



Review

Therapeutic strategies for miRNA delivery to reduce hepatocellular carcinoma

Bornika Roy ^a, Sampa Ghose ^b, Subhrajit Biswas ^{a,*}

^a Amity Institute of Molecular Medicine and Stem Cell Research (AIMMSGR), Amity University, Noida, Uttar Pradesh 201313, India
^b Department of Medical Oncology, All India Institute of Medical Sciences, New Delhi, India

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ABSTRACT

Malignancies of hepatocellular carcinoma (HCC) are rapidly spreading and commonly fatal. Like most cancers, the gene expression patterns in HCC vary significantly from patient to patient. Moreover, the expression networks during HCC progression are largely controlled by microRNAs (miRNAs) regulating multiple oncogenes and tumor suppressors. Therefore, miRNA-based therapeutic strategies altering these networks may significantly influence the cellular behavior enough for them to cure HCC. However, the most substantial challenges in developing such therapies are the stability of the oligos themselves and that of their delivery systems. Here we provide a comprehensive update describing various miRNA delivery systems, including virus-based delivery and non-viral delivery. The latter may be achieved using inorganic nanoparticles, polymer based nano-carriers, lipid-based vesicles, exosomes, and liposomes. Leaky vasculature in HCC-afflicted livers helps untargeted nanocarriers to accumulate in the tumor tissue but may result in side effects during higher dose of treatment. On the other hand, the strategies for actively targeting miRNA therapeutics to cancerous cells through nano-conjugates or vesicles by decorating their surface with antibodies against or ligands for HCC-specific antigens or receptors are more efficient in preventing damage to healthy tissue and cancer recurrence.

1. Introduction

1.1. Deregulation of miRNA signaling during HCC

Hepatocytes are the workhorse of the liver, which itself is a major immunometabolic hub of the body. Therefore, hepatocellular carcinoma (HCC), the most common form of liver cancer, can be catastrophic, and accounts for an increasing number of cancer-related deaths worldwide. The development of HCC is a complex process. Hepatotropic viruses are the leading cause of HCC in the developing world, but changing lifestyles are resulting in alcoholic or nonalcoholic fatty liver disease quickly catching up as a major etiological factor. All of these factors cause sustained inflammatory damage to hepatocytes, the type of cells that form the majority of the liver, and result in necrosis; an attempt to repair said damage and fibrotic deposition in the liver is a byproduct of the regenerative process.

A large part of the complications in coming up for a 'cure' for any cancer, including HCC, is the sheer complexity and heterogeneity of the disease, with diverse signaling pathway activation/deactivation. Gene

expression patterns could vary significantly from patient to patient, and therefore targeting a single tumor suppressor gene or oncogene in a one-size-fits-all therapy would be futile. However, gene expression networks are largely controlled by noncoding RNAs of between 21 and 25 bases, known as microRNAs or miRNAs. A single miRNA can control the expression of multiple genes involved in the main molecular pathways of development or progression of HCC. Therefore, the expression profiles of the miRNAs themselves act as valuable biomarkers, whether for diagnosis, evaluation of metastasis, prediction of therapeutic response or recurrence and overall survival. Their roles as master regulators of oncogene or tumor suppressive networks make them better targets for gene therapy than the oncogenes or tumor suppressor genes themselves.

1.2. MicroRNA-based gene therapeutics: possibilities & challenges

MicroRNAs play an important regulatory role in RNA silencing and post-transcriptional regulation of gene expression by targeting specific mRNAs for degradation or translation repression (Fig. 1). While siRNAs can inhibit the expression of only a specific target mRNA, miRNA can

* Correspondence to: Amity Institute of Molecular Medicine and Stem Cell Research, Research Laboratory 101, J3 Block Amity University Uttar Pradesh (AUUP), Sector 125, Noida, Uttar Pradesh 201313, India.

