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SUMMARY

Hepatocellular carcinoma (HCC) is a primary cancer of the liver, and HCC patients have an average survival of only 5% at 5-year post-diagnosis. This low survival has several identified causes, among which multidrug resistance i.e. resistance to chemotherapeutic treatment. These issues need be addressed in order to improve HCC management in the future. In this thesis we questioned the role of ABC transporters in HCC, and aimed at developing RNAi-based strategies to compensate their dysregulation. Two strategies were developed to modulate ABC transporter gene expression. The first one exploited endogenous regulation of ABC transporter genes by cellular miRNAs while the second strategy made use of shRNAs and artificial miRNAs for ABCC1 and ABCC2 knock-down. In conclusion, this research contributed to the development of two RNAi-based strategies to compensate ABC transporter gene dysregulation in HCC. At this moment the RNAi field faces toxicity-related and unanticipated off-targeting challenges that need to be overcome in order to take the technology forward, but once these limitations are fully apprehended, it will undoubtedly lead to significant advances for HCC diagnostics and therapeutics.

RNAi-BASED GENE THERAPY OF HEPATOCELLULAR CARCINOMA: TARGETING ABC TRANSPORTERS

Florie Borel

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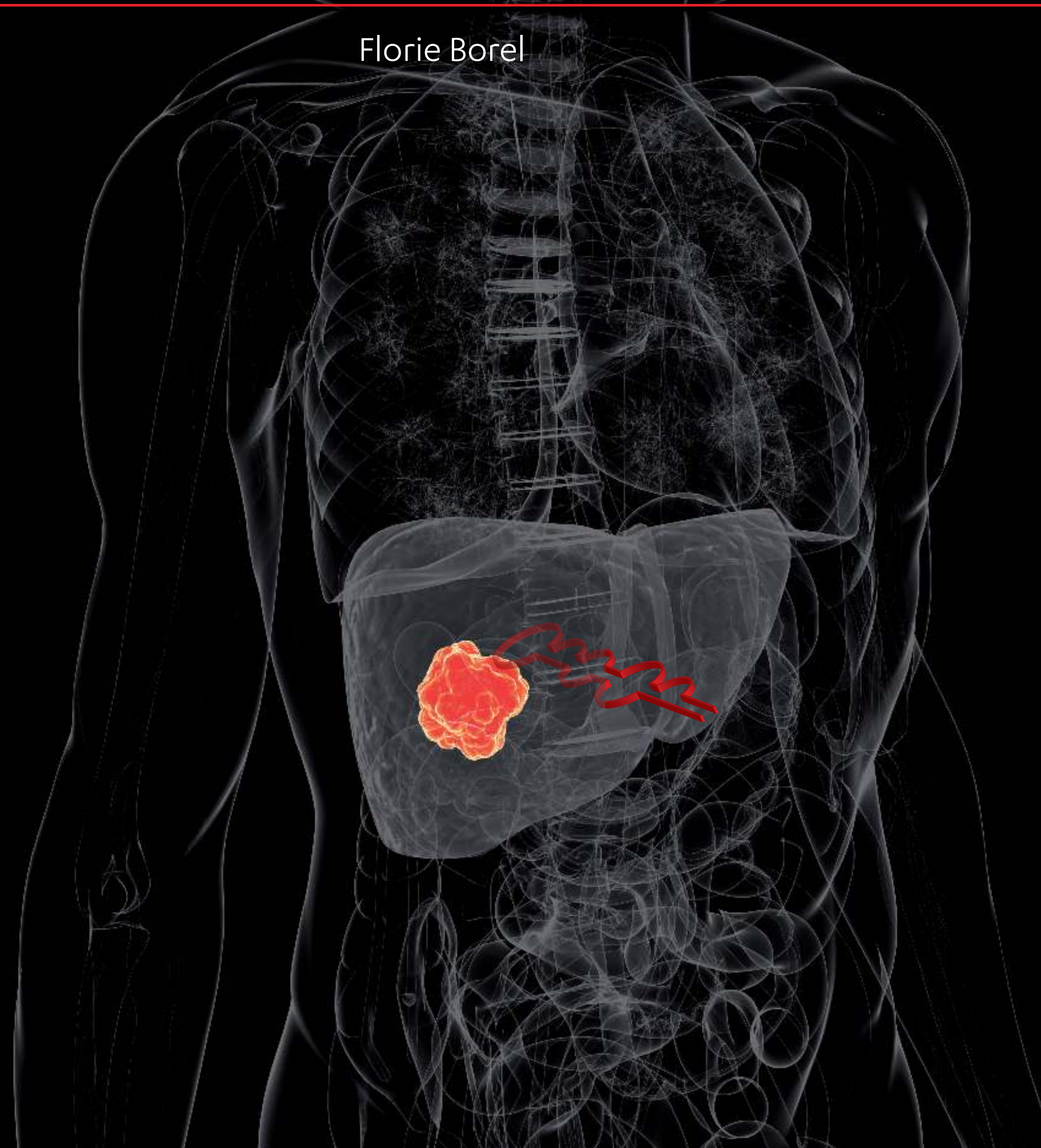

RNAI-BASED GENE THERAPY OF HEPATOCELLULAR CARCINOMA: TARGETING ABC TRANSPORTERS

Florie Borel

PhD thesis, University of Amsterdam, June 2012

RNAi-based gene therapy of hepatocellular carcinoma: targeting ABC transporters

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RNAI-BASED GENE THERAPY OF HEPATOCELLULAR CARCINOMA: TARGETING ABC TRANSPORTERS

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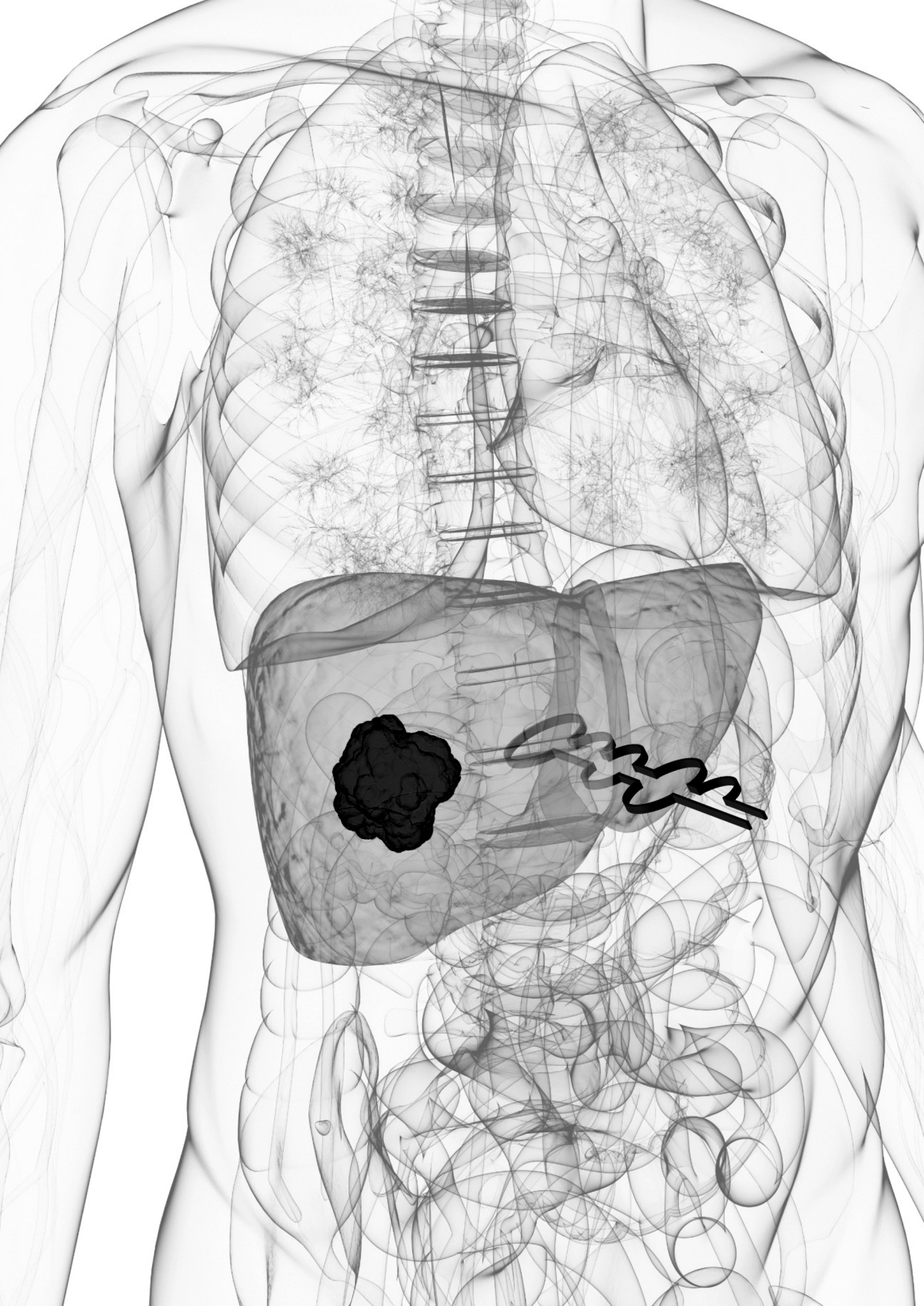

INTRODUCTION

List of abbreviations

HCC,	hepatocellular carcinoma
HBV,	hepatitis B virus
HCV,	hepatitis C virus
AFP,	alpha-feto protein
BCLC,	Barcelona Clinic Liver Cancer
TACE,	transarterial chemoembolization
ABC,	adenosine triphosphate binding cassette
RNA,	ribonucleic acid
RNAi,	RNA interference
miRNA,	microRNA
siRNA,	small interfering RNA
shRNA,	short-hairpin RNA
mRNA,	messenger RNA
3'UTR,	3' untranslated region
AAV,	adeno-associated virus
NHP,	nonhuman primate
AAT,	alpha 1-antitrypsin
ss,	single-stranded
ds,	double-stranded

Hepatocellular carcinoma (HCC) is a primary cancer of the liver, and HCC patients have an average survival of only 5% at 5-year post-diagnosis. This low survival has several identified causes, which should be addressed in order to improve HCC management in the future. One reason is multidrug resistance, in other words resistance to chemotherapeutic treatment. This is caused by overexpression of a family of drug transporters called ATP-binding cassette (ABC) transporters. Multidrug resistance has been considered to be induced by chemotherapeutic treatment, which would select a chemoresistant sub-population of tumor cells. ABC transporters overexpression, the regulation of their expression by cellular microRNAs (miRNAs) and their down-regulation in a therapeutic perspective, and the therapeutic and diagnostic potential of miRNAs have been addressed in this thesis. First, we describe that ABC transporters are up-regulated in HCC patient samples compared to the non-tumoral liver from the same patient. Subsequently, cellular miRNAs were profiled and we showed their down-regulation in HCC, which indicated a possible regulation mechanism, since miRNAs are post-transcriptional negative regulators implicated in many cell processes. Further verification of the hypothesis of miRNA regulation of ABC transporters expression revealed novel interaction mechanisms. Increasing cellular miRNA levels in HCC to a "healthy" physiological state may therefore constitute a first therapeutic approach to decrease the expression of ABC transporters. In a second therapeutic approach, the expression of ABC transporters may be directly down-regulated by short-hairpin RNAs (shRNAs). An adeno-associated virus (AAV) vector was used to deliver shRNA constructs targeting ABCC1 and ABCC2 genes in vivo. However, RNAi can be toxic via several mechanisms, among which the oversaturation of the RNAi machinery by an overload of exogenous RNAi effectors. Such toxicity was encountered during the in vivo testing of the shRNAs, and to counteract this shRNA-related toxicity, artificial miRNA constructs were developed, allowing expression from a weaker, liver-specific polII promoter. Finally, another reason for low survival is the late diagnosis of HCC, because of the lack of appropriate diagnosis tools. Dysregulation of cellular miRNAs in HCC may be reflected by their abundance in the circulation. To verify this hypothesis, two miRNAs identified in the initial HCC patient samples profiling were quantified in HCC undergoing orthoptic liver transplantation. In conclusion, this thesis addressed several points that are crucial for the future of HCC management: improving the knowledge about multidrug resistance, developing novel therapeutics against multidrug resistance and potentially against HCC itself, and developing novel diagnostics that may allow earlier detection of HCC.

1. HEPATOCELLULAR CARCINOMA

1.1. Etiology of hepatocellular carcinoma

Hepatocellular carcinoma (HCC) is a primary cancer of the liver. An estimated 80-90% of all HCC arise from cirrhotic liver. Major risk factors are chronic viral hepatitis B (HBV) or C (HCV), which account for 80-90% of all HCC worldwide (1), and alcoholic and non-alcoholic steatohepatitis-associated liver cirrhosis. Coexistence of etiologies increases the relative risk of developing HCC, *e.g.* patients with HBV and high aflatoxin B1 exposure - a mycotoxin present in some food products - and patients with HCV and alcohol abuse have more chances to develop HCC than those with HBV or HCV alone (2).

1.2. Diagnosis and surveillance of hepatocellular carcinoma

Serum markers for diagnosing HCC have been developed, among which α-feto protein (AFP), glypican-3 (GP-3) and des-gamma carboxyprothrombin (DCP) (3). However, the conventional biomarker AFP has very limited sensitivity and specificity and in current clinical practice guidelines has been discarded as instrument for either screening or diagnosis of HCC. AFP is used for surveillance, which is expectedly increasing with tumor recurrence. Currently the diagnosis of early HCC is based on radiological imaging and needle-biopsy, although national clinical guidelines are variable, *e.g.* this procedure is not encouraged. Radiological imaging has limited sensitivity and specificity and needle-biopsy is invasive and carries a risk of seeding tumor cells along the needle track. Thus, the development of other diagnostic markers for early HCC would be of interest.

1.3. Stages of hepatocellular carcinoma

The Barcelona Clinic Liver Cancer (BCLC) stage, which is an internationally recognized treatment allocation system, distinguishes 5 main stages in HCC (4). Very early stage HCC (0) includes HCC with a single nodule smaller than 2 cm in diameter. Early stage HCC (A) includes HCC with a maximum of 3 nodules whose diameters are smaller than 3 cm. Intermediate stage HCC (B) includes multinodular HCC without portal invasion. Advanced stage HCC (C) includes multinodular HCC with portal invasion. Finally, end stage HCC is defined as D.

1.4. Treatment of hepatocellular carcinoma

Liver transplantation is in principle the best therapeutic option with only ~20% recurrence (5), five-year survival is 70% (6). Resection is usually recommended for patients with not more than mild underlying liver disease (Child A), and five-year survival is 50% (6). Unresectable small HCCs are best treated by percutaneaous intervention, by either chemically or thermally ablating the tumor. When percutaneaous intervention is not efficient, a common treatment option is transarterial chemoembolization (TACE) using doxorubicin. For the last two treatment options, survival depends on the stage of liver disease. Finally sorafenib, an oral multikinase inhibitor, is indicated for advanced HCC patients for whom locoregional intervention and surgery are unsuitable or had been unsuccessful. Sorafenib is the first molecule which showed improved survival in a clinical trial (SHARP) in advanced HCC patients: increased survival from 7.9 to 10.7 months (7).

When using the BCLC stages, globally stages 0 and A represent 30% of the entire HCC patient population, and for these patients curative treatments are available (resection, transplantation, radiofrequency ablation / percutaneaous ethanol injection), giving a 5-year survival of 40-70%. Stages B and C represent 50% of the entire HCC patient population and the median survival is 11-20 months following (transarterial chemoembolization, sorafenib). Finally stage D represent 20% of the entire HCC patient population, and these patients have an expected survival of less than 3 months under symptomatic treatment.

With a 5-year survival of less than 5% (8) HCC remains one of the most deadly cancers, despite all the efforts made in disease management, and few treatments have proven their effectiveness. Major pitfalls are late diagnosis, tumor recurrence and metastasis, and multidrug resistance. This thesis approaches the need for new diagnostics, and focuses mainly on multidrug resistance.

2. MULTIDRUG RESISTANCE

2.1. Multidrug resistance and ABC transporters

The term multidrug resistance, or multiple drug resistance, designates a simultaneous resistance to several drugs. Multidrug resistance is frequently encountered in the clinic, *e.g.* in multidrug resistant microorganisms, but also in cancer and in epilepsy (9). In HCC, multidrug resistance is a major issue as it can compromise chemotherapy effectiveness, which is the major treatment received by intermediate and advanced stage patients, *i.e.* about 50% of all HCC patients. Multidrug resistance is mediated by so-called multidrug resistance transporters, which are drug efflux pumps. Multidrug resistance proteins belong to the adenosine triphosphate (ATP)-binding cassette (ABC) transporters family, which includes 48 transporters categorized into seven sub-families ranging from A to G. The most well-described ABC transporters are ABCB1 (ABC sub-family B member 1), also known as multidrug resistance protein 1 (MDR1) or P-glycoprotein (P-gp) (10, 11); ABCC1 (ABC sub-family C member 1), also known as multidrug resistance associated protein 1 (12), and ABCG2 (ABC sub-family G member 2), also known as breast cancer resistant protein (BCRP) (13, 14). ABC transporters include a nucleotide-binding domain and a transmembrane domain. The typical full ABC transporter includes two nucleotide-binding domains and two transmembrane domains (**Fig. 1**), but some ABC half-transporters include only one of each domain. Many knock-out mouse models for multidrug resistance proteins have been developed and are viable; however they are particularly sensitive to certain drugs (15).

The work presented here focused particularly on 7 out of the 48 ABC transporters, which will be described in more details. These transporters are ABCC1, ABCC2, ABCC4, ABCC5, ABCC10, ABCA1 and ABCE1.

ABCC1 is expressed at low levels in normal liver, and at high levels in most other tissues including colon, kidney, lung, heart, and muscle (RefExA, http://www.lsbm.org/site_e/

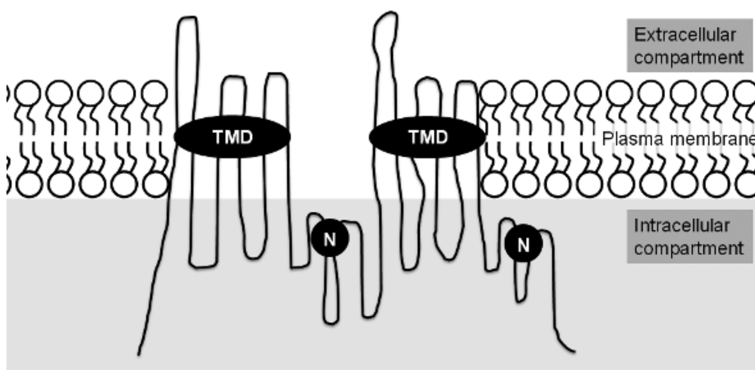

Figure 1: ABC transporters structure and drug efflux mechanism. The classical full multidrug resistance transporter consists of two transmembrane domains (TMD) and two nucleotide-binding domains (N). The structure described here is based on ABCB1 (MDR1). ATP will bind to the nucleotide-binding domain and be hydrolyzed, hence inducing conformational changes in the TMD and causing translocation of the substrate across the plasma membrane.

database/). ABCC1 is also expressed in tumor cells (16). In polarized cells ABCC1 is located on the basolateral membrane where it mediates the excretion of endogenous and xenobiotics compounds into the circulation. *Abcc1*$^{-/-}$ mice have normal viability and fertility, but were found to have increased sensitivity to anti-cancer drugs (17, 18). Interestingly, ABCC1 was shown to mediate *in vitro* resistance to doxorubicin (19), which is the major drug used nowadays in the treatment of HCC. Additionally, ABCC1 expression was reported to be higher in HCC and to be associated with a more aggressive HCC phenotype (20). Taken together, these facts indicate that ABCC1 could play a critical role in HCC and in multidrug resistance, making of *ABCC1* a relevant target for therapeutic development.
ABCC2 is expressed at high levels in normal liver, in intestine, and kidney (RefExA). ABCC2 expression has also been reported in human tumors (21). In polarized cells ABCC2 is present on the apical membrane where it mediates excretion into bile, urine and feces of endogenous and xenobiotic compounds, including conjugated bilirubin. Indeed patients with Dubin-Johnson syndrome, TR^{-} rats (22) and Eisai hyperbilirubinemic rats (23) where ABCC2 is not functional, present hyperbilirubinemia caused by defective export of bilirubin into the bile. *Abcc2*$^{-/-}$ mice have normal viability and fertility, however they present a 20-25% increase in liver weight compared to wild-type mice (24). Murine Abcc2 has been shown to transport various anti-cancer drugs *in vitro* including doxorubicin (24), and therefore *ABCC2* is a relevant target in the context of multidrug resistance in HCC.
Other ABCC transporters are less characterized, but since most of them are able to transport clinically relevant drugs, they may be desirable targets in HCC. ABCC4 is highly expressed in prostate and adipose, and at low levels in the liver (RefExA database). It transports methotrexate (25). ABCC5 is expressed at high levels in stomach and in fetal liver, and at low levels in adult liver (RefExA). It mediates transport of methotrexate (26), 6-mercaptopurine (6-MP) and thioguanine (27). ABCC10 distribution pattern is broad with highest expression in testes, bladder, kidney and ovary, and *Abcc10*$^{-/-}$ mice present increased sensitivity to paclitaxel (28).
ABC transporters are not all multidrug resistance transporters. ABCA1 is expressed ubiquitously, and at high levels in lung, colon, liver and fetal liver (RefExA). It is a cholesterol transporter which makes it interesting in the context of the liver. Mutated ABCA1 is responsible for Tangier disease, a rare autosomal recessive disease. Patients affected by this condition present various symptoms consequent to the accumulation of cholesterol in tissues, among which a mild hypertriglyceridemia, neuropathy, atherosclerosis, and cardiovascular diseases. ABCE1 is highly expressed in pancreas, skin, prostate and ovary and its expression in the liver is lower (RefExA). Though it does not have a transport activity but is a RNase L inhibitor, it has been previously associated with HCC (29) and its silencing inhibited the proliferation and invasiveness of small cell lung cancer cell line (30) highlighting its possible relevance as a target for cancer.

2.2. Current clinical trials on multidrug resistance

Reducing multidrug resistance is a goal pursued by many academic and industrial research groups, as it would have a great clinical impact. Desired effects include augmenting drug bioavailability, improving cancer chemotherapy and treatment of refractory epilepsy, and increase drug delivery to the brain through the blood-brain barrier in the case of brain metastases. In that perspective many ABCB1 inhibitors have been developed throughout 30 years of research and have shown very promising results *in vitro*, but most clinical trials

ended up in disappointment. Initially, first-generation inhibitors (verapamil, identified in 1981; cyclosporin A, in 1986) demonstrated high toxicity at physiologically relevant doses in clinical trials, because their affinity for ABCB1 was poor and high doses had to be used to efficiently compete with the active drug. Despite the development of second-generation inhibitors valspodar (PSC833; Novartis, Basel, Switzerland) (31) and biricodar (VX-710; Vertex Pharmaceuticals, Cambridge, MA), which presented a higher affinity for ABCB1, clinical trials were still inconclusive regarding the clinical benefit, as some toxicity was still observed. This toxicity issue might be solved with the development of third-generation, non-competitive inhibitors, which are more ABCB1-specific and generally do not affect drug metabolism or other transporters. This third-generation includes elacridar (GF120918; GSK, London, Great Britain) (32), zosuquidar (LY335979; Kanisa Pharmaceuticals, San Diego, CA), laniquidar (R101933, NCT00028873), CBT-1 (CBA Pharma, Lexington, KY). To our knowledge these ABCB1 inhibitors have demonstrated satisfying results to date and are progressing through clinical trials.

A few ABCB1 inhibitors demonstrated clinical benefit when combined with chemotherapy. However, inhibitors are only temporarily masking the multidrug resistance phenotype. Decreasing ABC transporters expression via RNA interference therefore appears a desirable alternative to directly inhibiting ABC transporters activity as it could potentially cause a long-term reversal of the multidrug resistance phenotype, which is one of the approaches followed in this thesis.

3. RNA INTERFERENCE

3.1. Discovery of RNAi and its applicability in biomedical sciences

The phenomenon of RNA interference (RNAi) was first described in April 1990 when two independent groups of Dutch and American plant scientists simultaneously published their observations in the journal Plant Cell. They both introduced the chalcone synthase gene in *Petunia*, initially with the goal to increase flower pigmentation. However they found out that the plants expressing the transgene actually presented a partial or total absence of floral pigmentation (33, 34). They reported that this phenotype could be caused by introduction of either genomic or coding sequences, and was mediated by a reduction in chalcone synthase mRNA level. This mechanism in plants was named co-suppression, thought it appeared later that high level of transcripts were processed by a RNA-dependant RNA polymerase into double-stranded RNA. RNAi is the silencing of gene expression, triggered by double-stranded RNA homologous to a part of the target gene. It will in most cases lead to degradation of the mRNA (35). The fact that RNAi is triggered by double-stranded RNA was identified in *C. elegans* in 1998 by Fire and Mello (36), work for which both received the Nobel Prize of Medicine in 2006. A few years after this work in worms, RNAi was identified in mammalian cells by Tuschl (37). Since then RNAi has generated increasing interest and publications in diverse research areas: from its natural role against viral infections in plants (38) to some interesting attempts at engineering novel plant-derived food products such as genetically-modified wheat for gluten-intolerant individuals (39), allergen-free peanut (40) or naturally decaffeinated coffee (41); and mainly in human health from gene function studies to its use as novel therapeutics (42). Some of these products are already in clinical studies; possible indications include various infections, *e.g.* human immunodeficiency virus (HIV) infection, initiated by City of Hope Medical Center (Duarte, CA)/Benitec (Sydney,

Australia) (43), hepatitis C virus infection, sponsored by Santaris Pharma (Copenhagen, Denmark) (44), and respiratory syncytial virus infection sponsored by Alnylam Pharma (Cambridge, MA) (45); but also against various diseases, *e.g.* hypercholesterolemia initiated by Tekmira Pharmaceuticals (Burnaby, Canada; NCT00927459), kidney disorders sponsored by Quark Pharmaceuticals (Fremont, CA; NCT00802347), glaucoma sponsored by Sylentis (Madrid, Spain; NCT01227291), macular degeneration initiated by Opko Health (Miami, FL; NCT00306904), Quark Pharmaceuticals (NCT01445899) and Sirna (San Francisco, CA; NCT00363714); and cancer, *e.g.* liver tumors sponsored by Alnylam Pharma (NCT01158079), leukemia sponsored by Santaris Pharma (NCT00285103), melanoma initiated by Duke University (Durham, NC; NCT00672542). It should be noted that in most of these clinical studies, synthetic RNAi inhibitors are used, except in the study for HIV infection initiated by City of Hope Medical Center which uses lentivirus and Benitec which uses adeno-associated virus. Many academic and industrial research groups see novel therapeutic promises in RNAi and its key players, microRNAs (miRNAs).

3.2. miRNAs, biogenesis, mechanism of action and circulating miRNAs

This topic has been discussed in the review presented in **Chapter 2**. Briefly, miRNAs are short, endogenous, non-coding RNAs. Following their cellular biogenesis, cellular miRNAs can be released into the circulation (circulating miRNAs) where they remain highly stable. Circulating miRNAs are currently being investigated as potential biomarkers.

3.3. siRNAs, shRNAs, artificial miRNAs, and applications for liver diseases

Small interfering RNAs (siRNAs) can be obtained by different manners. Several artificially engineered structures make use of the natural miRNA mechanism to generate siRNAs and are used for therapeutic applications. siRNAs (**Fig. 2A**) are double-stranded RNA of ~21 nucleotides. Short-hairpin RNAs (shRNAs, **Fig. 2B**) are double-stranded RNA with a perfectly complementary stem-loop structure. Artificial miRNAs (**Fig. 2C**) are similar double-stranded RNA with an imperfectly complementary stem-loop structure and additional single-stranded flanking regions. In this thesis shRNAs and artificial miRNAs have been used.

After processing, both shRNAs and miRNAs will produce siRNAs that can be designed to be perfectly complementary to the target mRNA, hence causing its cleavage and degradation (mechanism described in **Fig. 1**, **Chapter 2**). The RNAi approach is relevant for diseases caused by overexpression of one specific mRNA. An example of such disease is hypercholesterolemia, for which knock-down of ApoB could be therapeutic (46, 47). It can also be used against RNA-virus infections like HBV and HCV (48). Other possible therapeutic applications have been recently reviewed by Davidson and McCray (49). RNAi-based therapeutics open the door to new therapeutic areas which are not reachable via more traditional strategies, either regular drugs or classical replacement gene therapy strategies.

3.4. Toxicity issues related to RNAi therapeutics and possible counteractions

As appealing as RNAi-based therapeutics seem, toxicity is an issue and one should be conscious that RNAi presents some inherent risks that cannot always be anticipated and require extensive preliminary investigation. A first source of toxicity is off-targeting, *i.e.* the fact that an exogenous sequence will, in addition to its designated target, down-regulate mRNA(s) containing a partial, miRNA-like homology to the si/miRNA in their 3'UTR (50). The extend of the off-targeting can be reduced by using the lowest siRNA dose that produces

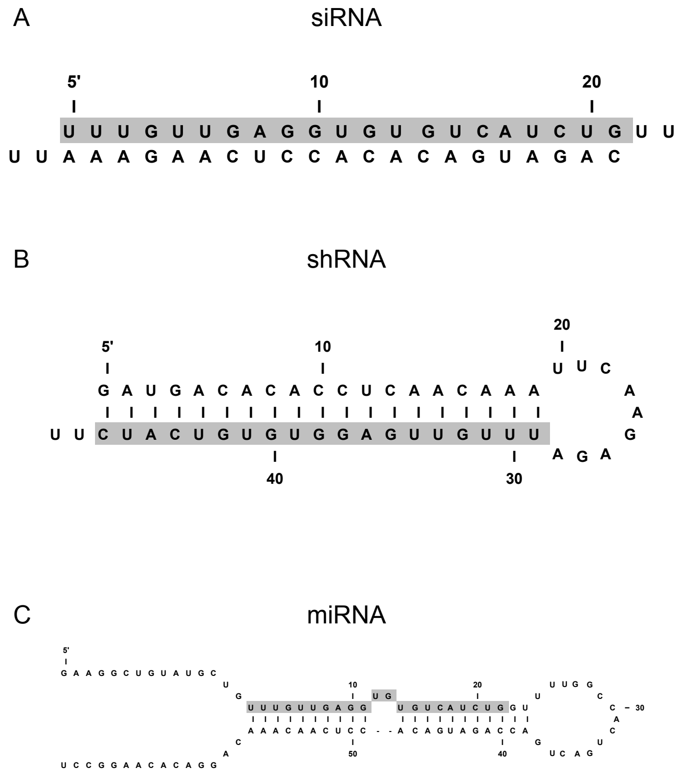

Figure 2: RNAi-inducing molecules. siRNA (A), shRNA (B), and artificial miRNA (C) can be designed to target and induce cleavage of a specific mRNA based on sequence homology. Guide strand is highlighted in grey.

maximal target silencing, but cannot be completely eliminated (51). Alternatively, using a pool of siRNAs against the same target, but having different sequences and therefore off-targets, can also contribute to a relative decrease of the off-targeting (52). Finally, a chemical modification of the siRNA - a 2'-*O*-methyl ribosyl substitution at position 2 in the guide strand - can also reduce ~80% of the off-targeting (53). However it should be noted that because of the differences in gene sequences between species, off-targeting is species-specific (54) which makes it hard to evaluate in preclinical studies. A second source of toxicity is via the activation of the interferon (IFN) response. Mammalian cells have Toll-like receptors (TLRs) which recognize pathogen-activated molecular patterns (PAMP) and activate immune cells. TLR3 can recognize siRNAs in a sequence-independent manner (55), TLR7 and TLR8 in a sequence-dependant manner, and respond by inducing IFN, TNFα and IL-6 (56, 57). Various chemical modifications of the siRNA, and selection of siRNAs without GU-rich motifs may be a way to avoid immune stimulation, though not all pro-inflammatory sequences have been identified (57-59). Such strategies, by making use of the endogenous miRNA pathway, can also create some competition between endogenous and exogenous si/miRNAs. Indeed, a high expression level of the exogenous si/miRNAs can possibly saturate the machinery, reduce

processing of the endogenous miRNAs, affect downstream regulations, and eventually lead to undesirable side-effects and in some cases to death, as it was showed in mice by Grimm *et al.* following delivery of a high dose of AAV-shRNA (60). Therefore the major drawback of RNAi-based gene therapy is the perspective of toxic side-effects caused by oversaturation of the RNAi machinery. Saturation of the RNAi machinery can be avoided by controlling tightly the siRNA levels, for instance by using tissue-specific polII promoters (61).

3.5. Therapeutic delivery of RNAi molecules

A major question raised by RNAi therapeutics is the delivery of the effector molecule, which is ideally controllable, sustained and tissue-specific. The first option is non-virally delivered synthetic molecules. siRNAs, miRNA mimics or miRNA antagomirs can be repeatedly delivered locally or systemically with the assumption that transient suppression of target gene expression is achieved. Possible synthetic molecules have been reviewed in **Chapter 2**. Long-lasting effect with synthetic molecules can only be achieved via frequently repeated administration, which is costly and not always suitable depending on the route of administration. The second option is virally-delivered RNAi triggers, which is what has been used in this thesis. Such approach is desirable when long-lasting effect is desired, for instance in the treatment of chronic and genetic diseases. Viral delivery indeed can offer sustained expression after single dosing. In a clinical perspective, the viral delivery approach raises more questions concerning safety than the non-viral, because it adds one parameter to control for. shRNAs and pri-miRNA can be delivered as an expression cassette using different types of viral vectors, which will be reviewed in more details under "Gene therapy vectors".

3.6. Current clinical studies based on RNAi in the liver and for liver cancer

HCV is a major target in the field of RNAi therapeutics. Cellular miR-122 stimulating HCV RNA accumulation and a preclinical study in nonhuman primates (NHPs) showed that inhibition of miR-122 leads to an efficient inhibition of HCV replication and stable reduction of viremia (62). In this study Landford *et al.* inhibited miR-122 expression in 4 chimpanzees using SPC3649 LNA-modified oligonucleotides (Miravirsen from Santaris Pharma). Following this study in NHPs, a phase I study for SPC3649 showed that it was well-tolerated and the drug is now in a phase II study. In this study, a robust dose-dependent anti-HCV activity is reported with a 3-log mean reduction of HCV RNA from baseline and a viral load below detection in 4 out of the 9 patients treated with the highest dose of 7mg/kg (Santaris Pharma, presentation The Liver Meeting 2011, San Francisco, CA). This approach holds promise for HCV patients, and because it targets a highly conserved region, it has the advantage that it should not allow the development of viral escape variants. Nevertheless Li *et al.* reported that mutations in miR-122 binding-site in HCV 5' UTR reduced SPC3649 treatment efficacy (63), which suggests that viral escape could still be possible.

In liver cancer patients who have been failed by chemotherapy and radiation, there are to our knowledge only two RNAi-based therapies being currently clinically tested. Developed by Tekmira, TKM-080301 consists of lipid nanoparticles (LPN) encapsulating siRNA targeting the proto-oncogene polo-like kinase 1 (PLK1). The goal of an ongoing phase I clinical study is to establish safety as primary endpoint and has included as secondary endpoint to determine the maximum tolerated dose. Alnylam Pharma ALN-VSP02 uses the Tekmira LPN formulation to deliver two siRNAs targeting respectively kinesin spindle protein (KSP) which is required for tumor proliferation; and vascular endothelial growth

factor (VEGF) which is required for tumor growth. The targets of ALN-VSP02 are solid tumors including those with liver involvement. Promising phase I clinical study showed that ALN-VSP02 was well-tolerated and studies continue at 1.0mg/kg dose (Alnylam Pharma, presentation ASCO 2011, Chicago, IL). These two studies show the overall feasibility and safety of RNAi-based therapies. But it should be noted that these two studies are based on non-viral delivery, which would imply a frequent re-administration of the drug. Such drawback could be counteracted by using viral delivery of the RNAi therapeutics, which would allow long-term treatment, but would raise additional safety concerns.

4. GENE THERAPY

4.1. Short history of gene therapy

Gene therapy encompasses three different ways of manipulating the genetic information of an individual. Firstly, one can introduce a new gene that is beneficial, thereby helping treat a disease. Secondly, to treat recessive diseases, the wild-type allele of a mutated gene can be introduced, in case the mutated protein is faulty or missing. Thirdly, to treat dominant diseases, the mutated gene can be silenced in order to decrease the level and toxic effect of detrimental mutated protein. In the early 90's, the first gene therapy studies treated young patients with severe combined immunodeficiency (SCID) by re-expressing adenosine deaminase (ADA) and induced some partial response (64, 65). But in 1999, a patient enrolled in a gene therapy clinical study dies. The liver of 18 year old Jesse Gelsinger, suffering from ornithine transcarbamylase (OT) deficiency, was infused with adenovirus vector carrying the OT gene. The patient experienced an inflammatory response, followed by multiple organs failure. During the subsequent investigation it appeared that the patient's condition should have excluded him from the study. Nevertheless this death, combined to the development of leukemia by an 11 year old boy who had received gene therapy for SCID (66), marked the memories and rendered the authorities and the public more cautious about gene therapy. However, this promising field of biomedical research is carefully moving forward. In 2003, Gendicine from Shenzen SiBiono GenTech (Shenzen, China) was approved in China for treatment of head and neck cancer. This promising development experienced a set-back when, in 2008, Advexin (adenovirus carrying p53 tumor suppressor gene) sponsored by Introgen (Austin, TX), and in 2010, Cerepro (adenovirus encoding HSV thymidine kinase gene) sponsored by Ark Therapeutics (Kuopio, Finland) received negative opinions from the authorities, for incompleteness and negative risk-benefit profile, respectively. On the positive side, in 2009, an 8 year old boy with Leber's congenital amaurosis recovered normal eyesight following injection AAV encoding retinal pigment epithelium-specific 65kDA protein gene. The 11 other subjects included in the study conducted by University of Pennsylvania (Philadelphia, PA) all showed clinical benefit, though less than the youngest subject (67). Unfortunately, in 2011, Glybera (AAV encoding lipoprotein lipase gene) from Amsterdam Molecular Therapeutics (Amsterdam, The Netherlands) received a negative opinion by the EMA, despite positive opinion from scientific experts on both clinical benefit and safety profile of the drug.

A large majority of current gene therapy studies target cancer diseases (64.6%). Other indications addressed include cardiovascular diseases (8.5%), monogenic diseases (8.3%), infectious diseases (8.1%), gene marking (2.9%), neurological diseases (2%), ocular diseases (1.3%), inflammatory (0.8%), other indications (1.1%), and healthy volunteers (2.3%) (source: Journal of Gene Medicine 2011).

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4.2. Gene therapy vectors

The most common viral vectors used in gene therapy clinical studies according to the Journal of Gene Medicine (2011) include adenovirus, retrovirus, *Vaccinia* and poxvirus, adeno-associated virus (AAV), *Herpes simplex*, and lentivirus. Their advantages and disadvantages are summarized in **Table 1.** Briefly, the main characteristic of lentivirus and retrovirus is their integration into the host genome, which raises several safety concerns. It has been reported that retroviral vectors integrate semi-randomly with different preferences, whereas lentiviral vectors are integrating preferentially in active genes (68). Currently non-integrating lentivirus are being developed (69). On the contrary, AAV genome remains episomal,, which gives it an advantageous safety profile for it would not cause insertional mutagenesis. However the episomal presence of AAV questions its relevance for cancer therapy. In this thesis, AAV vectors have been used as a delivery tool. Less variable transgene expression and increased safety could be the result of using recently developed integrating AAV vectors, that integrate stably and site-specifically (patent US 7122348). Up to now, adenovirus has been widely used in HCC gene therapy clinical studies (70-76), as well as *Vaccinia* virus (77). Despite the fact that many virus-delivered "classical" gene therapy products have been developed for HCC and are currently progressing through clinical study phases, no virus-delivered RNAi-based gene therapy has been tested in clinical studies yet. Indeed, more research still needs to be done to carefully evaluate potential risks

Table 1: Viral vectors used in gene therapy clinical studies. The viral vectors described could be used for delivery of RNAi-based gene therapy.

Viral vector	**Adenovirus**	**Retrovirus**	***Vaccinia* and poxvirus**	**Adeno-associated virus**	**Herpes simplex**	**Lentivirus**
% gene therapy clinical trials	24.2	20.7	13.6	4.7	3.3	2.3
Maximal transgene size (kb)	7.9	8	250	4.7	40	8
Transgene expression	Transient	Long lasting	Transient	Potential long lasting	Potential long lasting	Long lasting
Tropism	Broad	Dividing cells only	Broad. Natural tumor tropism	Broad	Broad	Both dividing and non-dividing cells
Host genome interaction	Non-integrating	Integrating	Non-integrating	Non-integrating	Non-integrating	Integrating
Immunogenicity	High	Low	High	Low	High	Low

Kb, kilobases. Non-viral gene therapy studies include: naked/plasmid DNA (18.7%), lipofection (6.4%), other vector (5.3%), unknown (3.2%).

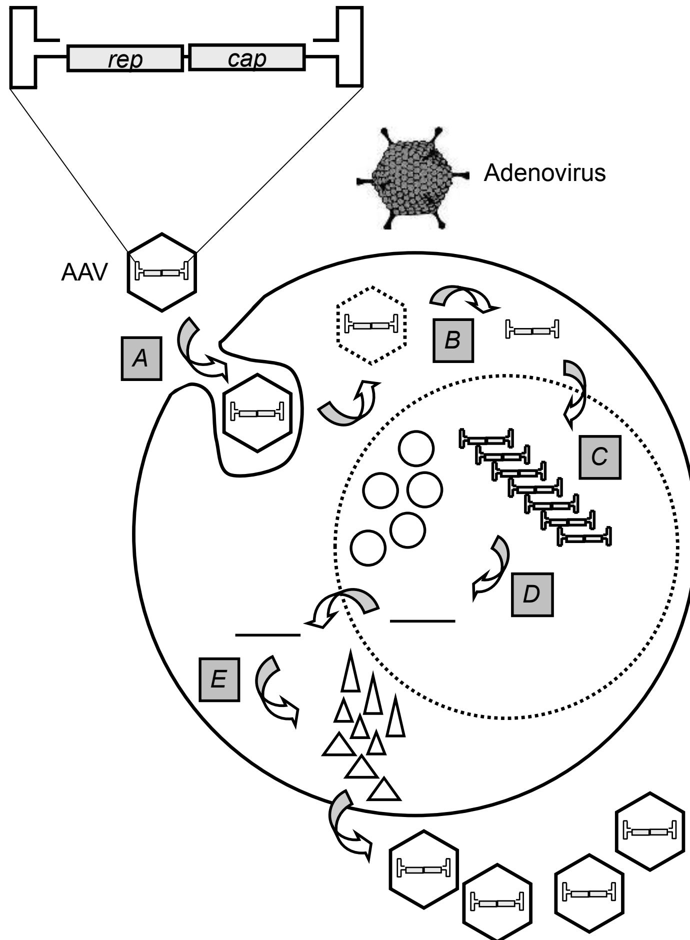

Figure 3: AAV lytic cycle. AAV initially binds to cell-surface receptors which will induce endocytosis (A) after which capsid will be hydrolyzed (B). Subsequently, the ssDNA genome enters the nucleus where the complementary strand will be synthesized, including episomes (C). dsDNA will be transcribed (D), and the transcript will be exported to the cytoplasm where it will be translated (E).

of this approach. As described earlier, shRNAs and miRNAs have become inevitable tools in biomedical science. Here we will focus on AAV-delivered shRNAs/miRNAs for therapy.

