RNAI-based gene therapy of hepatocellular carcinoma: targeting ABC transporters
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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
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.

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.
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 \textit{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 \textit{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 oversaturation 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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