E-mail address: sbiswas2@amity.edu (S. Biswas).

target multiple genes, and therefore help regulate whole pathways. Obviously, this is a very attractive prospect in translational medicine, since processes like development of a disease involve intricate pathways, with lots of redundancies to act as failsafe mechanisms. Modulating entire pathways could help cure complex diseases such as cancer, and using miRNAs to do so is emerging as a very real therapeutic option in HCC. Moreover, miRNAs can also influence targets otherwise immune to traditional small molecule chemotherapeutics or monoclonal antibodies, such as proteins that lack enzymatic function, or those with a conformation inaccessible to the relatively larger drug molecules.

MicroRNA therapy can nip the issue of chemoresistance in the bud, since the activation or inactivation of certain pathways are known to play a role in the development of acquired drug resistance. By corollary, miRNAs can also increase chemosensitivity towards traditional chemotherapeutic agents. Additionally, miRNAs are known to play a role in oncogenesis, tumor suppression, as well as the induction of chemoresistance. Therefore, miRNA mimics or antagomirs form the basis for what could one day be a personalized cure for cancer. As interesting as the prospect of microRNA-based gene therapy is, there exist major challenges in ensuring safe and efficient delivery of the miRNAs to tumor sites. The first is vulnerability of synthetic oligonucleotides to nucleases that are ever-present in the body. The second challenge concerns the efficiency with which miRNA therapeutic agents can target cancerous cells. How can the specificity in terms of the target tissue and cell type be ensured? Additionally, how can the oligonucleotides' inherently poor capacity to penetrate the host cell membrane be improved? To an extent, these challenges are addressed by introducing target sequences and multiple chemical modifications that maximize stability, delivery and cellular uptake efficiency of oligonucleotides *in vivo*. Meanwhile, the mode of delivery is also important in solving the

problems related to stability and tissue specificity of miRNA. These could be either viral-mediated or non-viral delivery systems (liposome or exosome-mediated, nanoparticle-based, or scaffold-based delivery). Viral vectors, derived from adenoviruses, adeno-associated viruses, lentiviruses, or retroviruses are usually the most common vehicles for gene therapy. However, certain drawbacks such as lack of cell-type specificity, and risk to patients due to a possibility of mutations *in vivo*, prevent viral-mediated miRNA therapy from being widely used. This is where non-viral delivery systems come into play.

Non-viral delivery systems have a higher capacity of release the encapsulated compounds as compared to viral vectors, and this is a distinct advantage when packaging multiple anti-miRNAs or pre-miRNA molecules together to form an efficient therapeutic strategy. Nonviral systems are also much less toxic and immunogenic than their viral counterparts, though their efficiency is also less. These nonviral delivery systems could be lipid-based, polymer-based, or inorganic carriers (Fig. 2), and while there are several excellent review articles discussing their use in cancers in general [1–6], their application in the treatment of hepatocellular carcinoma, focusing majorly on clinical and pre-clinical models, are discussed in this review.

2. Target specific miRNA alteration in regression of HCC

There exist multiple categories of microRNAs that could potentially be targeted in terms of miRNA-based therapeutics. While oncomiRs and metastamiRs (i.e., miRNA associated with tumor progression and metastasis, respectively) are overexpressed in tumor tissues, apopto-miRNAs (i.e., miRNAs involved in apoptosis) the tumor suppressive TS-miRNAs are usually downregulated, as they play a role in tumor suppression. Some of the best-studied oncomirs in HCC include microRNA-