4.3. Adeno-associated viral vectors, biology, serotype, tissue tropism and immune response

AAV belongs to the genus *Dependovirus*, family *Parvoviridae*. The viral particles of this non-enveloped, icosahedral capsid virus are about 22nm diameter. AAV has a 4.7kb linear ssDNA genome, which includes *cap* (capsid) and *rep* (replication) genes, respectively encoding via alternative splicing capsid proteins VP1 (87kDA), VP2 (72kDa) and VP3 (62kDa), and Rep proteins Rep40, Rep52, Rep68 and Rep78 (based on their molecular weight). AAV genome

is flanked by two 145nt-long inverted terminal repeats (ITRs) which are multipalindromic sequences able to fold on themselves and form a characteristic T-shape/hairpin structure (78). Upon adenovirus or *Herpes simplex* virus co-infection, AAV genome is replicated, yielding progeny virus (lytic cycle, **Fig. 3**).
AAV presents several advantages over other viral vectors. First of all, AAV naturally infects human and primates - approximately 80% of humans are seropositive for AAV2 - but the virus is currently not known to cause any disease, and it induces a very mild immune response. In the absence of helper virus co-infection (lysogenic cycle), wild-type AAV stably integrates in the genome on specific sites - AAVS1, on human chromosome 19 (C19), AAVS2 on C5, and AAVS3 on C3 - which makes it more predictable and safer than retroviruses. The DNA can later on be excised from the host genome upon helper virus co-infection, metabolic inhibitors or DNA damaging agents (UV irradiation, genotoxic compounds). However, as a gene therapy vector, removal of *cap* and *rep* genes eliminated its integrative abilities at specific loci. Such replication defective AAV is called recombinant AAV (rAAV). DNA remains mainly episomal, in which case it remains present for life of non-dividing cells, while it will be diluted in dividing cells; and an estimated 0.05% can be integrated in chromosomal DNA (79). rAAV can mediate stable long-term expression, as shown by several studies in various animal models (80-82). Another advantage of AAV as a gene therapy vector is that it offers the possibility to target a specific tissue by using a particular AAV serotype. AAV serotypes are defined as the inability of an antibody that is reactive to the viral capsid proteins of one serotype in neutralizing those of another serotype. Currently there are nine AAV serotypes having been described, from AAV1 to AAV9. AAV tropism is variable depending on the capsid serotype used, most commonly AAV2 genome will be packaged by capsids of other AAV serotype (for instance AAV2/1 designates capsids of AAV1 origin). A major limitation that should be noted is that tropism is variable depending on the animal model as well as the injection technique. Nevertheless, up to now some serotypes appear to have a marked superiority when it comes to certain tissues (**Table 2**). The liver is one of the tissues that can be the best targeted by AAV vectors. Serotypes that will preferentially transduce the liver following tail vein injection are AAV1, AAV2, AAV5, AAV7 and AAV9, followed by AAV3, AAV6 and AAV8 (83). Serotypes leading to the strongest expression in the liver are AAV7 and AAV9, followed by AAV1, AAV6 and AAV8 (83). AAV8, which was used in this thesis, is currently considered the best serotype for the liver as it uncoats fast and can therefore have a rapid expression onset (84), it very efficiently transduces hepatocytes in mice (85) and in nonhuman primates (86), and it leads to high transgene expression in mice (87). Besides the choice an AAV serotype, transgene expression can be to some extend restricted by using tissue-specific promoters. To restrict gene expression to the liver, common choices are α 1-antitrypsin (AAT) (88) and LP1 (89) promoters. In addition, a novel way of ensuring tissue-specific transgene expression is by using miRNA tissue de-targeting. By adding to the transgene target sites for a miRNA that is specifically expressed in the tissue to de-target, transgene expression can be locally repressed (90, 91). The major application of this de-targeting technique is to avoid the expression of potentially immunogenic transgenes in cells of hematopoietic lineage and prevent antigen expression in antigen-presenting cells, which can be achieved with the hematopoietic-specific miR-142 (92).
The genome of wild-type AAV is single-stranded DNA (ssDNA), and second-strand synthesis to obtain a dsDNA template for transcription is a rate-limiting step in AAV

Table 2: AAV tropism. Description based on literature of the tropism of the different AAV serotypes.

Serotype	Tissue(s) preferentially transduced
AAV1	Skeletal muscle with high transduction efficiency (Chao, ref 108)
AAV2	Wide tropism (liver with expression onset, muscle, lung, CNS) with moderate transduction efficiency
AAV3	
AAV4	Brain (astrocytes)
AAV5	Murine neurons (Alisky, ref 106)
AAV6	Skeletal muscle (Blankinship, ref 107), liver (quick expression onset)
AAV7	Skeletal muscle with high transduction efficiency (Gao, ref 86)
AAV8	Murine liver with high transduction efficiency and expression onset and level (Gao, ref 86), skeletal muscle, heart, pancreas, smooth muscle, brain
AAV9	Wide tropism in mouse (liver, muscle, lung, CNS) with high transduction efficiency (Gao, ref 86, Pacak, ref 111)
AAV10	Lymphoid tissues of *Cynomolgus* monkey (Mori, ref 110), liver, heart, muscle, lung, kidney, uterus of mouse (Mori, ref 109)
AAV11	Lymphoid tissues of *Cynomolgus* monkey (Mori, ref 110), muscle, kidney, spleen, lung, heart, stomach of mouse (Mori, ref 109)

transgene expression (93). This obstacle can be overcome by using an AAV vector containing a double-stranded transgene. Depending on the AAV production method, such double-stranded AAV (dsAAV) can be called either self-complementary AAV (scAAV) or monomeric duplex AAV (mdAAV). Self-complementary AAV is based on a mutation in the packaging signal (D-sequence) and the terminal resolution site (*trs*) of one of the ITR, which causes the polymerase to continue further and synthesize a second strand, resulting eventually in packaging of dsDNA (94). mdAAV can be obtained in insect cells by using different ratios of rep proteins (patent WO2011122950). dsAAV therefore allows a faster onset of transgene expression, as well as sustained expression at lower vector dose (95). Nevertheless using dsAAV reduces by half the maximal (coding) transgene size, which is a limiting factor for many applications where longer genes need to be expressed.
The main disadvantage of AAV is its small insert capacity (4.7kb) which renders it unsuitable for gene therapy approaches where the transgene is longer, or requires some transgene optimization (96). Nevertheless, this limited packaging capacity, even in the case of scAAV, is not an obstacle when it comes to RNAi-based gene therapy as the transgenes are globally only a few hundreds basepair-long.

4.4. rAAV production

The first main method of rAAV production is based on plasmid DNA transfection in HEK293T cells. Three plasmids are co-transfected: one carrying the transgene flanked by ITRs, one plasmid the *rep* and *cap* genes; and one plasmid with adenovirus genes expressing *E1A*, *E1B*, *E2A*, *E4ORF6* and *virus-associated* (*VA*) RNAs which provide the helper functions

required to induce AAV replication (**Fig. 4, left panel**). This method allows a relatively fast rAAV production which is suitable for research purposes but cannot really be used for large pre-clinical and for clinical applications due to several limitations. Firstly it relies on transfection of high amounts of DNA making it a difficultly scalable method, in addition its incompatibility with the use of suspension culture systems requires important handling and is therefore time-consuming and labor-intensive. Nevertheless this method is currently still marginally used to produce vectors for clinical studies.

The second main method of rAAV production is the baculovirus expression vector system in insect Sf9 cells. Here the transgene and the *rep* and *cap* genes are provided by three separate recombinant baculoviruses (**Fig. 4, right panel**). Upon co-infection of the Sf9 host cells with three baculoviruses, one carrying the transgene, one the *cap* gene and one the *rep* gene, rAAV is produced. Through the use of suspension culture, this method requires less handling and starting material compared to the HEK293T-based production system. The baculovirus-based production system is therefore suitable for large-scale applications. Though the whole process is much longer than the DNA transfection method, this production system allows much faster future productions and reduced handling, making

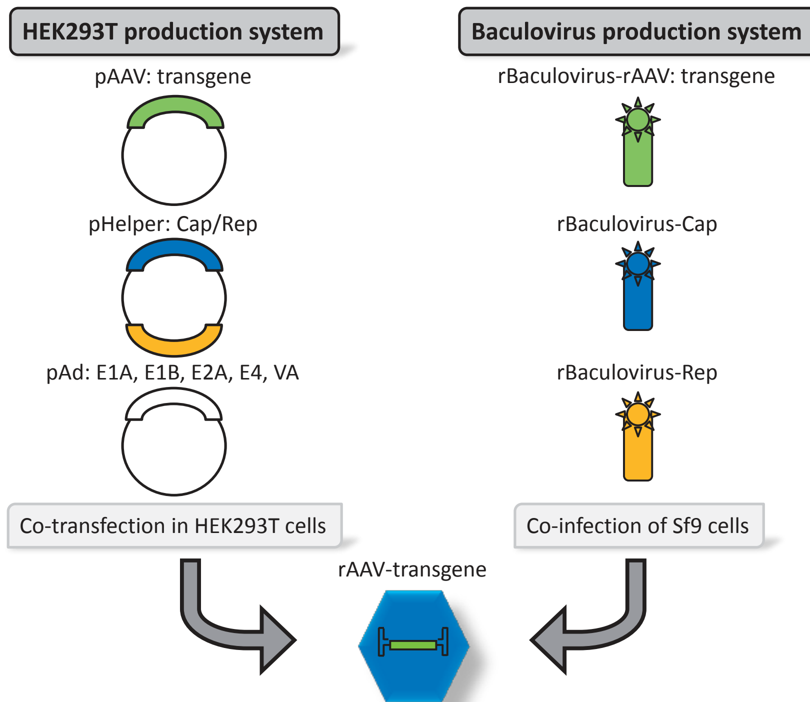

Figure 4: Principle of rAAV production. Production with the HEK293T system relies on co-transfection into HEK293T cells of three plasmids expressing respectively the transgene, the *cap* and *rep* genes, and some adenovirus genes. Production with the baculovirus system relies on co-transfection into Sf9 cells of three recombinant baculoviruses expressing respectively the transgene, the *cap* and the *rep* genes. In both systems, the transgene is replicated, viral capsid proteins are synthesized and assembled, and the transgene is packaged into a capsid.

it scalable for clinical applications, as reviewed in (97). The main disadvantage compared to the DNA transfection method is its increased complexity, making the latter method best suited for final clinical products than for research vector batches.

4.5. AAV-delivered shRNAs/miRNAs in the liver

As described earlier, shRNAs and miRNAs have become inevitable tools in biomedical science. Here we will focus on AAV-delivered shRNAs/miRNAs for therapy of liver diseases. Studies showing promising results will be reviewed hereafter, for indications such as alpha-1 antitrypsin deficiency, familial hypercholesterolemia, viral hepatitis and liver cancer.

Alpha-1 antitrypsin (AAT) deficiency is caused by a mutation in the AAT gene leading to a reduction in the serum levels of antiprotease AAT. This reduction of AAT is eventually leading to susceptibility to emphysema. Traditional gene therapy approaches therefore aim at introducing the wild-type gene in order to re-express the wild-type AAT protein to a known level at which the pulmonary disease would be treated. However it was also shown that mutant α-1 antitrypsin (mAAT) accumulation can lead to the development of a liver disease, where the mAAT polymerization in the endoplasmic reticulum of hepatocytes triggers liver cirrhosis. For the patients affected by this liver disease, down-regulation of mAAT may be a treatment. Following systemic injection of AAV8-shRNA2 targeting mAAT in the mouse model of the disease, Li *et al.* demonstrated 95% knock-down of mAAT mRNA in the liver and 95% reduction in serum protein levels (98). Such dramatic knock-down led to reversal of the liver fibrosis (98).

Apolipoprotein B100 is the structural protein of low-density lipoprotein cholesterol (LDL-C) and is therefore a therapeutic target for familial hypercholesterolemia, for its knock-down would reduce serum LDL-C levels. It was shown that scAAV8-shApoB could reduce ApoB by 95% and as a consequence serum cholesterol levels by 79% in mice (46). However at the highest dose of 1E11 genome copies (gc) per mouse, some toxicity was observed. In a subsequent work, the authors tried to overcome this toxicity by replacing the polIII H1 promoter by the polII LP1 promoter, expressing the shRNA sequence from a miRNA backbone (miApoB). *In vivo*, siRNA expression from miApoB was restricted to the murine liver, in addition miApoB showed an increased potency (47). This study demonstrates the superiority of artificial miRNAs over shRNAs for preclinical and clinical applications.

Treatment of viral hepatitis is a major application of RNAi-based therapy in the liver. The use of dsAAV7, dsAAV8 and dsAAV9 carrying an identical shRNA targeting the HBV genome led to significant decrease in liver HBV mRNA (99). Giering *et al.* engineered a shRNA expression cassette that allows liver-specific expression of shRNAs at low levels, in order to avoid RNAi-related toxicity (61). They used the liver-specific polII ApoE/hAAT promoter to express a shRNA targeting the envelope surface antigen (sAg) of HBV. dsAAV8-mediated delivery of ApoE/hAAT-shRNA to HBV transgenic mice resulted in the decrease of serum sAg by 85% without any sign of toxicity (61). The endogenous miR-17-92 polycistron was next used to generate a polycistronic primary miRNA that is processed into 5 mature artificial miRNAs targeting the HCV genome (100). Upon hydrodynamic tail vein co-injection of scAAV8-HCV-miR-Cluster1 and of the corresponding luciferase reporter carrying the 5 targets, 94% inhibition of the relative luciferase signal was observed (100).

Treatment of liver cancer appears as a major application for RNAi-based liver-directed gene therapy. Kota *et al.* showed that self-complementary AAV serotype 8 (scAAV8)-delivery of miR-26a in tumor-bearing *tet-o-myc/LAP-tTA* mice restored miR-26a expression (101). Re-

expression of miR-26a specifically reduced cancer cell proliferation, induced tumor-specific apoptosis, and suppressed tumorogenesis. At 3 weeks post-transduction most liver tissue in the control group was replaced with tumor, while in 8 out of 10 mice of the treated group no or small tumors only were found (101). This research demonstrated for the first time the therapeutic potential of restoring the expression of a dysregulated miRNA in the liver.

4.6. Current AAV clinical studies in the liver

There are few ongoing clinical studies targeting the liver using AAV. One therapeutic application of AAV gene therapy with the liver as target tissue is alpha anti-trypsin (AAT) deficiency. AAT deficiency is caused by a genetic mutation leading to production of an abnormal AAT protein. In a phase II clinical study sponsored by Applied Genetic Technologies Corp. (Alachua, FL), rAAV1-CB-AAT was administered intramuscularly to three groups of patients at 6.0x10E11, 1.9x10E12, and 6.0x10E12 vector genomes/kg (n=3 subjects/dose). Interim results showed a dose-dependent expression of the transgene that lasted for at least until the interim time point of 90 days (102). However the serum levels of transgene product were only about 30-50% of therapeutic level (102) and improvements would therefore be required in order to increase serum concentrations, for instance by administrating the vector intravenously instead of intramuscularly, using transient immunosuppression or choosing another serotype. Indeed it was shown in mice that AAV8 has a reduced ability to activate immune response (103), in addition AAV8 transduces hepatocytes more efficiently, hence leading to a better induction of tolerance to the transgene product in mice (104).
Thus far, the most advanced studies are those for hemophilia B. Hemophilia B is caused by a deficiency in blood clotting factor IX (FIX, cDNA=2.8kb). Interestingly, reaching FIX plasma concentrations of only 1% of the normal value is therapeutic for patients with severe hemophilia (<0.1%). Two phase I/II clinical studies are currently ongoing, both in adult patients. The first one, sponsored by the Children's Hospital of Philadelphia, delivers ssAAV2 via the hepatic artery (NCT00515710). However it was previously shown in another clinical study that AAV2 capsid is recognized and induces the host immune response (105). This recognition is most likely due to prior exposure to the capsid. As a result, transgene expression hence decreases under therapeutic levels after ~8 weeks (105). To counteract this issue, the protocol of the above mentioned study includes transient immunosuppression. However, as described above, a simple change of serotype might be an easier solution. The other study, which is sponsored by St. Jude Children's Research Hospital and uses intravenously-delivered scAAV8 (NCT00979238), might therefore offer better chances at a longer term expression, possibly without the need for immunosuppression.
Up to now, there are no clinical studies using RNAi-based AAV-mediated gene therapy, and pre-clinical studies are few. Since at this moment there is still no gene therapy product approved by western medicine agencies, adding an RNAi component to the therapeutic strategy is a double challenge. As described earlier, toxicity remains a concern when it comes to RNAi, and this would have to be carefully monitored in future preclinical and clinical studies. It is clear that more work is required to better control the safety profile, but also to fully demonstrate the high potential of this approach.

5. SCOPE OF THIS THESIS

This thesis focused on the role of ABC transporters and multidrug resistance in hepatocellular carcinoma. More specifically we were interested in ABC transporters and cellular miRNAs expression profiles in HCC patients as they could be predictors of disease progression. Regulations of ABC transporters expression by cellular miRNAs in association with HCC was further studies as a ground for RNAi-based AAV-mediated gene therapy for reducing multidrug resistance. Finally, circulating miRNAs associated with HCC in Dutch liver transplantation patients were screened and correlation with presence of HCC was assessed.

Literature on cellular miRNAs and their association with cancer has been increasing in the last years, and miRNAs signatures have now been developed for most cancers. In addition the recent discovery of circulating miRNAs holds great promises for cancer diagnosis and response-to-treatment monitoring. Focusing on HCC, we have reviewed in **Chapter 2** the changes in miRNA expression profile associated with HCC and the use of circulating miRNAs as diagnostic tools, and highlighted the potential of cellular miRNAs as therapeutics for HCC.

Multidrug resistance is one of the major problems in hepatocellular carcinoma management, and it is often viewed as a side-effect of or obstacle to chemotherapy, which triggers up-regulation of drug efflux transporters as a defence mechanism and renders the tumor resistant to chemotherapy. Nevertheless we showed in **Chapter 3** that 12 ABC transporter genes are up-regulated in HCC without prior chemotherapeutic treatment. Subsequent analysis of changes in miRNA expression in the same sample set led us to hypothesize that the up-regulation of several ABC transporters associated with HCC is mediated by cellular miRNAs.

Basing our work on the findings described in Chapter 3, we showed in **Chapter 4** that expression of ABCA1 and ABCC1 transporters, up-regulated in HCC, are regulated by cellular miRNAs. In addition, we show that mimicking miRNA down-regulation in HCC by decreasing miRNA levels *in vitro* leads to altered cholesterol transport by ABCA1.

As mentioned earlier, multidrug resistance is a common issue in HCC management. Nowadays, medicine is tending more and more towards personalized, custom-tailored therapeutics. Therefore when it comes to developing new strategies to overcome clinical multidrug resistance, a possibility appears in exploring RNAi-based gene therapy to down-regulate the expression of one or several specific multidrug resistance transporter genes. In **Chapter 5**, we have developed and tested *in vitro* shRNAs targeting two murine ABC transporters, Abcc1 and Abcc2. The best shRNA candidates were produced in scAAV8 and tested *in vivo* for their knock-down efficacy and safety profile. Finally, we have developed artificial miRNAs based on the sequences of the most efficient shRNAs and we compared side-by-side the shRNAs and the miRNAs activity *in vitro.*

Finally, we close this book with **Chapter 6** by discussing the future developments of RNAi-based gene therapy strategies and the potential for clinical applications against hepatocellular carcinoma.

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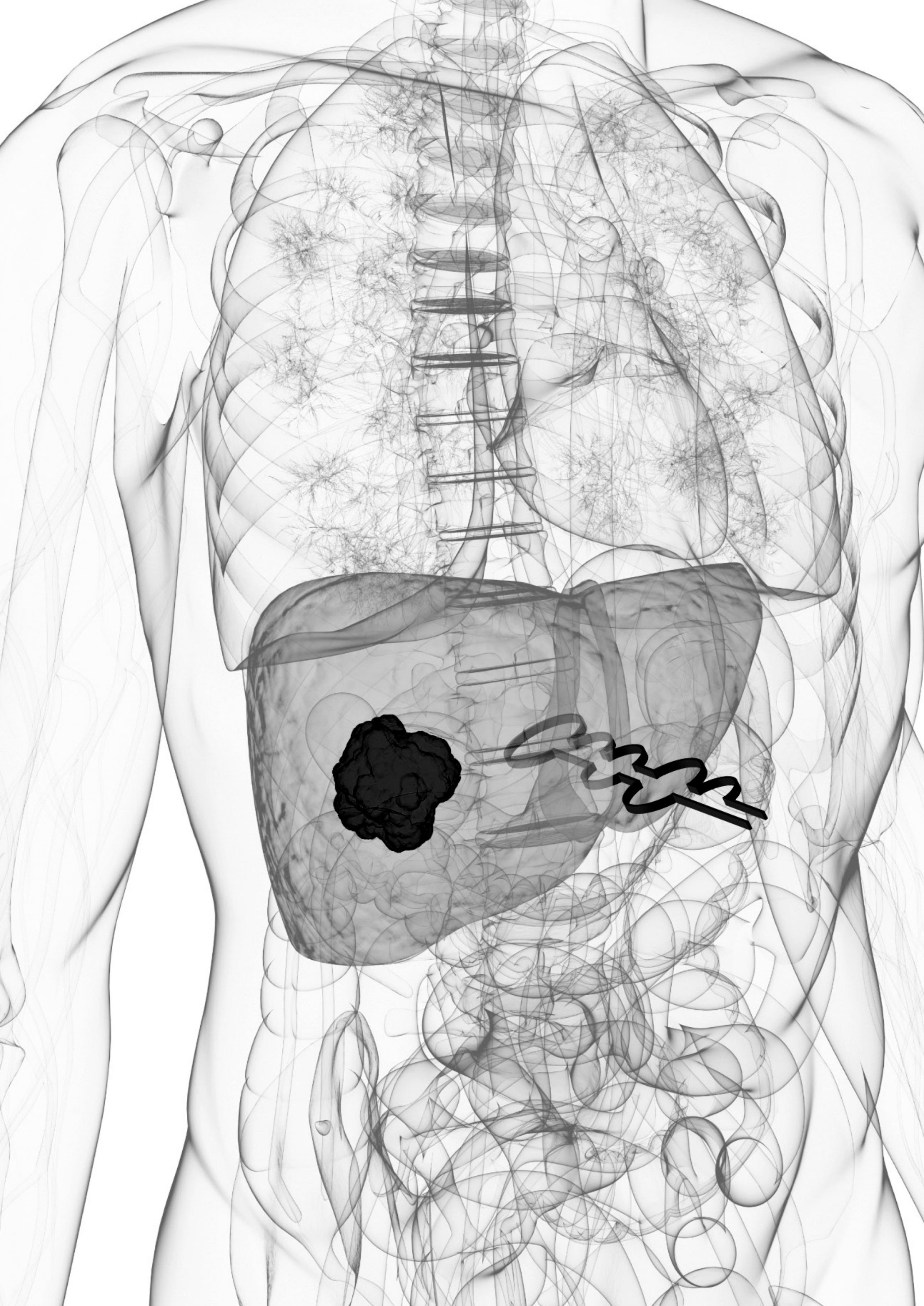

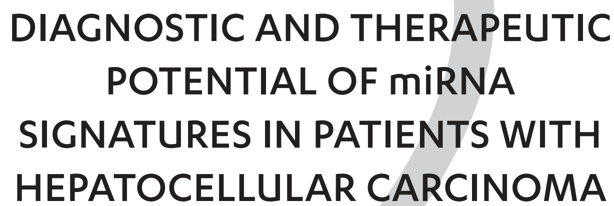

DIAGNOSTIC AND THERAPEUTIC POTENTIAL OF miRNA SIGNATURES IN PATIENTS WITH HEPATOCELLULAR CARCINOMA

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ABSTRACT

MicroRNAs (miRNAs) are evolutionary conserved small non-coding RNAs that regulate gene expression by mediating post-transcriptional silencing of target genes. Since miRNAs are involved in fine-tuning of physiological responses, they have become of interest for diagnosis and therapy of a number of diseases. Moreover, the role of dysregulated miRNAs in maintaining the malignant phenotype has profound implications for cancer therapy. We will review the best defined cellular miRNAs and changes in their expression profile in hepatocellular carcinoma (HCC). Cellular miRNAs can also be released into the circulation, and these miRNAs are detected in most body fluids. Circulating miRNAs are associated with HCC and are possible biomarkers. Finally, by affecting several clinically relevant targets, artificially increasing or decreasing the expression level of a given miRNA offers fascinating therapeutic perspectives. We will therefore highlight recent developments in miRNA-based gene therapy with a focus on their therapeutic potential for HCC.

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Hepatocellular carcinoma (HCC) is the major primary liver cancer, which is the fifth most common cause of cancer worldwide (1), with about 750.000 patients globally reported each year (International Agency for Research on Cancer, IARC). An estimated 80-90% of all HCC arise from cirrhotic liver. Major risk factors are chronic viral hepatitis B (HBV) or C (HCV), which account for 80-90% of all HCC worldwide (2), and alcoholic and non-alcoholic steatohepatitis-associated liver cirrhosis. Liver cancer is the third cancer-related cause of death, with an annual mortality of about 700.000 persons globally. Low survival is attributed to late diagnosis, resistance to treatment, tumor recurrence and metastasis, hence stressing the need for novel diagnostics and therapeutics. Specific miRNAs have been shown to be involved in various biological processes, including development, cellular proliferation, apoptosis, and oncogenesis (3). The finding that individual miRNAs may target several hundred genes, and that a large percentage of mRNAs may be subject to regulation by miRNAs, further underscores the emerging importance of miRNA-mediated regulation (4, 5). Here we review miRNA biogenesis and its alterations as well as miRNA polymorphisms linked to HCC, miRNA detection methods, the association of cellular and circulating miRNAs expression patterns with HCC, their predicted target genes, and discuss the diagnostic and therapeutic potential of some miRNAs.

miRNA EXPRESSION PROFILES ASSOCIATED WITH HCC

miRNA biogenesis and mechanism of action

miRNAs are endogenous ~22-nt long single stranded RNAs. There are currently 1492 human miRNA sequences registered in the miRBase database (http://www.mirbase.org). miRNAs are non-coding but are implicated in post-transcriptional regulation of genes involved in fundamental cell processes and in diseases (3). The miRNA gene is usually transcribed by RNA polymerase II in the nucleus into a primary transcript called pri-miRNA (**Fig. 1, A**) of approximately 1-4kb (6). These transcripts can be either monocistronic - a single miRNA gene behind a promoter - or polycistronic - expressed from one transcript as a cluster containing several miRNA gene products *e.g.* the miR-17~92 miRNA polycistron (7). Depending on their genome position, globally about 50% of all miRNA genes are intragenic, the so-called mirtrons - likely to be regulated through their host gene - but they can also be located in intergenic regions *i.e.* likely to be independent transcriptional units (8). The pri-miRNA is then cleaved by the microprocessor complex which consists of the nuclease Drosha and the double-stranded RNA-binding protein DiGeorge syndrome critical region gene 8 (DGCR8) into a precursor miRNA (pre-miRNA) (**Fig. 1, B**). This ~70-nt long pre-miRNA is exported to the cytoplasm via Exportin-5 (**Fig. 1, C**) where it will be cleaved by another nuclease, Dicer, into an imperfect miRNA-miRNA* duplex (**Fig. 1, D**) of ~18-25 nucleotides (9). While the passenger strand (miRNA*, marked in black in **Fig. 1**) is commonly degraded, the mature miRNA guide strand (marked in red in **Fig. 1**) is loaded into the RNA-induced silencing complex (RISC; **Fig. 1, E**) where further regulations will be undertaken, depending on the level of complementarity between the miRNA and its target in the 3' untranslated region (3' UTR) of the messenger RNA (mRNA). In case of perfect complementarity the mRNA will be cleaved by RISC and degraded; in case of imperfect complementarity, translation will be repressed (10). In mammalians decreased mRNA levels were shown to be preceding protein decrease in 84% cases (11). Functional target sites within the mRNA usually consist of a 6-7-nt long sequence which is complementary to the miRNA sequence, followed by

an adenosine, the so-called miRNA "seed" sequence. Target mRNAs end up in cytoplasmic processing-bodies (P-bodies) where they are degraded (12). Interestingly, other recently discovered classes of non-coding RNAs can also participate in regulation of gene expression and/or have been associated with HCC. Ender *et al.* showed that the small nucleolar RNA (snoRNA) ACA45 is processed by Dicer into RNAs of miRNA-like length (20- to 25-nt long) that will bind to Argonaute proteins (Ago), moreover they demonstrated the miRNA-like function of ACA45 by luciferase reporter assays (13). Yang *et al.* identified a long non-coding RNA (lncRNA) named High Expression in Hepatocellular Carcinoma (lncRNA-HEIH) that is differentially expressed in HCC and whose expression level is positively associated with tumor recurrence and negatively correlated with survival. In addition they showed that shRNA-mediated down-regulation of lncRNA-HEIH significantly inhibited the growth of tumors in a xenograft mouse model (14).

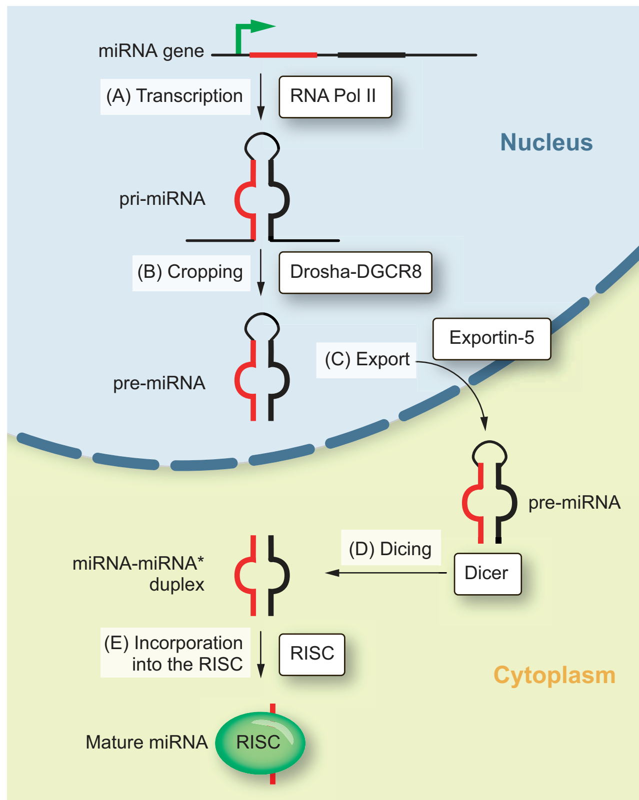

Figure 1. miRNA biogenesis involves multiple steps requiring (A) RNA Pol II for transcription of the 1-4kb primary transcript called pri-miRNA, (B) nuclease Drosha-DGCR8 for cropping of the single-stranded sequences flanking double-stranded stem-loop structure of the pre-miRNA precursor of ~70-nt long, (C) export of the pre-miRNA via Exportin-5 from the nucleus to the cytoplasm and (D) nuclease Dicer cleaving of the loop to generate the mature ~22-nt long miRNA that will be incorporated into the RISC (E). miRNA guide strand is represented in red, and passenger strand is represented in black.

Regulation of miRNA processing in association with HCC

Mature miRNA processing involves multiple steps, and each can potentially be affected, having an impact on the resulting net amount of produced mature miRNA. The process starts with the pri-miRNA transcription, which can be regulated by transcription factors or genes that are dysregulated in HCC, and that bind to the corresponding sequence located upstream in the promoter region. For example, the oncogenic transcription factor c-Myc binds upstream of miR-17 and up-regulates the transcription of the miR-17-92 tumor-promoting polycistron (15). Interestingly, c-Myc similarly binds upstream of other miRNAs, *e.g.* let-7 and miR-26a, this time by repressing their transcription, indicating that cellular miRNAs can have opposite functions in cancer development (16). In a similar fashion, the tumor-suppressor gene p53 up-regulates the transcription of miR-34, resulting in cell cycle arrest and apoptosis (17-20). Epigenetic mechanisms *e.g.* histone deacetylation and DNA methylation can result in miRNA silencing. For instance, Furuta *et al.* showed that methylation of miR-124 and miR-203 genes in HCC cell lines silenced their expression (21). The next step of miRNA processing, when Dicer cleaves out the mature miRNA, can be affected in HCC, *e.g.* Dicer expression is altered in many cancers (22). This results in miRNA dysregulation and as a consequence, in an abnormal gene expression that may lead to cancer phenotype. Finally, the stability of the mature miRNA molecule can be affected by differential polyadenylation modifications. miR-122, a liver-specific miRNA, is selectively 3' adenylated, which will result in a higher stability in the liver while it will be destabilized in fibroblasts due to poly(A) polymerase GLD-2 depletion (23, 24).

miRNA polymorphisms

Besides the possible alterations in the miRNA processing, miRNA polymorphisms can also be associated with an increased risk of HCC. A miRNA polymorphism consists of a single nucleotide polymorphism (SNP) in the miRNA gene. Although rare, a SNP in a miRNA can affect its transcription, processing, or target recognition. Since binding of a miRNA to its mRNA target is limited only to the seed sequence, even one nucleotide change would result in a different group of genes that would be regulated. Recently, two groups of investigators have described that a variant of miR-196a-2 is positively associated with HCC susceptibility, in two populations of distinctive ethnical background (25, 26). Yet the field of HCC-associated miRNA polymorphisms and their relevance to disease progression as a result of regulation of different pools of genes is only starting to develop.

Detection of miRNAs

Since miRNAs are involved in fine-tuning of physiological responses, they have become of interest for diagnosis and therapy of a number of diseases; nevertheless, reliable miRNA detection is a key requirement. Currently the three most commonly used detection methods are microarray, RT-qPCR and next-generation sequencing (NGS). Much less common is the use of northern blot, *in situ* hybridization and bead-based flow cytometry. Microarray is based on annealing of DNA oligonucleotides to the homologous sequences, on a microchip. Main advantages are the relatively low price and the high throughput, but the method has a low sensitivity and specificity, *i.e.* miRNAs with similar sequences (miRNA families) can hybridize with the same probe. The use of DNA locked nucleic acid (LNA) oligonucleotides in microarrays ensures a greater specificity by increasing the melting temperature. In addition, the sensitivity has also been increased (27). miRNA RT-qPCR is based on a

stem-loop primer binding to the mature miRNA during the reverse transcription, making it a highly specific technique that can distinguish 1-nt differences between related miRNAs (28). Although pre-amplification step sometimes required before the RT-qPCR can induce some bias and underestimate the concentration of lowly expressed miRNAs, this method is more sensitive than microarray. Despite its higher cost, it is currently the method of choice for validation of miRNA signatures. NGS is a high-throughput technology that provides global information on all miRNAs in a certain sample. Costs are much higher and data analysis is more laborious, but NGS provides quantitative data, allows miRNA discovery and provides data on miRNA polymorphisms and differential processing. Finally, the nCounter developed by Nanostring Technologies (Seattle, WA) is based on annealing of a fluorescent barcode probe followed by single molecule imaging, without pre-amplification step, offering high sensitivity and specificity. This technology offers high-throughput when using up to 800 multiplexed targets.

Key miRNAs dysregulated in HCC

Dysregulation of miRNAs in cancer has been repeatedly described , *e.g.* deregulated miRNAs in prostate, bladder and kidney cancer (29), breast cancer (30), colon cancer (31). Importantly, miRNAs are predominantly down-regulated in tumor tissue (32). Hepatocellular carcinoma is no exception and various HCC-specific miRNA signature have been described (**Table 1**). Screens of clinical samples are qualitatively heterogenic, firstly because of the variability in the technical procedure, from method of sampling (surgery or biopsy), time to and procedure of freezing, RNA isolation, to method of detection. Most miRNA screens are done using miRNA RT-qPCR, but some publications report microarray and NGS as described in **Table 1**. Secondly, the disease etiology is a significant factor of variation. This should be taken into account when pooling data, as the patient group can have a single etiology (alcohol or viral) or mixed etiologies (alcohol plus viral). Thirdly, the stage of the disease should also be considered, although miRNA dysregulations occur from an early stage (33), it is not clear how miRNA expression changes during disease progression. Finally, the control tissue used for normalization is also of importance, as it can be healthy liver from patients with a different pathology or no known pathology, or non-tumoral liver tissue from the same patient, *i.e.* with the same underlying liver disease (*e.g.* cirrhosis, viral infection), the latter allowing to look only at intra-individual changes (34). Nevertheless, dysregulation of several key miRNAs appear to be common to different screens, as described in **Table 1**.

Use of miRNAs in molecular classification of HCC and in prognostic

Key miRNAs are affected in HCC, and different dysregulation patterns can be used to discriminate tumors based on molecular characteristics. For instance, down-regulation of miR-375 has been associated with β-catenin mutation, and down-regulation of miR-107 with hepatocyte nuclear factor 1α (HNF1 α) (35). Toffanin *et al.* recently proposed a miRNA-based classification of HCC in three subclasses: the wingless-type MMTV integration site, interferon-related, and proliferation subclasses (36). Such miRNA-based determination of molecular subclasses of HCC could allow subtype-specific treatment. miRNA signatures can also be used to determine disease prognosis, *e.g.* Budhu *et al.* identified a 20-miRNA signature as a predictor of survival and recurrence (37). In addition, low tumoral miR-26 expression has been associated with high interleukin-6 (IL6) expression, and shorter

survival (38); Ji *et al*. showed a better response of these tumors to interferon therapy compared to tumors with high miR-26 levels (38). It hence appears that miRNA profiling may play a crucial role in the clinic, not only for HCC classification and subtype-specific treatment allocation, but also for prognosis.

GENE TARGETS OF MIRNAS AND THEIR ASSOCIATION WITH HCC

Oncogenic and tumor-suppressive miRNAs

Prior to inhibition of gene expression, mature miRNAs are loaded into RISC which will mediate recognition of the target mRNAs and lead to either mRNA degradation or translational repression. This negative regulation of gene expression by miRNAs leads to a balance between miRNA and gene expression level. In the context of HCC, miRNAs can act either as oncogenes, by inducing progression of a cell to cancer, or as tumor-suppressors, by preventing cell progression to cancer miRNAs (**Fig. 2**, marked in red) repress the expression of oncogenic targets; when down-regulated in HCC, a higher expression of their

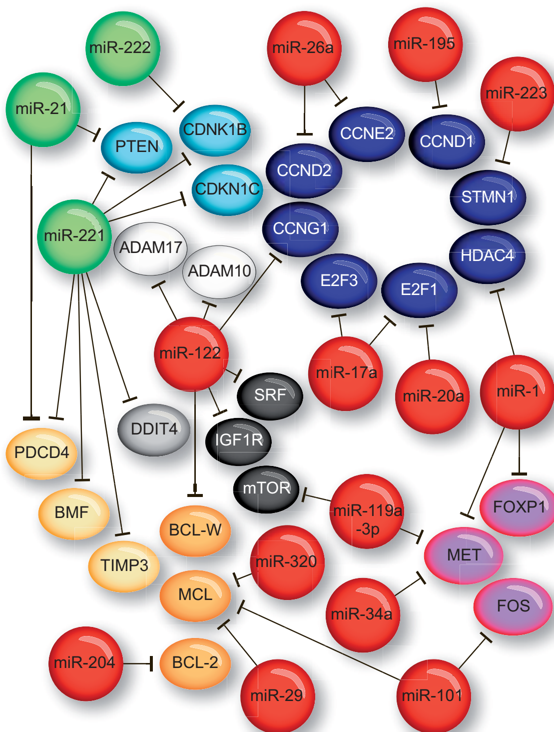

Figure 2. miRNAs and their oncogenic and tumor-suppressing targets associated with HCC. Tumor suppressing miRNAs which are down-regulated in HCC are marked in red and oncogenic miRNAs which are up-regulated in HCC are marked in green. miRNAs post-transcriptionally repress the expression of genes involved in cell cycle regulation, marked in blue; cell proliferation, marked in grey; apoptosis, marked in yellow; cell migration and invasion, marked in white; and of proto-oncogenes, marked in purple. Genes having a positive effect on the cell process are marked in the dark shade, while genes having a negative effect on the cell process are marked in the light shade. For example, *CCND1* is marked in dark blue, because it causes cell cycle progression, and has been linked to the development and progression of cancer. Vice-versa, PTEN is marked in light blue as it causes cell cycle arrest. Additionally, one gene can be targeted by several miRNAs, for example tumor-suppressor PTEN was shown to be simultaneously repressed by oncogenic miR-21 and miR-221. Bar-headed lines indicate post-transcriptional repression of gene expression. Data presented in this figure is non-exhaustive and based on literature.

