexpression of CAIX has been associated with increased chemoresistance, EMT, and poor prognosis in various cancer types including liver malignancies, making it as an attractive drug target. A small molecule inhibitor of CAIX, SLC-0111, has shown promising results in phase 1/2 clinical trials for treatment of advanced solid tumors (Nerella et al., 2022).

In our recent publication, we characterized CAIX expression in HB and studied the impact of SLC-0111 on this malignancy (Eloranta et al., 2023). We demonstrated that CAIX is expressed in the majority of HBs, while in healthy liver CAIX immunostaining was restricted to bile duct cells. CAIX-positive cells were clustered in the middle of viable tissue or adjacent to necrotic regions presumed to be hypoxic due to limited blood supply. In a patient cohort including 53 HBs and 14 normal liver samples, high *CA9* mRNA expression associated with events, distant metastases, and poor overall survival. Thus, CAIX may have potential as a prognostic marker.

Next, we assessed whether low oxygen tension induces CAIX expression in HB cells. We cultured HB cells under normoxic and hypoxic conditions and measured the CAIX expression levels. Our results showed little or no baseline CAIX expression when cultured under normoxia, while CAIX expression was markedly upregulated in hypoxic cells. SLC-0111 treatment caused marked reduction in *CA9* mRNA expression in hypoxic cells, while in normoxic cells *CAIX* expression remained unaltered after SLC-0111 treatment. To study the effect of SLC-0111 on HB cell survival, we investigated cell viability after SLC-0111 treatment. Interestingly, SLC-0111 decreased HB cell viability in both normoxia and hypoxia in both monolayer and 3D spheroid cultures. Next, we assessed the effect of SLC-0111 on HB cell migration by wound healing assay as several studies have reported decreased cell motility after pharmacological inhibition of CAIX. We found that migration rates significantly decreased after SLC-0111 treatment compared to control cells under both normoxic and hypoxic conditions. As mentioned above, SLC-0111 decreased viability and motility in HB cells even under normoxic conditions when CAIX expression was undetectable or extremely low, suggesting that the drug may have CAIX-independent effects. To clarify these mechanisms and transcriptomic changes induced by SLC-0111 treatment, we performed RNA sequencing

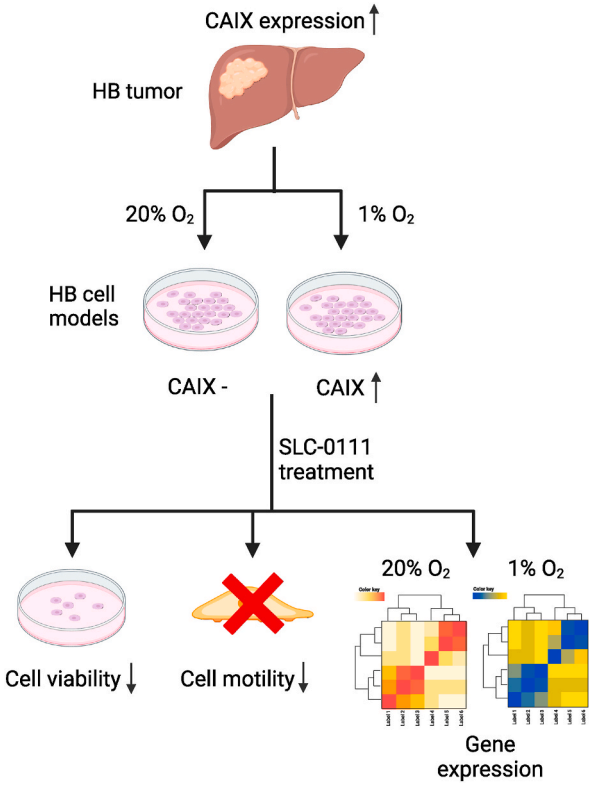

Fig. 5. Schematic view of SLC-0111 action in HB cells.

analysis for HB cells treated with SLC-0111 either in normoxic or hypoxic conditions. In normoxia, we observed 304 upregulated genes and 96 downregulated genes after SLC-0111 treatment. Under hypoxic conditions, SLC-0111 induced upregulation of 175 genes and downregulation of 312 genes. Altogether, 76 genes were differentially expressed in both normoxic and hypoxic HB cells treated with SLC-0111. Molecular functions associated with these overlapping genes included semaphorin binding, protein-arginine deaminase activity, and protease binding. Metal ion related biological processes were highly overrepresented in SLC-0111 treated cells. Finally, to identify other potential targets for SLC-0111, we performed *in silico* target prediction analysis. In addition to CAIX and CAXII, SLC-0111 had high expected probability of binding CAII. Other identified targets included histone deacetylase 3, thymidylate synthase, nuclear factor NF kappa-B inhibitor kinase alpha, mammalian target of rapamycin, cyclin dependent kinases 1/2/4/5, and phosphatidylinositol 3-kinases PK3CA, PK3CB, and PK3CG.