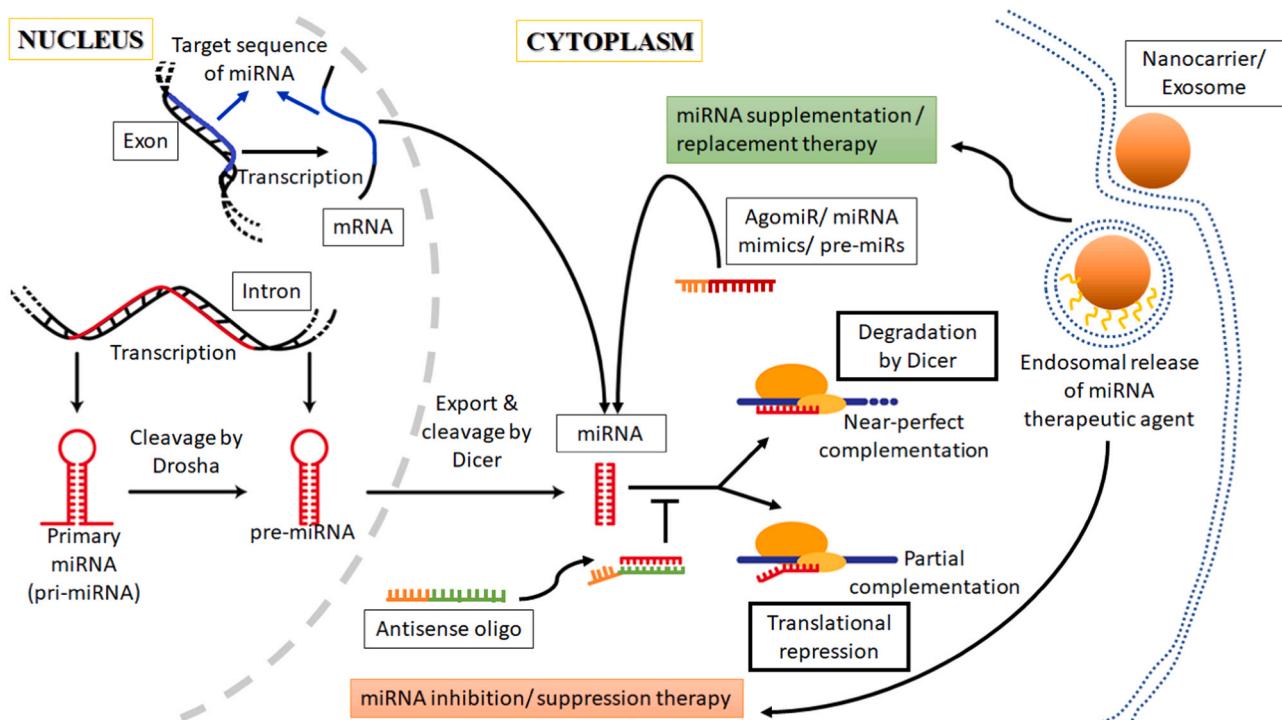


Fig. 1. Modulation of miRNA mechanism of action for miRNA based therapeutic approach through exosomes or nanocarriers. miRNA genes are transcribed into long primary miRNA transcripts, termed pri-miRNAs that are usually several kilobases long. Pri-miRNAs are processed in the nucleus by the nuclear Microprocessor complex, involving RNase III enzyme Drosha. Pre-miRNAs are exported to the cytoplasm by Exportin-5 and processed further by Dicer. During this process the mature miRNA is retained in the MiRISC, whereas the complementary strand, known as the miRNA star (miR*) is released. Chemically modified anti-miRNA oligonucleotides sequester the mature miRNA in competition with cellular target mRNAs leading to functional inhibition of the miRNA and derepression of the direct targets. The use of downregulated miRNA in tumor as replacement therapy or anti-miRNA therapies to block the onco-miR activities results in the upregulation of tumor-suppressor genes. The miRNA supplementation or supply of anti-miRs or TS-miRNAs (Tumor suppressive) could be possible by their endosomal release either using nanocarrier or exosomes. The effects of miRNA-based therapy indicate an increase in cell death concurrently with inactivation of tumor development.