Table 1. Key cellular miRNAs dysregulated in HCC (studies based on patient material) compared to healthy liver (HL). NI, no information; RT-qPCR, reverse-template quantitative PCR; NGS, next-generation sequencing, HCV, hepatitis C virus; HBV, hepatitis B virus.

miRNA	Dysregulation in HCC	Experimental settings	HCC etiology	miRNA detection method	Reference
let-7a	Down	19 paired HL and HCC	HBV	NGS, Northern blot	Connolly et al., 2008 [77]
let-7a	Down	21 HL, 17 HCC including 13 pairs	Mixed etiologies, mainly HCV	Microarray	Gramantieri et al., 2007 [39]
let-7a	Down	20 paired HL and HCC	Mixed etiologies	Microarray	Huang et al., 2009 [78]
let-7b	Down	21 HL, 17 HCC including 13 pairs	Mixed etiologies, mainly HCV	Microarray	Gramantieri et al., 2007 [39]
let-7c	Down	21 HL, 17 HCC including 13 pairs	Mixed etiologies, mainly HCV	Microarray	Gramantieri et al., 2007 [39]
let-7d	Down	21 HL, 17 HCC including 13 pairs	Mixed etiologies, mainly HCV	Microarray	Gramantieri et al., 2007 [39]
let-7e	Down	21 HL, 17 HCC including 13 pairs	Mixed etiologies, mainly HCV	Microarray	Gramantieri et al., 2007 [39]
let-7f	Down	21 HL, 17 HCC including 13 pairs	Mixed etiologies, mainly HCV	Microarray	Gramantieri et al., 2007 [39]
let-7g	Down	21 HL, 17 HCC including 13 pairs	Mixed etiologies, mainly HCV	Microarray	Gramantieri et al., 2007 [39]
miR-9-3p	Up	19 paired HL and HCC	NI	RT-qPCR	Wang et al., 2008 [85]
miR-9	Up	19 paired HL and HCC	NI	RT-qPCR	Wang et al., 2008 [85]
miR-10a	Up	3 HL, 43 HCC	HCV	RT-qPCR	Varnholt et al., 2008 [84]
miR-10b	Up	4 HL, 28 HCC	Mixed etiologies	RT-qPCR	Ladeiro et al., 2008 [35]
miR-15a	Up	19 paired HL and HCC	HBV	NGS, Northern blot	Connolly et al., 2008 [77]
miR-15a	Down	20 paired HL and HCC	Mixed etiologies	Microarray	Huang et al., 2009 [78]
miR-16	Up	20 paired HL and HCC	Mixed etiologies	Microarray	Huang et al., 2009 [78]

miR-17	Up	19 paired HL and HCC	HBV	NGS, Northern blot	Connolly et al., 2008 [77]
miR-17	Up	20 paired HL and HCC	Mixed etiologies	Microarray	Huang et al., 2009 [78]
miR-18a	Up	3 HL, 5 HCC	Mainly HBV	Microarray	Su et al., 2009 [83]
miR-18a	Up	19 paired HL and HCC	HBV	NGS, Northern blot	Connolly et al., 2008 [77]
miR-18b	Up	3 HL, 5 HCC	Mainly HBV	Microarray	Su et al., 2009 [83]
miR-18	Down	22 HL, 24 HCC including 22 pairs	Mixed etiologies	Microarray	Murakami et al., 2006 [82]
miR-18	Up	28 paired HL and HCC	Mixed etiologies	RT-qPCR	Jiang et al., 2008 [80]
miR-19b	Up	19 paired HL and HCC	HBV	NGS, Northern blot	Connolly et al., 2008 [77]
miR-20a	Up	19 paired HL and HCC	HBV	NGS, Northern blot	Connolly et al., 2008 [77]
miR-21	Up	3 paired HL and HCC	NI	Microarray	Meng et al., 2007 [81]
miR-21	Up	19 paired HL and HCC	NI	RT-qPCR	Wang et al., 2008 [85]
miR-21	Up	28 paired HL and HCC	Mixed etiologies	RT-qPCR	Jiang et al., 2008 [80]
miR-21	Up	4 HL, 28 HCC	Mixed etiologies	RT-qPCR	Ladeiro et al., 2008 [35]
miR-21	Up	19 paired HL and HCC	HBV	NGS, Northern blot	Connolly et al., 2008 [77]
miR-22	Down	19 paired HL and HCC	HBV	NGS, Northern blot	Connolly et al., 2008 [77]
miR-24	Up	20 paired HL and HCC	Mixed etiologies	Microarray	Huang et al., 2009 [78]
miR-25	Up	3 HL, 5 HCC	Mainly HBV	Microarray	Su et al., 2009 [83]
miR-25	Up	19 paired HL and HCC	NI	RT-qPCR	Wang et al., 2008 [85]
miR-25	Up	20 paired HL and HCC	Mixed etiologies	Microarray	Huang et al., 2009 [78]
miR-27a	Up	19 paired HL and HCC	HBV	NGS, Northern blot	Connolly et al., 2008 [77]
miR-27a	Down	20 paired HL and HCC	Mixed etiologies	Microarray	Huang et al., 2009 [78]
miR-29c	Down	3 HL, 5 HCC	Mainly HBV	Microarray	Su et al., 2009 [83]
miR-33	Up	28 paired HL and HCC	Mixed etiologies	RT-qPCR	Jiang et al., 2008 [80]
miR-34a	Up	3 paired HL and HCC	NI	Microarray	Meng et al., 2007 [81]

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miRNA	Dysregulation in HCC	Experimental settings	HCC etiology	miRNA detection method	Reference
miR-92	Down	3 paired HL and HCC	NI	Microarray	Meng et al., 2007 [81]
miR-92	Up	19 paired HL and HCC	HBV	NGS, Northern blot	Connolly et al., 2008 [77]
miR-93	Up	3 HL, 5 HCC	Mainly HBV	Microarray	Su et al., 2009 [83]
miR-93	Up	19 paired HL and HCC	HBV	NGS, Northern blot	Connolly et al., 2008 [77]
miR-96	Up	19 paired HL and HCC	NI	RT-qPCR	Wang et al., 2008 [85]
miR-99a	Down	3 HL, 5 HCC	Mainly HBV	Microarray	Su et al., 2009 [83]
miR-99a	Down	19 paired HL and HCC	HBV	NGS, Northern blot	Connolly et al., 2008 [77]
miR-100	Down	3 HL, 5 HCC	Mainly HBV	Microarray	Su et al., 2009 [83]
miR-100	Up	3 HL, 43 HCC	HCV	RT-qPCR	Varnholt et al., 2008 [84]
miR-101	Down	3 HL, 5 HCC	Mainly HBV	Microarray	Su et al., 2009 [83]
miR-101	Down	17 paired HL and HCC	Mainly HBV	Northern blot	Su et al., 2009 [83]
miR-101	Down	28 paired HL and HCC	Mixed etiologies	RT-qPCR	Jiang et al., 2008 [80]
miR-106b	Up	19 paired HL and HCC	HBV	NGS, Northern blot	Connolly et al., 2008 [77]
miR-107	Up	20 paired HL and HCC	Mixed etiologies	Microarray	Huang et al., 2009 [78]
miR-122a	Down	3 paired HL and HCC	NI	Microarray	Meng et al., 2007 [81]
miR-122a	Up	3 HL, 43 HCC	HCV	RT-qPCR	Varnholt et al., 2008 [84]
miR-122a	Down	4 HL, 28 HCC	Mixed etiologies	RT-qPCR	Ladeiro et al., 2008 [35]
miR-122a	Down	21 HL, 17 HCC including 13 pairs	Mixed etiologies, mainly HCV	Microarray	Gramantieri et al., 2007 [39]
miR-122a	Down	19 paired HL and HCC	HBV	NGS, Northern blot	Connolly et al., 2008 [77]
miR-122a	Down	20 paired HL and HCC	Mixed etiologies	Microarray	Huang et al., 2009 [78]

miR-122	Down	9 paired HL and T1-HCC (stage 1 TNM) and 11 paired HL and T3-HCC		Northern blot	Tsai et al., 2009 [41]
miR-124a	Down	21 HL, 17 HCC including 13 pairs	Mixed etiologies, mainly HCV	Microarray	Gramantieri et al., 2007 [39]
miR-124a	Down	20 paired HL and HCC	Mixed etiologies	Microarray	Huang et al., 2009 [78]
miR-124	Down	In 11 out of 19 paired HCC and HL	NI	RT-qPCR	Furuta et al., 2009 [21]
miR-125a	Down	20 paired HL and HCC	Mixed etiologies	Microarray	Huang et al., 2009 [78]
miR-125a	Down	3 paired HL and HCC	NI	Microarray	Meng et al., 2007 [81]
miR-125a	Down	20 paired HL and HCC	Mixed etiologies	Microarray	Huang et al., 2009 [78]
miR-125a	Down	22 HL, 24 HCC including 22 pairs	Mixed etiologies	Microarray	Murakami et al., 2006 [82]
miR-125b	Down	3 HL, 5 HCC	Mainly HBV	Microarray	Su et al., 2009 [83]
miR-125b	Down	3 paired HL and HCC	NI	Microarray	Meng et al., 2007 [81]
miR-125b	Down	20 paired HL and HCC	Mixed etiologies	Microarray	Huang et al., 2009 [78]
miR-126-3p	Down	20 paired HL and HCC	Mixed etiologies	Microarray	Huang et al., 2009 [78]
miR-126	Down	19 paired HL and HCC	HBV	NGS, Northern blot	Connolly et al., 2008 [77]
miR-126	Down	20 paired HL and HCC	Mixed etiologies	Microarray	Huang et al., 2009 [78]
miR-127-3p	Up	3 HL, 5 HCC	Mainly HBV	Microarray	Su et al., 2009 [83]
miR-128b	Down	20 paired HL and HCC	Mixed etiologies	Microarray	Huang et al., 2009 [78]
miR-129	Down	20 paired HL and HCC	Mixed etiologies	Microarray	Huang et al., 2009 [78]
miR-130a	Down	21 HL, 17 HCC including 13 pairs	Mixed etiologies, mainly HCV	Microarray	Gramantieri et al., 2007 [39]
miR-130a	Down	19 paired HL and HCC	HBV	NGS, Northern blot	Connolly et al., 2008 [77]
miR-130a	Down	20 paired HL and HCC	Mixed etiologies	Microarray	Huang et al., 2009 [78]

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miRNA	Dysregulation in HCC	Experimental settings	HCC etiology	miRNA detection method	Reference
miR-130b	Up	28 paired HL and HCC	Mixed etiologies	RT-qPCR	Jiang et al., 2008 [80]
miR-132	Down	21 HL, 17 HCC including 13 pairs	Mixed etiologies, mainly HCV	Microarray	Gramantieri et al., 2007 [39]
miR-135a	Up	28 paired HL and HCC	Mixed etiologies	RT-qPCR	Jiang et al., 2008 [80]
miR-136	Down	21 HL, 17 HCC including 13 pairs	Mixed etiologies, mainly HCV	Microarray	Gramantieri et al., 2007 [39]
miR-137	Up	19 paired HL and HCC	NI	RT-qPCR	Wang et al., 2008 [85]
miR-139	Down	19 paired HL and HCC	NI	RT-qPCR	Wang et al., 2008 [85]
miR-139	Down	28 paired HL and HCC	Mixed etiologies	RT-qPCR	Jiang et al., 2008 [80]
miR-141	Down	21 HL, 17 HCC including 13 pairs	Mixed etiologies, mainly HCV	Microarray	Gramantieri et al., 2007 [39]
miR-142	Down	21 HL, 17 HCC including 13 pairs	Mixed etiologies, mainly HCV	Microarray	Gramantieri et al., 2007 [39]
miR-143	Up	25 paired HL and HCC	12 HBV-, 13 HBV+	RT-qPCR	Zhang et al., 2009 [87]
miR-143	Down	21 HL, 17 HCC including 13 pairs	Mixed etiologies, mainly HCV	Microarray	Gramantieri et al., 2007 [39]
miR-143	Down	20 paired HL and HCC	Mixed etiologies	Microarray	Huang et al., 2009 [78]
miR-145	Down	21 HL, 17 HCC including 13 pairs	Mixed etiologies, mainly HCV	Microarray	Gramantieri et al., 2007 [39]
miR-145	Down	20 paired HL and HCC	Mixed etiologies	Microarray	Huang et al., 2009 [78]
miR-145	Down	3 HL, 43 HCC	HCV	RT-qPCR	Varnholt et al., 2008 [84]
miR-145	Down	19 paired HL and HCC	Mixed etiologies, mainly alcohol	RT-qPCR	Borel et al., 2011 [34]

miR-145	Down	19 paired HL and HCC	NI	RT-qPCR	Wang et al., 2008 [85]
miR-146	Down	21 HL, 17 HCC including 13 pairs	Mixed etiologies, mainly HCV	Microarray	Gramantieri et al., 2007 [39]
miR-148a	Up	19 paired HL and HCC	HBV	NGS, Northern blot	Connolly et al., 2008 [77]
miR-150	Down	21 HL, 17 HCC including 13 pairs	Mixed etiologies, mainly HCV	Microarray	Gramantieri et al., 2007 [39]
miR-150	Down	28 paired HL and HCC	Mixed etiologies	RT-qPCR	Jiang et al., 2008 [80]
miR-151	Up	19 paired HL and HCC	NI	RT-qPCR	Wang et al., 2008 [85]
miR-152	Down	20 paired HL and HCC	Mixed etiologies	Microarray	Huang et al., 2009 [78]
miR-155	Down	21 HL, 17 HCC including 13 pairs	Mixed etiologies, mainly HCV	Microarray	Gramantieri et al., 2007 [39]
miR-155	Up	19 paired HL and HCC	NI	RT-qPCR	Wang et al., 2008 [85]
miR-181a	Down	21 HL, 17 HCC including 13 pairs	Mixed etiologies, mainly HCV	Microarray	Gramantieri et al., 2007 [39]
miR-181c	Down	21 HL, 17 HCC including 13 pairs	Mixed etiologies, mainly HCV	Microarray	Gramantieri et al., 2007 [39]
miR-182-3p	Up	19 paired HL and HCC	NI	RT-qPCR	Wang et al., 2008 [85]
miR-182	Up	19 paired HL and HCC	NI	RT-qPCR	Wang et al., 2008 [85]
miR-183	Up	19 paired HL and HCC	NI	RT-qPCR	Wang et al., 2008 [85]
miR-185	Down	20 paired HL and HCC	Mixed etiologies	Microarray	Huang et al., 2009 [78]
miR-186	Up	19 paired HL and HCC	NI	RT-qPCR	Wang et al., 2008 [85]
miR-194	Down	20 paired HL and HCC	Mixed etiologies	Microarray	Huang et al., 2009 [78]
miR-195	Down	3 HL, 5 HCC	Mainly HBV	Microarray	Su et al., 2009 [83]
miR-195	Down	22 HL, 24 HCC including 22 pairs	Mixed etiologies	Microarray	Murakami et al., 2006 [82]
miR-195	Down	21 HL, 17 HCC including 13 pairs	Mixed etiologies, mainly HCV	Microarray	Gramantieri et al., 2007 [39]

Table 1. Key cellular miRNAs dysregulated in HCC (studies based on patient material) compared to healthy liver (HL). NI, no information; RT-qPCR, reverse-template quantitative PCR; NGS, next-generation sequencing, HCV, hepatitis C virus; HBV, hepatitis B virus.

miRNA	Dysregulation in HCC	Experimental settings	HCC etiology	miRNA detection method	Reference
miR-195	Up	10 paired HL and HCC	HBV-, HCV-	Microarray	Huang et al., 2008 [79]
miR-195	Down	20 paired HL and HCC	Mixed etiologies	Microarray	Huang et al., 2009 [78]
miR-198	Down	3 HL, 43 HCC	HCV	RT-qPCR	Varnholt et al., 2008 [84]
miR-199a-3p	Down	19 paired HL and HCC	Mixed etiologies, mainly alcohol	RT-qPCR	Borel et al., 2011 [34]
miR-199a-3p	Down	22 HL, 24 HCC including 22 pairs	Mixed etiologies	Microarray	Murakami et al., 2006 [82]
miR-199a-3p	Down	28 paired HL and HCC	Mixed etiologies	RT-qPCR	Jiang et al., 2008 [80]
miR-199a	Down	3 HL, 5 HCC	Mainly HBV	Microarray	Su et al., 2009 [83]
miR-199a	Down	22 HL, 24 HCC including 22 pairs	Mixed etiologies	Microarray	Murakami et al., 2006 [82]
miR-199a	Down	21 HL, 17 HCC including 13 pairs	Mixed etiologies, mainly HCV	Microarray	Gramantieri et al., 2007 [39]
miR-199a	Down	3 paired HL and HCC	NI	Microarray	Meng et al., 2007 [81]
miR-199a	Down	28 paired HL and HCC	Mixed etiologies	RT-qPCR	Jiang et al., 2008 [80]
miR-199a	Down	19 paired HL and HCC	Mixed etiologies, mainly alcohol	RT-qPCR	Borel et al., 2011 [34]
miR-199a	Down	20 paired HL and HCC	Mixed etiologies	Microarray	Huang et al., 2009 [78]
miR-199b-3p	Down	3 HL, 5 HCC	Mainly HBV	Microarray	Su et al., 2009 [83]
miR-199b	Down	21HL, 17 HCC including 13 pairs	Mixed etiologies, mainly HCV	Microarray	Gramantieri et al., 2007 [39]
miR-199b	Down	19 paired HL and HCC	Mixed etiologies, mainly alcohol	RT-qPCR	Borel et al., 2011 [34]
miR-199b	Down	28 paired HL and HCC	Mixed etiologies	RT-qPCR	Jiang et al., 2008 [80]

miR-200a	Down	22 HL, 24 HCC including 22 pairs	Mixed etiologies	Microarray	Murakami et al., 2006 [82]
miR-200a	Down	20 paired HL and HCC	Mixed etiologies	Microarray	Huang et al., 2009 [78]
miR-200b	Down	21 HL, 17 HCC including 13 pairs	Mixed etiologies, mainly HCV	Microarray	Gramantieri et al., 2007 [39]
miR-200b	Down	28 paired HL and HCC	Mixed etiologies	RT-qPCR	Jiang et al., 2008 [80]
miR-203	Down	In 8 out of 14 paired HL and HCC	NI	RT-qPCR	Furuta et al., 2009 [21]
miR-203	Down	4 HL, 28 HCC	Mixed etiologies	RT-qPCR	Ladeiro et al., 2008 [35]
miR-205	Up	20 paired HL and HCC	Mixed etiologies	Microarray	Huang et al., 2009 [78]
miR-207	Up	20 paired HL and HCC	Mixed etiologies	Microarray	Huang et al., 2009 [78]
miR-210	Up	3 HL, 5 HCC	Mainly HBV	Microarray	Su et al., 2009 [83]
miR-210	Up	3 paired HL and HCC	NI	Microarray	Meng et al., 2007 [81]
miR-213	Up	3 paired HL and HCC	NI	Microarray	Meng et al., 2007 [81]
miR-214	Down	21 HL, 17 HCC including 13 pairs	Mixed etiologies, mainly HCV	Microarray	Gramantieri et al., 2007 [39]
miR-214	Down	19 paired HL and HCC	NI	RT-qPCR	Wang et al., 2008 [85]
miR-214	Down	28 paired HL and HCC	Mixed etiologies	RT-qPCR	Jiang et al., 2008 [80]
miR-215	Down	3 HL, 5 HCC	Mainly HBV	Microarray	Su et al., 2009 [83]
miR-216a	Up	3 HL, 5 HCC	Mainly HBV	Microarray	Su et al., 2009 [83]
miR-216	Up	19 paired HL and HCC	NI	RT-qPCR	Wang et al., 2008 [85]
miR-221	Up	21 HL, 17 HCC including 13 pairs	Mixed etiologies, mainly HCV	Microarray	Gramantieri et al., 2007 [39]
miR-221	Up	19 paired HL and HCC	NI	RT-qPCR	Wang et al., 2008 [85]
miR-221	Up	3 paired HL and HCC	NI	Microarray	Meng et al., 2007 [81]
miR-221	Up	28 paired HL and HCC	Mixed etiologies	RT-qPCR	Jiang et al., 2008 [80]
miR-221	Up	20 paired HL and HCC	Mixed etiologies	Microarray	Huang et al., 2009 [78]

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miRNA	Dysregulation in HCC	Experimental settings	HCC etiology	miRNA detection method	Reference
miR-222	Up	3 HL, 5 HCC	Mainly HBV	Microarray	Su et al., 2009 [83]
miR-222	Up	42 paired HL and HCC	Mixed etiologies, mainly HBV	RT-qPCR	Wong et al., 2008 [86]
miR-222	Up	3 paired HL and HCC	NI	Microarray	Meng et al., 2007 [81]
miR-222	Up	19 paired HL and HCC	NI	RT-qPCR	Wang et al., 2008 [85]
miR-222	Up	4 HL, 28 HCC	Mixed etiologies	RT-qPCR	Ladeiro et al., 2008 [35]
miR-222	Up	20 paired HL and HCC	Mixed etiologies	Microarray	Huang et al., 2009 [78]
miR-223	Down	3 HL, 5 HCC	Mainly HBV	Microarray	Su et al., 2009 [83]
miR-223	Down	42 paired HL and HCC	Mixed etiologies, mainly HBV	RT-qPCR	Wong et al., 2008 [86]
miR-223	Down	21 HL, 17 HCC including 13 pairs	Mixed etiologies, mainly HCV	Microarray	Gramantieri et al., 2007 [39]
miR-223	Down	28 paired HL and HCC	Mixed etiologies	RT-qPCR	Jiang et al., 2008 [80]
miR-224	Up	3 HL, 5 HCC	Mainly HBV	Microarray	Su et al., 2009 [83]
miR-224	Up	22 HL, 24 HCC including 22 pairs	Mixed etiologies	Microarray	Murakami et al., 2006 [82]
miR-224	Up	19 paired HL and HCC	NI	RT-qPCR	Wang et al., 2008 [85]
miR-224	Up	4 HL, 28 HCC	Mixed etiologies	RT-qPCR	Ladeiro et al., 2008 [35]
miR-224	Up	20 paired HL and HCC	Mixed etiologies	Microarray	Huang et al., 2009 [78]
miR-292-3p	Down	3 paired HL and HCC	NI	Microarray	Meng et al., 2007 [81]
miR-294	Up	3 paired HL and HCC	NI	Microarray	Meng et al., 2007 [81]
miR-301	Up	19 paired HL and HCC	NI	RT-qPCR	Wang et al., 2008 [85]

miR-301	Up	28 paired HL and HCC	Mixed etiologies	RT-qPCR	Jiang et al., 2008 [80]
miR-324	Up	19 paired HL and HCC	NI	RT-qPCR	Wang et al., 2008 [85]
miR-324	Up	19 paired HL and HCC	HBV	NGS, Northern blot	Connolly et al., 2008 [77]
miR-338	Down	20 paired HL and HCC	Mixed etiologies	Microarray	Huang et al., 2009 [78]
miR-362	Up	3 HL, 5 HCC	Mainly HBV	Microarray	Su et al., 2009 [83]
miR-365	Down	3 HL, 5 HCC	Mainly HBV	Microarray	Su et al., 2009 [83]
miR-373	Up	3 paired HL and HCC	NI	Microarray	Meng et al., 2007 [81]
miR-374	Up	19 paired HL and HCC	NI	RT-qPCR	Wang et al., 2008 [85]
miR-376a	Up	3 paired HL and HCC	NI	Microarray	Meng et al., 2007 [81]
miR-378	Down	3 HL, 5 HCC	Mainly HBV	Microarray	Su et al., 2009 [83]
miR-382	Up	3 HL, 5 HCC	Mainly HBV	Microarray	Su et al., 2009 [83]
miR-422a	Down	3 HL, 5 HCC	Mainly HBV	Microarray	Su et al., 2009 [83]
miR-422b	Down	4 HL, 28 HCC	Mixed etiologies	RT-qPCR	Ladeiro et al., 2008 [35]
miR-424	Down	3 HL, 5 HCC	Mainly HBV	Microarray	Su et al., 2009 [83]
miR-491	Up	3 HL, 5 HCC	Mainly HBV	Microarray	Su et al., 2009 [83]
miR-500	Up	40 paired HL and HCC	Mixed etiologies, mainly viral	RT-qPCR	Yamamoto et al., 2009 [91]
miR-519	Up	3 HL, 5 HCC	Mainly HBV	Microarray	Su et al., 2009 [83]
miR-520c-3p	Down	3 HL, 5 HCC	Mainly HBV	Microarray	Su et al., 2009 [83]
miR-527	Up	3 HL, 5 HCC	Mainly HBV	Microarray	Su et al., 2009 [83]

targets is allowed, hence promoting the malignant phenotype. Alternatively, up-regulation of oncogenic miRNAs (**Fig. 2**, marked in green) in HCC will cause down-regulation of their gene targets, again promoting the malignant phenotype.

Targets of tumor-suppressing miRNAs down-regulated in HCC

Being down-regulated in HCC, tumor-suppressor miRNAs cause up-regulation of oncogenic target genes, stimulate and/or increase cellular mechanisms such as cell proliferation, cell cycle regulation, cell migration and invasion, apoptosis, and hence participate in the establishment and maintenance of the cancer phenotype, as described in **Fig. 2**. Cyclin G1 (CCNG1) is one of the most well-characterized targets of miR-122 (39). However, miR-122 also targets the anti-apoptotic BCL-W (40) and ADAM17 (a disintegrin and metalloprotease family 17) involved in metastasis (41). Additional validated miRNA targets are described in **Fig. 2**.

Targets of oncogenic miRNAs up-regulated in HCC

Oncogenic miRNAs are up-regulated in HCC, thus causing down-regulation of target genes and decrease of cell mechanisms such as apoptosis, which eventually leads to onset and progression of the disease, as described in **Fig. 2**. Overall less miRNAs are up-regulated than down-regulated in cancer (32). miR-221 targets the cyclin-dependent kinase inhibitors CDKN1B/p27 (42) and CDKN1C/p57 (43), which results in an increase in the G1 to S phase shift and induces cell growth. Another target of miR-221 is the pro-apoptotic BMF of the BCL-2 family (44), therefore in HCC BMF down-regulation inhibits apoptosis. Enforced miR-221 expression also induces down-regulation of DNA damage-inducible transcript 4 (DDIT4), leading to modulation of the mTOR pathway (45); and of the tumor suppressors thymidylate synthase (TIMP3) and PTEN (46), which results in enhanced cell migration.

CIRCULATING MIRNAS

Origin and clinical relevance of circulating miRNAs

Recently it has been described that miRNAs are found in many body fluids including plasma (47). Vesicles (microvesicles or exosomes) released from cells to the circulation do contain miRNAs (48). A positive correlation between cellular and exosomal miRNAs levels was reported for a subset of 8 miRNAs, both presenting a profile significantly different in ovarian cancer compared to benign ovarian disease (49). Circulating miRNAs can therefore be considered representative of some pathological conditions. Moreover their accessibility and high stability in the circulation system (50) make them perfect biomarkers, especially for surveillance of early stage, pre-symptomatic diseases in at-risk patients. For example, a serum diagnostic test based on a 34-miRNAs signature, could diagnose early stage lung cancer with 80% accuracy (51). This study underscores the remarkable potential of circulating miRNAs in early, pre-symptomatic disease diagnosis.

Circulating miRNAs associated with HCC

As described above, many miRNAs are dysregulated in HCC. Therefore it is anticipated that circulating miRNAs are also affected during HCC progression. A few studies reported altered levels of circulating miRNAs in association with HCC (**Table 2**). For instance, the serum level of miR-221 was shown to be 4.8 fold elevated in HCC patients (52). Additionally,

high level of miR-221 positively correlated with cirrhosis, tumor size and tumor stage, and negatively correlated with overall survival. These promising results should be validated in a larger patient cohort; nevertheless, miR-221 serum level monitoring could be of clinical relevance as a potential diagnosis tool and biomarker of treatment efficacy. Indeed, no optimal blood tumor marker has been developed so far, the performance of α-fetoprotein (AFP), *Lens culinaris* agglutinin-reactive AFP (AFP-L3) and des-γ-carboxyprothrombin (DCP) (53) is limited in a surveillance mode and for early HCC detection. In addition,

Table 2. Circulating miRNAs candidate biomarkers for HCC. HL, healthy liver; HCC, hepatocellular carcinoma; CH, chronic hepatitis; LC, liver cirrhosis, CLD, chronic liver diseases.

miRNA	Dysregulation in plasma	Experimental settings	Result	Reference
miR-16	Down	71 HL, 105 HCC, 107 CLD	Significant association with HCC, combination with traditional markers improves diagnostic	Qu et al., 2011 [54]
miR-21	Up	89 HL, 101 HCC, 48 CH	Unspecific marker of liver injury	Xu et al., 2010 [90]
miR-21	Up	20 HL, 46 HCC	Elevated in HCC	Li et al., 2011 [52]
miR-92a	Down	10 HL, 10 HCC	Decreases post-resection	Shigoka et al., 2010 [89]
miR-122	Up	89 HL, 101 HCC, 48 CH	Unspecific marker of liver injury	Xu et al., 2010 [90]
miR-195	Down	71 HL, 105 HCC, 107 CLD	Significant association with HCC	Qu et al., 2011 [54]
miR-199a	Down	71 HL, 105 HCC, 107 CLD	Significant association with HCC	Qu et al., 2011 [54]
miR-221	Up	20 HL, 46 HCC	Elevated in 35/46 HCC, correlates with HCC stage and prognosis	Li et al., 2011 [52]
miR-222	Up	20 HL, 46 HCC	Elevated in HCC	Li et al., 2011 [52]
miR-223	Up	89 HL, 101 HCC, 48 CH	Unspecific marker of liver injury	Xu et al., 2010 [90]
miR-224	Up	20 HL, 46 HCC	Elevated in HCC	Li et al., 2011 [52]
miR-500	Up	10 HCC	Levels increased in 3/10 HCC, reduced 6 months postoperation	Yamamoto et al., 2009 [91]
miR-885	Up	10 HL, 15 HCC, 10 LC	Marker of liver cirrhosis	Gui et al., 2011 [88]

the American Association for the Study of Liver Diseases (AASLD) Practice Guidelines (July 2010) discarded AFP for surveillance and diagnosis. Therefore there is a need for novel markers that would combine the less invasiveness of a blood test and serve as a reliable early detection method. miRNAs definitely have this potential because not only can they be detected in plasma, but their sensitivity and stability is suitable for a clinical setting. Depending on the method, as little as one copy can be detected (see paragraph on detection). Nevertheless appropriate controls should be used, since HCC is most often accompanied by viral infection, cirrhosis, or other underlying liver condition. Therefore in order to assess the HCC-specificity of a miRNA, it is critical to ensure not only an age- and gender-matched control group but they should also be matched for etiology and severity of underlying liver disease. For instance, the miRNA profile of 3 patient groups was compared: 105 patients with HCC (19.1% HBV, 62.9% HCV, 17.1% other etiology), 107 with chronic liver disease (CLD; 7.5% HBV, 55.1% HCV, 37.4% other etiology) and 71 normal controls (54). In another study, miR-16 and miR-199a levels were decreased in serum and significantly associated with HCC (54). miR-16 was more sensitive as HCC detection marker than AFP, DCP and AFP-L3. Combination of miR-16 with AFP, DCP and AFP-L3 allowed detection of 92.4% HCC cases with a high specificity (78.5%), and interestingly, it could detect tumors ≤ 3cm with the same sensitivity (92.4%). This research demonstrates the feasibility of plasma markers for diagnosis of HCC. Circulating miRNAs could therefore be used as a first-line testing in HCC patients if they would outperform the currently used tumor markers. The discovery of circulating miRNAs offers interesting clinical perspectives but this field of research is quite recent and more work has to be done. It remains to be established which miRNA can sensitively and reliably be correlated with the presence of HCC at early stages of disease development and prognosis.

CELLULAR MIRNAS AS THERAPEUTIC TARGETS IN HCC

Potential of miRNA-based gene therapy

RNAi was identified in *C. elegans* in 1998 by Fire and Mello (55) and in mammalian cells in 2001 by Tuschl (56). Since then RNAi has generated increasing interest and publications in diverse research areas. The combination of RNAi with the latest developments in the field of gene therapy which rendered it safer, and the delivery more efficient, opens the door to novel therapeutic perspectives. Among the many possibilities currently investigated, the use of cellular miRNAs as therapeutic agents is one of the most promising from a clinical point of view. Many miRNAs are down-regulating genes that are highly relevant to HCC and therefore contribute to disease progression. Because a single miRNA could potentially affect several clinically relevant targets, artificially increasing or decreasing the expression level of a given miRNA offers interesting therapeutic perspectives. Such therapy could even be combined with local chemotherapy via the transarterial route (transarterial chemoembolization, TACE) to increase the treatment effectiveness. Nevertheless, because a miRNA can affect the expression of several downstream targets, modulating the expression of a miRNA of interest could also lead to undesirable off-target effects.

miRNA-based gene therapy for HCC

The main question raised by RNAi-based gene therapy is the delivery of the effector molecule, which should preferably be controllable, sustained and tissue-specific. Several

groups have chosen for non-viral delivery of synthetic miRNA molecules. miRNA mimics or miRNA antagomirs can be repeatedly delivered locally or systemically and that would cause transient suppression of target gene expression. To prevent rapid degradation of naked molecules, miRNAs are modified or conjugated to improve stability or target delivery to a specific tissue. They can be incorporated into stable nucleic acid lipid particles (SNALPs), a lipid bilayer coated by polyethylene glycol (PEG) which will protect them from degradation, prevent immunostimulation and facilitate their uptake in endosomes (57). Similarly, 2'O-Methyl modifications increase the stability of synthetic molecules, additionally preventing off-targeting (58). Mimicking the external viral protein structure, virus-like particles (VLP) can also be used for synthetic miRNA delivery, yet they are not suitable for all applications since they stimulate the immune response (59). However, VLP vehicles take advantage of the natural virus tropism, *e.g.* HBV in the liver, and can efficiently mediate hepatic gene transfer (60). Finally, miRNA conjugation to HDL, LDL or cholesterol will also lead to hepatic uptake (61). However, even with the described improvements, miRNAs would need to be delivered monthly or bimonthly. Landford *et al.* inhibited miR-122 expression in 4 chimpanzees using SPC3649 LNA-modified oligonucleotides. Because miR-122 stimulates HCV RNA accumulation, miR-122 inhibition leads to an efficient suppression of HCV replication and stable reduction of viremia in chimpanzees (62). A phase I trial for SPC3649 (Miravirsen from Santaris Pharma) showed that SPC3649 was well-tolerated and the drug is now in phase II trial. This approach holds promise for HCV patients and has the advantage that it should not allow the development of viral escape variants. Nevertheless Li *et al.* reported that mutations in miR-122 binding-site in HCV 5' UTR reduced SPC3649 treatment efficacy (63). This indicates that viral escape could still be possible.

Alternatively, gene therapy using virally-delivered miRNAs is desirable when chronic and genetic diseases need to be treated. Viral delivery indeed can offer sustained expression after single dosing, however, in a clinical perspective, it raises several questions concerning safety. Pri-miRNA can be delivered as an expression cassette using different types of viral vectors. The advantages and disadvantages of the viral vectors used in gene therapy clinical trials are summarized in **Table 3**. Briefly, the main disadvantage of lentivirus and retrovirus is their integration into the host genome, which raises safety issues. On the contrary, adeno-associated virus (AAV) genome remains episomal, which gives it an advantageous safety profile. However the episomal presence of AAV questions its relevance for cancer therapy. Up to now, adenovirus has been widely used in HCC gene therapy clinical trials (64-70), as well as *Vaccinia* virus (71). Despite the fact that many virus-delivered "classical" gene therapy products have been developed for HCC and are currently progressing through clinical trial phases, no virus-delivered miRNA-based gene therapy has been tested in clinical trials yet. Indeed, more research still needs to be done to carefully evaluate potential risks of this approach. Kota *et al.* showed that self-complementary AAV serotype 8 (scAAV8)-delivery of miR-26a in tumor-bearing tet-o-myc; LAP-tTA mice restored miR-26a expression (72). Re-expression of miR-26a specifically reduced cancer cell proliferation, induced tumor-specific apoptosis, and suppressed tumorogenesis. At 3 weeks post-transduction most liver tissue in the control group was replaced with tumor while in 8 out of 10 mice of the treated group no or small tumors only were found (72). This research demonstrated for the first time the therapeutic potential of restoring the expression of a dysregulated miRNA in the liver. Additional advantages of this approach is that the miRNA is well-tolerated, given

Table 3. Gene therapy vectors used in clinical trials. Kb, kilobases. [1] Source: Gene Therapy Clinical Trials Worldwide Database, The Journal of Gene Medicine, 2011.

Viral vector	% gene therapy clinical trials [1]	Maximal transgene size (kb)	Transgene expression	Tropism	Host genome interaction	Immunogenicity
Adenovirus	24.1	7.9	Transient	Broad	Non-integrating	High
Retrovirus	20.8	8	Long lasting	Dividing cells only	Integrating	Low
Vaccinia and poxvirus	13.5	250	Transient	Broad. Natural tumor tropism	Non-integrating	High
Adeno-associated virus	4.8	4.7	Potential long lasting	Broad	Non-integrating	Low
Herpes simplex	3.3	40	Potential long lasting	Broad	Non-integrating	High
Lentivirus	2.2	8	Long lasting	Both dividing and non-dividing cells	Integrating	Low

that it is only down-regulated in tumor cells, and therefore only tumor cells are affected. However, its significance in patients still needs to be determined.
With the first successes of RNA agents in clinical trials, it becomes clear that miRNAs and their inhibitors hold a great potential as therapeutics for different cancers including HCC.

CONCLUSION

It is now well-established that miRNAs are key players in many various biological processes, including development, cellular proliferation, apoptosis, and oncogenesis (3). In HCC, miRNAs have aberrant processing and expression profiles, in addition, the profile of circulating miRNAs is also affected, which renders them potential biomarkers, with possible applications in diagnosis, especially for early, pre-symptomatic disease, and prognosis of HCC. One miRNA may target several genes that are involved in the development and maintenance of the HCC phenotype. Therefore miRNA-based gene therapy offers promising perspectives compared to classical gene therapy for HCC. An additional advantage of miRNAs is that since they encode no protein, they are generally not immunogenic. However activation of Toll-like receptors (TLRs), involved in initiation of inflammatory responses to pathogens, can occur, as reviewed by O'Neill *et al.* (73) indicating that unanticipated off-target effects can occur in a clinical setting. For instance the let-7 family regulates the expression of TLR4 and this can create off-target effects (74). A positive aspect of gene therapy for HCC is that the delivery route seems not to be questioned, up to now direct imaging-guided intratumoral injection has been the most used strategy in clinical trials, but tumor-selective intra-arterial administration could be a good alternative (70). In HCC, combination of classical and miRNA-based therapies appears a desirable goal. First, chemo- or radiation therapy can improve gene transfer efficiency and transgene expression (75, 76). Second, in the case of combined chemo- and gene- therapies, a direct co-injection of both via the intravenous route used for the TACE procedure, could be suitable, offering a gene therapy delivery route already clinically approved and in practice. The discovery of miRNA-mediated gene regulation as a fundamental post-transcriptional mechanism increases the complexity of cancer genetics. However, understanding the molecular mechanisms by which miRNAs regulate development and tumorogenesis may lead to novel concepts in the diagnosis and treatment of cancer. Besides the fact that miRNAs have shown promising results in pre-clinical studies, miRNA-based gene therapy is not yet suitable for routine clinical practice.

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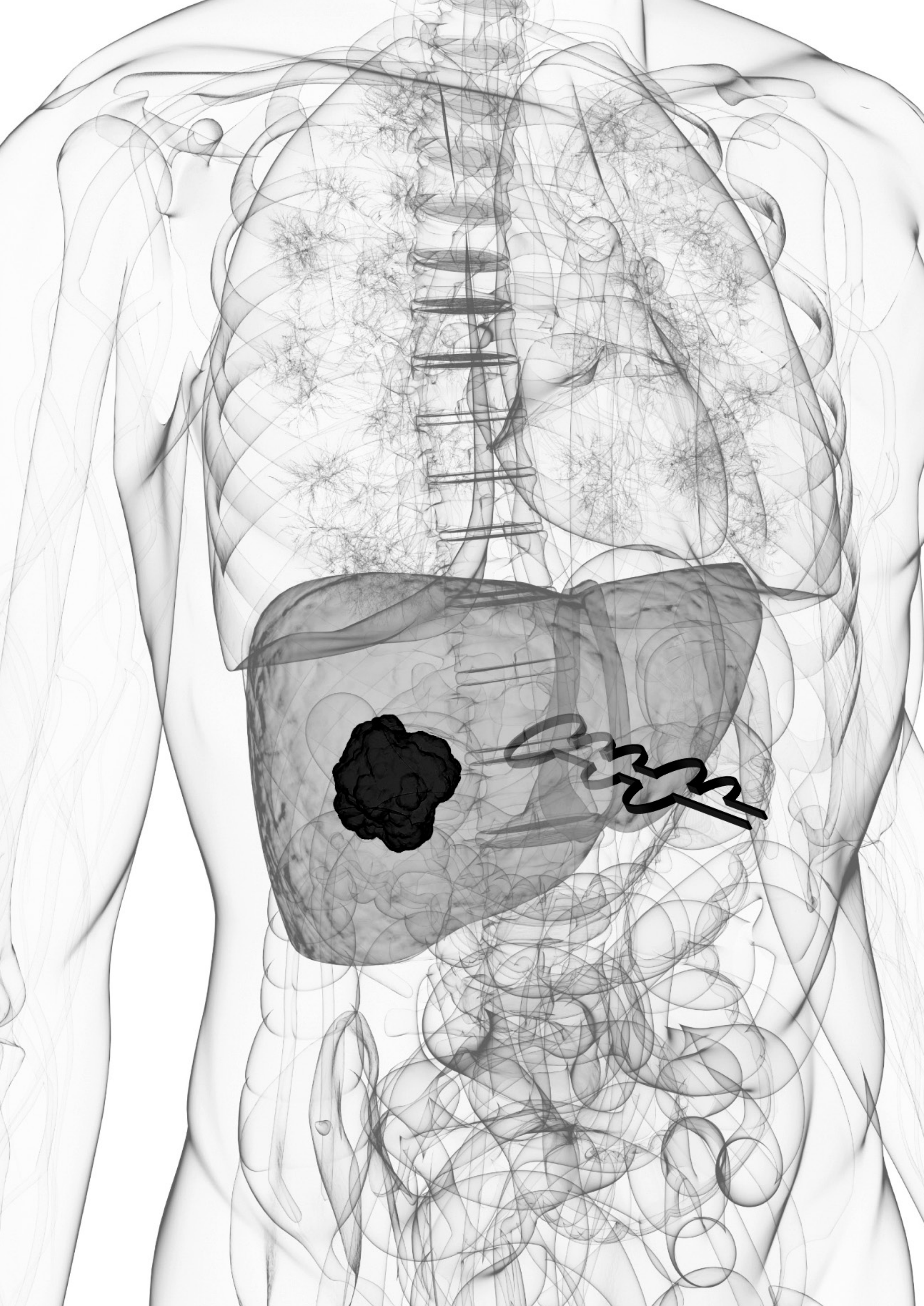

ADENOSINE TRIPHOSPHATE-BINDING CASSETTE TRANSPORTER GENES UP-REGULATION IN UNTREATED HEPATOCELLULAR CARCINOMA IS MEDIATED BY CELLULAR MicroRNAs

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ABSTRACT

ATP-binding cassette (ABC) transporters are drug efflux pumps responsible for the multidrug resistance phenotype causing hepatocellular carcinoma (HCC) treatment failure. Here we studied the expression of 15 ABC transporters relevant for multidrug resistance in 19 paired HCC patient samples (16 untreated, 3 treated by chemotherapeutics). Twelve ABC transporters showed up-regulation in HCC compared to adjacent healthy liver. These include ABCA2, ABCB1, ABCB6, ABCC1, ABCC2, ABCC3, ABCC4, ABCC5, ABCC10, ABCC11, ABCC12 and ABCE1. The expression profile and function of some of these transporters have not been associated with HCC thus far. Since cellular microRNAs (miRNAs) are involved in post-transcriptional gene silencing, we hypothesized that regulation of ABC expression in HCC might be mediated by miRNAs. To study this, miRNAs were profiled and dysregulation of 90 miRNAs was shown in HCC compared to healthy liver, including up-regulation of 11 and down-regulation of 79. miRNA target sites in ABC genes were bioinformatically predicted and experimentally verified *in vitro* using luciferase reporter assays. In total 13 cellular miRNAs were confirmed that target ABCA1, ABCC1, ABCC5, ABCC10 and ABCE1 genes and mediate changes in gene expression. Correlation analysis between ABC and miRNA expression in individual patients revealed an inverse relationship providing indication for miRNA regulation of ABC genes in HCC. ***Conclusion*****:** Up-regulation of ABC transporters in HCC occurs prior to chemotherapeutic treatment and is associated with miRNA down-regulation. Up-regulation of 5 ABC genes appears to be mediated by 13 cellular miRNAs in HCC patient samples. miRNA-based gene therapy may be a novel and promising way to affect the ABC profile and overcome clinical multidrug resistance.