Our findings demonstrate that CAIX is expressed in majority of HBs and is associated with unfavorable clinical outcome. Furthermore, we found that hypoxia induces CAIX expression *in vitro* and the CAIX inhibitor SLC-0111 reduces HB cell survival and motility and thus holds potential as a novel treatment modality in HB patient care. Our results also suggest that SLC-0111 may have

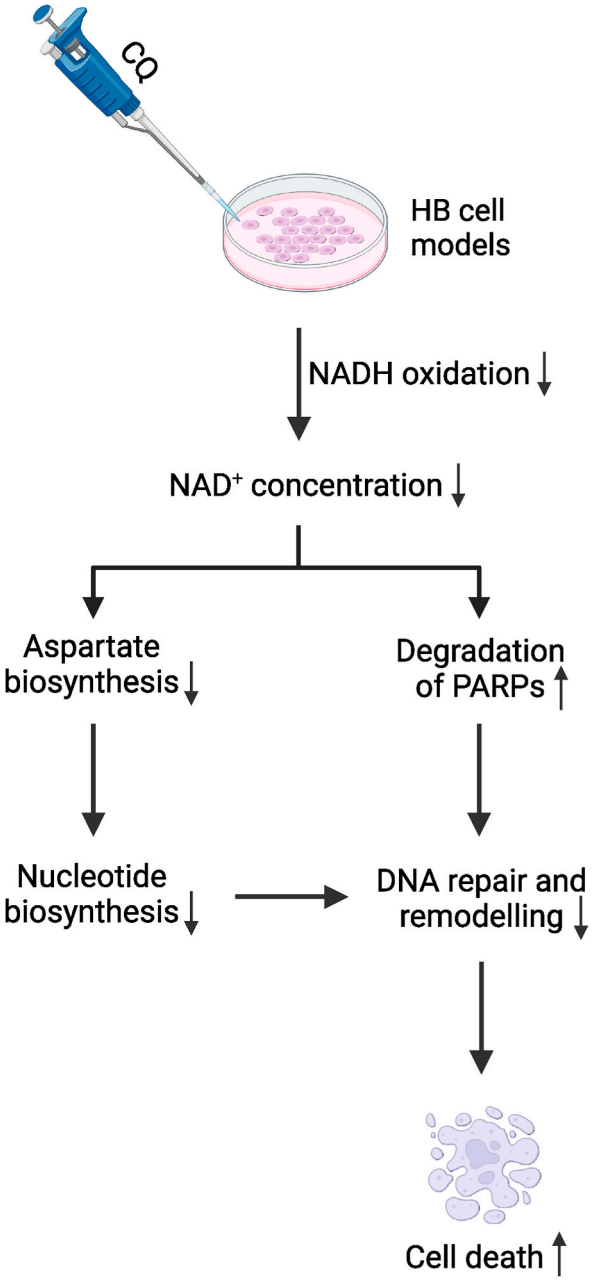

Fig. 6. Schematic view of CQ action in HB cells.

CAIX-independent modes of action. The main results are summarized in Fig. 5.

4.5. Chloroquine triggers cell death and inhibits PARPs in HB cells

Chloroquine (CQ) has previously been used in the treatment of malaria, rheumatoid arthritis, and systemic lupus erythematosus. CQ has also been shown to exhibit anticancer activity in various cancer types including HCC (Abdel-Aziz et al., 2022; Hu et al., 2016). Furthermore, CQ has shown potential to sensitize cancer cells to conventional therapy after primary treatment has failed (Cocco et al., 2022). Several mechanisms of action for the antitumoral effects of CQ have been suggested, including autophagy inhibition, G2/M cell cycle arrest, increased apoptosis, altered inflammatory responses, and tumor vessel normalization.

In our published study we demonstrated the efficacy of CQ in HB cell models and shed new light on the molecular mechanisms of CQ action in these cells (Eloranta et al., 2020). In 2D and 3D cultured HB models CQ treatment caused a significant decrease in cell viability and induction of apoptosis. Furthermore, the morphology monitoring revealed that CQ treatment triggered a time and dose-dependent increase in the necrotic non-viable zone and loss of proliferative edge in 3D cultured HB spheroids. To further investigate the mechanism of action of CQ in HB cells the metabolomic profiling was conducted. It revealed a statistically significant decrease in 12 metabolites and an increase in 4 metabolites, of which nicotine adenine dinucleotide (NAD^+) was the most significantly altered. Moreover, NAD^+/NADH ratio was significantly lower in CQ treated cells compared to control cells. Pathway analysis implicated alanine, aspartate, and glutamate metabolism as having the highest impact. Aspartate demonstrated marked decrease in concentration when comparing CQ treated cells to control cells, and aspartate supplementation was able to prevent cell death triggered by CQ treatment. Interestingly, aspartate depletion induced by CQ treatment has previously been demonstrated to limit nucleotide biosynthesis and subsequently predispose cells to replication stress. Aspartate biosynthesis requires electron acceptors, e.g., NAD^+, and in the presence of oxygen their pools are maintained by electron transport chain (ETC) reactions. ETC is carried out by four complexes (I–IV) and ATP synthase. Complex I regenerates NAD^+ from NADH and pharmacological inhibition of that reaction is linked with disturbed NAD^+/NADH balance and subsequent reduction in aspartate synthesis. The drop in aspartate and NAD^+ concentrations in HB cells treated with CQ indicates that limited aspartate availability may be a consequence of ETC malfunction. Next, we assessed the changes in gene expression caused by CQ treatment utilizing an RT^2 Profiler Cell Death Pathway Finder array. 16/84 genes were found to be statistically significantly differentially expressed in CQ treated cells compared to control cells. Poly(ADP-Ribose) Polymerases (PARPs) are multifunctional enzymes activated by DNA damage, which facilitate DNA repair. In aggressive HB, PARP1 is shown to be aberrantly activated, promoting expression of nonfunctional tumor suppressor proteins. Our gene expression analyses showed a significant downregulation of both PARP1 and PARP2 in HB cells after CQ treatment. As the catalytic activities of PARP1 and PARP2 are NAD^+ dependent, we suggest that the reduced NAD^+ pools caused by CQ trigger degradation of PARPs.