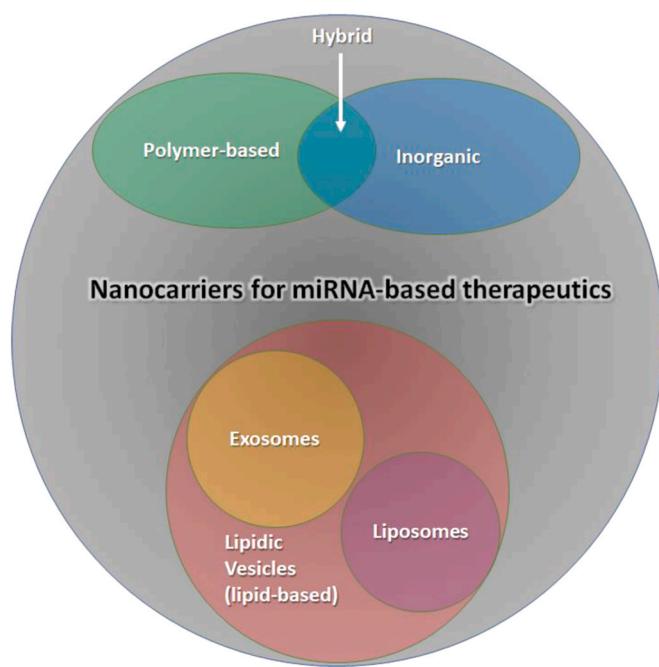


Fig. 2. Schematic showing major nanoparticle-based platforms for miRNA delivery.

21 [7,8], which was one of the first mammalian microRNAs to be identified and is one of the most frequently upregulated miRNAs in solid tumors, miR-221 [9–11] which has the oncogene CD117 or KIT as one of its targets, and miR224 that regulates AKT signaling [12,13]. Well characterized TS-miRNAs include miR-122, which also regulates fatty acid metabolism [14,15], and miR-375, which regulates metabolism

through one of its targets- pyruvate dehydrogenase kinase [16,17]. Certain other miRNAs, that may or may not fall under any particular category, play a role in the development of chemoresistance or escaping immuno-surveillance. Moreover, these categories have a certain amount of overlap, some miRNAs may function as oncogenic or tumor suppressor in a context-dependent manner (Fig. 3). Therefore, depending on the dysregulation status of the target microRNA either by miRNA inhibition or miRNA replacement therapy could improve outcomes for HCC patients. This approach has certainly worked in mice models, wherein the miRNAs can be targeted through viral transfection [18], or nonviral delivery systems like nanoparticles.

Till date, there have been a few successful miRNA-based therapies for liver diseases in humans (Table 1). The most success work is that of miravirsen, a locked nucleic acid-modified phosphorothioate oligonucleotide inhibiting miR-122, which, while a tumor-suppressing miRNA, aids the replication of Hepatitis-C virus [19]. While this approach was originally intended to decrease the target miRNA, it served as a proof of concept for miRNA therapeutics. The molecular heterogeneity of cancers due to the sheer diversity of pathways involved in the disease etiology, especially in HCC arising in a hyperinflammatory background, present a challenge to which miRNA-based therapeutics are ideally suited [20]. Another human study, this time a Phase I clinical trial targeting miRNA-34a through an miRNA mimic packaged in a liposomal vehicle, had promising preliminary results against solid tumors in the liver, including HCC [21,22]. However, the clinical trial encountered serious immune-mediated adverse events, which only serves to underscore the importance of organ- and cell-specific targeting. Targeting nanocarriers specifically to the hepatocytes in the liver might be possible as indicated by preclinical research, and can effectively nullify these side effects and increase efficacy of such miRNA-based therapeutics [23].