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the 5th most common type of cancer worldwide. With a 5-year survival of less than 5% (1), HCC remains one of the most fatal cancers, and few treatments have proven to be effective. Major pitfalls are late diagnosis, tumor recurrence, and resistance to chemotherapeutic treatment. This is caused by a phenomenon called multidrug resistance, mediated by high expression of ATP-binding cassette (ABC) transporter family members that decrease the intracellular concentration of chemotherapeutic agents (2-6). There is limited information in the literature on the expression profile of ABC genes in HCC. For example, ABCB1 (MDR1) (7, 8) and ABCC3 (MRP3) (9) have been shown to be up-regulated in HCC of undetermined treatment status and a high expression of ABCC1 (MRP1) has been associated with an aggressive HCC phenotype in untreated patients (10). ABCA1 and ABCG2 down-regulation and ABCC4 (MRP4) up-regulation was shown in HCC of undetermined treatment status (11). The conventional model of multidrug resistance describes a genetically altered, highly resistant sub-population of cells selected under pressure of chemotherapeutic agents (12). Therefore profiling HCC tissues of untreated patients is of interest as it addresses the question of inherent multidrug resistance of HCC that has developed in the absence of chemotherapy. The regulation of ABC genes expression in HCC could be mediated by microRNAs (miRNAs), a family of small RNAs which is often dysregulated in cancer (13-15).

miRNAs are ~22 nucleotide (nt) long endogenous, single-stranded, non-coding RNAs (16). miRNAs are loaded into the RNA-induced silencing complex (RISC) where further regulations will be undertaken. If the complementarity is perfect in the “seed region” (nt 2-7 from the 5’ end of the miRNA) between the miRNA and its target in the messenger RNA (mRNA), the mRNA will be cleaved by RISC and degraded; in case of imperfect complementarity, translation will be repressed (17-20). Specific miRNAs have been shown to be involved in various biological processes, including development, cellular proliferation, apoptosis, and oncogenesis (21, 22). The finding that individual miRNAs may target several hundred genes, and that a large percentage of mRNAs may be subject to regulation by miRNAs, further underscores the emerging importance of miRNA-mediated regulation (23, 24).

Since miRNAs are involved in a great number of cellular processes and pathological conditions, it is thus possible that miRNAs regulate the expression of ABC transporters. Evidence was provided by Kovalchuk *et al.*, who showed that miR-451 may regulate ABCB1 in MCF7 breast cancer cells (25). Additionally, both miR-451 and miR-27a regulate ABCB1 expression in multidrug-resistant A2780DX5 and KB-V1 cancer cell lines (26). Re-expression of miR-203 *in vitro* in the liver cancer cell lines Hep3B, HuH7 and HLF was shown to induce down-regulation of ABCE1 (27). These results indicate that cellular miRNAs are implicated in mediating the regulation of the expression of at least 2 ABC genes, including ABCE1 in liver cancer cells.

In the current study, we hypothesized that ABC transporter gene expression is regulated by cellular miRNAs, resulting in a specific HCC phenotype. We show up-regulation of 12 ABC transporters in HCC including 8 which have not been previously associated with HCC. Subsequently up-regulation of 11 cellular miRNAs and down-regulation of 79 was shown. Interestingly 25 down-regulated miRNAs had predicted targets in 6 up-regulated ABC genes, of which we confirmed ABCA1, ABCC1, ABCC5, ABCC10 and ABCE1.

MATERIAL AND METHODS

Patients and samples

Samples were received as frozen tissue from the Centre de Ressources Biologiques Foie, France (courtesy of Prof. Dr. F. Degos, Hôpital Beaujon, Paris, and Prof. Dr. B. Clément, INSERM UMR991, Rennes, France). They were taken from 19 HCC patients and included paired tumor tissue (HCC) and adjacent healthy liver (AHL) tissue from each patient, frozen 15-105 min post-sampling. RNA preparations from healthy liver (HL) samples from 3 patients with pancreatic cancer were obtained from Dr. F. Schaap, Academic Medical Center, Amsterdam (28). All patients provided informed consent in writing. No donor organs were obtained from executed prisoners or other institutionalized persons.

RNA isolation

Total RNA was isolated from frozen tissue with Trizol (Invitrogen, Carlsbad, CA). RNA quality was assessed by checking for presence of 28S and 18S RNA on a 1.2% agarose gel (data not shown).

RT-qPCR for ABC genes

RNA was reverse-transcribed using random hexamer primers with the Dynamo kit (Finnzymes, Espoo, Finland) using 1.4µg of total RNA. Expression of ABC genes was analyzed using custom-designed FAM-labeled 384-well Taqman Gene Expression Array (Applied Biosystems, Foster City, CA). cDNA input per loading port (48 wells) was 1µg. Custom array included 15 ABC genes and internal control 18S. Arrays were run in triplicates on a 7900HT RT-qPCR system (Applied Biosystems).

RT-qPCR for miRNAs

miRNA RT reactions were performed with the TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems) according to manufacturer's instructions using 1µg RNA. Specific miRNA targets were pre-amplified using Taqman PreAmp MasterMix and Megaplex PreAmp Primers (Applied Biosystems). Cellular miRNA expression from 10 HCC and 3 HL samples was analyzed using 384-well Taqman Array Human MicroRNA A cards v2.0 (Applied Biosystems) including 378 miRNAs and 6 controls (mammalian U6 in quadruplicates, RNU44, RNU48) as described in manufacturer's instructions, including a pre-amplification step using Megaplex Primer, Human Pools Set v3.0 (Applied Biosystems). Arrays were run on a 7900HT RT-qPCR system (Applied Biosystems). For individual miRNA assay, RT reactions were performed with the TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems) using 10ng RNA and 3µl miRNA-specific RT-stem-loop primer (Applied Biosystems) according to manufacturer's instructions. Taqman assay was done in 20µl using 9µl cDNA (5x diluted), 1µl miRNA-specific primer with FAM-labeled fluorogenic probe (Applied Biosystems) and 10µl Taqman 2x Universal PCR Master Mix (Applied Biosystems) and run in duplicates on a 7500 RT-qPCR system (Applied Biosystems).

Data analysis

Raw data were analyzed with RQ Manager (Applied Biosystems). Normalization was performed with DataAssist v2.0 (Applied Biosystems): for ABC genes HCC were normalized to their paired AHL, for miRNA HCC were normalized to HL (screen) or AHL

(validation), and the relative gene expression $2^{-\Delta\Delta Ct}$ method of Livak and Schmittgen (29) was used. We set a 2-fold threshold for changes in gene expression per individual patient, *i.e.* changes lower than 0.5 fold were considered down-regulation and higher than 2 fold were considered up-regulation. Statistical analysis was performed using two-tailed paired t-test and Wilcoxon matched-pairs test and differences were considered significant when the p-value was lower than 0.05. For miRNA data analysis an additional manual screen was performed in order to check that the control group has consistent Ct values (Ct obtained for all 3 HL samples and less than 1.5 Ct variation between all 3). All miRNAs which had deviating Ct values between the 3 HL samples were excluded from the analysis. Statistical analysis was performed using two-tailed t-test and differences were considered significant when the p-value was lower than 0.05.

miRNA target prediction

Softwares TargetScan (24) and PicTar (23) were used for ABC 3'untranslated region (3'UTR) target prediction of cellular miRNAs. Additionally, 3'UTR sequences were manually screened for miRNA seed-matching sequences. Predictions are presented in **Table S1** and **Table S2**.

DNA constructs

Luciferase reporters were made by cloning of ABC 3'UTR sequences (**Table S3** and **Table S4**), in the renilla luciferase gene in the psiCheck-2 vector (Promega, Madison, WI). Contructs with mutated miRNA seed sequence in the ABC genes (nt 2 to 6) were synthesized by IDT (Coralville, IA). Primary miRNA (pri-miRNA) sequences were amplified (primer sequences, **Table S3**) from human adult normal breast tissue genomic DNA (Biochain, Hayward, CA). miRNA expression plasmids were made by cloning of the pri-miRNAs in the pcDNA6.2 vector (Invitrogen). All constructs were verified by sequencing (Macrogen, Seoul, Korea).

Cell lines and transfections

Human embryonic kidney (HEK) 293T cells were cultured according to the ATCC's instructions. Cells were plated in 6-, 24- or 96-well plates one day prior to transfection. Transfections were performed with Lipofectamine 2000 or LTX reagent (Invitrogen) according to manufacturer's instructions. For luciferase assays, HEK293T cells were co-transfected with 5ng of Luc-ABC reporter that contains both firefly and renilla luciferase genes and 150ng of the corresponding miRNA expression constructs. Expression values when the miR-Control (miR-Ctrl) was transfected were set at 1.

Luciferase assays

Transfected cells were assayed at 72hr post-transfection and firefly and renilla luciferase activities were measured with the Dual-Luciferase Reporter Assay System (Promega) according to manufacturer's instructions. The relative luciferase activity was calculated as the ratio between the renilla and firefly luciferase activities.

RESULTS

Clinical data

In order to perform ABC gene and miRNA expression profiling, tissues were sampled from HCC and adjacent healthy liver (AHL) from 19 patients. Three patients received chemotherapy (FR06, FR16 and FR17) prior to sampling while 16 were untreated. Seventy-four percent of the HCCs included in the study arose in patients with alcoholic cirrhosis and 16% in patients with hepatitis C virus (HCV)-associated cirrhosis. Thirty-nine percent of the tumors had a size of 31-50mm, 28% of 51-100mm, and 22% of the tumors were smaller than 30mm (**Table 1**).

12 ABC transporters are up-regulated in HCC compared to AHL

To obtain ABC expression signatures in HCC patients, RNA was isolated from 19 paired HCC and AHL samples, and expression of 15 ABC genes was determined by custom-made ABC Taqman microfluidic array. Biological function of the 15 ABC genes is described in **Table 2**. Student's two-tailed paired t-test and Wilcoxon matched-pairs test were performed to determine whether changes in gene expression between AHL and HCC were significant ($P<0.05$). Both tests indicated that twelve ABC genes were significantly up-regulated in HCC compared to the paired AHL control samples (**Fig. 1**). From these genes, ABCA2, ABCC11 and ABCE1 showed a mild up-regulation of 1.6-1.7 fold on average. Higher changes in expression profiles were detected for ABCB1, ABCB6, ABCC1, ABCC2, ABCC3, ABCC4, ABCC5, ABCC10 and ABCC12 genes, which were on average 2.0-5.3 fold up-regulated (**Fig. 1**). Expression of ABCA1, ABCC6 and ABCG2 was not significantly changed. However there was heterogeneity in ABC gene regulation between patients as not 100% of the HCC samples showed an up-regulated profile (**Table S5**). Globally, up-regulation higher than 2 fold was observed in more than 15% of the patients for ABCC6 and ABCG2 and in more than 30% of the HCC patients for ABCA1. Interestingly an up-regulation higher than 2 fold was observed in more than 50% of the HCC patients for ABCB6, ABCC1, ABCC4, ABCC5, ABCC10 and ABCC12. A majority of the patient samples presented an up-regulation higher than 1.5 fold change (**Table S4**).

Association of changes in ABC expression profile with clinical parameters

We subsequently determined whether the changes in expression of ABC genes correlated with clinical parameters *e.g.* treatment, patient gender, differentiation state and size of the tumor. First we determined the effect of HCC treatment, as we included 16 untreated and 3 treated patients in this study. When excluding the 3 treated patients (FR06, FR16 and FR17) from the analysis, up-regulation was significant for 10 ABC genes (ABCA2, ABCB1, ABCB6, ABCC2, ABCC3, ABCC4, ABCC5, ABCC10, ABCC11 and ABCE1). Expression of ABCA1, ABCC1, ABCC6, ABCC12 and ABCG2 was not significantly changed in untreated patients. Out of the 19 patients, only one, FR06, had a stable or down-regulated expression for the entire gene set, with 0.3-1.3 fold changes for all genes. Patient FR06 had a well-differentiated 50mm initial tumor and was previously treated with TACE.

We then determined if there was an association between ABC up-regulation and gender. Expression of ABCC6 was significantly higher in females than in males (female, $n = 4$, $AFC = 2.7$; male, $n = 15$, $AFC = 1.2$; $P = 0.0341$) while for the expression of other ABC genes there was no gender difference. We then correlated the ABC expression data with the

Table 1. Clinical data of the 19 patients from which paired HCC and adjacent healthy liver (AHL) tissue samples were analyzed. Obtained from the CRB-Foie, France.

Patient information (n = 19)		
Age		
Average	67	
Min-max	42-85	
Gender		
Male	15	FR01-03, FR05-08, FR11-FR16, FR18-19
Female	4	FR04, FR09-10, FR17
Etiology		
Alcohol	14	FR01-03, FR05-08, FR10-11, FR14-16, FR18-19
HCV	3	FR04, FR09, FR17
HBV	1	FR12
Alcohol, HCV	1	FR13
Tumor		
Initial	16	FR01-06, FR08-11, FR13-17, FR19
Relapse	3	FR07, FR12, FR18
Treatment		
Portal embolization	1	FR02
TACE/lipiodol	1	FR06
Portal embolization, TACE/lipiodol	2	FR16-17
Non-treated	15	FR01, FR03-05, FR07-15, FR18-19
Tumor size		
NA	1	FR15
< 30mm	4	FR02, FR09, FR12-13
31-50mm	7	FR01, FR05-07, FR11, FR17-18
51-100mm	5	FR03, FR10, FR14, FR16, FR19
> 100mm	2	FR04, FR08
Differentiation		
NA	1	FR15
Intermediate	14	FR01-04, FR07-08, FR11-14, FR16-19
Well-differenciated	4	FR05-06, FR09-10
Peri-tumoral capsule		
NA	1	FR15
Partially circumferential	2	FR16, FR18
Fully circumferential	3	FR07-08, FR10
Over circumferential	2	FR04, FR14
Absent	11	FR01-03, FR05-06, FR09, FR11-13, FR17, FR19
Vascular embolization		
NA	1	FR15
No	18	FR01-14, FR16-19

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Table 2. Biological function and cellular localization of the 15 profiled ABC transporters as described in OMIM and UniProtKB databases.

ABC transporter	(Putative) cellular localization	(Putative) function
ABCA1 (CERP)	Plasma membrane	Cholesterol efflux, anion transporter
ABCA2	Plasma membrane	Sterol transport, reactive oxygen species (ROS) protection
ABCB1 (MDR1)	Apical plasma membrane	Multidrug resistance, transport of organic anions and cations
ABCB6	Outer mitochondrial membrane	Binds heme and porphyrins and functions in their ATP-dependent uptake into the mitochondria
ABCC1 (MRP1)	Basolateral plasma membrane	Multidrug resistance, organic anion transport
ABCC2 (MRP2, cMOAT)	Apical plasma membrane	Multidrug resistance, organic anion transport
ABCC3 (MRP3)	Basolateral plasma membrane	Multidrug resistance, organic anion transport
ABCC4 (MRP4)	Basolateral plasma membrane	Multidrug resistance, organic anion transport
ABCC5 (MRP5)	Plasma membrane	Multidrug resistance, transport of chemotherapeutic agents, 6MP, Platinum
ABCC6 (MRP6)	Basolateral plasma membrane	Multidrug resistance, resistance against inhibitors of topoisomerase II etoposide and teniposide, and the anthracyclines doxorubicin and daunorubicin.
ABCC10 (MRP7)	Plasma membrane	Putatively involved in cellular detoxification through lipophilic anion extrusion
ABCC11 (MRP8)	Plasma membrane	Stimulates the ATP-dependent efflux of a range of physiological and synthetic lipophilic anions, steroid sulfates, glucuronides, the monoanionic bile acids glycocholate and taurocholate, and methotrexate
ABCC12 (MRP9)	Plasma membrane	Putative transporter
ABCE1 (RNS4I)	Cytoplasm, mitochondrion	No putative transport function. Antagonizes the binding of 2-5A (5'-phosphorylated 2',5'-linked oligoadenylates) by RNase L through direct interaction with RNase L and therefore inhibits its endoribonuclease activity, antagonizes the anti-viral effect of the interferon-regulated 2-5A/RNase L pathway
ABCG2 (BCRP)	Apical plasma membrane	Transports broad spectrum of chemotherapeutic agents (mitoxantrone, daunorubicin, doxorubicin, daunorubicin)

differentiation state, and found that up-regulation of ABCC10 was significantly lower in well-differentiated HCC (well-differentiated, n = 4, AFC = 1.40; intermediate differentiation, n = 15, AFC = 3.25; P = 0.0081). Finally, we determined a possible association of ABC expression with tumor size. Up-regulation of ABCB6 and ABCC2 was significantly higher in patients with tumors <30mm than in patients with tumors >31mm (<30mm, n = 4; >31mm, n = 15), with AFC values of respectively 4.6 and 2.3 for ABCB6 (P = 0.0144) and 4.2 and 1.5 for ABCC2 (P = 0.0022).

Total miRNA profiling reveals that 79 miRNAs are down-regulated and 11 are up-regulated in HCC patient samples

We hypothesized that ABC gene expression might be regulated by cellular miRNAs, *i.e.* ABC genes up-regulation in HCC would be the consequence of the down-regulation of cellular miRNAs. In order to obtain miRNA expression signatures, RNA was isolated from 10 HCC and 3 healthy liver (HL) samples. To minimize variation in the miRNA profile, only 10 HCC with alcohol etiology were selected from the 19 available (FR01, FR03, FR05, FR06, FR07, FR08, FR10, FR11, FR14 and FR18). miRNA expression was determined by Taqman 384-well microfluidic array including 378 cellular miRNAs and 6 control wells, and data were normalized to mammalian U6 RNA. In total 361 out of 378 miRNAs were detectable. Changes in miRNA expression between 2 and 40 fold were considered up-regulation and changes between 0 and 0.5 fold were considered down-regulation. Average miRNA expression was compared in HCC and HL groups by two-tailed t-test. miRNA expression in HCC compared to HL control was significantly higher for 11 cellular miRNAs, and lower for 79 miRNAs, which accounted for respectively 3 and 22% of the detectable miRNAs (**Fig. 2** and **Fig. S2**). Analysis of the conservation of the 90 dysregulated miRNAs revealed that 87 were conserved up to the mouse, and 25 up to the chicken (**Table S6**). Next a subset of miRNAs quantified with the microfluidic array was cross-examined on all samples: 19 paired HCC and AHL, and 3 HL. Six miRNAs were selected: miR-135b, miR-145, miR-199a-3p/a/b, and miR-296 because they were consistently down-regulated in the 10 HCC patient samples (low standard deviation). Expression of these 6 miRNAs was quantified using single miRNA Taqman assays (**Fig. 3**). First, miRNA expression in HL from pancreatic cancer patients, and AHL from liver cancer patients was similar (**Fig. 3**). This indicated that the miRNA profile is not affected in healthy liver tissues despite the different background of these samples. Second, differences in miRNA expression observed between paired AHL and HCC samples were significant for miR-145, miR-199a-3p, miR-199a-5p and miR-199b, hence confirming the miRNA signature in HCC from the microfluidic array. These differences were also significant for miR-135b when the 2 patients presenting the highest variation in each group are excluded (**Fig. 3**, miR-135b; FR01; FR12, FR13 and FR19; P = 0.0070). miR-296 presented a down-regulated profile, but differences were not statistically significant. When comparing all 6 miRNA profiles, there was a trend for FR13 to recurrently present the highest variation among the AHL group, for miR-135b, miR-145, miR-199a-3p/a/b. Patient FR13 was an untreated HCV-infected male with a history of alcohol abuse.

Verification of ABC miRNA target prediction

miRNAs can induce post-transcriptional down-regulation of target genes, *i.e.* genes which have in their 3'UTR sequences complementary to a miRNA seed sequence. We

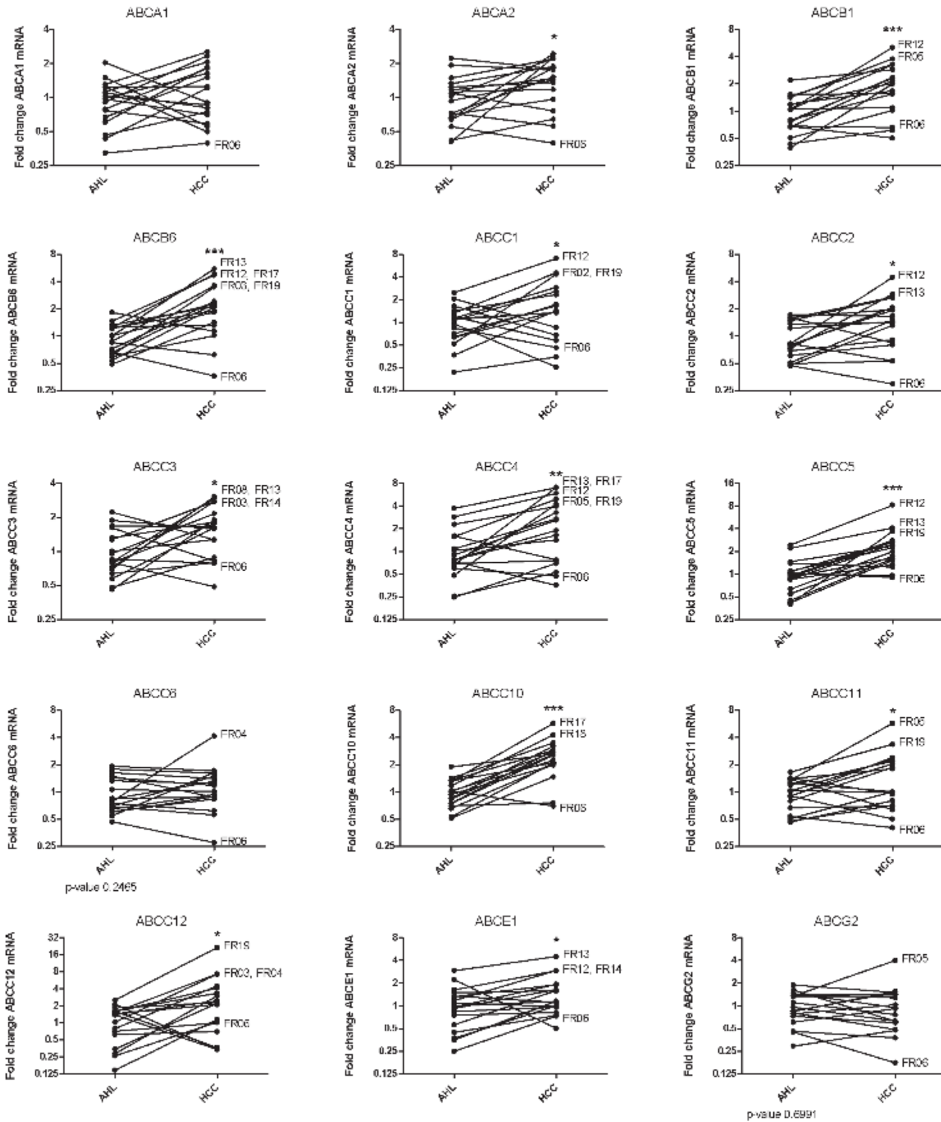

Figure 1. ABC gene profiling in 19 paired HCC patient samples. Expression of 15 ABC genes was determined by custom-made ABC Taqman microfluidic array in 19 paired adjacent healthy liver (AHL) and HCC samples. Ct values were normalized with control 18S rRNA (dCt), each sample was normalized with the average dCt of the AHL group (ddCt), and fold change in expression was calculated according to the $2^{-\Delta\Delta Ct}$ method of Livak and Schmittgen (29). Significant gene up-regulation in HCC is marked by *P<0.05, **P<0.01, ***P<0.001.

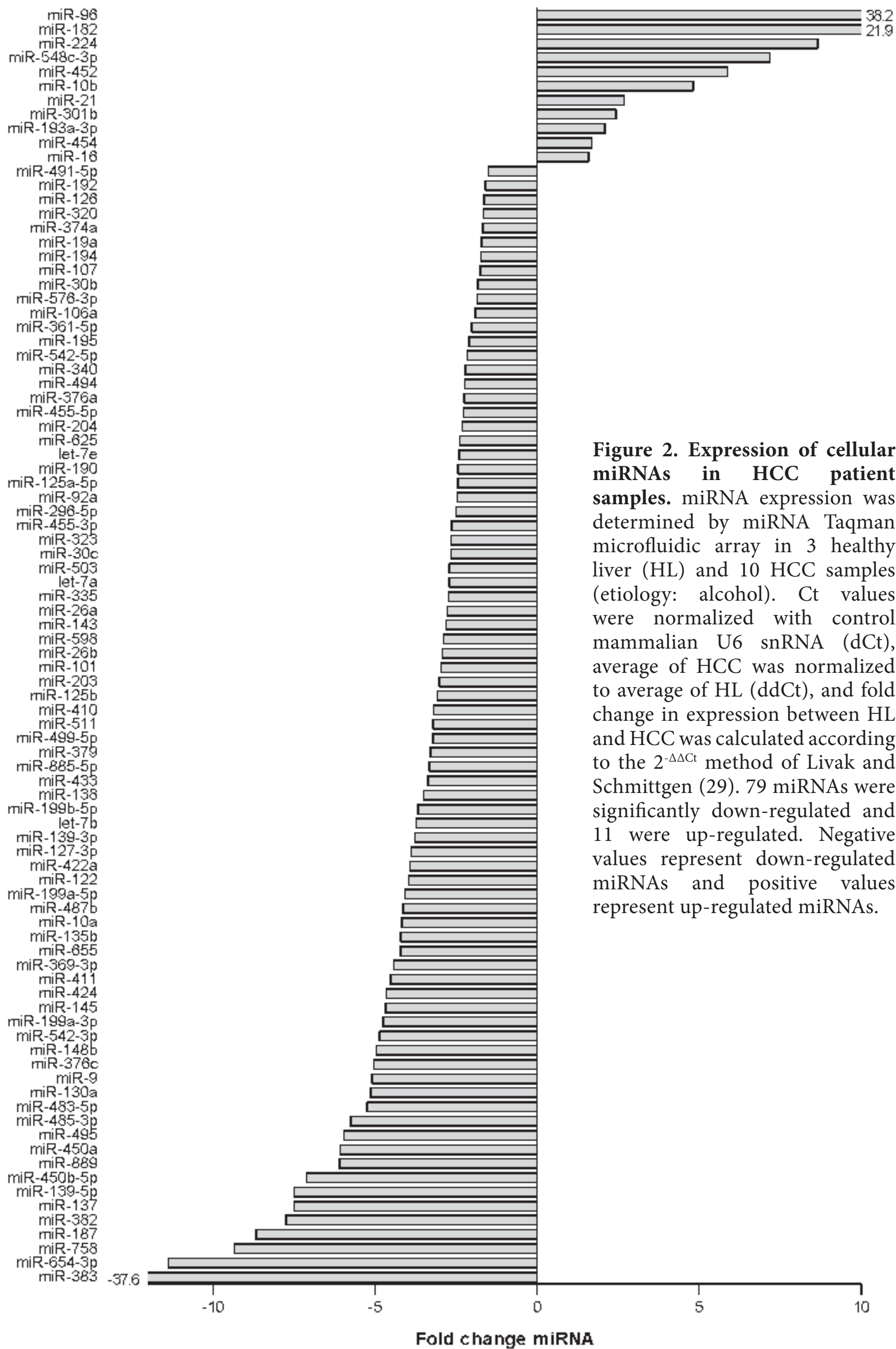

Figure 2. Expression of cellular miRNAs in HCC patient samples. miRNA expression was determined by miRNA Taqman microfluidic array in 3 healthy liver (HL) and 10 HCC samples (etiology: alcohol). Ct values were normalized with control mammalian U6 snRNA (dCt), average of HCC was normalized to average of HL (ddCt), and fold change in expression between HL and HCC was calculated according to the $2^{-\Delta\Delta Ct}$ method of Livak and Schmittgen (29). 79 miRNAs were significantly down-regulated and 11 were up-regulated. Negative values represent down-regulated miRNAs and positive values represent up-regulated miRNAs.

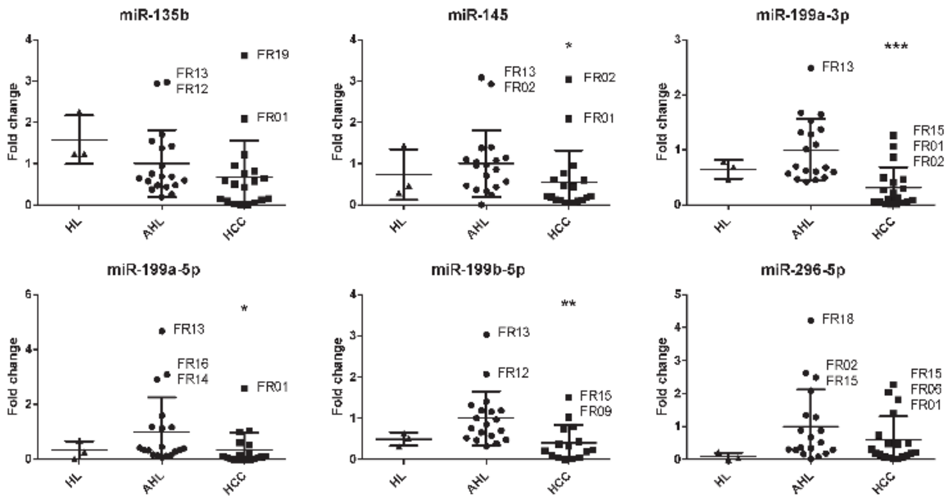

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Figure 3. Verification of miRNA expression profile on selected miRNAs in HCC patient samples. Among the 79 down-regulated miRNAs, miR-135b, miR-145, miR-296, miR-199a-3p, miR-199a, miR-199b were consistently down-regulated (low standard deviation among 10 HCC). Therefore miRNA expression was determined on the entire sample set (3 healthy liver, HL; 19 adjacent healthy liver, AHL and 19 HCC) by small RNA Taqman. Each sample was measured in triplicate and fold change in expression between AHL and HCC was calculated according to the $2^{-\Delta\Delta Ct}$ method of Livak and Schmittgen (29). Down-regulation of miR-145, miR-199a-3p, miR-199a, miR-199b, miR-135b in HCC compared to paired AHL was confirmed except for miR-296. Significant miRNA down-regulation in HCC is marked by *P<0.05, **P<0.01, ***P<0.001.

hypothesized that some down-regulated miRNAs are regulating ABC expression and that this is associated with HCC. Since most of the ABC genes were up-regulated in HCC samples, we concentrated on the down-regulated miRNAs as potentially influencing ABC expression. Interestingly, from the 79 down-regulated miRNAs, 25 had predicted targets in up-regulated ABC genes. We therefore determined *in silico* miRNA target sequences in the 3'UTRs of the up-regulated ABC genes, and cross-analyzed this data with the down-regulated miRNAs (**Table S1** and **Table S2**). Twenty-four cellular miRNAs were cloned in the expression vector pcDNA6.2 and Luc-ABC reporters where the 3'UTR of the 6 ABC genes was cloned into the dual luciferase vector psiCheck-2 were made for 6 ABC genes. Because the 3'UTR of ABCA1 is 3.3kb, we made three Luc-ABCA1 variants: Luc-ABCA1-5' contains the 5' end of the 3'UTR, ABCA1-3' the 3' end, and ABCA1 is a composite containing 246 nt from the 5' end and 303 nt from the 3' end of the 3'UTR (**Fig. S2**). We subsequently validated the *in silico* miRNA target predictions *in vitro* using a luciferase reporter system. Based on the *in silico* predictions, we co-transfected HEK293T cells with the Luc-ABC reporter plasmids that contained the miRNA predicted targets and the respective miRNAs and measured luciferase expression. Knock-down of luciferase expression would indicate that the ABC transporter is a possible target for the specific miRNA. To determine which miRNAs were efficiently down-regulating the corresponding Luc-ABC reporters, we considered all miRNAs that inhibited luciferase expression below a set threshold of 0.5 as possibly targeting the ABC gene. With this method we were able to experimentally

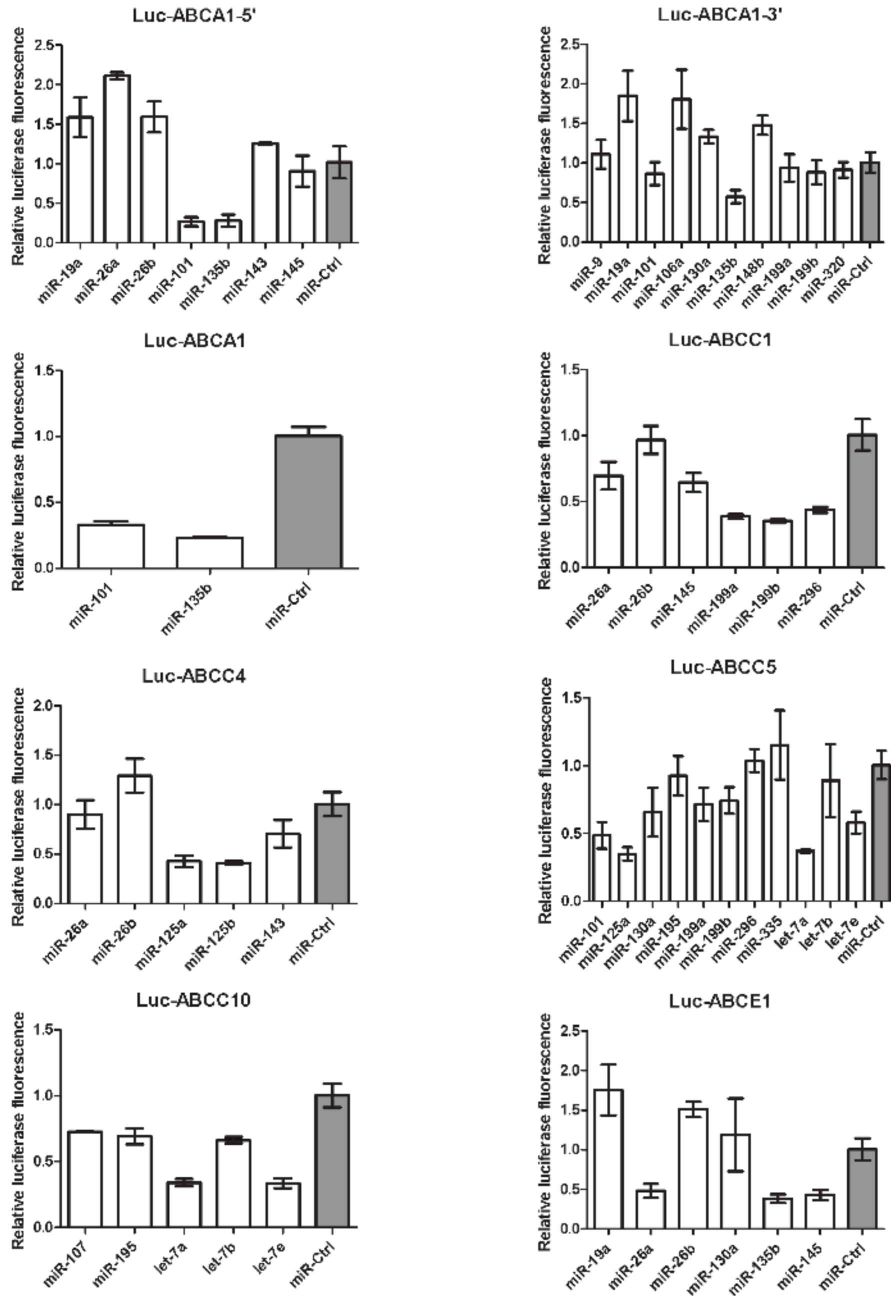

Figure 4. Down-regulation of Luc-ABC reporters by cellular miRNAs associated with HCC phenotype. HEK293T cells were transfected with 5ng Luc-ABC reporter plasmid and 150ng miRNA expression plasmid. Luciferase assay was performed 72hr post-transfection. Renilla luciferase was normalized with firefly luciferase (relative luciferase fluorescence, RLF), then RLF of each miRNA was normalized to RLF of miR-Control (miR-Ctrl) which was set at 1. Results are presented as the average of 3-4 experiments ± SD.

confirm 15 ABC miRNA targets out of the 51 associations that were bioinformatically predicted (**Fig. 4**). miR-101 and miR-135b down-regulated Luc-ABCA1-5', however they had no effect on Luc-ABCA1-3' expression. Interestingly, the composite Luc-ABCA1 was down-regulated by miR-101 and miR-135b by respectively 67% and 77% indicating an additive effect of the targets at 5' and 3' ends of the 3'UTR. Several other miRNA targets were verified with the Luc-ABC assay: Luc-ABCC1 was down-regulated by miR-199a/b and miR-296, Luc-ABCC4 was down-regulated by miR-125a/b, Luc-ABCC5 was down-regulated by miR-101, miR-125a and let-7a, Luc-ABCC10 was down-regulated by let-7a/e, and Luc-ABCE1 was down-regulated by miR-26a, miR-135b and miR-145 (**Fig. 4**). To experimentally verify the miRNA targets, we next mutated the predicted miRNA targets by changing the seed sequences of miRNAs in the 6 Luc-ABC reporters. We were able to confirm all Luc-ABC as *bona fide* miRNA targets, except for Luc-ABCC4 which was not clearly affected when the miR-125a/b sites were mutated (**Fig. 5**).
Finally, for each paired HCC patient sample we tested the correlation between a specific ABC expression profile and a corresponding validated miRNA (**Fig. 6** and **Fig. S3**). We expected an inverse ABC-miRNA correlation, therefore tumors with a high ABC expression should simultaneously present a low validated miRNA levels and *vice versa*. As anticipated, our positive control, the previously published ABCE1/miR-203 pair presented a good qualitative correlation with 9 out of 10 tumors having high ABCE1 and low miR-203 levels (**Fig. S3**). However the correlation coefficient R^2=0.6433 indicating that the samples do not fit a linear regression, likely due to the low number of samples (n=10) and the absence of samples displaying down-regulated ABCE1 expression in the sample set. We therefore discarded R^2 as a quantitative readout and determined only a qualitative response, *i.e.* if for each ABC/miRNA pair, a majority of tumors present a high ABC expression and a low validated miRNA level. ABCC5/miR-101 pair presented a good correlation with 9 out of 10 HCC samples being high for ABCC5 and low for miR-101 (**Fig. 6**). ABC/miRNA pairs ABCC5/let-7a, ABCC5/mir-125a and ABCC5/miR-125b showed similar results (**Fig. 6**). The other verified ABC/miRNA pairs also showed inverse correlations in expression profile in each individual patient tumor (**Fig. S3**). This negative correlation would require validation on a larger sample set but provides indication of a miRNA regulation of ABC genes in HCC.

DISCUSSION

In the current study, we have quantified the expression of 15 ABC transporters in 19 paired HCC and AHL patient samples. The majority had not received chemotherapy prior to sampling (16/19 untreated patients) and in most (14/19) the etiology was alcoholic cirrhosis. We showed that 12 ABC genes were up-regulated in HCC. In several patients the ABC genes were up-regulated up to two-fold and the physiological relevance of such a mild regulation needs additional attention. We speculate that in the context of chemotherapy, even changes of 1.5 fold may tip the toxic concentration of the drug due to changes in efflux activity of the ABC genes in the tumor cells, therefore resulting in a significant physiological effect. Up-regulation of some of these transporters has been described previously, *e.g.* ABCB1 (7, 8) and ABCC3 (9) were up-regulated in HCC. The expression of three ABC genes, namely ABCA1, ABCC6 and ABCG2, was not significantly changed in this study. Interestingly, ABCA1 and ABCG2 down-regulation was shown in HCC compared to adjacent healthy liver in patients of unknown treatment status (11), and the two genes were

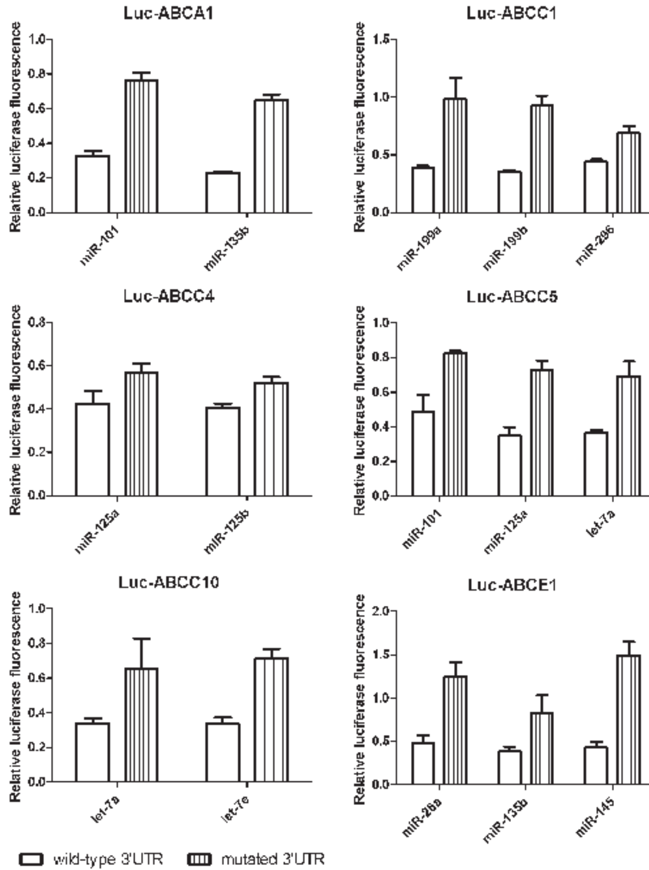

Figure 5. Mutation of miRNA target sites abrogates inhibition of Luc-ABC reporters. The miRNAs seed sequences were mutated to create mutated Luc-ABC reporters. HEK293T cells were co-transfected with 5ng mutated Luc-ABC reporter plasmid (wild-type or mutated) and 150ng miRNA expression plasmid. Luciferase assay was performed 72hr post-transfection. Renilla luciferase was normalized with firefly luciferase (relative luciferase fluorescence, RLF), then RLF of each miRNA was normalized to RLF of miR-Control (miR-Ctrl) which was set at 1. Wild-type reporters are represented as solid bars; mutated reporters are represented as striped bars. Results are presented as the average of 3-6 experiments ± SD.

respectively 14.6 and 9.3 fold up-regulated in TACE-treated samples (30). These mixed results may indicate a high variability in the expression of ABCA1 and ABCG2 in HCC patients, possibly linked to treatment status. In our study, only one patient presented a stable or down-regulated expression of all 15 transporters. This patient, FR06, had received transarterial chemoembolization (TACE) treatment, but as the other two TACE-treated patients did not present such profile, we cannot make any conclusion on the effect of TACE on ABC expression. Second, we were able to demonstrate that in the 16 untreated patients, the expression of 10 ABC transporters was significantly up-regulated in HCC compared to paired AHL samples. In untreated patients, only up-regulation of ABCC1 has been shown to be associated with a more aggressive HCC phenotype (10).