Our results suggest that CQ has potential as a novel treatment modality for aggressive HB, which acts through disturbing the NAD^+ and aspartate metabolism exposing cells to impaired DNA repair and histone remodeling by PARPs (main findings summarized in Fig. 6). Thus, our study sets the basis for further investigations in HB and offers novel potential applications for CQ re-purposing strategies.

4.6. New treatment options

Although the life expectancy of HB patients has become quite favorable with current treatments, approximately 20–30% of cases do not respond to treatment. The five-year survival rate for high-risk patients is only 60%. Additionally, in cases where the tumor is extensively spread, liver-sparing surgery is not feasible. New safer, more effective, and targeted treatment options are urgently needed, especially for high-risk HB patients.

In recent years, alongside conventional drugs, targeted therapies have emerged for cancer treatment. These therapies are based on the specific cellular structure and abnormalities of certain tumor types. Molecules present in HB, such as sonic hedgehog, isocitrate dehydrogenase 1, and various tyrosine kinases, already have existing targeted drugs. Several clinical trials based on these target molecules are ongoing, and the results of these studies are expected to contribute to the future of HB treatment (https://clinicaltrials.gov). We believe that also our recent findings outlined above may offer novel putative targets for future HB therapy. Such new potential targets, however, still await testing of their efficacy in HB in *in vivo* settings.

Personalized therapy is becoming more common in cancer treatment. This involves tailoring treatment based on the patient's individual genetic and molecular information. By sequencing mutations in DNA and RNA from the tumor tissue, suitable drug targets are identified. In the ongoing NCI-MATCH study, patients are recruited into different arms of the study based on mutations found in their tumors and the corresponding targeted drugs (Flaherty et al., 2020). HB is involved in thirteen clinical trials testing targeted therapies. A newly published study utilized computational methods trained on pan-cancer datasets to predict drug sensitivity from a tumor's transcriptome. In this study, the efficacy of drugs in HB patients with the aggressive subtype and poor clinical outcome were computationally screened starting from their transcriptome. The results revealed two CDK9 inhibitors, alvocidib and dinaciclib, as potent novel drugs for HB treatment (Failli et al., 2023).

Functional drug sensitivity testing can also customize patient treatment by investigating how cancer cells respond to drugs in the laboratory. Cells are exposed to a panel containing approved and experimental drugs, and the results are combined with the patient's other molecular and clinical information to identify potentially effective drugs. Preclinical studies related to HB are ongoing, uncovering new potential drugs through drug sensitivity testing (Nousiainen et al., 2022).

As for immunological treatments, CAR-T cell therapy is a notable example. In this treatment, the patient's T cells are isolated and genetically modified to better recognize and destroy cancer cells. While CAR-T cell therapy has mainly been used for leukemia and

lymphoma treatment, it has been developed to treat solid tumors as well (Zhang et al., 2022). Several clinical trials are ongoing to test this therapy in the treatment of refractory or recurrent solid cancers in children and young adults.

Despite the active development of targeted therapies, these treatments are not yet a part of HB treatment protocol studies. Due to the significant internal heterogeneity of these tumors, the use of targeted drug therapies as single-agent treatments will likely be limited in the future, and these treatments will probably be used in combination with other drug therapies.

Author contributions

Marjut Pihlajoki: Conceptualization, Writing - original draft, Writing - Review & Editing, Visualization, Project administration; Katja Eloranta: Conceptualization, Writing - Review & Editing; Ruth Nousiainen: Writing - Review & Editing; Ville Väyrynen: Writing - Review & Editing; Tea Soini: Writing - Review & Editing; Antti Kyrönlahti: Writing - Review & Editing; Seppo Parkkila: Writing - Review & Editing; Jukka Kanerva: Writing - Review & Editing; David B Wilson: Writing - Review & Editing; Mikko P. Pakarinen: Writing - Review & Editing; Markku Heikinheimo: Conceptualization, Writing - Review & Editing, Funding acquisition.

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Declaration of competing interest

None.

Data availability

No data was used for the research described in the article.

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