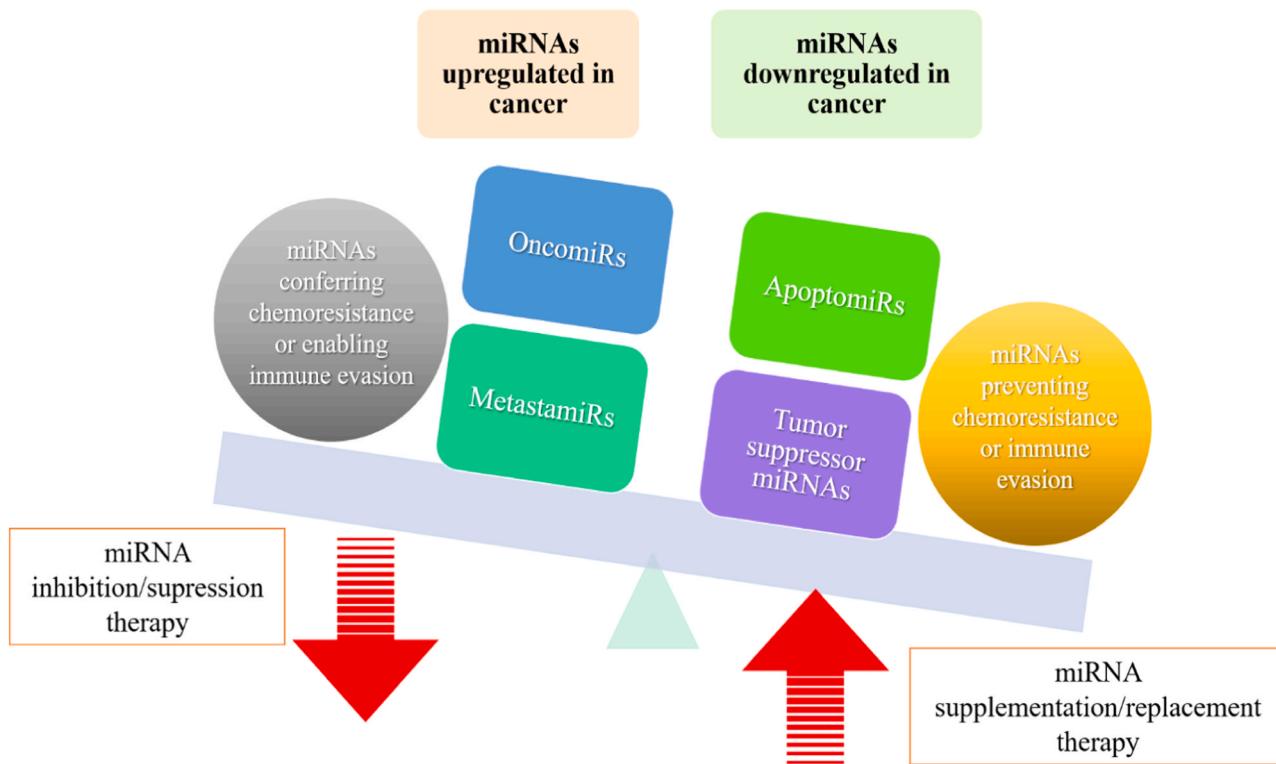


Fig. 3. Scheme outlining the ability of categorized miRNAs during cancer progression. In tumor tissues oncomiRs and metastamiRs are overexpressed where as apoptomiRs and tumor suppressive TS-miRNAs are usually downregulated. Certain other miRNAs, that may or may not fall under any particular category, play a role in the development of chemoresistance and immune evasion.

Table 1

Differentially regulated miRNAs in HCC can be targeted through delivery of pre-miRNA or antisense oligonucleotide. (source: dbDEMC 2.0 [91] & mirPath v.3 [92]) The dbDEMC database was searched for datasets of differentially expressed miRNA in HCC as compared to normal primary hepatocytes or normal biopsies. The miRNAs that consistently showed the same deregulation pattern by at least 1.5-fold in two or more datasets (upregulation or downregulation) have been tabulated below. Due to the limitations of datasets, certain miRNAs that are proven targets for HCC may not be included in this table, such as miR-223 [93], let-7c family members [94], miR-21 [95], etc. Also tabulated are the top 5 pathways that these miRNA's gene targets were involved in. Though upregulated in the datasets as they are known oncomiRs, there are no studies proving the efficacy of downregulating miRNAs 224 or 452 in HCC, *in vitro* or otherwise.