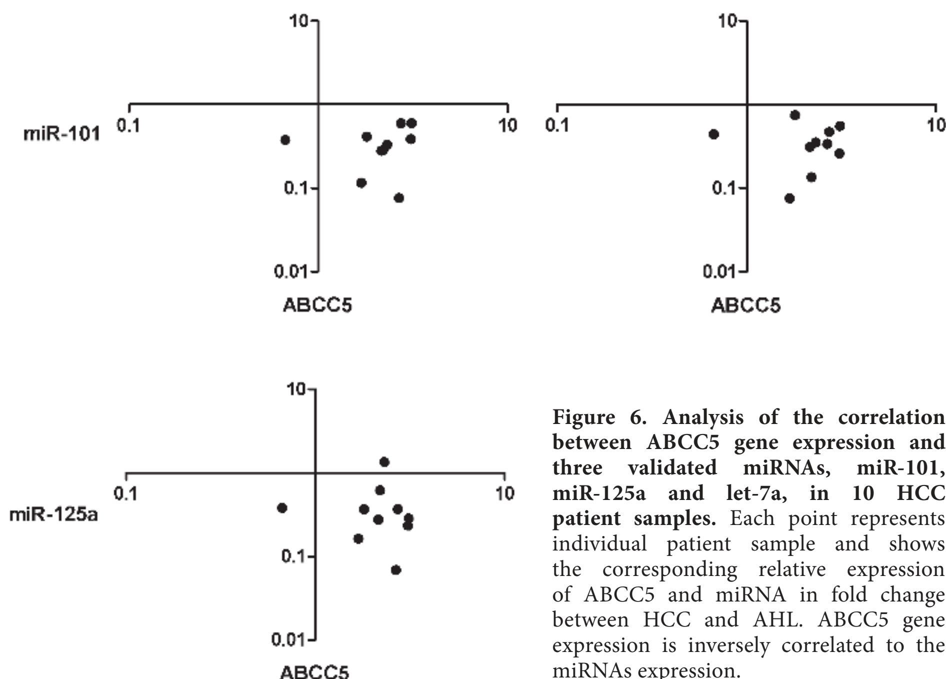

Figure 6. Analysis of the correlation between ABCC5 gene expression and three validated miRNAs, miR-101, miR-125a and let-7a, in 10 HCC patient samples. Each point represents individual patient sample and shows the corresponding relative expression of ABCC5 and miRNA in fold change between HCC and AHL. ABCC5 gene expression is inversely correlated to the miRNAs expression.

To our knowledge this is the first publication reporting up-regulation of a broad range of ABC transporters in untreated HCC patients. This includes 8 ABC genes, namely ABCA2, ABCB6, ABCC2, ABCC3, ABCC5, ABCC10, ABCC11 and ABCE1, whose association with HCC has not been reported so far. Due to the size of some sub-populations, the described associations between ABC profile and clinical parameters will have to be confirmed on a larger patient population. Nevertheless, the results of the ABC profiling raise the question of the possible regulation pathways implicated in the phenomenon of ABC genes up-regulation.

We chose to further investigate the possibility of miRNA-mediated ABC gene regulation in association with HCC. So far only miR-203 has been reported to specifically target ABCE1 (27) but its implication in HCC still needs to be determined. Our miRNA screen in 10 HCC patients identified significant changes in expression of 90 miRNAs, including 11 up-regulated miRNAs and 79 down-regulated miRNAs, of which 25 had predicted ABC targets. Interestingly, 97% of the dysregulated miRNAs were highly conserved in mammals (up to the mouse), indicating their possible association with HCC, as has been shown for several diseases (31). Seventy-nine of 90 dysregulated miRNAs are down-regulated and this is in agreement with the evidence that miRNAs are generally down-regulated in cancer (32). Many miRNAs identified during the current screen confirmed findings obtained with larger sample sets or sample sets coming from patients with a different cancer etiology. Similar to other publications, we observed down-regulation of miR-101 (33), miR-122 (34-37), miR-125a (37, 38), miR-130a (34, 39), miR-145 (39-41), miR-199a (33, 36, 38, 39), miR-199b (38, 40) and up-regulation of miR-21 (33-37, 40-42). Seventy four percent of our patient had alcoholic cirrhosis, and ethanol-treatment has been linked in the literature to the miR-199

family (43). However, no correlation between the miRNA profiles and viral HCC etiology could be determined, probably due to the small patient size (14 alcohol, 3 HCV, 1 HBV, and 1 alcohol/HCV). Our screen also identified 12 miRNAs with predicted ABC targets that have not been previously associated with HCC. Down-regulated miRNAs were reported to be repressed by oncogenes: miR-145 by Ras (44), and miR-26a and miR-195 by Myc (45) as well as let-7 (45, 46). The most down-regulated miRNAs, miR-383 and miR-654-3p, had no putative targets in any of the 15 ABC genes. These 2 miRNAs, together with the 2 most up-regulated miRNAs, miR-96 and miR-182, require further study for understanding their relevance in HCC. Down-regulated miRNAs are of interest because they can act as tumor suppressors (39). Cellular miRNAs can also act as oncogenes (47), and their up-regulation in cancer will cause down-regulation of their tumor-suppressive targets. In general, these miRNAs are potentially relevant for HCC therapy: tumor suppressor miRNAs can be introduced back in a cancer cell, thereby repressing tumorogenesis, and oncogenic miRNAs can be inhibited by using synthetic miRNA antagonists or virally-delivered sponge-like sequences (48, 49). This brings exciting possibilities for the use of miRNAs as therapeutics.

In the current study, we experimentally verified 13 predicted miRNAs targets in 5 ABC genes using luciferase reporter assays. We were able to prove that the miRNA effect was sequence-specific by mutating the targets in the reporters and by co-transfecting miRNAs not having targets in ABC genes (data not shown). Except for ABCC4, our mutational analysis revealed some new miRNA targets in ABC genes. Strikingly, we were able to show that for several miRNA-ABC pairs, a very high proportion of the analyzed tumors have an increased ABC gene expression level together with a reduced level of miRNA. Thus far the only evidence of miRNA-mediated regulation of ABC gene expression in HCC has been provided by Furuta *et al.* (27) who showed that miR-203 regulates the expression of ABCE1, which is involved in translation initiation, but has not been linked to multidrug resistance. In this perspective, we are currently working on *in vitro* validation of the miRNA-mediated regulation of endogenous ABC gene expression with a special focus on the ABCC subfamily. Future research will concentrate on delivery of these miRNAs as gene therapy, either in miRNA-replacement therapy for HCC or as a novel indirect strategy to induce down-regulation of ABC transporters instead of direct shRNA- or artificial miRNA-mediated gene therapy approaches (50). The focus should be on ABCB6, ABCC1, ABCC4, ABCC5, ABCC10 and ABCC12 as these genes were up-regulated in more than 50% of the patients.

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SUPPLEMENTARY MATERIAL

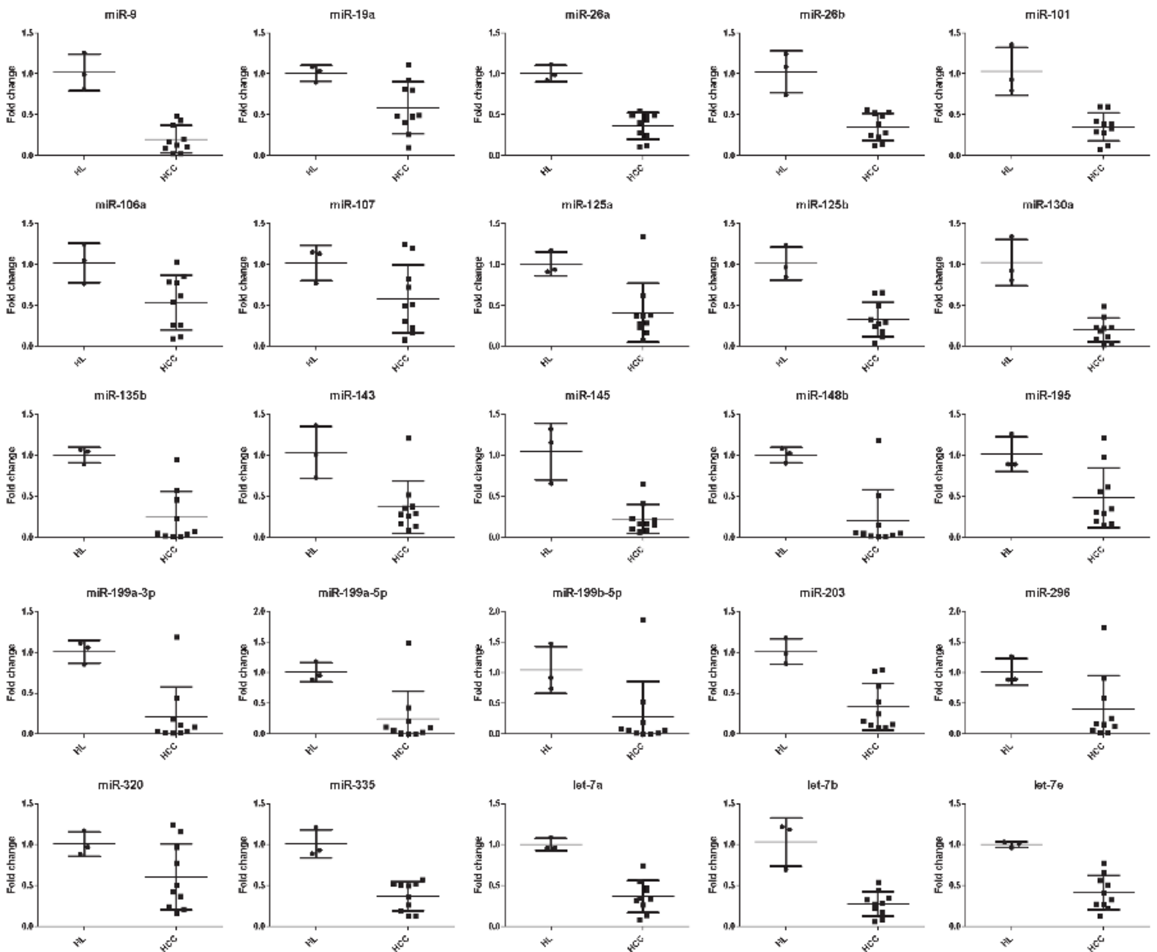

Figure S1. Down-regulation of a subset of 25 cellular miRNAs in HCC patient samples. miRNA expression was determined by miRNA Taqman microfluidic array in 3 healthy liver (HL) and 10 HCC samples (etiology: alcohol). Ct values were normalized with control mammalian U6 snRNA (dCt), average of HCC was normalized to average of HL (ddCt), and fold change in expression between HL and HCC was calculated according to the $2^{-\Delta\Delta Ct}$ method of Livak and Schmittgen (29). 79 miRNAs were significantly down-regulated; a subset of 25 is presented here.

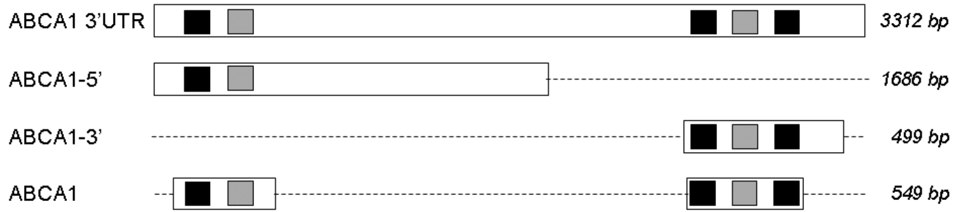

Figure S2. Schematic representation of ABCA1 3'UTR (middle part), the fragments used in Luc-ABCA1-5' and Luc-ABCA1-3' (upper part) and the fragments used in the composite Luc-ABCA1 (lower part).

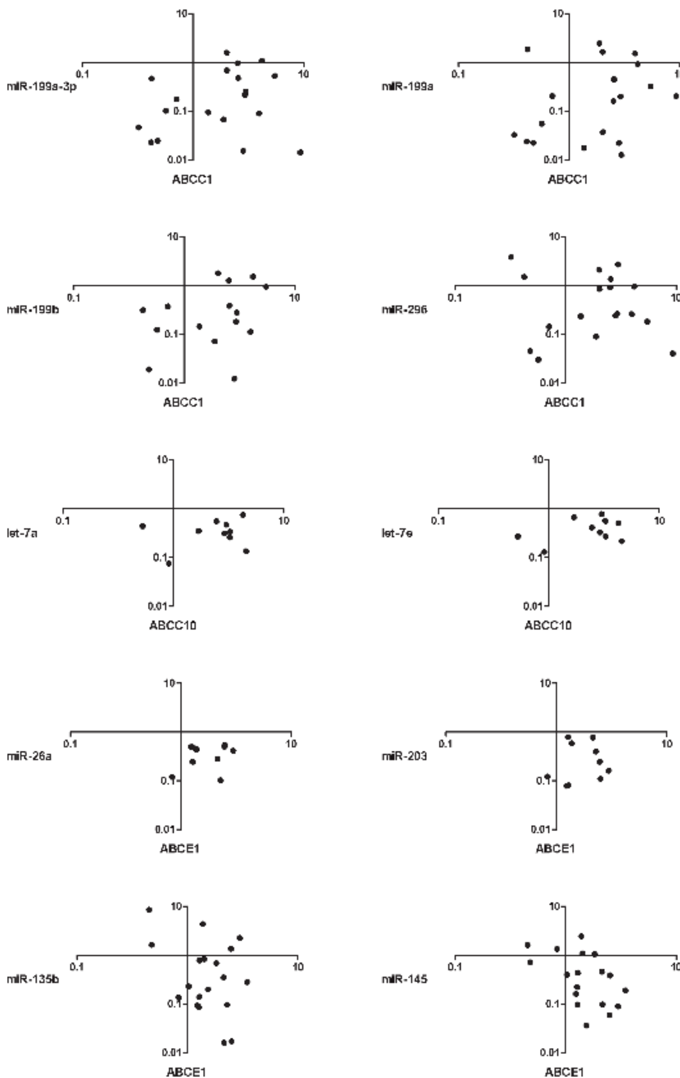

Figure S3. Analysis of the correlation between ABC genes expression and the expression of validated miRNAs, in 10 or 19 patient samples (miRNA profiling in all 19 patient samples was done only for miR-135b, miR-199a-3p, miR-199a, miR-199b and miR-296). Each point represents one patient sample and shows the corresponding gene and miRNA relative expression (fold change between HCC and adjacent healthy liver (AHL)).

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Table S1. miRNA target predictions in ABC genes. 51 miRNA targets were predicted in the 3'UTR of 6 out of 15 ABC genes using TargetScan and PicTar algorithms. miRNAs targets are summarized in the table, where x represent a single target sequence in the 3' untranslated region (3'UTR); xx, two targets sequences in the 3'UTR; xxx, three targets sequences. Detailed prediction analysis is presented in **Table S2**. miR-203-mediated regulation of ABCE1 was verified (27).

	Predicted targets					
miRNA	**ABCA1**	**ABCC1**	**ABCC4**	**ABCC5**	**ABCC10**	**ABCE1**
miR-9	x					
miR-19a	xx					x
miR-26a/b	x	x	x			xxx
miR-101	xxx			x		
miR-106a	x					
miR-107					x	
miR-125a/b			x	x		
miR-130a	x			x		x
miR-135b	xx					x
miR-143	x		x			
miR-145	x	x				xx
miR-148b	x					
miR-195				x	x	
miR-199a-3p	xxx			x		
miR-199a/b	x	x		x		
miR-203						x
miR-296		x		x		
miR-320	x					
miR-335				x		
let-7a/b/e				x	x	

Table S2. miRNA targets were predicted in the 3' untranslated region (3'UTR) of the ABC genes, using algorithms TargetScan, PicTar, and additional manual screening. Targets were identified in the 3'UTR of 6 ABC genes. miRNAs selected for further validation are bold.

Target	miRNA	Match	Position	Sequence	Prediction		
ABCA1	miR-145	7mer-m8	109-115	AACUGGA	TargetScan	PicTar	
	miR-144	8mer	115-121	AUACUGUA	TargetScan	PicTar	
	miR-33a/miR-33b	8mer	134-140	CAAUGCAA	TargetScan		
	miR-33a/miR-33b	8mer	139-145	CAAUGCAA	TargetScan		
	miR-33a/miR-33b	8mer	149-155	CAAUGCAA	TargetScan		
	miR-124/miR-506	7mer-m8	184-190	GUGCCUU	TargetScan		
	miR-26a	7mer-1A	226-232	ACUUGAA	TargetScan	PicTar	
	miR-26b/miR-1297	7mer-1A	226-232	ACUUGAA	TargetScan	PicTar	
	miR-23a/miR-23b	7mer-1A	254-260	AUGUGAA	TargetScan		
	miR-96/miR-1271	7mer-m8	498-504	GUGCCAA	TargetScan	PicTar	
	miR-183	7mer-m8	1366-1372	GUGCCAU	TargetScan	PicTar	
	miR-27a/miR-27b	8mer	2592-2598	ACUGUGAA	TargetScan	PicTar	
	miR-128	7mer-1A	2592-2598	ACUGUGA	TargetScan	PicTar	
	miR-101	7mer-1A	2686-2692	UACUGUA	TargetScan	PicTar	
	miR-144	7mer-1A	2686-2692	UACUGUA	TargetScan	PicTar	
	miR-9	7mer-1A	2832-2838	CCAAAGA	TargetScan	PicTar	
	miR-140	7mer-m8	2860-2866	AACCACU	TargetScan	PicTar	
	miR-1/miR-206/miR-613	7mer-1A	3070-3076	CAUUCCA	TargetScan		
	miR-17/miR-20a/miR-20b/ miR-93/miR-106a/ miR-106b/miR-519d	8mer	3078-3084	GCACUUUA	TargetScan	PicTar	
	miR-19a/miR-19b	7mer-m8	3109-3115	UUUGCAC	TargetScan	PicTar	
	miR-130a/miR-130b/ miR-301a/miR-301b/ miR-454	7mer-m8	3110-3116	UUGCACU	TargetScan	PicTar	
	miR-148a/miR-148b/ miR-152	7mer-m8	3111-3117	UGCACUG	TargetScan	PicTar	
	miR-199a/miR-199b	7mer-1A	3113-3119	CACUGGA	TargetScan		
	miR-145	7mer-1A	3114-3120	ACUGGAA	TargetScan	PicTar	
	miR-340	7mer-m8	3223-3229	CUUUAUA	TargetScan		
	miR-200b/miR-200c/ miR-429	7mer-1A	3233-3239	AGUAUUA	TargetScan		
	miR-758	8mer	3271-3277	GUCACAAA	TargetScan		
	miR-135b	7mer-m8		AAGCCAU			Manual
	miR-143	7mer-m8		UCAUCUC			Manual

Table S2. miRNA targets were predicted in the 3' untranslated region (3'UTR) of the ABC genes, using algorithms TargetScan, PicTar, and additional manual screening. Targets were identified in the 3'UTR of 6 ABC genes. miRNAs selected for further validation are bold.

Target	miRNA	Match	Position	Sequence	Prediction		
ABCC1	miR-133a/miR-133b	7mer-1A	366-372	GACCAAA	TargetScan		
	miR-7	8mer	590-596	GUCUUCCA	TargetScan		
	miR-199a/miR-199b	8mer	1726-1732	ACACUGGA	TargetScan		
	miR-145	7mer-1A	1728-1734	ACUGGAA	TargetScan		
	miR-217	7mer-1A	1746-1752	UGCAGUA	TargetScan		
	miR-26a	7mer-1A		ACUUGAA			Manual
	miR-26b/miR-1297	7mer-1A		ACUUGAA			Manual
	miR-296	7mer-m8		GGGCCCU			Manual
ABCC4	miR-124/miR-506	8mer	260-266	GUGCCUUA	TargetScan		
	miR-125a/miR-125b	7mer-1A	376-382	UCAGGGA	TargetScan	PicTar	
	miR-218	7mer-m8	1398-1404	AAGCACA	TargetScan		
	miR-143	7mer-m8	1731-1737	UCAUCUC	TargetScan		
	miR-26a	7mer-1A		ACUUGAA			Manual
	miR-26b/miR-1297	7mer-1A		ACUUGAA			Manual
ABCC5	miR-130a	7mer-m8	223-229	UUGCACU		PicTar	
	miR-125a/miR-125b	7mer-1A	238-244	UCAGGGA	TargetScan	PicTar	
	miR-128	7mer-m8	373-379	CACUGUG	TargetScan		
	miR-296	7mer-m8	547-553	GGGCCCU		PicTar	
	miR-195	7mer-m8	927-933	UGCUGCU		PicTar	
	miR-335	7mer-m8	1084-1090	GGUCUUG		PicTar	
	miR-199a/miR-199b	7mer-m8	1104-1110	ACACUGG	TargetScan	PicTar	
	miR-101	8mer	1124-1130	GUACUGUA	TargetScan	PicTar	
	miR-144	7mer-1A	1125-1131	UACUGUA	TargetScan	PicTar	
	miR-98/let-7a/let-7b/let-7c/ let-7d/let-7e/let-7f/let-7g/ let-7i	8mer	1139-1145	CUACCUCA	TargetScan	PicTar	
ABCC10	miR-98/let-7a/let-7b/let-7c/ let-7d/let-7e/let-7f/let-7g/ let-7i	8mer	98-105	CUACCUCA		PicTar	
	miR-107	7mer-m7		UGCUGCU			Manual
	miR-195	7mer-m8		UGCUGCU			Manual

Table S2. miRNA targets were predicted in the 3' untranslated region (3'UTR) of the ABC genes, using algorithms TargetScan, PicTar, and additional manual screening. Targets were identified in the 3'UTR of 6 ABC genes. miRNAs selected for further validation are bold.

Target	miRNA	Match	Position	Sequence	Prediction	
ABCE1	miR-135a/miR-135b	7mer-m8	22-28	AAGCCAU	TargetScan	
	miR-203	8mer	235-241	CAUUUCAA	TargetScan	
	miR-141/miR-200a	7mer-m8	326-332	CAGUGUU	TargetScan	
	miR-182	7mer-m8	378-384	UUGCCAA	TargetScan	
	miR-130a/miR-130b/ miR-301a/miR-301b/ miR-454	8mer	495-501	UUGCACUA	TargetScan	
	miR-29a/miR-29b/miR-29c	7mer-m8	595-601	UGGUGCU	TargetScan	
	miR-145	8mer	1118-1124	AACUGGAA	TargetScan	
	miR-19a/miR-19b	7mer-m8	3109-3115	UUUGCAC		Manual
	miR-26a	7mer-1A		ACUUGAA		Manual
	miR-26b/miR-1297	7mer-1A		ACUUGAA		Manual

Table S3. Oligonucleotides used in this study. Genome sequences around the primary miRNA (pri-miRNA) in the PCR primers are in bold.

Name	Sequence (5’-3’)	Target	
Luc-ABCA1-5’-f	GTACTCGA**GTAAAGAGGAACTAGAC**	ABCA1-5’	1686bp
Luc-ABCA1-5’-r	ATGGCGGCC**GCTCCATATGGAAGTATT**		
Luc-ABCA1-3’-f	GTACTCGA**GCCCAGCACAATTCATTGTT**	ABCA1-3’	499bp
Luc-ABCA1-3’-r	ATGGCGGCCG**CTAGTTTTGCAATTCTCAG**		
Luc-ABCC1-f	GTACTCGAG**CTGCAGTTTTGTGGTTGAG**	ABCC1	1254bp
Luc-ABCC1-r	ATGGCGGCCGC**TACTGCAAATGTTCGCAT**		
Luc-ABCC4-f	GTACTCGAG**GAGTAGGACAAAGTTGTCA**	ABCC4	1447bp
Luc-ABCC4-r	ATGGCGGCCGC**TCTGAATGGAGATGAAAAC**		
Luc-ABCC5-f	GTACTCGAG**GGAGAGTCATATTTTGA**	ABCC5	1026bp
Luc-ABCC5-r	ATGGCGGCCGC**AACACACCAAACCACACAGC**		
Luc-ABCC10-f	GTACTCGAG**TGCTAGAGCCCAGTTCAG**	ABCC10	761bp
Luc-ABCC10-r	ATGGCGGCCGC**CACCAAGGAGTGATTTCCA**		
Luc-ABCE1-f	GTACTCGAG**ACTGACTCTGAGAATATTG**	ABCE1	1898bp
Luc-ABCE1-r	ATGGCGGCCGC**GGTAGTATAATAAACTGTTCC**		
miR-9-f	GTAGGATCC**TCTGGATCGGGGTCAACTCC**	miR-9	720bp
miR-9-r	ATGCTCGAG**GACTGTGACTCCTACCTGT**		
miR-19a-f	GTAGGATCC**AACAGGACAGTTTGATTGGG**	miR-19a	508bp
miR-19a-r	ATGCTCGAG**ATTATGGTGACAGCTGCCTC**		
miR-26a-f	GTAGGATCC**ACGTGACTGTAAGCATGACT**	miR-26a	466bp
miR-26a-r	ATGCTCGAG**CAACAGCTGCAGACTCCCA**		
miR-26b-f	GTAGGATCC**AGGCGCACAGGAAGGAGACT**	miR-26b	529bp
miR-26b-r	ATGCTCGAG**AAGAGGCAGTGGTGGGCTT**		
miR-101-f	GTAGGATCC**GTTCATCCTCATTAATATGG**	miR-101	462bp
miR-101-r	ATGCTCGAG**CAGAATAACTCTCCCTATG**		
miR-106a-f	GTAGGATCC**AACCTAGCTTAGACTCTGTA**	miR-106a	443bp
miR-106a-r	ATGCTCGAG**TGCTGTATATTAGGCAGCCT**		
miR-107-f	GTAGGATCC**ACAACTACATATCCTTAGG**	miR-107	578bp
miR-107-r	ATGCTCGAG**TGTTCCATGCCTCAACTCCT**		
miR-125a-f	GTAGGATCC**TCTGACTCCCTCTTATTCT**	miR-125a	435bp
miR-125a-r	ATGCTCGAG**TCTCAAGGCCAGGGGAGAAG**		
miR-125b-f	GTAGGATCC**AGATACTGCGTATGTGTGT**	miR-125b	407bp
miR-125b-r	ATGCTCGAG**AGCTGCCACTCTCTGGTCA**		
miR-130a-f	GTAGGATCC**AAGCCCGAAGTATCCCTGCA**	miR-130a	510bp
miR-130a-r	ATGCTCGAG**TGTCTATCACAAGCACTGCA**		
miR-135b-f	GTAGGATCC**TGGAGTGAGGACCATTGTGT**	miR-135b	545bp

3

Table S3. Oligonucleotides used in this study. Genome sequences around the primary miRNA (pri-miRNA) in the PCR primers are in bold.

Name	Sequence (5'-3')	Target	
miR-135b-r	ATGCTCGAG**AATCTCCCTCTGGAATCAC**		
miR-143-f	GTAGGATCC**TCCTGGGTGCTCAAATGGCA**	miR-143	467bp
miR-143-r	ATGCTCGAG**CTCATGCTAAGATGGACACA**		
miR-145-f	GTAGGATCC**TTGAAGTTCCGGTCACTACT**	miR-145	506bp
miR-145-r	ATGCTCGAG**CCTGGTTCAACCGCTGCTCA**		
miR-148b-f	GTAGGATCC**GATTCATTTGCAGCAGCCTA**	miR-148b	416bp
miR-148b-r	ATGCTCGAG**AATAACTCCCAGCTACTGAG**		
miR-195-f	GTAGGATCC**ATCCTGGAGAAGTGACAC**	miR-195	514bp
miR-195-r	ATGCTCGAG**AGCAAGGCCTGCACGAGAGA**		
miR-199a-f	GTAGGATCC**TAGGATTTCCTGAAAACCCA**	miR-199a	473bp
miR-199a-r	ATGCTCGAG**TGGGGATGGCAGACTGATAG**		
miR-199b-f	GTAGGATCC**ACGTCAAAGGAGGCAGAAG**	miR-199b	455bp
miR-199b-r	ATGCTCGAG**ATCCTCTCAGTCTTCCTCGG**		
miR-203-f	GTAGGATCC**TCTAAGGCGTCCGGTACGGC**	miR-203	617bp
miR-203-r	ATGCTCGAG**ATTCCACGGAGTTTCGAGGT**		
miR-296-f	GTAGGATCC**TCCTTGGAGCTGAGATGGAG**	miR-296	466bp
miR-296-r	ATGCTCGAG**CACGGCGAGTTCACCATTC**		
miR-320-f	GTAGGATCC**GTCACAACCTCACCTGCAA**	miR-320	507bp
miR-320-r	ATGCTCGAG**TCCAAGGTCACCATGGAGAA**		
miR-335-f	GTAGGATCC**TCTCTCTCCTTACCATCCCT**	miR-335	692bp
miR-335-r	ATGCTCGAG**ACTCAGCTCTGTGTTGCTT**		
let-7a-f	GTAGGATCC**AACCACAGCATAGATTATGC**	let-7a	604bp
let-7a-r	ATGCTCGAG**CTACTACCTCACTCTGATAG**		
let-7b-f	GTAGGATCC**CACATGGCACAATCTGAA**	let-7b	487bp
let-7b-r	ATGCTCGAG**TTCATGGTCAGAACAGCTT**		
let-7e-f	GTAGGATCC**TGGACTCCTGGGTTCCTTGG**	let-7e	641bp
let-7e-r	ATGCTCGAG**CACAGAGACACAGCAGAGAT**		

Table S4. Sequences of the mutated ABC 3' untranslated region (3'UTR) that were synthesized and cloned into psiCheck-2 vector to construct mutated Luc-ABC reporters and composite ABCA1 3'UTR.

Name	Sequence (5'-3')	Size
Luc-ABCA1	GCGGCCGCAAATCCTTATGGAAATAGAAACATTCTAAG- GGGGATGCAACAATTTTGAAAAGAATTAGAGCAATATT- TCTACAGTATTACATTATTACTAGTAGATAATAACAAGGGTA- CAAATTAATGTGATATATACAACCACAAGAAGAAAACACA- GACAAATGGCTTTAGTCAATGATTACTATACAGTGAAT- GAATGATGTGCAACATTTAATAGTCACAAAGCATTTGCTT- TCAGTACAGATAATGAAATACAGTAGTGTGAGGTTTGGTT- GTTTTTTAACAATGAATTGTGCTGGGCATTGCCAGCAAT- GGATGTGTCACCGGGAACCAGTCTCCTGGGCATGGCAT- GGCTTAGTGAATGAGGATGTGCATTACCTTTTGATTTT- GATCAATAATCGCTGGCCATGATTACTTGATCTGTAAT- GGAATTTTGTTTTCATTGCATTGAATTGCATTGCATTGAAT- AGTATCAGTACAGTATCCAGTTTACTTCTTCCCACATCAACT- TCTGGCTCTTTTCTCCACAACACTTCACATCTCGAG	549bp
Luc-mABCA1	CTCGAGATGTGAAGTGTTGTGGAGAAAAGAGCCAGAAGTT- GATGTGGGAAGAAGTAAACTGGATCAGTATCTGATACTAT- TCAATGCAATGCAATTCAATGCAATGAAAACAAAATTCCAT- TACAGATCAAGTAATCATGGCCAGCGATTATTGATCAAAAT- CAAAAGGTAATGCACATCCTCATTCACTAGACCTAGCCAT- GCCCAGGAGACTGGTTTCCCGGTGACACATCCATTGCT- GGCAATGCCCAGCACAATTCATTGTTAAAAAACAACCAAAC- CTCACACTCAGTATTTTCATTATCTGTACTGAAAGCAAAT- GCTTTGTGACTATTAAATGTTGCACATCATTCATTCACTGTAT- AGTAATCATTGACTAAGACCTATTGTCTGTGTTTTCTTCTT- GTGGTTGTATATATCACATTAATTTGTACCCTTGTTATTATC- TACTAGTAATAATGTAATCAGTATGAAATATTGCTCTAAT- TCTTTTCAAAATTGTTGCATCCCCCTTAGAATGTTTCTATT- TCCATAAGGATTTGCGGCCGC	549bp
Luc-mABCC1	CTCGAGCTGCAGTTTTGTGGTTGAGGGGCCTGGA- GAAAATCATTTTCTCCCCTTGGCAGTGTCCCAGAACAAT- GGATGGTCCTCTTACCAACATCTGGTCTTCCAGGCACT- CAAAAGCTGGGAACCAGCATCTCAGCGCCACACATTAGA- TAAACTAAGTTTTGGGGGATCCTTTTGTAATGACTTAACAAT- GCAATGCGAACATTTGCAGTAGCGGCCGC	221bp
Luc-mABCC4	CTCGAGGAGTAGGACAAAGTTGTCACAGGTTTTTGTT- GTTGTTTTTATTGCCCCCAAAATTACATGTTAATTTCCATT- TATATACGGAGTTCTATTTACTTGAAGACTGTGAAGTT- GCCATTTTGTCTCATTGTTTTCTTTGACATAACTAGGATC- CATTATTTCCCCTGAAGGCTTCTTGTTAGAGCGGCCGC	194bp
Luc-mABCC5	CTCGAGTATTCCATATTCATGTAAACAAAATTTAGTTTTT- GTTCTTAATTGCACTCTAAAAGGTTACGGAGACCGTTAT- TATAATTGTATCAGAGGCCTATAATGA AGCTTTATACGTGTAGCTATGCAGCTCTTGCTAATCAGTGTCT- CACACTGGCGTAGAAGTTTTTGTCAGTATAAGAGACCATC- CCTAGGTTGCTGGTTGCTGTGTGGTTTGGTGTGTTGCG- GCCGC	232bp

Table S4. Sequences of the mutated ABC 3' untranslated region (3'UTR) that were synthesized and cloned into psiCheck-2 vector to construct mutated Luc-ABC reporters and composite ABCA1 3'UTR.

Name	Sequence (5'-3')	Size
Luc-mABCC10	CTCGAGTGCTAGAGCCCAGTTCAGGGCGAGTCGGTTCG-GACGGCGTGGACACCAGCCAGCTTGTTTACATTCTCCTCT-GGGGCTCATCCCTTCCACACTTCCCCAGAAGGGAAAAG-GGCACCCTGGATTACTCTTTGGAAATCACTCCTTGGT-GGGCGGCCGC	163bp
Luc-mABCE1	GCGGCCGCGGTAGTATAATAAACTGTCTCGATTTTTT-GTTTTTTTAAGTATTTGAGACTTTTTCCAATTAGGACAT-AAGCCATTATTTGCATATTAATCAAGACTTCTGTTATTCAC-GATAGCTCTTAAAGTTTGTCAAACAGTTTCTAATTTCCTC-CTCAATTTTGCTTGCCCCAGTCATTTATTTAGTAAACACTCT-TCCTCACTCATAATCTCGATTTAGTCAGAAGTACAACT-TAAAGTGGCTCCAGGCTCCACATTTTCTGTGTTTCAACTA-CAATTTAGAACCCAACTGGCTTTTTATGTTATATTAAGTATAT-TATACTCACGATTCCAGAGTATGTGGGGCATAAAATCCTGAT-CACGATTTTATATGACAAAAAATTCTAGTAAATACTCCTTT-TAATAATAGGTCTATCAATATTCTCAGAGTCAGTCTCGAG	443bp

Table S5. ABC gene profiling in 19 paired HCC patient samples. Expression of 15 ABC genes was determined by custom-made ABC Taqman microfluidic array in 19 paired adjacent healthy liver (AHL) and HCC samples. Ct values were normalized with control 18S rRNA (dCt), each sample was normalized with the average dCt of the AHL group (ddCt), and fold change in expression (FC) was calculated according to the $2^{-\Delta\Delta Ct}$ method of Livak and Schmittgen (29). Up-regulations (FC≥1.5) are marked in light grey and down-regulations (FC≤0.5) are marked in dark grey.

	FR01T	FR02T	FR03T	FR04T	FR05T	FR06T	FR07T	FR08T	FR09T	FR10T	FR11T	FR12T	FR13T	FR14T	FR15T	FR16T	FR17T	FR18T	FR19T
ABCA1	1,5	2,6	2,4	1,0	0,6	0,9	1,7	2,6	0,6	2,4	0,6	3,5	1,8	0,9	1,6	2,1	0,8	0,4	0,2
ABCA2	1,0	3,4	1,6	1,2	1,2	0,5	4,2	3,2	1,1	1,3	1,4	2,0	1,4	0,8	1,6	1,7	1,2	0,9	1,4
ABCB1	1,1	4,7	3,4	1,2	1,5	0,7	3,9	1,7	1,3	1,2	1,0	6,7	2,2	2,1	1,6	3,8	3,3	1,5	1,0
ABCB6	0,5	5,5	4,3	1,2	1,0	0,4	2,5	1,4	2,0	2,5	3,8	5,4	5,5	2,3	1,5	4,8	3,4	1,8	2,8
ABCC1	2,0	5,5	2,8	0,6	0,7	0,4	3,0	1,9	2,0	1,4	9,3	3,9	0,4	0,3	2,5	2,9	2,6	0,5	4,2
ABCC2	0,4	3,9	1,4	1,2	0,7	0,5	2,2	0,9	1,5	2,4	2,2	7,5	4,0	2,1	1,4	3,3	1,8	1,1	0,7
ABCC3	1,0	6,6	4,0	2,6	0,8	0,7	2,1	3,7	0,6	1,6	4,0	3,0	3,6	2,6	1,3	1,5	0,9	0,7	0,5
ABCC4	2,0	2,2	4,6	2,9	1,8	0,6	2,1	0,4	2,1	1,8	1,5	2,8	1,9	3,5	2,3	2,6	11,4	0,6	4,4
ABCC5	2,3	4,1	3,1	1,1	1,7	0,7	2,7	2,7	2,0	3,1	2,2	4,7	1,9	2,2	1,9	3,9	2,2	1,8	4,6
ABCC6	0,5	2,4	1,0	6,3	0,5	0,4	1,0	0,7	1,1	2,6	1,9	1,3	1,6	1,2	1,7	1,8	0,9	0,9	1,1
ABCC10	1,7	4,9	3,2	2,5	0,9	0,5	3,0	3,3	1,7	2,5	2,9	3,8	2,2	4,6	2,3	4,3	4,6	4,2	1,2
ABCC11	0,6	1,7	2,4	3,1	2,4	0,6	1,5	1,5	1,2	1,9	2,2	3,3	1,8	1,5	1,2	1,8	0,7	0,5	1,0
ABCC12	1,0	10,7	5,0	8,2	3,2	1,1	4,9	0,5	2,1	9,0	2,7	2,8	1,4	3,1	0,1	38,3	1,9	0,2	4,3
ABCE1	1,4	1,8	3,0	1,3	0,8	1,3	2,5	2,3	1,4	2,5	1,3	3,5	1,5	2,1	0,5	1,0	2,1	1,2	0,5
ABCG2	0,5	1,6	0,9	1,5	1,7	0,3	0,5	0,7	1,0	1,4	2,1	1,0	0,9	2,2	1,4	2,2	0,4	1,2	0,8

Table S6. Conservation among species of dysregulated miRNAs. miRNA conservation was determined using the UCSC Genome Browser. An x marks the miRNA conservation in the stated specie.

	Conservation between species						
miRNA	**Rhesus**	**Mouse**	**Dog**	**Elephant**	**Opossum**	**Chicken**	**Xenopus**
miR-383	x	x	x	x		x	x
miR-654-3p	x	x	x	x			
miR-758	x	x	x	x			
miR-187	x	x	x	x	x		
miR-382	x	x	x	x			
miR-137	x	x	x	x	x	x	x
miR-139-5p	x	x	x	x	x		
miR-450b-5p	x	x	x	x			
miR-889	x	x	x	x			
miR-450a	x	x	x	x			
miR-495	x	x	x	x			
miR-485-3p	x	x	x	x			
miR-483-5p		x	x				
miR-130a	x	x	x	x	x		
miR-9	x	x	x	x	x	x	x
miR-376c	x	x	x	x			
miR-148b	x	x	x	x			x
miR-542-3p	x	x	x	x			
miR-199a-3p	x	x	x	x	x	x	x
miR-145	x	x	x	x	x		x
miR-424	x	x	x	x			
miR-411	x	x	x	x			
miR-369-3p	x	x	x	x			
miR-655	x	x	x	x			
miR-135b	x	x	x	x	x	x	x
miR-10a	x	x	x	x	x		x
miR-487b	x	x	x	x			
miR-199a-5p	x	x	x	x	x	x	x
miR-122	x	x	x	x	x		
miR-422a	x	x	x	x			
miR-127-3p	x	x	x	x			x
miR-139-3p	x	x	x	x	x		
let-7b	x	x	x	x	x		

Table S6. Conservation among species of dysregulated miRNAs. miRNA conservation was determined using the UCSC Genome Browser. An x marks the miRNA conservation in the stated specie.

	Conservation between species						
miRNA	**Rhesus**	**Mouse**	**Dog**	**Elephant**	**Opossum**	**Chicken**	**Xenopus**
miR-199b-5p	x	x	x	x	x	x	x
miR-138	x	x	x	x	x	x	x
miR-433	x	x	x	x			x
miR-885-5p	x		x	x			
miR-379	x	x	x	x			
miR-499-5p	x	x	x	x	x	x	x
miR-511	x	x	x	x			
miR-410	x	x	x	x			
miR-125b	x	x	x	x	x		x
miR-203	x	x	x		x	x	
miR-101	x	x	x	x	x	x	x
miR-26b	x	x	x	x	x		x
miR-598	x	x	x	x			
miR-143	x	x	x	x	x		x
miR-26a	x	x	x	x	x	x	x
miR-335	x	x	x	x			
let-7a	x	x	x	x	x	x	x
miR-503	x	x	x	x			
miR-30c	x	x	x	x	x		x
miR-323	x	x	x	x			
miR-455-3p	x	x	x	x	x	x	x
miR-296-5p	x	x	x	x			
miR-92a	x	x	x	x	x	x	x
miR-125a-5p	x	x	x	x			
miR-190	x	x	x	x	x	x	
let-7e	x	x	x	x			x
miR-625	x	x					
miR-204	x	x	x	x	x		x
miR-455-5p	x	x	x	x	x		x
miR-376a	x	x	x	x			
miR-494	x	x	x	x			
miR-340	x	x	x	x			
miR-542-5p	x	x	x	x			

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Table S6. Conservation among species of dysregulated miRNAs. miRNA conservation was determined using the UCSC Genome Browser. An x marks the miRNA conservation in the stated specie.

miRNA	Conservation between species						
	Rhesus	**Mouse**	**Dog**	**Elephant**	**Opossum**	**Chicken**	**Xenopus**
miR-195	x	x	x	x	x		x
miR-361-5p	x	x	x	x			
miR-106a	x	x	x	x	x	x	x
miR-576-3p	x	x		x			
miR-30b	x	x	x	x	x	x	
miR-107	x	x	x	x	x	x	x
miR-194	x	x	x	x		x	x
miR-19a	x	x	x	x	x	x	x
miR-374a	x	x					
miR-320	x	x	x	x			
miR-126	x	x	x	x	x	x	x
miR-192	x	x	x	x			
miR-491-5p	x	x	x				
miR-16	x	x	x	x	x	x	x
miR-454	x		x	x	x		
miR-193a-3p	x	x	x	x	x		
miR-301b	x	x	x	x	x		x
miR-21	x	x	x	x	x	x	
miR-10b	x	x	x	x	x	x	x
miR-452	x	x	x	x			
miR-548c-3p	x						
miR-224	x	x	x	x			
miR-182	x	x	x	x	x		x
miR-96	x	x	x	x	x		x

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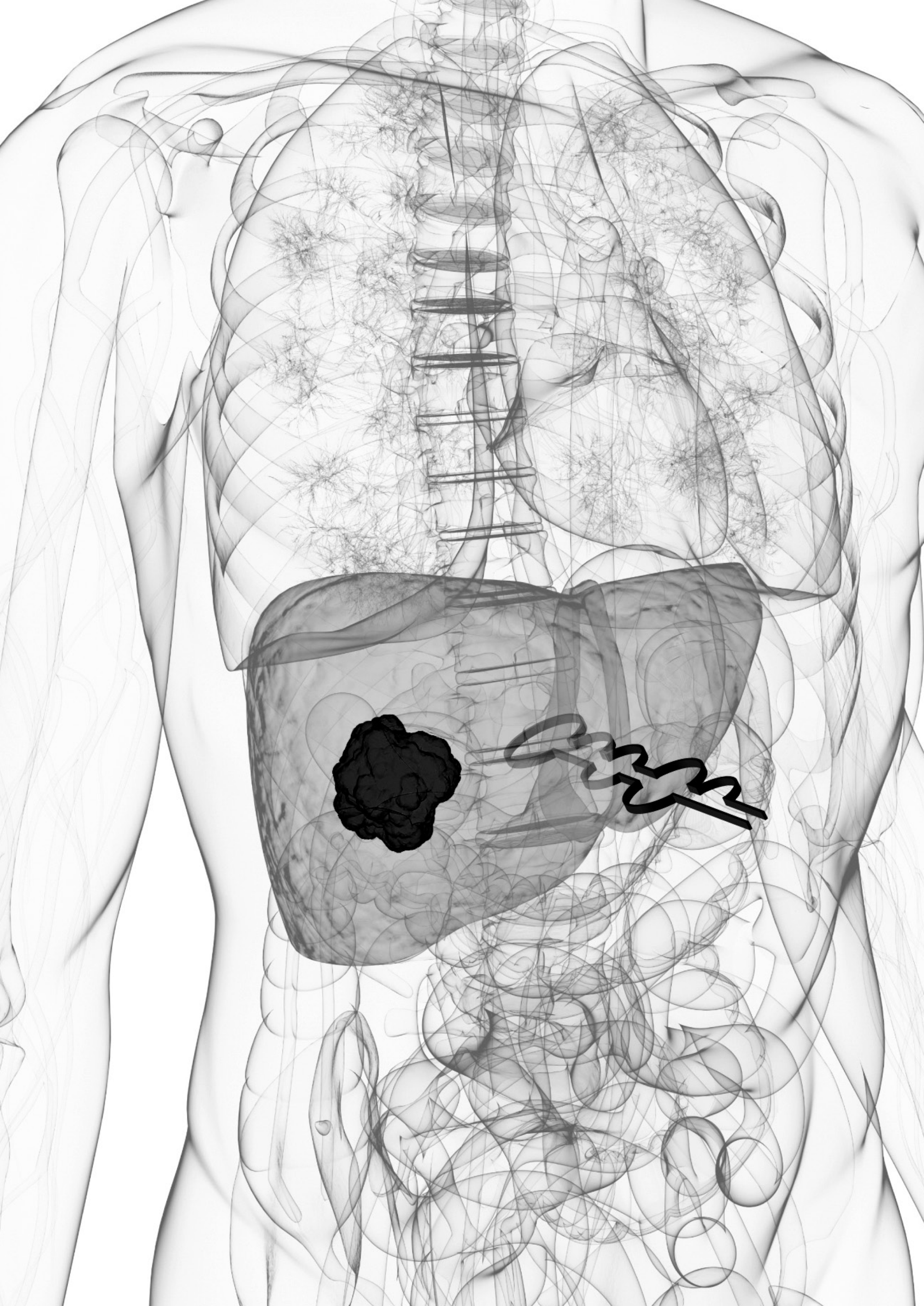

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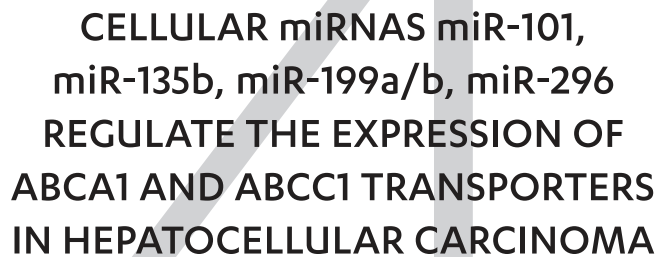

CELLULAR miRNAS miR-101, miR-135b, miR-199a/b, miR-296 REGULATE THE EXPRESSION OF ABCA1 AND ABCC1 TRANSPORTERS IN HEPATOCELLULAR CARCINOMA

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ABSTRACT

ATP-binding cassette (ABC) transporters are drug efflux pumps responsible for the multidrug resistance phenotype causing hepatocellular carcinoma (HCC) treatment failure. Our previous data indicates that the expression of ABC transporters is regulated by cellular miRNAs in HCC patients. Here we demonstrate that miR-101 and miR-135b regulate ABCA1 expression, and miR-199a/b and miR-296 regulate ABCC1 expression. Luciferase reporter analyses revealed that the cellular miRNA have true targets in the 3'UTR of ABCA1 and ABCC1. We further verified the miRNA regulation of ABCA1 and ABCC1 by analysis of endogenous mRNA and protein expression. Moreover we show that miR-296 is up-regulated upon sorafenib treatment while ABCC1 is down-regulated which may indicate a direct post-transcriptional regulation mechanism. By potentially down-regulating ABCC1 via mir-296 up-regulation, this may be the mechanism by which sorafenib sensitizes tumor cells to doxorubicin treatment.

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INTRODUCTION

The expression of ATP-binding cassette (ABC) transporters is dysregulated in HCC prior to any chemotherapeutic treatment (1), suggesting that the selection of a multidrug resistant sub-population by chemotherapy may not be the only cause of ABC transporter genes overexpression in HCC. The regulation of ABC genes expression in HCC could be mediated by microRNAs (miRNAs), a family of small RNAs which is often dysregulated in cancer (2-4). Moreover, it was shown that miR-33 inhibits the expression of ABCA1 resulting in reduced cellular cholesterol efflux to ApoA1 acceptor (5).

miRNAs are ~22 nucleotide (nt) long endogenous, single-stranded, non-coding RNAs (6). miRNAs are loaded into the RNA-induced silencing complex (RISC) where further regulations will be undertaken. If the complementarity is perfect in the “seed region” (nt 2-7 from the 5’ end of the miRNA) between the miRNA and its target in the messenger RNA (mRNA), the mRNA will be cleaved by RISC and degraded; in case of imperfect complementarity, translation will be repressed (7-10). Specific miRNAs have been shown to be involved in various biological processes, including development, cellular proliferation, apoptosis, and oncogenesis (11, 12). The finding that individual miRNAs may target several hundred genes, and that a large percentage of mRNAs may be subject to regulation by miRNAs, further underscores the emerging importance of miRNA-mediated regulation (13, 14).

We previously identified 12 up-regulated ABC genes and 79 down-regulated miRNAs in HCC patient samples (1). Subsequently, we showed using luciferase reporters that miRNAs down-regulated the expression of 5 out of the 12 up-regulated ABC genes. Here we confirm *in vitro* that miR-101 and miR-135b regulate the expression levels of endogenous cholesterol transporter ABCA1 and miR-199a/b and miR-296 of multidrug resistance transporter ABCC1.

MATERIALS AND METHODS

miRNA target prediction

Softwares TargetScan (14) and PicTar (13) were used for ABCA1 and ABCC1 3’UTR target prediction of cellular miRNAs previously identified as down-regulated in HCC (1). Additionally, 3’UTR sequences were manually screened for miRNA seed-matching sequences.

Luciferase reporters and miRNA expression contructs

Cloning of wild-type and mutated luciferase reporters Luc-ABCA1, Luc-ABCC1, mLuc-ABCA1 and mLuc-ABCC1, and miRNA expression plasmids were described previously (1). Briefly, luciferase reporters were made by cloning of ABCA1 and ABCC1 3’UTR sequences behind the renilla luciferase gene in the psiCheck-2 vector (Promega, Madison, WI), and miRNA expression plasmids were made by cloning of the pri-miRNAs sequences in the pcDNA6.2 vector (Invitrogen, Carlsbad, CA).

Cell lines and transfection

Human embryonic kidney (HEK) 293T and human hepatocellular carcinoma HuH7 cell lines were obtained from ATCC and maintained in Dulbecco’s modified Eagle’s medium (DMEM, Invitrogen) containing 10% (v/v) fetal calf serum, 100U/ml penicillin and 100U/

ml streptomycin, at 37°C and 5% CO_2. Cells were plated in 6- or 96-well plates. Cells were transfected using Lipofectamine 2000 reagent (Invitrogen) according to manufacturer's instructions. For RT-qPCR analysis of ABC genes and miRNAs expression and for immunoblotting, 1.2µg miRNA expression plasmid or 125pmol miRNA inhibitor (Exiqon, Vedbaek, Denmark) were transfected in 6-well plates. For cell viability assays in HuH7 cells, 200ng miRNA expression plasmids or 4.2pmol miRNA inhibitors were transfected in 96-well plates. To assess the effect of sorafenib on ABCC1 gene and miRNAs expression, HuH7 were cultured for 7 days in 6-well plates with 2µl DMSO or 1mM sorafenib (S-8502, LC Laboratories, Woburn, MA).

RNA isolation, RT-qPCR for ABC genes and for miRNAs

For ABC genes and miRNA expression, cells were harvested 72-hr post-transfection with miRNA expression plasmid. Total RNA was isolated with Trizol (Invitrogen). For ABCA1 and ABCC1 expression, RT reactions were performed with the High Capacity RNA-to-cDNA kit (Applied Biosystems, Foster City, CA) using 900ng RNA template. RT-qPCR was performed using 4ng cDNA, 3µl 2x Fast SYBR Green Master Mix (Applied Biosystems) and 0.2µl of each primer (primers sequences, **Table S1**) and run on a 7500 RT-qPCR system (Applied Biosystems). For miRNA expression, RT reactions were performed with the TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems) using 10ng RNA and 3µl miRNA-specific RT-stem-loop primer (Applied Biosystems) according to manufacturer's instructions. Taqman assay was done in 20µl using 9µl cDNA (5x diluted), 1µl miRNA-specific primer with FAM-labeled fluorogenic probe (Applied Biosystems) and 10µl Taqman 2x Universal PCR Master Mix (Applied Biosystems) and run in duplicates on a 7500 RT-qPCR system (Applied Biosystems).

Luciferase assay

HEK293T cells were transfected with 5ng luciferase reporter and 150ng miRNA expression plasmid or 4.2pmol miRNA inhibitor, and were assayed at 72-hr (miRNA) or 48-hr post-transfection (miRNA inhibitor). Firefly and renilla luciferase activities were measured with the Dual-Luciferase Reporter Assay System (Promega) according to manufacturer's instructions, and the relative luciferase activity was calculated as the ratio between the renilla and firefly luciferase activities.

Immunoblotting

Cells were harvested 72-hr post-transfection with miRNA expression plasmid and homogenized in 10mM KCl, 1.5mM MgCl2, 10mM Tris-HCl pH7.4, 0.5% SDS w/v, and one Complete Protease Inhibitor tablet (Roche Diagnostics, Basel, Switzerland) per 10ml buffer, completed by mechanical lysis through a 25G-needle. Protein concentration was determined using the QuickStart Bradford protein assay (Biorad, Hercules, CA). Antibody dilutions were as follows: 1:1,000 anti-ABCA1 (AB18180, Abcam, Cambridge, MA), 1:1,000 anti-ABCC1 (EB08734; Everest Biotech, Oxfordshire, UK), 1:10,000 anti-αTubulin (SC5286, Santa Cruz Biotechnologies, Santa Cruz, CA), 1:1,000 anti-Actin (4968, Cell Signaling Technology, Beverly, MA), 1:10,000 rabbit anti-mouse P0260 and rabbit anti-goat P0449 (Dako, Glostrup, Denmark). Antibody binding was detected by the Lumi-LightPLUS chemiluminescent detection kit (Roche Diagnostics, Basel, Switzerland).

Cell viability assay

HuH7 cells were transfected with miRNA expression plasmids, and 48-hr later, 0.2 or 0.5μl 1mM doxorubicin (44583, Sigma) or equal volume of DMSO (D2650, Sigma) was added. Viability of HuH7 cells was measured 24-hr later according to the manufacturer's protocol (CellTiter-Glo Luminescent Cell Viability Assay, Promega).

RESULTS

miRNA target sites predictions in the 3'UTR of ABCA1 and ABCC1

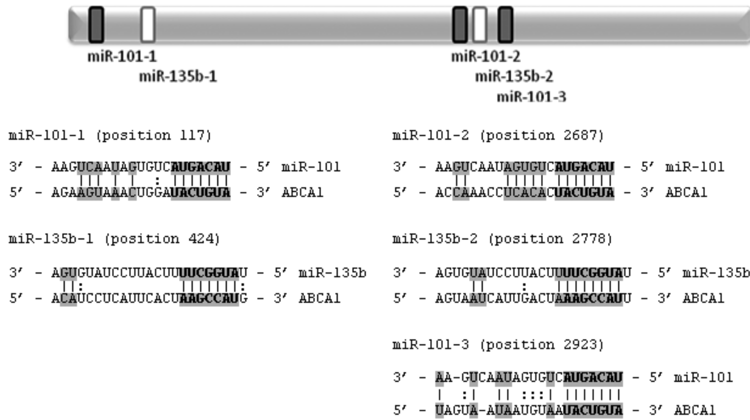
For the 79 miRNAs previously identified as down-regulated in HCC (1), miRNA targets in ABCA1 and ABCC1 were predicted using algorithms PicTar and TargetScan. ABCA1 3'UTR is 3312-bp long and contains 3 putative miR-101 target sites, and 2 putative miR-135b target sites (**Fig. 1A**, **upper panel**). The close proximity of miR-101-2, miR-135b-2 and miR-101-3 target sites indicates functionality of ABCA1 3'UTR. In addition, the complementarity between the miRNAs and ABCA1 was high in this region, with multiple complementary bases outside of the seed region. Taking into account that in RNA G can pair with U, miR-101 binds via its 7-nt seed and via an additional 6, 7 and 9 bases complementary to the 3'UTR, for respectively miR-101-1, -2 and -3 target sites. miR-135b binds via its 7-nt seed and via 4 additional bases complementary to the 3'UTR (miR-135b-1) and via its 7-nt+A seed and 3 additional bases complementary to the 3'UTR (miR-135b-2). ABCC1 3'UTR is 1792-bp long and contains 1 miR-199a/b target site (7-nt seed + 6 additional bases), and 1 miR-296 target site (7-nt seed + 6 additional bases), (**Fig. 1A**, **lower panel**).

Validation of miR-101 and miR-135b targets in ABCA1 gene

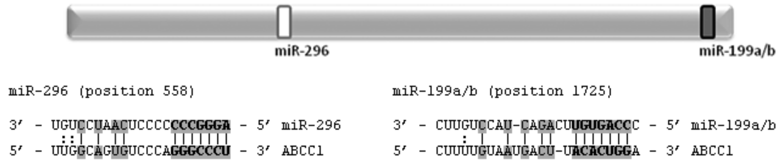
In order to verify the predicted miRNA targets in ABCA1, a luciferase reporter was made that contained the 3'UTR of the ABCA1 gene (Luc-ABCA1). HEK293T cells were co-transfected with Luc-ABCA1 and miR-Scr, miR-101 or miR-135b. Seventy-two hours post-transfection firefly and renilla luciferase activities were measured and relative luciferase fluorescence (RLF) was calculated. RLF was reduced by more than 50% upon co-transfection of miR-101 or miR-135b with Luc-ABCA1 (**Fig. 1B**), indicating that the gene is a true target for both miRNAs. Next, we wanted to confirm that direct miRNA binding was responsible for the observed effect. All miRNA binding sites in the 3'UTR of Luc-ABCA1 were mutated to produce a mutated luciferase reporter (mLuc-ABCA1). Upon co-transfection of miR-101 or miR-135b with mLuc-ABCA1, inhibition of RLF was alleviated (**Fig. 1B**), hence confirming that the knock-down effect is a consequence of direct miRNA binding to ABCA1 target sequences. To further verify the miRNA targets in ABCA1, inhibitors of miR-101 and miR-135b were co-transfected with Luc-ABCA1. miRNA inhibitors bind to cellular miRNAs and therefore prevent their binding and inhibition of target gene expression, resulting in a reversal of the knock-down effect. 48-hr post-transfection firefly and renilla luciferase activities were measured and RLF was calculated. miR-101 and miR-135b sequestration lead to a significant increase in RLF (**Fig. 1B**), which shows that reducing endogenous miRNA levels alleviates the negative regulation exerted by miR-101 and miR-135b on Luc-ABCA1 and confirming those miRNAs as inhibitors of ABCA1 gene expression in HCC.

A

ABCA1 3’UTR (3312bp)



ABCC1 3’UTR (1792bp)



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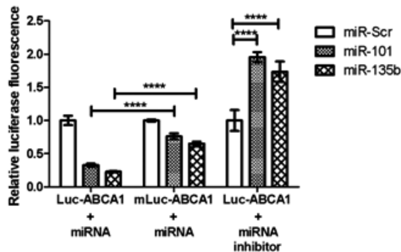

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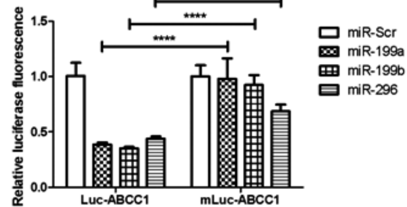

Figure 1. Prediction and validation of ABCA1 and ABCC1 as targets of respectively miR-101 and miR-135b; and miR-199a/b and miR-296. **A**. Schematic of ABCA1 and ABCC1 3’UTR with predicted targets of miR-101 and miR-135b, and miR-199a/b and miR-296, respectively. The 3’UTR of ABCA1 contains three miR-101 and two miR-135b target sites. The 3’ UTR of ABCC1 contains one miR-199a/b and one miR-296 target sites. The seed sequence is represented with bold characters, base-complementarity with grey highlighting, and G-U base-pairing with semi-colons. **B.** Knock-down of Luc-ABCA1 by miR-101 and miR-135b. **C.** Knock-down of Luc-ABCC1 by miR-199a/b and miR-296. HEK293T cells were transfected with 5ng Luc-ABCA1, Luc-ABCC1, mLuc-ABCA1 or mLuc-ABCC1 reporter plasmid, 150ng miRNA expression plasmid or 4.2pmol miRNA inhibitor. mLuc-ABCA1 and mLuc-ABCC1 carry mutations in the miRNA binding sites. Luciferase assay was performed 72-hr post-transfection. Renilla luciferase was normalized with firefly luciferase (relative luciferase fluorescence, RLF), then RLF of each miRNA was normalized to RLF of miR-Scramble (miR-Scr) which was set at 1. Data are presented as average of 3-4 technical replicates ± SD. ****: $P < 0.0001$, **: $P < 0.01$.

Validation of miR-199a/b and miR-296 targets in ABCC1 gene

As described for ABCA1, Luc-ABCC1 reporter was designed to contain the 3'UTR of the ABCC1 gene. HEK293T cells were co-transfected with Luc-ABCC1 and miR-Scr, miR-199a, miR-199b or miR-296, and 72-hr post-transfection firefly and renilla luciferase activities were measured and RLF was calculated. RLF was reduced by more than 50% upon co-transfection of miR-199a, miR-199b or miR-296 with Luc-ABCC1 (**Fig. 1C**), indicating that it is a true target for both miRNAs. Mutating the ABCC1 target sites in Luc-ABCC1 (mLuc-ABCC1) resulted in a similar effect as for ABCA1, and inhibition of RLF was alleviated (**Fig. 1C**), hence confirming that the knock-down is a direct consequence of the miRNA binding. Inhibiting miR-199a/b or miR-296 expression with miRNA inhibitors did not lead to a significant increase in RLF (data not shown).

Endogenous ABC transporter genes and miRNAs expression in HEK293T and HuH7

As a next step, endogenous ABCA1 and ABCC1 knock-down by the previously described cellular miRNAs was determined. miRNAs regulate gene expression by causing mRNA degradation or translational repression. The effect of a give miRNA can therefore be assessed both at mRNA and protein levels. Initially, endogenous expression of ABCC1 and ABCA1 in HEK293T and HuH7 cells was determined by RT-qPCR. ABCC1 was expressed in both cell lines and ABCA1 was expressed in HuH7 only (**Fig. 2A**). Next, mature miRNA expression was quantified by RT-qPCR in HEK293T and HuH7 cells following plasmid transfection to determine if there was a significant increase in expression following miRNA transfection, indicating proper processing of the mature miRNA from the pri-miRNA expression plasmid. Cellular miRNAs predicted to target ABCA1 were detectable in non-transfected cells at Ct~30. Expression was significantly increased by 90 and 60000 in HEK293T and by 15 and 2000 in HuH7 upon transfection with miR-101 or miR-135b expression plasmid (**Fig. 2B**). Cellular miRNAs predicted to target ABCC1 were detectable in non-transfected cells at Ct~30-35, with the exception of miR-199a which was undetectable in both cell lines. Expression was significantly enhanced upon transfection with miR-199a/b or miR-296 expression plasmid (**Fig. 2B**). The endogenous expression of miR-199a/b and miR-296 is very low, and it would be difficult to artificially decrease it with miRNA inhibitors. This could be the reason why the previously described Luc-ABCC1 experiment using miRNA inhibitors was unsuccessful in alleviating miRNA inhibition.

Cellular miR-101 and miR-135b regulate endogenous ABCA1 expression *in vitro*

We previously showed that the 3'UTR of ABCA1 presents target sites for miR-101 and miR-135b. Cellular miRNAs regulate the expression of their targets either by causing degradation of the mRNA, or by repressing its translation. To determine the effect of these miRNAs on endogenous ABCA1 expression, HuH7 cells were transfected with 4µg miR-101, miR-135b, and miR-Scr expression plasmid or 125pmol of the respective miRNA inhibitor, and RNA and protein were isolated 72-hr post-transfection. ABCA1 expression was determined by RT-qPCR and significant differences in mRNA levels were measured between treatments. A negative correlation between miRNA and ABCA1 mRNA levels was observed. Increased miRNA expression reduced ABCA1 mRNA levels. On the contrary, miRNA inhibition increased ABCA1 mRNA levels (**Fig. 3A**). Next, ABCA1 protein

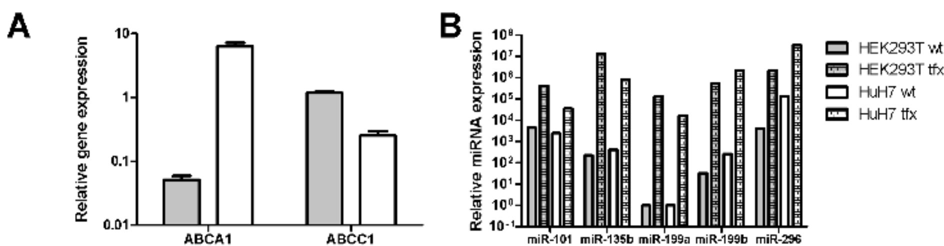

Figure 2. miRNAs and ABC genes expression levels in HEK293T and HuH7 cells. **A**. Endogenous ABCA1 and ABCC1 gene expression in HEK293T and HuH7. Expression of actin was set at 1. Data are represented as average of three technical replicates ± SD. **B.** miRNA expression levels in wild-type (wt) and transfected (tfx) HEK293T and HuH7 cells. Cells were transfected with the corresponding miRNA expression plasmid and expression was measured 72-hr later. Data are represented as average of two technical replicates.

expression was determined by immunoblotting. Endogenous ABCA1 protein levels were reduced upon transfection with miR-101 or miR-135b expression plasmid (**Fig. 3B**). miR-135b transfection lead to the strongest effect throughout 3 biological replicates (data not shown).

Cellular miR-199a, miR199b and miR-296 regulate endogenous ABCC1 expression *in vitro*

We previously showed on luciferase reporters that the 3'UTR of ABCC1 presents target sites for miR-199a, miR-199b and miR-296. To determine the effect of these miRNAs on endogenous ABCC1 expression, HEK293T cells were transfected with 4μg miR-199a, miR-199b, miR-296 and miR-Scr expression plasmid, and RNA and protein were isolated 72-hr post-transfection. ABCC1 expression was determined by RT-qPCR, but no significant

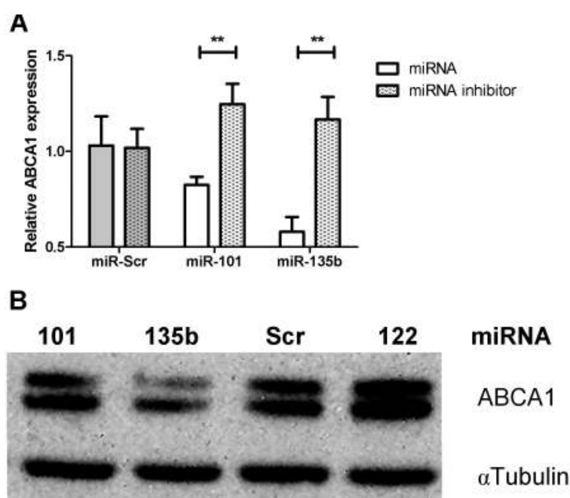

Figure 3. Cellular miR-101 and miR-135b regulate endogenous ABCA1 *in vitro*. A. Endogenous ABCA1 mRNA levels upon transfection of HuH7 cells with 1.2μg miRNA expression plasmid or 125pmol miRNA inhibitor. ABCA1 gene expression was normalized with actin, and miR-Scr was set at 1. **: P-value < 0.01. **B.** Levels of endogenous ABCA1 protein levels upon transfection of HuH7 cells with 1.2μg of miR-101 and miR-135b expression plasmid.

differences in mRNA levels were measured between treatments (data not shown). Knock-down of ABCC1 protein expression by miR-199a/b and miR-296 was determined by immunoblotting. Endogenous ABCC1 protein levels were reduced upon transfection with miR-199a, miR-199b or miR-296 expression plasmid (**Fig. 4A**). miR-199a transfection lead to the strongest effect throughout 3 biological replicates (data not shown).
Subsequently, because ABCC1 effluxes the cytotoxic drug doxorubicin, we determined if decreasing ABCC1 levels by transfecting HuH7 cells with miRNA expression plasmid would decrease cell viability. HuH7 cells were transfected with 20 and 200ng miRNA expression plasmid, increasing dose of doxorubicin was added to the cells 48-hr later, and cell viability was measured after 24-hr. Surprisingly, no significant differences in cell viability were observed between transfections with 20ng (**Fig. 4B**) or 200ng miRNA (data not shown).

miR-296 up-regulation in response to sorafenib treatment may down-regulate ABCC1

To date sorafenib is the only drug proved to be efficient for treatment of HCC. Having previously shown that ABCC1 is up-regulated in untreated HCC (1), we postulated that this up-regulation might be a hallmark of HCC rather than a consequence of chemotherapeutic treatment. Though there is limited literature on sorafenib transport, it should be noted that up to now sorafenib was not shown to be a substrate for ABCC1. However, we hypothesized that sorafenib may change cellular miRNA expression levels, resulting in altered target gene regulation. Since ABCC1 is targeted by miR-199a/b and miR-296, we determined the expression of those miRNAs and of ABCC1 in HuH7cells cultured on sorafenib. RNA

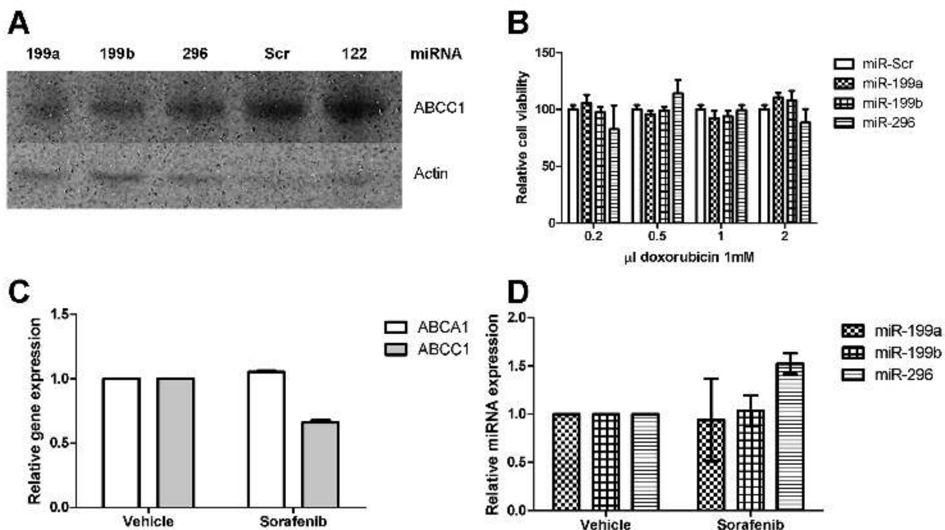

Figure 4. Cellular miR-199a/b and miR-296 regulate endogenous ABCC1 *in vitro*. A. Endogenous ABCC1 protein knock-down upon transfection of HuH7 cells with 1.2µg of miR-199a/b and miR-296 expression plasmid. **B**. Cell viability of HuH7 cells in response to doxorubicin treatment upon transfection with miRNA expression plasmid. Cells were transfected with 200ng miRNA expression plasmid, doxorubicin was added 48-hr later, and cell viability was measured after 24-hr. **C.** Expression of ABCC1 and miR-296 in HuH7 cells treated with 1mM sorafenib.

was isolated from cells cultured with 1 mM sorafenib for 1 week and the expression of ABCA1 and ABCC1 was determined by RT-qPCR. ABCA1 expression was not affected by sorafenib treatment; however, ABCC1 was significantly down-regulated (**Fig. 4C**). Next, expression of cellular miRNAs predicted to target ABCC1 was subsequently determined in the same samples. Interestingly, miR-296 was up-regulated 1.5 times while miR-199a/b expression didn't change. That could be due to the extremely low expression levels in Huh7 cells (**Fig. 4D**). This finding indicates that sorafenib may chemosensitize HuH7 cells via up-regulation of cellular miR-296 that subsequently down-regulates ABCC1 by a post-transcriptional silencing mechanism.

DISCUSSION

miRNA dysregulation has been clearly linked to cancer and HCC, but the molecular mechanisms by which miRNAs modulate tumorigenesis are poorly described. In this study we focused on miR-101, miR-135b, miR-199a/b and miR-296 and evaluated their effect on their targets ABCA1 and ABCC1. Decreased expression of miR-101 (1, 15, 16) and miR-199a/b in HCC has been repeatedly shown (1, 15-20).

Bioinformatics prediction that ABCA1 is a target of cellular miR-101 and miR-135b, with respectively three and two targets in the 3'UTR of ABCA1, was confirmed in the current study on luciferase reporters, on endogenous mRNA levels, and endogenous protein levels. ABCA1 is a cholesterol transporter whose expression was previously shown to be regulated by a cellular miRNA. miR-33a represses ABCA1 expression, thereby reducing cholesterol efflux to ApoA1 (5). Our data indicate that miR-101 and miR-135b, together with miR-33a and miR-122 (21), belong to the small group of cellular miRNAs involved in cholesterol metabolism. To clearly prove this relationship, radiolabeled cholesterol efflux study would provide a definitive functional validation of the miRNA regulation. For this, hepatocytes or macrophages should be used, which was not feasible in our current research project.

Bioinformatics prediction that ABCC1 is a target of cellular miR-199a/b and miR-296 was confirmed on luciferase reporters in the current study and on endogenous protein levels. miRNAs can regulate gene expression via two mechanisms, either mRNA degradation or translational repression. This could explain why no differences were observed on endogenous mRNA levels, yet endogenous protein levels were reduced upon miRNA expression plasmid transfection. ABCC1 gene is highly relevant to HCC, e.g. increase in ABCC1 expression has been associated with a more aggressive HCC phenotype (22), and ABCC1 transports clinically relevant drugs for HCC such as doxorubicin (23). However, we were unable to show any differences in viability of miRNA-transfected HuH7 cells upon doxorubicin treatment. When one ABC gene is down-regulated, its function may be taken over by other member(s) of the family. The absence of a measurable effect on cell viability may be due to functional overlapping of ABC transporters. In this case, doxorubicin is a substrate not only for ABCC1 but for several other ABC transporters including ABCB1 (24), ABCC2 (24) and ABCC10 (25). If doxorubicin efflux is taken over by other transporter(s), it would mask the effect of ABCC1 knock-down. Assessing a functional effect of miRNA-mediated endogenous ABCC1 knock-down may hence require to also inhibit and/or down-regulate other ABC transporters with overlapping transport capacities.

Another clinically relevant drug for HCC treatment is sorafenib. Sorafenib treatment reduces ABCC1 expression levels *in vitro*, as it was shown in this study and previously for

several ABC transporters (26). Here we demonstrated that ABCC1 expression is regulated by cellular miR-199a/b and miR-296. In addition we showed that in sorafenib-treated cells, miR-296 is up-regulated, which may cause ABCC1 down-regulation. This finding, may indicate a novel involvement of miR-296 regulation of ABCC1 in the multidrug resistance phenotype in HCC. Further research in primary hepatocytes and in murine models of HCC will characterize this interesting potential mechanism of sensitizing cells to chemotherapy by sorafenib-induced miRNA regulation of gene expression. Further work is warranted to evaluate the effect of modulating miRNA expression, using gene therapy vectors encoding the cellular miRNAs in an animal model of multidrug resistant HCC. Artificially increasing miR-199a/b and miR-296 levels in HCC patients may reduce ABCC1 levels, and may improve chemotherapy effectiveness and/or render HCC tumors less aggressive.

Table S1. List of primers used in this study.

Name	Sequence	Gene
ABCA1-f	GTTGCTGCTGTGGAAGAACC	ABCA1
ABCA1-r	TCAGCCGAACAGAGATCAGG	
ABCC1-f	TTACCTCTGGGCCTGTTTCC	ABCC1
ABCC1-r	GTTGGGCTGACCAGAAACAC	
ACTIN-f	CAGCCATGTACGTTGCTATCCAGG	βactin
ACTIN-r	AGGTCCAGACGCAGGATGGCATG	

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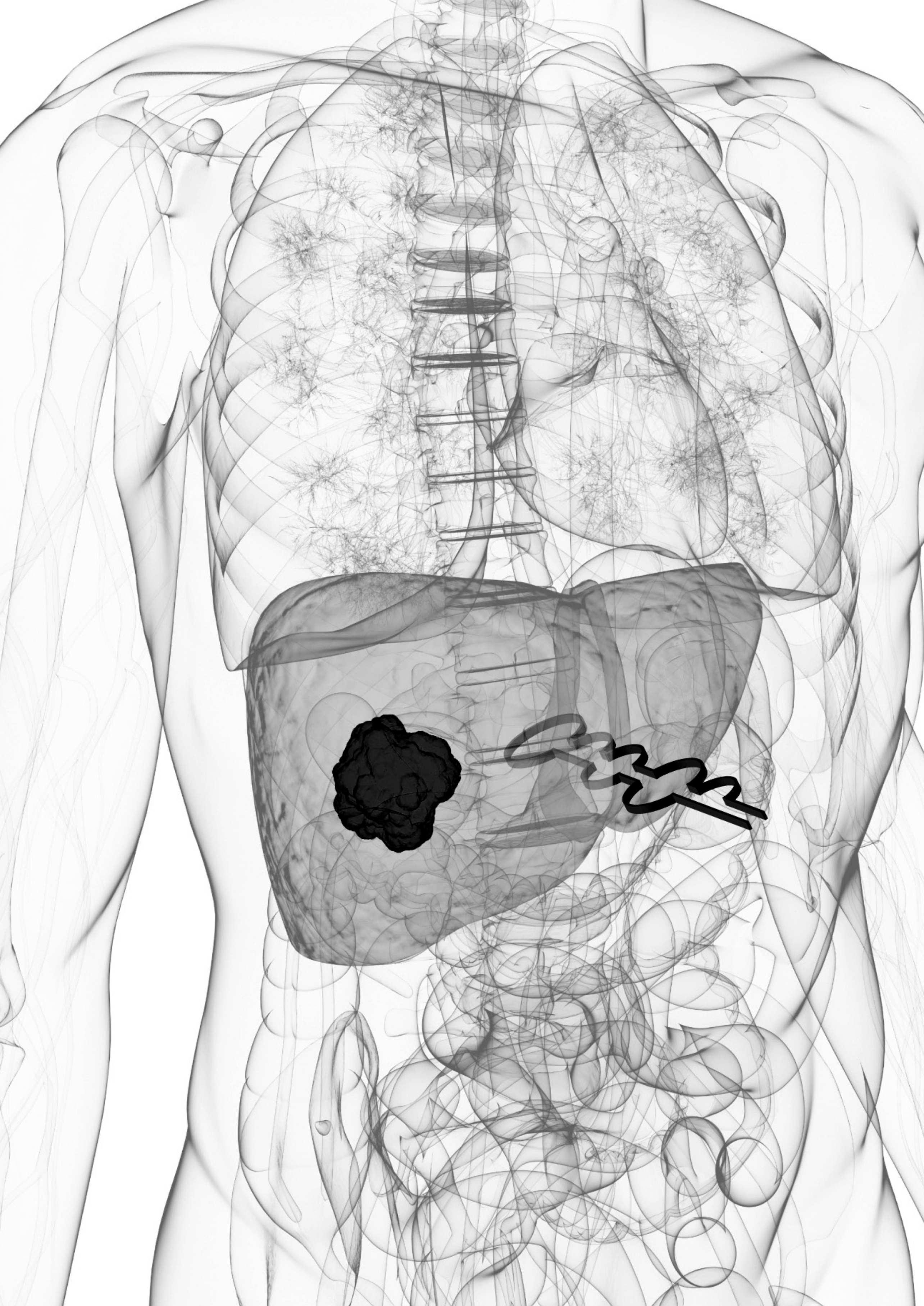

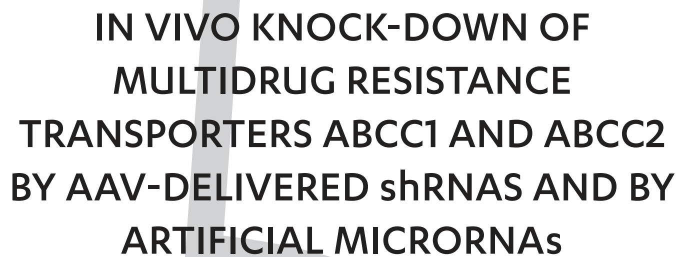

IN VIVO KNOCK-DOWN OF MULTIDRUG RESISTANCE TRANSPORTERS ABCC1 AND ABCC2 BY AAV-DELIVERED shRNAS AND BY ARTIFICIAL MICRORNAs

Florie Borel, Richard van Logtenstein,
Annemart Koornneef, Tita Ritsema, Piotr Maczuga,
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ABSTRACT

ABC transporters export clinically relevant drugs and their over-expression causes multidrug resistance. In order to knock-down ABC transporters ABCC1 and ABCC2, 13 shRNAs were developed. Four shRNA candidates were tested *in vivo* using self-complementary adeno-associated virus serotype 8. A strong, specific knock-down of *Abcc2* was observed in mice liver, but at the cost of toxicity caused by over-saturation of the RNAi machinery due to high shRNA expression. Subsequent generation of artificial miRNAs showed better efficacy profile. These results demonstrate the feasibility of knocking down *Abcc2* via AAV-delivered shRNAs to the liver, but encourage towards the use of miRNA in further therapeutics development.

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the fifth most common cause of cancer worldwide, with about 750,000 patients globally reported each year (International Agency for Research on Cancer, website: http://globocan.iarc.fr). Poor survival of HCC patients has several causes, among which resistance to chemotherapeutic treatment which frequently is a problem in HCC management. Many ATP-binding cassette (ABC) transporter family members can decrease the intracellular concentration of toxic compounds (1-5). These transmembrane pumps are over-expressed in tumor cells, hence causing the multidrug resistance phenotype. ABCB1 and ABCC3 have been shown to be up-regulated in HCC (6, 7) and the up-regulation of ABCC1 has been associated with a more aggressive HCC phenotype (8). Thus far ABCB1 inhibitors failed to show benefit during clinical trials, but more are being tested (9). Decreasing the expression of other ABC transporters is a desirable alternative as it could potentially reverse the multidrug resistance phenotype.