S. No.	miRNA ID	GEO ID of dataset where the miRNA is significantly dysregulated	Top 5 KEGG pathways the gene targets are involved in (in descending order of number of targets involved)	Reference(s) for miRNA-based therapeutic strategy
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Potential targets for miRNA supplementation/replacement therapy (through agomiRs, miRNA mimics, or pre-miRNAs)

1.	hsa-miR-122	GSE20077, GSE40744	Pathways in cancer	[50,63,96–98]
2.	hsa-miR-139	GSE36915, TCGA_LIHC	PI3-Akt Pathway MAPK signaling pathway Proteoglycans in cancer Viral carcinogenesis Pathways in cancer	[99,100]
3.	hsa-miR-199a	GSE21362, TCGA_LIHC, GSE41874, GSE69580,	PI3-Akt signaling pathway MAPK signaling pathway Proteoglycans in cancer Viral carcinogenesis Pathways in cancer	[23,101,102]
4.	hsa-miR-375	GSE20077, GSE21362, TCGA_LIHC	MAPK signaling pathway PI3-Akt signaling pathway Proteoglycans in cancer Regulation of actin cytoskeleton Pathways in Cancer	[16,31,51,103]

Potential targets for miRNA suppression/inhibition therapy (through antagomir)

1.	hsa-miR-221	GSE20077, GSE10694, GSE40744, TCGA_LIHC	PI3K-AKT pathway	[33,56,104]
2.	hsa-miR-224	GSE40744, TCGA_LIHC, GSE39678	MAPK signaling pathway Pathways in Cancer Viral carcinogenesis Proteoglycans in cancer PI3K-AKT pathway	N/A

Table 1 (continued)

S. No.	miRNA ID	GEO ID of dataset where the miRNA is significantly dysregulated	Top 5 KEGG pathways the gene targets are involved in (in descending order of number of targets involved)	Reference(s) for miRNA-based therapeutic strategy
3.	hsa-miR-452	GSE40744, GSE36915, TCGA_LIHC, GSE39678	MAPK signaling pathway Pathways in Cancer Epstein-Barr virus infection Regulation of actin cytoskeleton Pathways in Cancer	N/A

3. Recent advances in nonviral miRNA delivery systems

Nonviral nanoparticle (NP) carriers offer unprecedented opportunities for a cell-specific and controlled delivery of either surface receptor specific ligand conjugated or vesicle mediated encapsulated miRNA for the purpose of gene therapy. AntimiRs and miRNA mimics can be successfully delivered *in vitro* using commercially available transfecting agents such as Lipofectamine, or by electroporation. However, for reasons discussed in 1.2, these strategies may not work *in vivo*, and therefore cannot effectively be used to treat HCC. Nanoparticles can shield their relatively unstable microRNA conjugates from external influences, and therefore reduce chances of their inactivation or degradation while also enhancing their spatiotemporal specificity in terms of increasing circulation time and targeted accumulation. These nanocarriers are an attractive therapeutic option owing to their biocompatibility and, in some cases, biodegradability.

Nanoparticles not only shield the negative charge of free miRNAs that would otherwise hinder cellular uptake, but they can also display targeting ligands and release encapsulated miRNA only upon action of certain specific environmental triggers. Moreover, addition of a stealth coating of polyethylene glycol (PEG) around NPs in siRNA or drug delivery systems are known to prevent clearance by the reticuloendothelial system (RES), therefore avoiding unfavorable immune cell stimulation [24,25]. Nanoparticle can also be functionalized by modification of the surface with ligands for specific receptors on the target cells. This is done by conjugating NPs to proteins, antibodies and carbohydrates by modifying the surface with amino and carboxy groups, respectively. Such surface functionalization opens up the possibility of programmed cell-specific uptake and transfection technique that is also non-immunogenic.