RNA interference (RNAi) is a suitable approach towards this goal, as it would allow combinatorial, possibly patient-tailored targeting of ABC transporters. RNAi is a naturally-occurring post-transcriptional gene silencing mechanism which can induce sequence-specific degradation of a messenger RNA (mRNA), thus reducing gene expression. RNAi can be induced by synthetic small interfering RNAs (siRNAs), or by intracellular expression of short hairpin RNAs (shRNAs) and artificial microRNAs (miRNAs). shRNAs and miRNAs are processed by Dicer into siRNAs, which are loaded in the RNA-induced silencing complex (RISC) where they mediate sequence-specific mRNA recognition, ultimately causing its degradation. *In vivo* siRNA-mediated knock-down of *Abcc1* was demonstrated after intratumoral siRNA injection followed by *in situ* electroporation of the tumor; this led to decreased tumor weight in response to epirubicin (10). In addition, *Abcc2* was inhibited *in vitro* and *in vivo* by plasmid-, adenovirus- and lentivirus-delivered shRNAs which respectively resulted in reversed cisplatin and paclitaxel resistance (11), decreased bilirubin transport (12), and reduced growth of cisplatin-treated tumors (13). Despite these findings, the major problem of RNAi applications still lies in sustained and tissue-specific delivery of the effector molecules *in vivo*. Recombinant adeno-associated virus (AAV) has emerged as the vector of choice for gene therapy and for RNAi-mediated therapy as it yields long-term, tissue-specific expression without any apparent pathogenicity (14). In the current study we assessed the feasibility of *in vivo* AAV-mediated knock-down of two murine endogenous ABC transporters: ABCC1 and ABCC2. Initially, we verified the knock-down activity of 13 shRNA constructs and selected two candidates targeting Abcc2 for further *in vivo* testing. A single injection of self-complementary AAV8 (scAAV8) carrying shAbcc2 resulted in efficient Abcc2 knock-down in murine liver. Concomitant signs of toxicity including elevated transaminases were attributed to high levels of shRNAs being processed into siRNAs, causing over-saturation of the RNAi machinery. Nevertheless subsequent translocation of the validated shRNA sequences into a miRNA scaffold offers the perspective of safe AAV-mediated *in vivo* knock-down.

MATERIAL AND METHODS

DNA constructs

Six shRNA constructs targeting murine *Abcc1* and seven targeting murine *Abcc2*, and control shRNAs targeting eGFP (shGFP) and Luciferase (shLuc) and a scramble sequence (shScr) were made by annealing of complementary oligonucleotides and ligating them into the pSuper vector containing the H1 Pol III promoter (OligoEngine, Seattle, WA). The sequence for constructing the negative control hairpin shScr has been described previously (15). The sequences of all oligonucleotides used in this study are listed in **Table S1**. Luciferase reporters *Luc-Abcc11/17* and *Abcc22/28* were made by cloning of *Abcc1/2* sequences behind renilla luciferase in the siCheck vector (Promega, Madison, WI).

For cloning in the AAV backbone, the H1-shRNA expression cassettes were subcloned in pCR-Blunt II-TOPO vector (Invitrogen, Carlsbad, CA), and then ligated in the pVD287 vector. pVD287 contains the *egfp* gene under the control of the liver-specific LP-1 promoter and generates scAAV due to a mutation in one terminal repeat (16).

Cell culture and transfections

Human embryonic kidney (HEK) 293T and murine hepatoma (Hepa1-6) cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) containing 10% (v/v) fetal calf serum, 100U/ml penicillin and 100U/ml streptomycin, at 37°C and 5% (v/v) CO_2. Cells were plated in 6-, 24- or 96-well plates one day prior to transfection. Transfections were performed with Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's instructions. For the interferon response experiment we transfected 2μg of a synthetic analog of dsRNA, polyinosinic:polycytidylic acid (poly I:C), as a positive control.

Luciferase assays

For luciferase assays, cells were co-transfected with 10ng *Luc-Abcc* reporter that contains both firefly and renilla luciferase genes, and 0, 0.5, 2.5, 10 or 50ng of the corresponding shAbcc expression construct. Transfected cells were assayed at 48hr post-transfection and firefly and renilla luciferase were measured with the Dual-Luciferase Reporter Assay System (Promega). Relative luciferase activity was calculated as the ratio between the renilla and firefly luciferase activities, and transfection with shScr was set at 100%. Data are represented as mean values ± SD from a representative experiment conducted with three technical replicates.

RNA isolation, quantitative RT-PCR (RT-qPCR), siRNA and miRNA Taqman assays

For *in vitro* experiments, total RNA was isolated from cells 18hr or 72hr post-transfection using the Nucleospin kit (Clontech, Mountain View, CA). For *in vivo* experiments, total RNA was isolated from frozen liver sections at two weeks post-injection (p.i.) using Trizol (Invitrogen) according to the manufacturer's protocol. DNAse-treatment and RT-qPCR were performed as described previously (17) and data are represented as mean values ± SD from a representative experiment conducted with three technical replicates. siRNA and miRNA expression was quantified with custom-made siRNA assays or miRNA-specific Taqman assays (Applied Biosystems, Foster City, CA) according to manufacturer's instructions.

AAV vector production and in vivo experiments

Self-complementary AAV8 vectors were produced and purified as described previously (17-19) with an added fractionation step yielding higher virus concentration. Final concentration was determined by qPCR with LP1 primers. All animal experiments were conducted according to the guidelines of the local animal welfare committee. Six-to-eight-week-old male C57BL/6 mice received $2.2x10^{11}$gc AAV-shScr, $2.6\text{-}3x10^{12}$gc AAV-shAbcc22 or -shAbcc28 per animal intravenously via the tail vein. Heparinized blood samples were taken by retro-orbital bleeding at 1 and 2 weeks p.i. for plasma analysis. Mice were sacrificed on day-15 p.i. and livers were examined for *Abcc1* and *Abcc2* knock-down. Plasma levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST) and total bilirubin were analyzed on Modular Analytics P800 analyzer (Roche Diagnostics, Basel, Switzerland). Data are represented as mean values ± SEM (n = 4-5).

RESULTS AND DISCUSSION

Design and validation of shRNAs targeting murine Abcc1 and Abcc2

Six short hairpin RNAs (shRNAs) were designed against murine *Abcc1* mRNA (shAbcc11-shAbcc17) (**Fig. 1A** and **Fig. 1C**), and seven against murine *Abcc2* mRNA (shAbcc22-shAbcc28) (**Fig. 1B** and **Fig. 1D**). The ability of these constructs to knock-down endogenous *Abcc1* and *Abcc2* was assessed *in vitro* in Hepa1-6 cells using shGFP and shLuc as negative controls. shAbcc11, shAbcc17, and all 7 shAbcc2 constructs respectively inhibited endogenous *Abcc1* and *Abcc2* expression by more than 50% (**Fig. 1E** and **Fig. 1F**). For each target we selected the 2 most efficient shRNAs: shAbcc11 and shAbcc17, shAbcc22 and shAbcc28.

The specificity of *Abcc1* and *Abcc2* knock-down by these 4 constructs was tested on luciferase reporters containing the *Abcc1* or *Abcc2* target sequences. For all 4 constructs increasing concentrations of the shRNA construct induced dose-dependent inhibition of the specific luciferase reporter (**Fig. 1G**). These results support the sequence-specificity of endogenous *Abcc1* and *Abcc2* knock-down by these shRNAs.

siRNA processing was examined *in vitro* in two cell lines and compared to the siRNA processing of a validated active shRNA, shApoB (17). Efficient processing of the shRNA would indicate efficacy of the shRNA *in vivo*. HEK293T and Hepa1-6 cells were transfected with increasing amounts of plasmids encoding shAbcc22, shAbcc28 and shApoB. All shRNAs expressed similar amounts of siRNAs in HEK293T (**Fig. 1H**) and Hepa1-6 cells (**Fig. 1I**), indicating that they do not suffer from any misprocessing that could impair the anticipated *in vivo* knock-down.

Double-stranded RNA (dsRNA) including siRNAs can induce the interferon response (20). We determined that none of the 4 selected shRNAs induced expression of marker genes of the interferon response *in vitro* following transfection of Hepa1-6 cells (**Fig. 2**).

In vivo knock-down of Abcc1 and Abcc2 via scAAV-delivered shRNAs

C57BL/6 mice were injected with $4x10^{12}$gc/kg scAAV8 encoding shAbcc11, shAbcc17, shAbcc22, shAbcc28 and the shScr and PBS as controls. Animals were sacrificed at two weeks p.i. and *Abcc1*, *Abcc2* knock-down and siRNA expression in liver was determined, as well as alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels in plasma and

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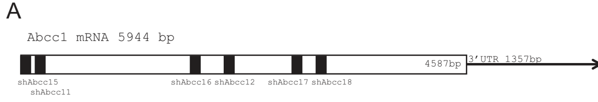

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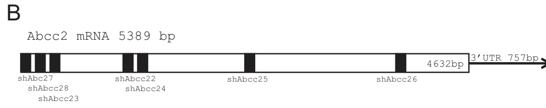

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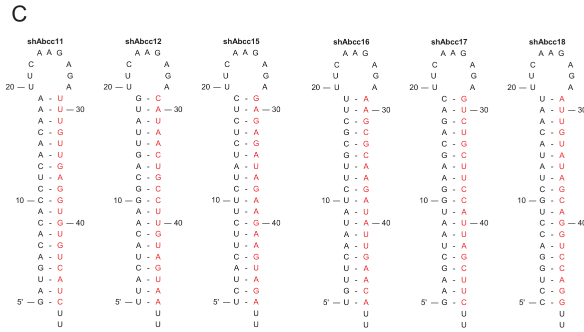

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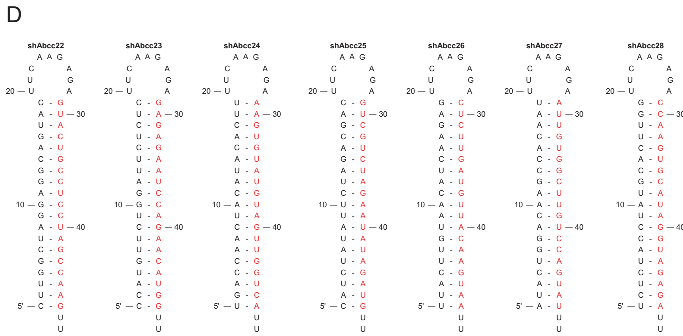

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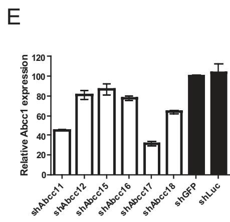

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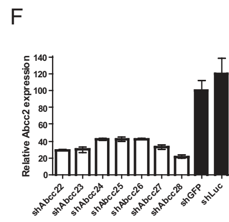

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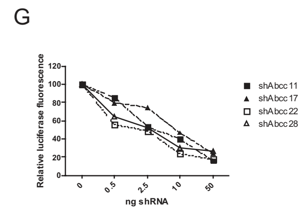

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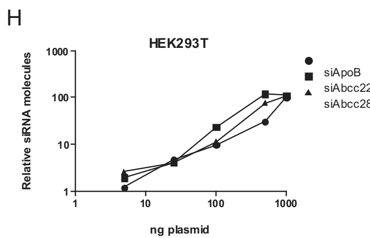

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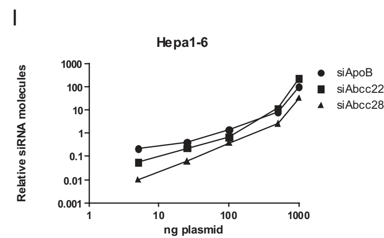

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interferon pathway genes expression in white blood cells. Transduction efficiency by scAAV8-shAbcc was greater than 90% as determined by fluorescence microscopy (data not shown). However, *Abcc1* and *Abcc2* mRNA expression was not significantly affected and we could determine only very low levels of siRNA (data not shown). We concluded that at this viral dose, these shRNAs were not expressed at a level sufficient to show a detectable effect. We subsequently narrowed our focus on the highly expressed *Abcc2* gene and injected C57 BL/6 mice with a 10-30 fold higher dose of 1.2-1.3x10^{14}gc/kg AAV-shAbcc22, AAV-shAbcc28, and AAV-shScr and PBS as controls. Mice injected with AAV-shAbcc22 and AAV-shAbcc28 were therefore sacrificed on day-13 p.i. in agreement with the guidelines of the local animal welfare committee as the animals presented some signs of physiological stress and one AAV-shAbcc22-injected mouse died. Animals from AAV-shScr and PBS groups were sacrificed on day-15 p.i. Gene expression analysis by RT-qPCR in the livers revealed a profound knock-down of *Abcc2* mRNA of 83% by shAbcc22 and shAbcc28 (**Fig. 3A**). Since there were signs of toxicity,

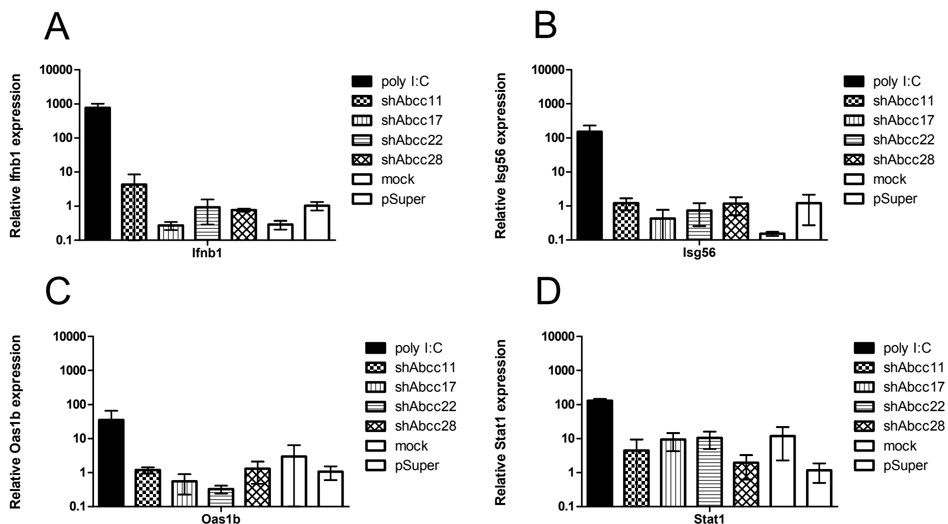

Figure 2. Four interferon pathway genes are not activated by the shAbcc constructs. Expression of interferon pathway genes. **(A)** Interferon beta 1 (*Ifnb1*), **(B)** Interferon-stimulated gene 56 (*Isg56*), **(C)** 2'-5' oligoadenylate synthetase 1B (*Oasb1b*), **(D)** Signal transducer and activator of transcription 1 (*Stat1*). Gene expression was quantified by RT-qPCR and normalized to β-actin (pSuper was set at 1).

◀ **Figure 1. shRNAs design and *in vitro* studies on endogenous *Abcc* mRNA and *Abcc* luciferase reporters.** Schematic representation of *Abcc1* and *Abcc2* mRNA and target sequences of the shRNAs. **(A)** shAbcc11, shAbcc12, shAbcc15, shAbcc16, shAbcc17, shAbcc18 targeting *Abcc1* mRNA (NM_013806.2) and **(B)** shAbcc22, shAbcc23, shAbcc24, shAbcc25, shAbcc26, shAbcc27, shAbcc28 targeting *Abcc2* mRNA (NM_008576.2). Predicted stem-loop structure with guide strand in red of **(C)** shAbcc1 and **(D)** shAbcc2. **(E)** Endogenous *Abcc1* mRNA knock-down by shAbcc1 constructs and **(F)** endogenous *Abcc2* mRNA knock-down by shAbcc2 constructs in Hepa1-6 cells (shGFP-treated cells were set at 100%). **(G)** *Luc-Abcc1* and *Luc-Abcc2* knock-down by shAbcc1 and shAbcc2 constructs in HEK293T cells (shScr-treated cells were set at 100%). Relative amount of siRNA molecules after transfection of HEK293T **(H)** and Hepa1-6 cells **(I)** with shAbcc22, shAbcc28 and shApoB (the highest siApoB value was set at 100%).

we determined whether the *Abcc2* knock-down was sequence-specific rather than due to general toxicity. Therefore the expression of the housekeeping genes *β-actin*, glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) and hypoxanthine guanine phosphoribosyl transferase (*Hprt*) was determined in RNA isolated from mouse livers. Our results indicated no detectable down-regulation of expression of the housekeeping genes between the 3 AAV-injected groups (data not shown) thus indicating the sequence-specificity of *Abcc2* knock-down by AAV-shAbcc22 and AAV-Abcc28 *in vivo*. Further quantification of the amount of siAbcc22 and siAbcc28 by Taqman revealed a higher number of siRNA in the AAV-shAbcc22-injected mice than in the AAV-shAbcc28-injected mice (**Fig. 3B**).

Interestingly, the mouse injected with AAV-shAbcc22, which first showed signs of toxicity and died was indeed the one presenting the highest levels of siRNA. While *in vitro* the amount siRNA produced by shAbcc22 and shAbcc28 was similar, *in vivo* there were differences between mice injected with the same dose of AAV-shAbcc22 and AAV-shAbcc28. We conclude that our *in vitro* studies were insufficient predictors of the shRNAs processing *in vivo*. Nevertheless *in vitro* efficacy correlated well with *in vivo* results, which following highly efficient transduction of hepatocytes with AAV8 achieved 83% knock-down of the target mRNA.

During the first week p.i. the mice from the AAV-shAbcc22 and AAV-shAbcc28 groups did not gain any weight, while the AAV-shScr and the PBS groups did (data not shown). It appears that the over-expression of shAbcc22 and shAbcc28 *in vivo* resulted in severe toxicity. Mice presented several indications of physiological stress. Analysis of plasma sampled on day-8 p.i. revealed elevated levels of AST, ALT and total bilirubin indicating liver toxicity in the AAV-shAbcc groups (**Fig. 4A**). On day-13 p.i. one of the AAV-shAbcc22-injected mice died and subsequently all mice from both AAV-shAbcc22 and AAV-shAbcc28 groups were sacrificed on the same day. Plasma analysis at 2 weeks p.i. revealed highly-elevated AST and ALT levels in the AAV-shScr group (data not shown) and weight loss was also observed during the second week p.i., indicating a slower onset of the toxicity, most likely due to the lower viral dose that was administered to this group.

It has been reported that high siRNA expression from shRNA cassettes containing a strong promoter can lead to lethal toxicity by over-saturating the RNAi machinery and

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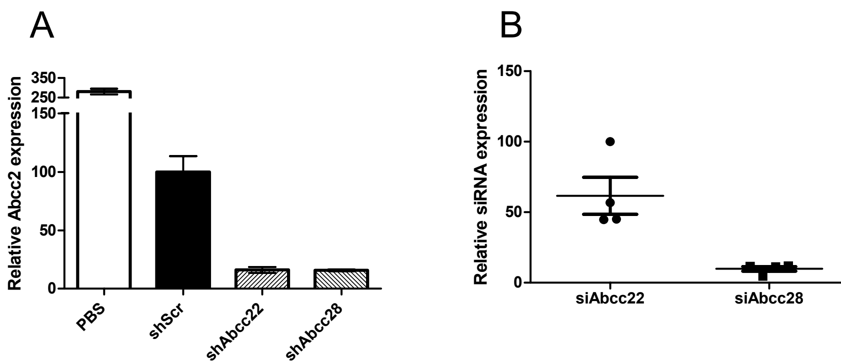

Figure 3. *In vivo* knock-down of *Abcc2* and quantification of siAbcc2. **(A)** Relative *Abcc2* expression was quantified by RT-qPCR and normalized to *β-actin* (shScr group was set at 100%). **(B)** Relative quantification of siAbcc22 and siAbcc28 molecules was quantified by RT-qPCR and normalized to *β-actin* (the highest value was set at 100%).

affecting endogenous miRNA processing (21). We determined the expression levels of 3 cellular miRNAs, namely miR-122, miR-29a and let-7a, in the livers of the injected animals. If saturation of the RNAi machinery occurred to the point where endogenous metabolic processes are expected to be significantly affected, expression of these liver miRNAs should be significantly decreased. Taqman assays for the 3 cellular miRNAs revealed down-regulation in the mouse livers injected with AAV-shScr, AAV-shAbcc22 and AAV-shAbcc28 compared to the PBS group (**Fig. 4B**), indicating over-saturation of the RNAi machinery.

Circumventing shRNA toxicity

Expression of shRNA from a weaker promoter and from a miRNA scaffold can circumvent toxicity problems (22). Therefore, shAbcc11 and shAbcc28 sequences were expressed from a miRNA backbone (**Fig. 5A** and **Fig. 5B**) and their ability to knock-down *Luc-Abcc11* and *Luc-Abcc28* luciferase reporters was assessed *in vitro* in HEK293T cells. Comparison of knock-down induced by shAbcc11 and miAbcc11, and by shAbcc28 and miAbcc28 showed that both constructs have a similar efficiency, the miRNA being slightly better (**Fig. 5C** and **Fig. 5D**). Furthermore quantification of the amount of siRNA molecules necessary to achieve this knock-down effect by Taqman assay revealed that miAbcc28 produced 55%-75% less siAbcc28 molecules than shAbcc28 (**Fig. 5E**). Since these miRNAs were expressed from a significantly weaker Pol II promoter, less siRNA molecules were being produced while retaining equal inhibition properties compared to the shRNA. This indicates that miRNAs may be more potent molecules for induction of RNA silencing than shRNAs. Considering the toxicity issues raised in this study, the possibility of expressing siRNA sequences from a miRNA backbone which would render similar or increased efficiency but would present reduced toxicity risks in *in vivo* studies is of considerable interest. We are currently comparing the long-term efficacy and toxicity profile of siRNAs expressed from AAV-shRNA and AAV-miRNA backbone *in vivo*. Future research will therefore focus on expressing siRNA from a miRNA backbone to achieve efficient and safe *in vivo* target knock-down.

To our knowledge, this is the first report to show AAV-mediated knock-down of Abcc2 *in vivo*. Concomitant toxicity was observed and was attributed to a previously described mechanism of over-saturation of the RNAi machinery (21). Subsequent generation of miRNAs showed a better efficacy profile, i.e. the same effect mediated by significantly less

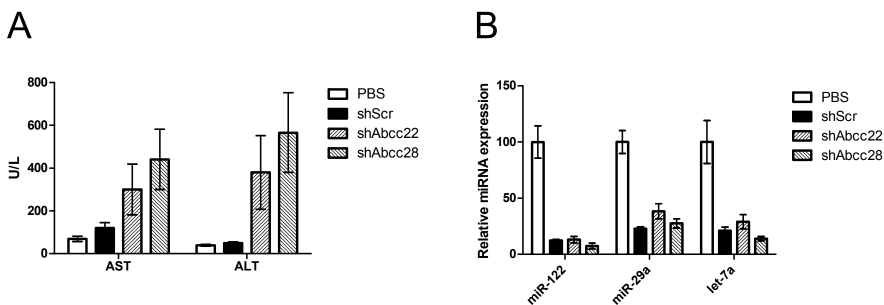

Figure 4. Over-expression of shAbcc2 induces liver toxicity. (A) Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels in plasma at 8 days p.i. **(B)** Relative expression of three cellular miRNAs, miR-122, miR-29a and let-7a was quantified by RT-qPCR and normalized to *β-actin* (PBS group was set at 100%).

Figure 5. Expression of shAbcc11 and shAbcc28 from a miRNA scaffold and siRNA quantification. Predicted stem-loop structure with guide strand in red of **(A)** miAbcc11 and **(B)** miAbcc28. *Luc-Abcc1* knock-down by *Abcc1*-targeting **(C)** and *Luc-Abcc2* knock-down by *Abcc2*-targeting **(D)** shRNAs and miRNAs (shScr and miScr were set at 100%). **(E)** Relative amount of siRNA molecules produced by shRNA and miRNA constructs (the lowest shRNA dose was set at 100%).

siRNA molecules. We therefore expect that these constructs will yield a strong and safe knock-down following liver transduction with AAV.

ACKNOWLEDGEMENTS

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SUPPLEMENTARY MATERIAL

Table S1. Oligonucleotide sequences used in this study. mmu-*Abcc1* (NM_008576), mmu-*Abcc2* (NM_013806), *luc* and *gfp* sequences in the shRNA oligos are in bold.

Name	Sequence (5′-3′)	Target	
shAbcc11-f	GATCCCC**GATGACACACCTCAACAAA**TTCAAGA-GA**TTTGTTGAGGTGTGTCATC**TTTTTA	Abcc1	207-225
shAbcc11-r	TCGAGAAAAAGATGACACACCTCAACAAATCTCTT-GAATTTGTTGAGGTGTGTCATCGGG		
shAbcc12-f	GATCCCC**TTACTACAAGGCAGTTATG**TTCAAGA-GA**CATAACTGCCTTGTAGTAA**TTTTTA	Abcc1	2214-2232
shAbcc12-r	TCGAGAAAAATTACTACAAGGCAGTTATGTCTCTT-GAACATAACTGCCTTGTAGTAAGGG		
shAbcc15-f	GATCCCC**TCTACTTCTTCTATCTCTC**TTCAAGA-GA**GAGAGATAGAAGAAGTAGA**TTTTTA	Abcc1	169-182
shAbcc15-r	TCGAGAAAAATCTACTTCTTCTATCTCTCTCTCTT-GAAGAGAGATAGAAGAAGTAGAGGG		
shAbcc16-f	GATCCCC**TGTTCAATATCTTGCGCTT**TTCAAGA-GA**AAGCGCAAGATATTGAACA**TTTTTA	Abcc1	1175-1793
shAbcc16-r	TCGAGAAAAATGTTCAATATCTTGCGCTTTCTCTT-GAAAAGCGCAAGATATTGAACAGGG		
shAbcc17-f	GATCCCC**GAAGCTAATGGAAGCAGAC**TTCAAGA-GA**GTCTGCTTCCATTAGCTTC**TTTTTA	Abcc1	2832-2850
shAbcc17-r	TCGAGAAAAAGAAGCTAATGGAAGCAGACTCTCTT-GAAGTCTGCTTCCATTAGCTTCGGG		
shAbcc18-f	GATCCCC**CCTGGACCTGCTATACAAT**TTCAAGA-GA**ATTGTATAGCAGGTCCAGG**TTTTTA	Abcc1	3147-3165
shAbcc18-r	TCGAGAAAAACCTGGACCTGCTATACAATTCTCTT-GAAATTGTATAGCAGGTCCAGGGGG		
shAbcc22-f	GATCCCC**CTTGGCTAGGAGGCAGTAC**TTCAAGA-GA**GTACTGCCTCCTAGCCAAG**TTTTTA	Abcc2	1221-1239
shAbcc22-r	TCGAGAAAAACTTGGCTAGGAGGCAGTACTCTCTT-GAAGTACTGCCTCCTAGCCAAGGGG		
shAbcc23-f	GATCCCC**CCATGTTCTGGATTCTCTC**TTCAAGA-GA**GAGAGAATCCAGAACATGG**TTTTTA	Abcc2	395-413
shAbcc23-r	TCGAGAAAAACCATGTTCTGGATTCTCTCTCTCTT-GAAGAGAGAATCCAGAACATGGGGG		
shAbcc24-f	GATCCCC**TGACCAACTACTACACTT**TTCAAGA-GA**AAGTGTAGTAGTTGGTCA**TTTTTA	Abcc2	1295-1313
shAbcc24-r	TCGAGAAAAATGACCAACTACTACACTTTCTCTT-GAAAAGTGTAGTAGTTGGTCAGGG		
shAbcc25-f	GATCCCC**CATCTATATTCTAGACGAC**TTCAAGA-GA**GTCGTCTAGAATATAGATG**TTTTTA	Abcc2	2334-2352
shAbcc25-r	TCGAGAAAAACATCTATATTCTAGACGACTCTCTT-GAAGTCGTCTAGAATATAGATGGGG		

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Table S1. Oligonucleotide sequences used in this study. mmu-*Abcc1* (NM_008576), mmu-*Abcc2* (NM_013806), *luc* and *gfp* sequences in the shRNA oligos are in bold.

Name	Sequence (5'-3')	Target	
shAbcc26-f	GATCCCC**TTACTTGTAACATCAAGAG**TTCAAGA-GA**CTCTTGATGTTACAAGTAA**TTTTTA	Abcc2	3953-3971
shAbcc26-r	TCGAGAAAAATTACTTGTAACATCAAGAGTCTCTT-GAACTCTTGATGTTACAAGTAAGGG		
shAbcc27-f	GATCCCC**ATACTGGACAAGCCACAAT**TTCAAGA-GA**ATTGTGGCTTGTCCAGTAT**TTTTTA	Abcc2	266-284
shAbcc27-r	TCGAGAAAAAATACTGGACAAGCCACAATTCTCTT-GAAATTGTGGCTTGTCCAGTATGGG		
shAbcc28-f	GATCCCC**TCTCTACCTATGCACTTGG**TTCAAGA-GA**CCAAGTGCATAGGTAGAGA**TTTTTA	Abcc2	312-330
shAbcc28-r	TCGAGAAAAATCTCTACCTATGCACTTGGTCTCTT-GAACCAAGTGCATAGGTAGAGAGGG		
shScr-f	GATCCCC**GATCGAATGTGTACTTCGA**TTCCAAGA-GA**TCGAAGTACACATTCGATC**TTTTTGCATGCC	Doege et al. 2008	
shScr-r	TCGAGGCATGCAAAAAGATCGAATGTGTACTTC-GATCTCTTGGAATCGAAGTACACATTCGATCGGG		
shGFP-f	GATCCCC**AGCTGGAGTACAACTACAA**CCTTCCT-GTCAG**TTGTAGTTGTACTCCAGCT**TTTTGCATGCC	eGFP	
shGFP-r	TCGAGGCATGCAAAAAGCTGGAGTACAACTA-CAACTGACAGGAAGGTTGTAGTTGTACTCCAGCT-GGG		
shLuc-M-f	GATCCCC**CTGCCCTGCTGGTGCCCACAC**TTCCAA-GAGA**GTGTGGGCACCAGCAGGGCAG**TTTTTGCAT-GCC	Luc	
shLuc-M-r	TCGAGGCATGCAAAAACTGCCCTGCTGGT-GCCCACACTCTCTTGGAAGTGTGGGCACCAGCAG-GGCAGGGG		
Luc-Abcc11-f	TCGAG**TCTCTCTCGCCATGACCGGGGCTACATC-CAGATGACACACCTCAACAAAACCAAAACTGCCT-TAGGATTCTTTCT**GC	Abcc11	
Luc-Abcc11-r	GGCCGC**AGAAAGAATCCTAAGGCAGTTTTGGTTTT-GTTGAGGTGTGTCATCTGGATGTAGCCCCGGT-CATGGCGAGAGAGA**C		
Luc-Abcc17-f	TCGAG**GCAGAAGGCTGGAGCTAAGGAGGAGACGT-GGAAGCTAATGGAAGCAGACAAGGCCCAGACAG-GGCAGGTGCAGCT**GC	Abcc17	
Luc-Abcc17-r	GGCCGC**AGCTGCACCTGCCCTGTCTGGGCCTT-GTCTGCTTCCATTAGCTTCCACGTCTCCTCCT-TAGCTCCAGCCTTCTGC**C		
Luc-Abcc22-f	TCGAG**TATATAAGAAGGCACTAAC-CCTATCTAACTTGGCTAGGAGGCAGTACACGATT-GGAGAGACGGTGAACTTGAT**GC	Abcc22	

Table S1. Oligonucleotide sequences used in this study. mmu-*Abcc1* (NM_008576), mmu-*Abcc2* (NM_013806), *luc* and *gfp* sequences in the shRNA oligos are in bold.

Name	Sequence (5′-3′)	Target	
Luc-Abcc22-r	GGCCGC**ATCAAGTTCACCGTCTCTCCAATCGTG-TACTGCCTCCTAGCCAAGTTAGATAGGGTTAGT-GCCTTCTTATATA**C		
Luc-Abcc28-f	TCGAG**ACCTCTCACAGAAGATACT-GGACAAGCCACAATTCCTCCTGTTAAATATA-CAAATCCAATTCTCTACCTATGCACTTGGCTCCT-GGTGTTGGTGGCAG**GC	Abcc28	
Luc-Abcc28-r	GGCCGC**CTGCCACCAACACCAGGAGCCAAGTGCAT-AGGTAGAGAATTGGATTTGTATATTTAACAGGAG-GAATTGTGGCTTGTCCAGTATCTTCTGTGAGAGGT**C		
Abcc1-f	GAGTCAAAGCCGGTGGAAAAT	Abcc1	qRT-PCR
Abcc1-r	TTAGCTCCAGCCTTCTGCAGTT		
Abcc2-f	TGAAAAACAGAATGGGACCGA	Abcc2	qRT-PCR
Abcc2-r	TTGGATGCATTTCTGCAAGC		
Actin-f	ACGGCCAGGTCATCACTATTG	βactin	qRT-PCR
Actin-r	CAAGAAGGAAGGCTGGAAAAGA		
Gapdh-f	ACATGTTCCAGTATGACTCCACTCA	Gapdh	qRT-PCR
Gapdh-r	GCCTCACCCCATTTGATGTT		
Hprt-f	AAGACTTGCTCGAGATGTCATGAAG	Hprt	qRT-PCR
Hprt-r	TCCAGCAGGTCAGCAAAGAA		
Ifnb1-f	CTGCCTTTGCCATCCAAGAG	Ifnb1	qRT-PCR
Ifnb1-r	ACTGTCTGCTGGTGGAGTTC		
Igs56-f	TGCACAACCTCCTGGCCTAC	Isg56	qRT-PCR
Igs56-r	TCGCCAGGCTTCTCTTGCTC		
Oas1b-f	TGATGTGCTGCCAGCCTATG	Oasb1b	qRT-PCR
Oas1b-r	GATAACTTGCCCTCCTTCCC		
Stat1-f	GTGAGAGCCAGTCGTTTCAG	Stat1	qRT-PCR
Stat1-r	GCAGGTTCGGGATTCAACAC		
LP1-f	CCCTGTTTGCTCCTCCGATAA	LP1	qRT-PCR
LP1-r	GTCCGTATTTAAGCAGTGGATCCA		

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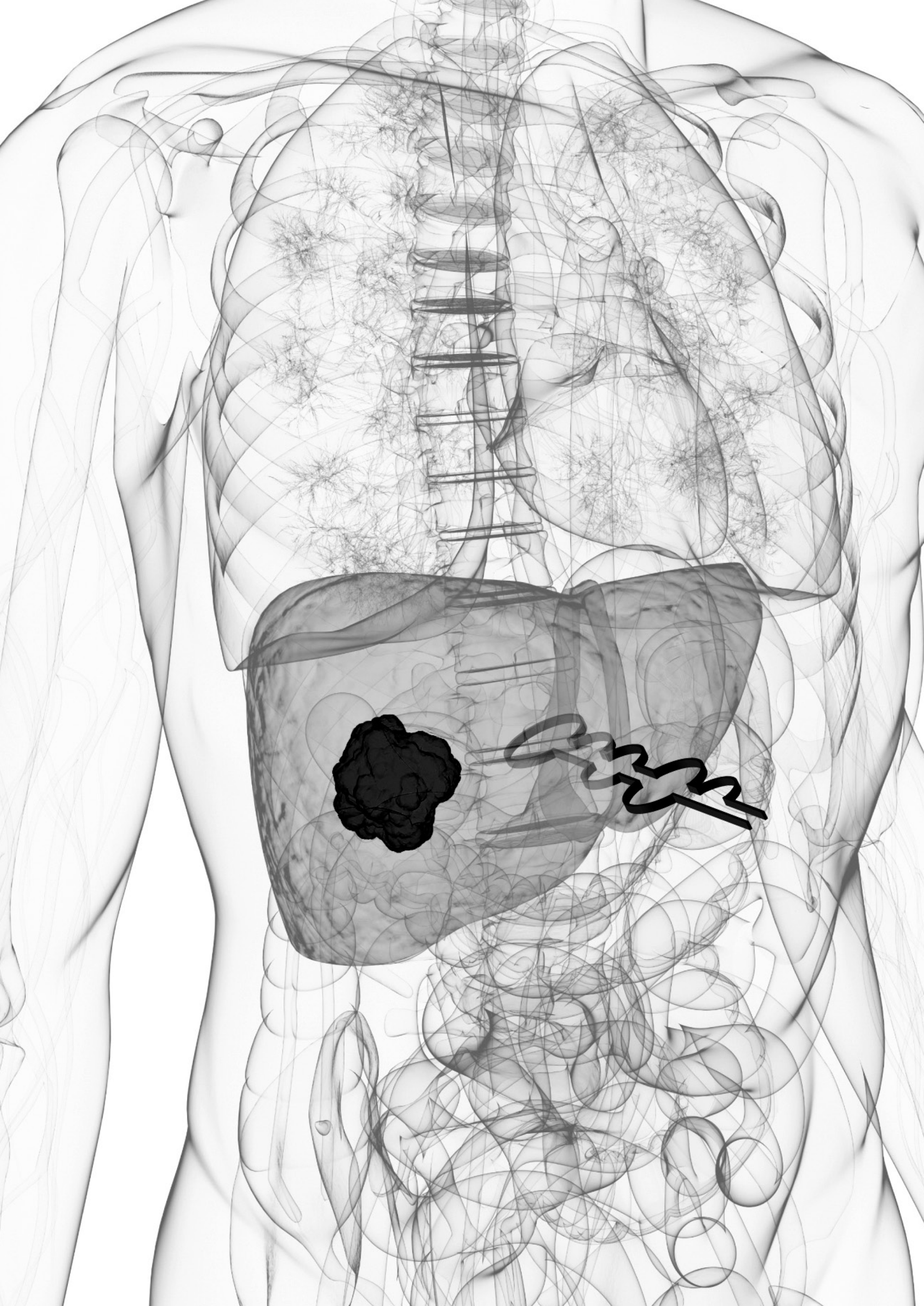

GENERAL DISCUSSION

List of abbreviations

HCC,	hepatocellular carcinoma
ABC,	ATP-binding cassette
RNA,	ribonucleic acid
RNAi,	RNA interference
siRNA,	small interfering RNA
shRNA,	short-hairpin RNA
miRNA,	microRNA
mRNA,	messenger RNA
AFP,	α-fetoprotein
DCP,	des-γ carboxyprothrombin
OLT,	orthoptic liver transplantation

The current research aimed at characterizing the ABC transporter gene and cellular miRNA profiles of untreated hepatocellular carcinoma (HCC). Employing the molecular profiling of HCC patient samples, two strategies were then developed for RNAi-based gene therapy of HCC, one harnessing the endogenous regulation of ABC transporters by cellular miRNAs, the other one making use of shRNAs and artificial miRNAs to directly knock-down the ABC transporters. An overview of the PhD work is presented in **Fig. 1**.

1. Molecular profiling of hepatocellular carcinoma: up-regulation of ABC transporter genes, concomitant down-regulation of cellular miRNAs, and implications

1.1. Up-regulation of ABC transporter genes in HCC

Multidrug resistance, a major cause of chemotherapeutic treatment failure, is acknowledged as one of the three main causes of low survival of HCC patients, together with tumor recurrence and late diagnosis of the disease. Multidrug resistance is a critical issue for many diseases besides HCC, as it can affect patients with a variety of hematological malignancies and solid tumors, including head and neck, lung, ovarian, breast, prostate, colorectal, bladder cancers (1), but also patients with rheumatoid arthritis (2) or epilepsy (3). Multidrug resistance is therefore a very well studied field of biomedical research, with several axes of investigation being developed to better understand and counteract this phenomenon, for instance generation and characterization of knock-out mouse models, development of inhibitors, and the emerging field of gene therapy. Nevertheless, literature on multidrug resistance transporters expression in HCC patients is limited, though expression profiling of several ABC transporters is well-implemented (4), and studies performed on clinical samples have been published for instance in melanoma (5) and pseudoxanthoma elasticum (6). Most studies performed on HCC patient samples report the profiling of a limited subset of genes only, *e.g.* ABCC3 (7); ABCB1, ABCC1, ABCC2 and ABCC3 (8); ABCB1, ABCC1, ABCC3 and ABCG2 (9); ABCB1, ABCB4, ABCC1, ABCC2, and ABCC3 (10).

The only notable exception is a study by Moustafa *et al.* which determined the expression of ABCA1, ABCA2, ABCB1, ABCB2, ABCB3, ABCB4, ABCC1, ABCC2, ABCC3, ABCC4, ABCC5, ABCG1 and ABCG2 in 21 HCC patients undergoing surgical resection (11). This study reported up-regulation of ABCC4 and ABCG1 and down-regulation of ABCA1, ABCB4 and ABCG2 in tumoral compared to non-tumoral tissues. However no clinical data was reported for those 21 included patients. In the present work described in **Chapter 3**, expression of 15 ABC transporters was determined in 19 HCC patients undergoing surgical resection, namely ABCA1, ABCA2, ABCB1, ABCB6, ABCC1, ABCC2, ABCC3, ABCC4, ABCC5, ABCC6, ABCC10, ABCC11, ABCC12, ABCE1 and ABCG2. The study revealed that in HCC 12 ABC transporter genes are significantly up-regulated in tumoral compared to non-tumoral tissue, namely ABCA2, ABCB1, ABCB6, ABCC1, ABCC2, ABCC3, ABCC4, ABCC5, ABCC10, ABCC11, ABCC12 and ABCE1 (12). In addition, the patient samples obtained from the Réseau Centre de Ressources Biologiques Foie (French Liver Biobanks Network) were provided with detailed clinical data which allowed us to analyze possible correlations between expression profile and clinical characteristics. Moreover the inclusion criteria was the absence of chemotherapeutic treatment prior to surgery, a criterion which could be met for 16 of the patients included in the study. This work is hence the first extensive ABC expression profiling in untreated HCC patients, revealing that in the

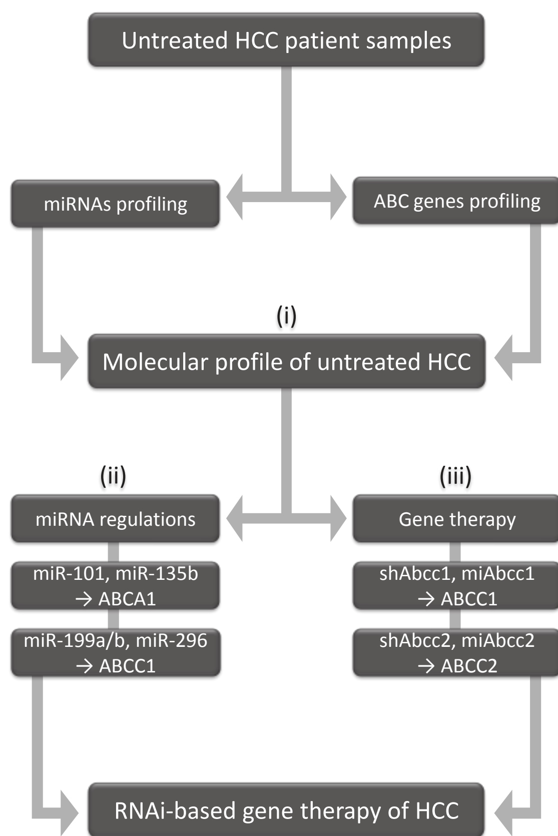

Figure 1: Overview of the presented PhD work. Based on the established molecular profile of ABC and miRNA expression in HCC (i), two strategies were developed for RNAi-based gene therapy. The first one relied on modulating ABC transporters expression by changing cellular miRNA expression (ii). The second one (iii) directly targeted ABCC1 or ABCC2 using shRNAs or artificial miRNAs.

absence of prior chemotherapeutic treatment, 10 ABC transporter genes are significantly up-regulated in tumoral compared to non-tumoral tissue, namely ABCA2, ABCB1, ABCB6, ABCC2, ABCC3, ABCC4, ABCC5, ABCC10, ABCC11 and ABCE1 (12). For instance ABCB1 was 2.6 fold up-regulated, ABCC1 2.9 fold, ABCC10 3.4 fold. Though changes in expression can appear to be mild, such up-regulation of so many transporters in tumoral tissue could be physiologically significant. In addition, our findings go in the direction of a novel hypothesis stating that beyond their role in drug efflux, ABC transporters may have a fundamental role in tumor biology (13). Strengthening this thesis is the fact that some ABC transporters, namely ABCB1, ABCC1, ABCC3 and ABCG2, were detected in hepatic progenitor cells (14, 15), moreover ABCC1 expression was higher in HCC with poor survival (9). To definitely corroborate this hypothesis, larger cohorts of untreated HCC patients would be needed, in order to correlate the expression of ABC transporters to clinical parameters such as tumor aggressiveness, prognosis, and response to therapy.

1.2. Down-regulation of cellular miRNAs in HCC

A general down-regulation of cellular miRNAs is observed in tumor tissues. This prevents tumor differentiation and indicates a role for miRNAs in initiation and maintenance of tumors(16). In HCC, several studies have been done and the miRNA profile is well-described, as reviewed in **Chapter 2** (17). In the present work described in **Chapter 3** (12), we determined global changes in miRNA expression between HCC patient samples and non-tumoral liver samples, in order to correlate miRNA expression data with those from the ABC genes, in the same sample set. The goal was to determine the miRNA profile of untreated HCC patients, as it may differ from the profile of treated HCC described in literature. Though performed on a small sample set, the current profiling appeared consistent with literature, with the identification of HCC hallmarks miR-122 and miR-21 among the down- and up-regulated miRNAs, respectively, and a majority of miRNAs being down-regulated. Out of 378 miRNAs whose expression was determined, 90 were significantly dysregulated in HCC, among which 11 were up-regulated and 79 down-regulated. This finding led us to formulate the hypothesis that cellular miRNAs regulate the expression of ABC transporter genes, and therefore that the observed general down-regulation of miRNAs may be the cause of the general up-regulation of ABC transporter genes. The physiological consequences of such inverse changes of cellular miRNAs and ABC transporters needed to be further justified and validated.

2. miRNA target validation: ABC transporter genes expression is regulated by cellular miRNAs in hepatocellular carcinoma

miRNA regulation is based on target recognition. If in the seed region (nt 2-7 from the 5' end of the miRNA) the complementarity between the miRNA and its target in the mRNA is perfect, the mRNA will be cleaved by RISC and degraded; in case of imperfect complementarity translation will be repressed (18-21). In the present work described in **Chapter 4**, predictions of ABC transporter genes regulations by cellular miRNAs were made by bioinformatics. With the development of bioinformatics tools, *in silico* miRNA target predictions can easily be made for a gene or a group of genes of interest based on profiling data. When entering a gene of interest, the algorithm creates a list of all miRNAs that could bind to the 3'UTR, based on sequence complementarity. However, more delicate is the next step of miRNA target validation, *i.e.* the demonstration that in a given biological system, modulating miRNA levels does affect the levels of the predicted target gene(s). The biological validation is frequently performed on luciferase reporters initially, then on the endogenous target *in vitro*, and finally on the endogenous target *in vivo*, and must present evidence of the bioinformatically predicted regulation (22). A significant percentage of the predictions cannot be biologically validated *in vitro* because the miRNA-3'UTR binding relies on a 7-8 nt homology, the so called "seed" sequence. As one can imagine, there would be multiple 7-8 nt random matches of the cellular miRNAs within the human genome. For instance, the false positive rate alone of two frequently used algorithms is estimated at 31% for TargetScan for targets identified in mammals (23), and at 30% for PicTar (24). While often the miRNAs at the top of the list are chosen for further validation, *i.e.* those with the most predicted target sites and/or strongest predicted binding, here we chose to take into account only those miRNAs which were identified during our screen as down-regulated in HCC. At the same time, we selected only ABC transporter genes identified during our screen – performed on the same clinical samples – as up-regulated. Out of the 79 cellular

miRNAs identified as down-regulated in HCC, 25 had predicted targets in 6 of the ABC transporter genes. In total, 13 regulations of ABC genes by cellular miRNAs were verified on specific luciferase reporters. During the next step, the confirmation of miRNA regulation of endogenous ABC gene expression was performed for ABCA1 and ABCC1, 100% of the targets validated on luciferase reporters were confirmed. Cellular miR-101 and miR-135b targeted ABCA1, while miR-199a/b and miR-296 targeted ABCC1. In addition, it would be extremely interesting to provide functional validation for the miRNA regulation of ABCA1 and ABCC1 in relation with HCC. ABCA1 is a cholesterol transporter, thus for ABCA1, functional validation could be achieved by cholesterol efflux assay, and possibly further by *in vivo* measurements of circulating HDL cholesterol and plasma triglycerides. ABCC1 is a multidrug resistant transporter with a broad range of substrates among which cytotoxic drugs, therefore for ABCC1, functional validation could be provided by cytotoxicity assay, provided that an ABCC1-specific cytotoxic drug would be used in order to overcome functional compensation due to overlapping in ABCC substrates. These experiments should be carried on in the future in order to confirm the role of cellular miRNAs in regulating ABCA1 and ABCC1 in HCC. Unfortunately confirmation of the miRNA targets in ABCC5, ABCC10 and ABCE1, faced some technical difficulties related to protein levels detection. Technical improvements are needed to verify the regulation of those genes by cellular miRNAs. ABCC5 and ABCC10 are not very well characterized, but they both are multidrug resistance transporters, hence relevant in the context of HCC. ABCE1 is not a transporter but a RNase L inhibitor, however it has been previously associated with HCC (25), and its silencing inhibited the proliferation and invasiveness of a small cell lung cancer cell line (26) underlining its possible relevance as a target for cancer.

Another commonly encountered problem when it comes to target validation is reproducibility. For instance, it was previously shown that endogenous ABCE1 expression is regulated by miR-203 in HuH7 cells (25). Unsurprisingly, this regulation was identified among our pool of 51 predictions, since we used the same algorithms. However we were unable to further validate this regulation due to technical difficulties to detect ABCE1 protein. Therapeutic applicability is another limitation of target validation. The effect of miRNAs on the target genes is usually in the range of 2 fold changes, so one could argue that the modulation of their expression in a therapeutic perspective may be insufficient to cause a significant physiological effect that would be clinically assessable. However, the effect of miRNAs on ABC transporters should not be underestimated, as was recently demonstrated by Regulus Therapeutics (San Diego, CA), a biopharmaceutical company involved in development of medicines targeting miRNAs. They demonstrated that ABCA1 expression was regulated by miR-33a (27). Based on studies performed *in vitro* and in mice, showing that decreasing miR-33a up-regulates ABCA1 (27), preclinical studies were initiated and a recent presentation stated that these findings have been successfully translated to non-human primates (Keystone Symposium, Sante Fe, NM, January 2012). Based on these data, one would expect that artificially increasing ABCA1 expression would promote the efflux of cholesterol to ApoA1, hence increasing plasma HDL and reducing atherosclerosis. According to the press release, this study indeed demonstrated that "systemic delivery of an anti-miR-33a/b oligonucleotide increased hepatic expression of ABCA1, induced a sustained increase in circulating HDL cholesterol and decreased plasma triglycerides". Hence it seems that miRNA modulation is able to lead to clinically assessable effects. In the Regulus project, ABCA1 contains 3 target sites for miR-33a. Therefore our

study, where miR-101 and miR-135b have respectively 3 and 2 target sites in ABCA1, may hold therapeutics promises as well.

Looking further than ABC transporters, a spectacular study by Kota *et al.* showed that miRNA modulation can affect liver cancer. Instead of decreasing endogenous miRNA levels as described previously for miR-33a, the authors adopted a miRNA replacement therapy, *i.e.* systemic administration of miR-26a to compensate for its down-regulation in HCC. The authors show that the predicted targets of miR-26a CCND2 and CCNE2 are reduced upon enforced miR-26a expression *in vitro* (28). Moreover AAV-miR-26a delivery in a mouse model of liver cancer protected the animals from tumor development (28). The only limitation of this study is the fact that miR-26a was initially identified as the most down-regulated miRNA in tumors of the same mouse model that what used for validation. This may be the reason why such profound effects were observed in this model. Though miR-26a down-regulation was later on confirmed in HCC patient samples, the effect of its up-regulation in human may be more limited. This work shows how modulating the expression of one miRNA only can have dramatic effects on tumor development. In conclusion, if in the future the role of ABC transporters in the tumor phenotype is confirmed, modulating the expression of miRNAs targeting ABC transporters could have similarly dramatic effects. Nevertheless, independently of the possible role of ABC transporters in tumors but making use of their primary transporter function, it would be extremely interesting to further explore the potential of modulating ABC transporters expression via miRNA regulations. Based on the results presented here for ABCA1 and ABCC1, this approach could possibly allow adjustment of the cholesterol pathway or decrease of the resistance to chemotherapeutics.

3. Challenges and future perspectives for RNAi therapeutics

RNAi has been described as a posttranscriptional gene regulation mechanism in mammalian cells and holds the promise to be used as a therapy of a wide range of indications including cancer, infectious, genetic and autoimmune diseases (29). Initially discovered in 1990 (30, 31), RNAi was awarded the Nobel Prize of Medicine in 2006 (32), and has been used since then for various biomedical applications, with more than a dozen of RNAi-based therapeutics currently undergoing clinical studies. Nevertheless, the RNAi field faces several challenges that need to be overcome in order to take the technology forward. Several of those challenges are the activation of the immune response by RNAi molecules, the toxicity that can be caused by overexpression of shRNAs, and induction of unanticipated off-target effects.

In the present work described in **Chapter 5** (33), shRNAs against ABCC1 and ABCC2 were developed and tested *in vitro*, where the constructs giving the strongest knock-down were selected. It has been described that Toll-like receptors (TLRs) are able to recognize siRNAs of specific sequences as pathogen-activated molecular patterns and activate immune cells by inducing IFN, TNFα and IL-6 (34, 35). Therefore in the present work, additional *in vitro* studies were performed which demonstrated non-activation of the interferon pathway. This encouraged us to proceed to the next step of *in vivo* proof-of-concept following AAV-mediated delivery to wild-type mice. At the highest viral dose, a knock-down was observed; unfortunately some toxicity was also encountered. Following the report in 2006 by Grimm *et al.* that shRNAs can cause fatality in mice due to oversaturation of the cellular RNAi pathway (36), toxicity was openly established as one of the main issues of RNAi therapeutics. The authors described that systemic injection of high doses of AAV-shRNA

led to extremely high shRNA expression levels, which when processed into siRNA by the cellular RNAi machinery impeded the processing of endogenous miRNAs, eventually leading to severe toxicity or fatality. In the present work, cellular miRNA levels were similarly down-regulated, and the observed toxicity was attributed to oversaturation of the RNAi machinery. This unexpected event prevented the initiation of further *in vivo* studies in a sub-cutaneous tumor model. Because ABCC1 and ABCC2 are drug efflux transporters, it is expected that their down-regulation in multidrug resistant tumors would lead to an increase in chemotherapy efficacy. Tumor growth rates upon chemotherapeutic treatment could therefore have been compared in RNAi-treated and untreated tumors.
Currently the scientific community acknowledges that to avoid toxicity related to oversaturation of the RNAi pathway, tissue-specific and/or regulated pol II promoters with a moderate level of activity should be used. There are two possibilities, either expressing shRNAs from a pol II promoter (37) or embedding the shRNA sequences within a miRNA scaffold also transcribed from a pol II promoter (38). When delivery vehicles are used at equal doses, an artificial miRNA rather than a shRNA would result in reduced toxicity caused by overloading of the cellular RNAi machinery (38, 39). Therefore in the present work, artificial miRNAs were next developed in order to counteract toxicity, and *in vitro* tests revealed their efficacy in terms of luciferase reporter knock-down. In a subsequent work, a shRNA and an artificial miRNA targeting ApoB were directly compared *in vivo*. To our surprise, it appeared that despite predictions, embedding the same siRNA sequence into either a shRNA or a miRNA scaffold does not lead to the same siRNA products due to differential processing patterns. Next Generation Sequencing (NGS) analysis of small RNAs revealed that the length of the siRNA products varied, with shApoB products between 19 and 23 nt-long, 21-nt being the most abundant, and miApoB products between 23 and 25 nt-long, 24-nt being the most abundant (40). The abundance of guide and passenger strands also varied between shApoB and miApoB. For shApoB, 75.9% of the reads corresponded to the guide strand, and 11% matched the passenger strand. For miApoB, only 29.3% of the reads corresponded to the guide strand, and 61.8% matched the passenger strand (40). Moreover, significant changes in the cellular miRNA profile were observed in shApoB- compared to miApoB-transfected cells indicating that the two hairpins had different effects on the cellular miRNA machinery. Future studies have been set up to look at the significance of those effects in vivo. Based on this work, we concluded that careful analysis of the siRNA products is necessary in preclinical studies, in order to screen for changes in the cellular miRNA profile and/or unexpected off-targeting effects. Off-targeting designates the knock-down of undesired genes, often based on a miRNA-like sequence complementarity. Common strategies to avoid off-targeting include chemical modifications (41), use of the lowest siRNA dose that gives the maximum effect (42) or of several siRNAs against the same target, hence dividing the off-targeting (43). In addition, Boudreau *et al.* recently showed that a careful screen of predicted miRNA-like targets of a given siRNA translates into changes in global expression profile (44), which suggests that attention to target predictions should be paid early on.
RNAi is a very potent technology which targets a niche market, and it therefore offers great perspectives for biomedicine. However, it appears to be a double-edged sword, as its dramatic potential could also translate into dramatic side-effects. Every new project should therefore be carefully designed starting from the very early steps, as has been described above. First the desired expression pattern should be determined, for instance regulated

expression and/or a tissue-specific expression. A more sophisticated manner of regulating transgene expression is to harness endogenous miRNA. It has been known that different cell types express a specific pool of cellular miRNAs (45). For example antigen-presenting cells (APC) specifically express mir-142-3p (46). Linking mir-142-3p targets to a transgene will result in recognition of the sequences by the cellular miRNAs in APC and hence inhibition of expression due to mRNA degradation. As a result, transgene expression will be restricted to cell types, which do not express this miRNA and antigen presentation will be avoided (47, 48). Next, early development stages should include determination of the extend of off-targeting via global gene expression profile analysis, and screen for immune response activation. Later development stages should then only screen for knock-down efficacy among those pre-selected candidates. In the recent years, the field of RNAi therapeutics developed considerably. Learning from the mistakes that have been made and adapting the development steps accordingly will contribute to making the field more reliable in the future.

4. Biomarkers for hepatocellular carcinoma

The last years have witnessed the appearance and development of a novel field of research, that of circulating miRNAs and their applications in biomedicine. Endogenous miRNAs are extremely stable in the circulation, and represent potentially informative biomarkers for a range of diseases including HCC, as reviewed in **Chapter 2** (17). Circulating miRNAs are detectable in a broad range of body fluids including plasma, saliva, tears, urine, and seminal fluid (49), the most commonly collected from HCC patients being plasma. Though the field is still in its infancy, a few circulating miRNAs have already been proposed as biomarkers for liver disease and/or HCC, including miR-16 (50), miR-21 (51, 52), miR-92a (53), miR-122 (52), miR-195 (50), miR-199a (50), miR-221 (51), miR-222 (51), miR-223 (52), miR-224 (51), miR-500 (54), miR-885 (55). Notably, miR-16 was shown to be more sensitive as HCC detection marker than traditional markers α-fetoprotein (AFP), *Lens culinaris* agglutin-reactive AFP (AFP-L3), and des-γ-carboxyprothrombin (DCP) (50). The combination of miR-16 with AFP, AFP-L3 and DCP allowed detection of 92.4% of HCC cases, including tumors ≤ 3cm. This study shows that utilizing plasma markers for HCC detection is possible, and can be very sensitive even for very small tumors. These promising results should be validated on larger patient cohorts. If confirmed, circulating miRNAs may be novel markers for detection of early-stage HCC.
We wanted to verify those interesting results on a different population, in this case HCC patients that underwent orthoptic liver transplantation (OLT) at the Leiden University Medical Center. We aimed at determining whether circulating miRNAs would allow identification of the presence of HCC in the pre-OLT samples. Their dysregulation in the post-OLT samples would be a verification of their cancer-related specificity. For that a small-scale retrospective study was implemented in which 15 HCC patients were included. From those 15 patients, 3-4 plasma samples were provided by the LUMC biobank, 1-2 samples pre-OLT and 2 samples post-OLT. Study design is presented in **Fig. 2**. For each plasma sample, RNA was isolated, and based on the results of our HCC profiling and on literature, expression of mir-16, mir-122, mir-221, mir-222, mir-142-3p and RNU48 was determined. Relative miRNA expression was calculated by normalizing miRNA expression to the expression control miR-142-3p, and miRNA expression at t0 was set at 1 per patient. A limitation of this study is that the expression of some miRNAs (miR-221, miR-222) and

small RNAs (RNU48) could not be detected in the plasma samples, while it was detectable in a pilot study performed on fresh plasma from healthy volunteers with the exact same methodology. On the LUMC plasma samples, only miR-16 and miR-122 could be reliably detected. Therefore, we established miR-16 and miR-122 relative expression at t0 and t1 pre- and t2 and t3 post-OLT, and analyzed the profile over time per patient. Unfortunately no trend could be detected post-OLT for both miRNAs, and no consistent changes in miRNA expression between the pre- and post-OLT samples could be identified. Next, correlations between relative miRNA expression at t1, t2 and t3 and clinical parameters were determined.

No correlation could be identified between relative miRNA expression and etiology, MELD score, or treatment pre-OLT. This could be due to several reasons: (i) most of the samples were 20 years old, which compromises RNA quality; (ii) with n = 15, the patient sample set was rather small; (iii) it is difficult to determine a time point at which the miRNA expression should be taken as a reference, because of the random sampling times. For example, in the current study we took t0 pre-OLT as a reference but the first sample post-OLT could have been used, or expression could have been arbitrarily normalized. More sensitive methods such as NGS or nCounter might be more suited for identification of miRNA as biomarkers. Nevertheless, we believe that circulating miRNAs hold great promises for the future of HCC diagnostics, and studies performed on large patient cohorts worldwide support this idea.

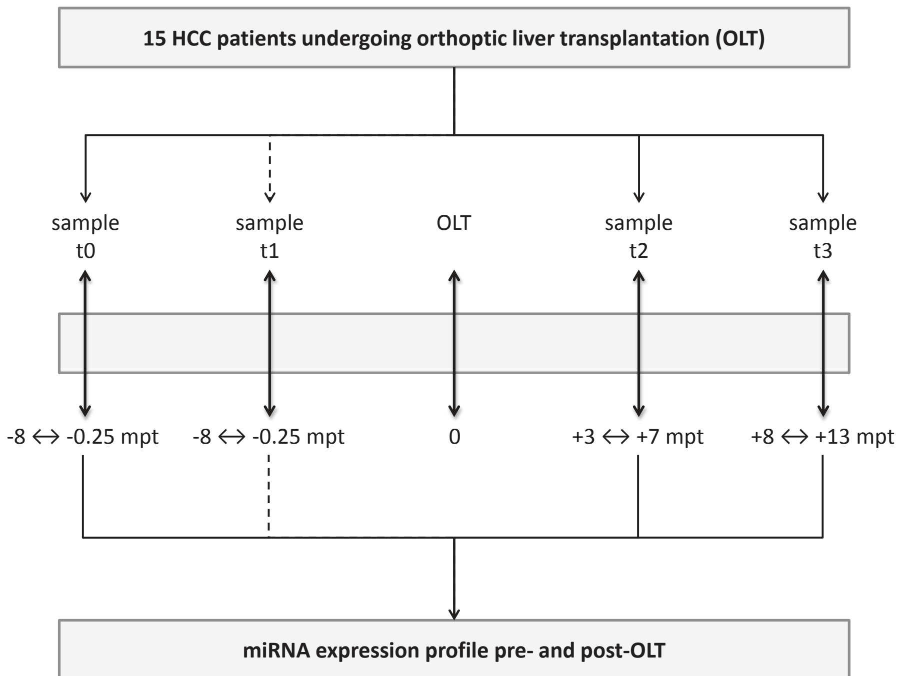

Figure 2. Study design. Fifteen HCC patients undergoing OLT were included in the study. For each patient 1 or 2 plasma samples pre-OLT and 2 plasma samples post-OLT were analyzed for miR-16 and miR-122 expression.

Late diagnosis is a major issue in HCC management, and one of the reasons for low survival. Biomarkers would be extremely useful for diagnosis, in particular for detection of early-stage HCC. In addition, novel markers would also be useful for monitoring response to treatment and for surveillance. If circulating miRNA confirm their status of first-in-class, as it now seems to be, without any doubt their use in the clinic will dramatically impact HCC management.

5. Conclusions and perspectives

In this thesis, the ABC transporter genes profile and the miRNA profile of untreated HCC was established. Based on this molecular profiling, two RNAi-based strategies were developed to modulate ABC transporter genes expression.

First of all, up-regulation of 10 ABC transporters in HCC occurs prior to chemotherapeutic treatment and is associated with a global miRNA down-regulation. Up-regulation of 5 ABC transporters, ABCA1, ABCC1, ABCC5, ABCC10, and ABCE1, appears to be mediated by 13 cellular miRNAs in HCC patient samples. Regulation of ABCA1 by miR-101 and miR-135b, and that of ABCC1 by miR-199a/b and miR-296 was confirmed *in vitro*. Modulation of miRNA levels could be of clinical significance, by affecting cholesterol levels in the case of ABCA1, or by decreasing the efflux of chemotherapeutics hence the multidrug resistance in the case of ABCC1.

Next, shRNAs were developed in order to knock-down ABCC1 and ABCC2 *in vivo*. A strong knock-down of ABCC2 was obtained, but at the cost of toxicity caused by over-saturation of the RNAi machinery due to high shRNA expression. Subsequent generation of artificial miRNAs showed better efficacy profile. These results demonstrate the feasibility of knocking-down ABCC2 via AAV-delivered shRNAs to the liver, but encourages towards the use of artificial miRNAs for further therapeutics development.

In conclusion, the field of RNAi-based gene therapy holds promises for modulating ABC transporter genes in the context of HCC therapy. Once the limitations will have been fully apprehended, this extremely potent technology will undoubtedly lead to significant advances for HCC diagnostics and therapeutics.

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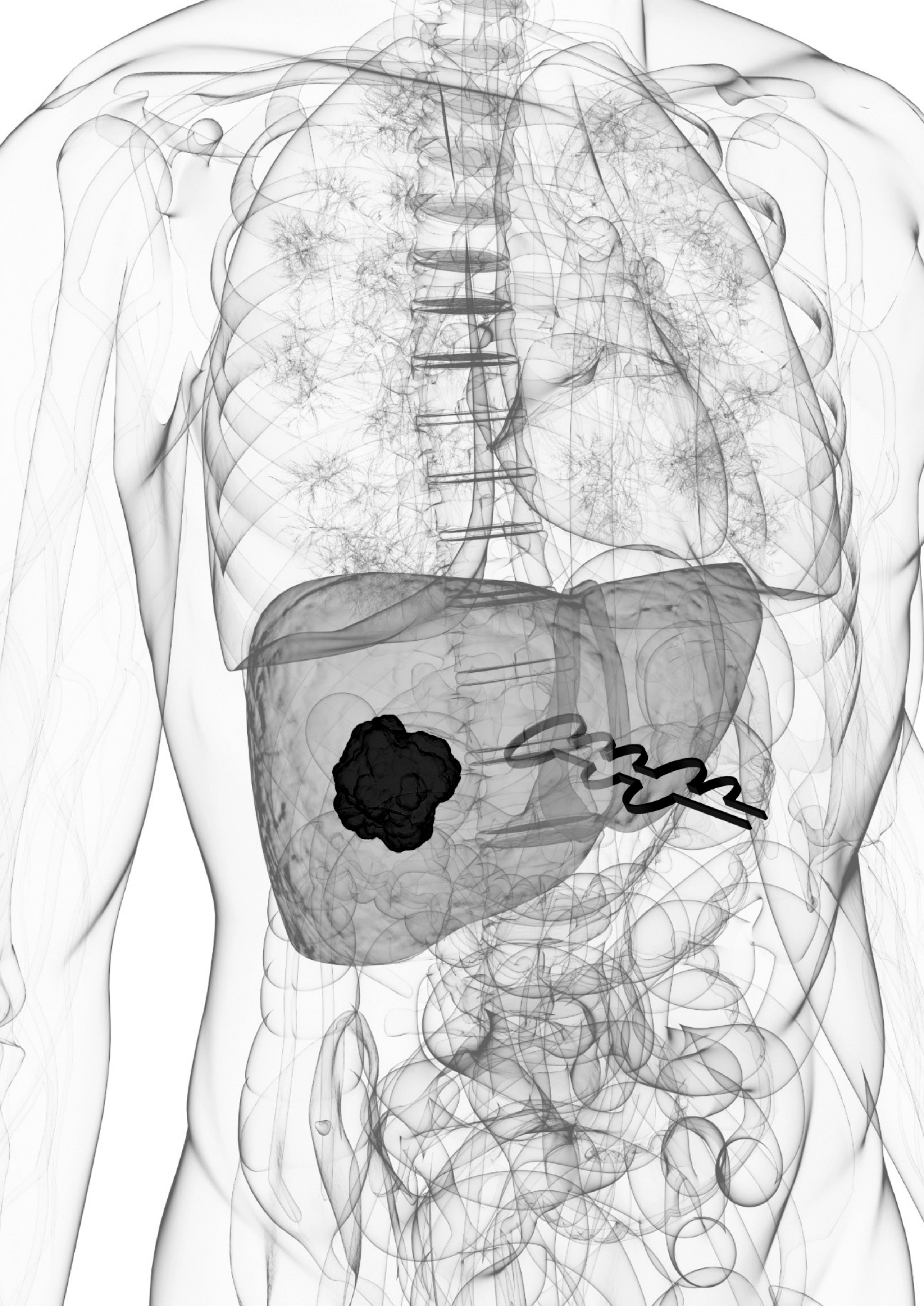

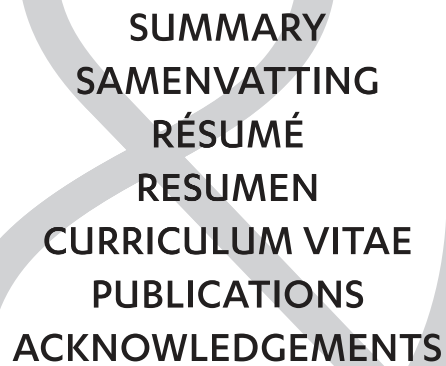

SUMMARY
SAMENVATTING
RÉSUMÉ
RESUMEN
CURRICULUM VITAE
PUBLICATIONS
ACKNOWLEDGEMENTS

SUMMARY

Hepatocellular carcinoma (HCC) is a primary cancer of the liver, and HCC patients have an average survival of only 5% at 5-year post-diagnosis. This low survival has several identified causes, among which multidrug resistance *i.e.* resistance to chemotherapeutic treatment. These issues need be addressed in order to improve HCC management in the future. In this thesis we questioned the role of ABC transporters in HCC, and aimed at developing RNAi-based strategies to compensate their dysregulation. Two strategies were developed to modulate ABC transporter gene expression. The first one exploited endogenous regulation of ABC transporter genes by cellular miRNAs while the second strategy made use of shRNAs and artificial miRNAs for ABCC1 and ABCC2 knock-down.

First of all, the up-regulation of ABC transporter genes and the concomitant global down-regulation of cellular miRNAs in untreated HCC patients were described in **Chapter 3**. This up-regulation concerns 10 ABC transporters, namely ABCA2, ABCB1, ABCB6, ABCC2, ABCC3, ABCC4, ABCC5, ABCC10, ABCC11 and ABCE1 and suggests that besides their transporter role, ABC transporters may be playing a role in cancer. This work provides the first insights into the importance of ABC transporter genes in untreated HCC patient samples and implies that they might be used as therapeutic targets in the future.

Based on the molecular profiling of HCC, we explored the hypothesis of the regulation of ABC transporter genes by cellular miRNAs. After *in silico* target predictions, the novel regulations of the expression of five ABC transporter genes (ABCA1, ABCC1, ABCC5, ABCC10, and ABCE1) by cellular miRNAs were predicted and confirmed by luciferase reporter assays in **Chapter 3**. Regulation of ABCC1, ABCC5, ABCC10, and ABCE1 by cellular miRNAs was a novel finding that has potential relevance for HCC. Regulation of ABC transporter genes by miRNAs was biologically validated in **Chapter 4**, the cholesterol transporter ABCA1 being regulated by miR-101 and miR-135b, and the multidrug resistance transporter ABCC1 being regulated by miR-199a/b and miR-296. This validation work opens the perspective of a miRNA-based therapeutic approach to modulate ABC transporter gene expression, which could be relevant to decrease clinical multidrug resistance for most ABC transporters, but also to reduce atherosclerosis in the case of ABCA1.

Next, we explored another approach to decrease multidrug resistance transporters in **Chapter 5**, by using shRNAs targeting ABCC1 and ABCC2. *In vitro*, the shRNA constructs demonstrated sequence-specific, dose-dependent target knock-down, proper processing into siRNA, and non-activation of the immune response. ABCC2 expression was significantly reduced in wild-type mice upon AAV-mediated shRNA delivery. However, we observed viral dose-dependent hepatotoxicities and corroborated that shRNA overexpression can adversely perturb the RNAi machinery *in vivo*. Artificial miRNAs that were subsequently developed in order to counteract toxicity, demonstrated target knock-down efficiency similar to that of shRNA while expressing less siRNA molecules.

In conclusion, this research contributed to the development of two RNAi-based strategies to compensate ABC transporter gene dysregulation in HCC. At this moment the RNAi field faces toxicity-related and unanticipated off-targeting challenges that need to be overcome in order to take the technology forward, but once these limitations are fully apprehended, it will undoubtedly lead to significant advances for HCC diagnostics and therapeutics.

SAMENVATTING

Hepatocellulair carcinoom (HCC) is een primaire kanker van de lever HCC patiënten hebben een gemiddelde overlevingskans van slechts 5%, 5 jaar na de diagnose. Deze lage overlevingskans heeft verschillende oorzaken, zoals, de weerstand tegen chemotherapeutische behandeling (multidrug resistance). Deze oorzaken zullen moeten worden aangepakt om HCC behandeling in de toekomst te verbeteren.

In dit proefschrift hebben we de rol van ABC transporters in HCC bekeken, en getracht RNAi-gebaseerde strategieën te ontwikkelen om hun ontregeling te compenseren. Voor het moduleren van ABC transporter gen expressie zijn twee strategieën ontwikkeld. De eerste maakt gebruik van endogene regulatie van ABC transporter genen door middel van cellulaire miRNAs, terwijl de tweede strategie gebruik gemaakt van shRNAs en kunstmatige miRNAs om een knock-down van ABCC1 en ABCC2 te bewerkstelligen.

Allereerst is de up-regulatie van ABC transporter genen en de daarmee gepaard gaande globale down-regulatie van cellulaire miRNAs bij onbehandelde HCC patiënten beschreven in **Hoofdstuk 3**. Deze up-regulatie betreft 10 ABC transporters, namelijk ABCA2, ABCB1, ABCB6, ABCC2, ABCC3, ABCC4, ABCC5, ABCC10, ABCC11 en ABCE1 en suggereert dat ze naast hun rol als transporter, een rol zouden kunnen spelen bij kanker. Dit werk geeft als eerste enig inzicht in het belang van ABC transporter genen in monsters van onbehandelde HCC patiënten en impliceert dat zij in de toekomst gebruikt kunnen worden als therapeutische targets.

Op basis van de moleculaire profilering van HCC, hebben we de hypothetische regulatie van ABC transporter genen door cellulaire miRNAs onderzocht. Na *in silico* target voorspellingen werden de nieuwe regulaties van de expressie van vijf ABC transporter genen, ABCA1, ABCC1, ABCC5, ABCC10 en ABCE1, door cellulaire miRNAs bevestigd door luciferase reporter assays. Dit staat beschreven in **Hoofdstuk 3**. Regulering van ABCC1, ABCC5, ABCC10 en ABCE1 door cellulaire miRNAs was een nieuwe vaststelling, die mogelijk relevant is voor HCC. Regulering van ABC transporter genen door miRNAs is later biologisch gevalideerd in **Hoofdstuk 4**, waarbij de cholesterol transporter ABCA1 gereguleerd wordt door miR-101 en miR-135b en de multidrug resistance transporter ABCC1 gereguleerd wordt door miR-199 a/b en miR-296. Deze validatie opent perspectieven voor een miRNA-gebaseerde therapeutische benadering voor het moduleren van ABC transporter genexpressie, die relevant kan zijn voor de verlaging van klinische multidrug resistance voor de meeste ABC transporters, maar in het geval van ABCA1 ook om atherosclerose te verminderen.

Vervolgens hebben we in **Hoofdstuk 5** een andere benadering om multidrug resistance transporters te verminderen onderzocht, door gebruik te maken shRNAs tegen ABCC1 en ABCC2. shRNAs toonden *in vitro* een target knock-down aan in een sequentie-specifieke en dosis-afhankelijke manier, even als de juiste processing in siRNA, en non-activering van de immuunrespons. ABCC2 expressie werd in wild-type muizen significant verminderd na AAV-gemedieerde shRNA toediening. Toch zagen we virale dosis-afhankelijke hepatotoxiciteit die bevestigde dat shRNA overexpressie de RNAi "machine" *in vivo* nadelig kan verstoren. Kunstmatige miRNAs die vervolgens werden ontwikkeld om de toxiciteit tegen te gaan, lieten een knock-down zien met een zelfde efficientie als die van shRNA, maar met minder productie van siRNA moleculen.

In conclusie, dit onderzoek heeft bijgedragen aan de ontwikkeling van twee RNAi-gebaseerde strategieën om de ABC transporter gen ontregeling in HCC te compenseren. Op dit moment is staat het RNAi veld voor toxiciteit-gerelateerde en onvoorziene off-targeting uitdagingen die moeten worden overwonnen om de technologie vooruit te helpen. Zodra deze beperkingen volledig zijn verholpen, zal dit ongetwijfeld leiden tot een aanzienlijke vooruitgang voor HCC diagnose en therapie.

RÉSUMÉ

Le carcinome hépatocellulaire (CHC) est un cancer primaire du foie, et les patients CHC ont une survie moyenne de seulement 5% à 5 ans après le diagnostic. Ce faible taux de survie a plusieurs causes identifiées, parmi lesquelles la résistance multidrogue aux traitements de chimiothérapie. Ces questions doivent être abordées en vue d'améliorer la gestion du CHC dans le futur.

Dans cette thèse, nous avons cherché à connaître le rôle des transporteurs ABC dans le CHC et tenté de développer des stratégies basées sur l'ARN interférence (ARNi) pour compenser leur dérégulation. Deux stratégies ont été élaborées pour moduler l'expression des transporteurs ABC. La première exploite la régulation endogène des transporteurs ABC par les miARNs cellulaires tandis que la seconde stratégie fait usage de shRNAs et de miARNs artificiels pour obtenir un knock-down de ABCC1 et ABCC2.

Tout d'abord, la surexpression des transporteurs ABC et la sous-expression concomitante des miARNs cellulaires chez les patients CHC non-traités ont été décrit dans le **Chapitre 3**. Cette surexpression concerne 10 transporteurs ABC, à savoir ABCA2 ABCB1, ABCB6, ABCC2, ABCC3, ABCC4, ABCC5, ABCC10, ABCC11 et ABCE1, et suggère que, outre leur rôle de transporteur, les transporteurs ABC pourraient jouer un rôle direct dans le cancer. Ce travail donne pour la première fois un aperçu de l'importance des transporteurs ABC chez des patients CHC non-traités et implique qu'ils pourraient être utilisés à l'avenir en tant que cibles thérapeutiques.

En nous basant sur le profil moléculaire obtenu, nous avons exploré l'hypothèse de la régulation de l'expression des transporteurs ABC par les miARNs cellulaires. Après des prédictions de cibles *in silico*, les nouvelles régulations par les miARNs cellulaires de l'expression de cinq transporteurs ABC, à savoir ABCA1, ABCC1, ABCC5, ABCC10 et ABCE1, ont été confirmées par des tests avec des rapporteurs de la luciférase dans le **Chapitre 3**. La régulation de l'expression de ABCC1, ABCC5, ABCC10, et ABCE1 par les miARNs cellulaires était une conclusion nouvelle qui a un intérêt potentiel pour le CHC.

La régulation de l'expression des transporteurs ABC par les miARNs cellulaires a ensuite été validée biologiquement dans le **Chapitre 4**. Nous avons confirmé que le transporteur de cholestérol ABCA1 est régulé par miR-101 et miR-135b tandis que le transporteur multidrogue ABCC1 est régulé par miR-199a/b et miR-296. Ce travail de validation ouvre la perspective d'une approche thérapeutique basée sur les miARNs pour moduler l'expression des transporteurs ABC. Cette approche pourrait être pertinente pour réduire la résistance multidrogue observée pour la plupart des transporteurs ABC mais aussi de réduire l'athérosclérose dans le cas de ABCA1.

Dans le **Chapitre 5,** nous avons exploré une autre approche pour réduire l'expression des transporteurs multidrogues en utilisant des shRNAs ciblant ABCC1 et ABCC2. *In vitro*, les shRNAs développés ont démontré leur capacité à réduire de manière séquence-spécifique et dose-dépendante les transcrits des gènes cibles. De plus, les shRNAs sont correctement transformés en siARNs et n'activent pas la réponse immunitaire. Chez les souris de type sauvage, l'expression de ABCC2 a été considérablement réduite après injection de AAV-shRNA. Toutefois, nous avons observé des toxicités hépatiques dose virale-dépendantes et montré que la surexpression de shRNAs pouvait perturber la machinerie ARNi *in vivo*. Des miARNs artificiels ont ensuite été développés afin de contrecarrer la toxicité et ont démontré une efficacité similaire à celle des shRNAs au niveau de la diminution des transcrits des gènes cibles tout en exprimant moins de molécules siRNAs.

En conclusion, cette recherche a contribué à l'élaboration de deux stratégies basées sur l'ARNi pour compenser la dérégulation des transporteurs ABC dans le CHC. En ce moment, le domaine de l'ARNi est confronté à des challenges liés à des problèmes de toxicité et de spécificité (« off-targeting »).Une fois surmontés, ces problèmes permettront à la technologie de l'ARNi de devenir un traitement de choix pour le diagnostic et la thérapeutique du CHC.

RESUMEN

El carcinoma hepatocelular (CHC) es un cáncer primario del hígado, y los pacientes con CHC tienen una supervivencia en torno al 5% a los 5 años del diagnóstico. Esta baja sobrevivencia tiene varias causas identificadas, entre las cuales se encuentra la resistencia multifarmaco al tratamiento con quimioterapia. Estos temas deben ser abordados con el fin de mejorar el manejo del CHC en el futuro.

En esta tesis se cuestionó el papel de los transportadores ABC en el CHC, y se intento crear estrategias basadas en el ARNi para compensar su desregulación. Se desarrollaron dos estrategias con el fin de modular la expresión de los transportadores ABC. La primera de ellas aprovecha la regulación endógena de los transportadores ABC por parte de los miRNAs celulares, mientras que la segunda estrategia hace uso de shRNAs y miRNAs creados artificialmente para obtener un knock-down de ABCC1 y ABCC2.

En primer lugar, la sobre-expresión de los genes transportadores ABC y la concomitante sub-expresión de miRNAs celulares en pacientes con CHC tratados se han descrito en el **Capítulo 3**. Esta sobre-regulación implica a 10 transportadores ABC, a saber, ABCA2, ABCB1, ABCB6, ABCC2, ABCC3, ABCC4, ABCC5, ABCC10, ABCC11, ABCE1, y sugiere que, además de su papel primario como transportador, los transportadores ABC pueden estar involucrados en el desarrollo del cáncer. Este trabajo sugiere por primera vez la idea de la importancia de los genes de transportadores ABC en muestras de CHC de pacientes no tratados y su posible utilización en el futuro como dianas terapéuticas.

Con base en el perfil molecular del carcinoma hepatocelular, se exploró la hipótesis de la regulación de la expresión de los transportadores ABC por miRNAs celulares. Después de realizar las predicciones de dianas *in silico*, las nuevas regulaciones por parte de las miRNAs celulares de la expresión de cinco genes transportadores ABC, ABCA1, ABCC1, ABCC5, ABCC10 y ABCE1 se confirmaron mediante pruebas de marcado con luciferasa en el **Capítulo 3**. La expresión de ABCC1, ABCC5, ABCC10 y ABCE1 por miRNAs celulares es un hallazgo novedoso que tiene potencial relevancia en el manejo del CHC. La regulación de los genes transportadores ABC por los miRNAs fue posteriormente validado biológicamente en el **Capítulo 4**; el transportador de colesterol ABCA1 está regulado por miR-101 y miR-135b y el transportador de la resistencia multifármaco ABCC1 está regulado por miR-199a/b y miR-296. Este trabajo de validación abre la perspectiva de un enfoque terapéutico basado en miARNs para modular la expresión de los genes de transportadores ABC, que podría ser relevante para disminuir la resistencia clínica a múltiples fármacos en el caso de la mayoría de los transportadores ABC y también para reducir la aterosclerosis en el caso de ABCA1.

A continuación, exploramos en el **Capítulo 5** otro enfoque para reducir los transportadores de resistencia multifármaco, mediante el uso de shRNAs dirigidos contra ABCC1 y ABCC2. *In vitro*, los shARNs creados demostraron un knock-down secuencia-específica y dosis-dependiente de los genes diana, la transformación correcta de shRNAs en siARN, y la ausencia de activación de la respuesta inmune. En ratones de tipo salvaje, la expresión de ABCC2 se redujo significativamente después de la aplicación de shRNA mediada por AAV. Sin embargo, se observó hepatotoxicidad dosis viral-dependiente y se corroboró que la sobre-expresión de shRNAs puede perturbar el mecanismo de ARNi *in vivo*. Posteriormente, se desarrollaron miRNAs artificiales con el fin de contrarrestar la toxicidad y demostraron una eficacidad similar a la de los shRNA en cuanto al knock-down de genes diana expresando menos moléculas de siRNA.

En conclusión, esta investigación ha contribuido al desarrollo de dos estrategias basadas en el ARNi para compensar la desregulación de los genes de transportadores ABC en el CHC. En este momento el campo del ARNi se enfrenta al problema de la toxicidad y al de la aparición no prevista de "off-targeting", obstaculos que deben superarse con el fin de llevar la mejorar la tecnología disponible, lo cual, sin duda, dará lugar a avances significativos en el diagnóstico y el tratamiento del carcinoma hepatocelular.

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