Despite the excitement in the scientific community regarding their use, certain challenges exist in successful miRNA delivery by nanoparticles. Firstly, due to their high affinity to water, miRNAs easily diffuse into the aqueous phase when nanoprecipitation or emulsion-based preparation methods are used, resulting in a low encapsulation efficiency. Secondly, the NP surface has to be modified with ligands for specific receptors on target cells in order to facilitate uptake by receptor-mediated endocytosis, and to reduce the required dosage and side effects of treatment [26]. Such biofunctionalization is expensive [27]. Another problem is to do with the release of conjugated or encapsulated miRNA into the cytoplasm, since miRNAs can degrade in the low pH of the lysosome if released from NPs too late. Moreover, selection of the material and encapsulated miRNA loading process for a nanoparticle is of utmost importance. For e.g., the size and zeta potential of NPs have to be considered in order for cellular uptake to be efficient, since NPs

measuring in the range 100–200 nm can be more easily internalized by target cells. Research on increasing the efficiency of NP uptake is still in its nascent stages, and there is as yet no one-size-fits-all targeting strategy, even for all cancers originating from the same tissue.

4. Inorganic nanoparticles in HCC therapeutics

The material constituting the nanoparticle could be of an organic or inorganic origin. Where inorganic nanocarriers are used in conjugation with miRNA, gold nanoparticles (AuNPs) comprise the majority of such therapeutics, at least, for the treatment of HCC [28]. This can be ascribed to various properties of gold that make it amenable for such use, including ease of synthesis of NPs, a non-reactive nature, and large surface area of the nanoparticles that can support a larger number of functionalization or encapsulated molecules. The microRNA 181b was successfully inhibited by gold nanoparticles with multiple surface functionalization moieties [29]. More recently, functionalized gold nanocages have been utilized for photothermal delivery of a combination of the chemotherapeutic drug Doxorubicin (DOX) and a miRNA 122 mimic to treat HCC [30]. Gold nanoparticles have also been used to deliver miR-375 to successfully treat HCC in mice models [31]. Studies have also used gold nanoparticles to carry miRNA therapeutics targeting other miRNAs such as miR-326 [32], miR-221 [33], miR-21 [7] and 181b [34] *in vitro*.

Magnetic nanoparticles are also an attractive possibility, since magnetic fields can be applied to cause targeted sedimentation (and therefore transfection of miRNAs), reducing the need for additional surface functionalization [35]. Though successfully used for imaging [36] and diagnosis [37,38], there are no studies yet on the efficacy of miRNA-conjugated magnetic NPs in HCC. However, the system has proven to be effective against mice xenograft models of breast cancer [39], and to deliver the chemotherapeutic drugs like sorafenib [40,41] and doxorubicin [37], or to upregulate tumor suppressor genes [42] in HCC.

Other inorganic nanoparticles that have been successfully used *in vitro*, but not *in vivo*, are quantum dots (QDs), graphene-based nanoparticles, silica-based nanoparticles, and calcium carbonate NPs. Quantum dots, also called “designer atoms” are semiconductor-based nanosized crystals that present the possibility of multiplexing miRNA delivery with bioimaging, an exciting concept known as traceable drug delivery. MicroRNA delivery using QDs, such as PEI-coated QDs used by G. Liang, Y. Li, and colleagues to supplement miR-26a in HepG2 cells [43], helps enhance our understanding of miRNA biology in tumors. Quantum dots, in conjugation with anti-P-Glycoprotein antibody-modified graphene oxide were successful in inducing apoptosis of the multidrug resistant human hepatoma cell line HepG2/ADM [44]. Carbon-based inorganic nanoparticles, on the whole, are a virtually unexplored vehicle for miRNA-based therapeutics in HCC, even though its efficacy as a chemogene therapy and molecular imaging capabilities were tested in glioblastoma a while back [45]. The first papers exploring the use of graphene oxide nanoparticles to treat HCC through tumor suppressive cargo such as siRNA or C6-ceramide were published only as recently as 2019. However, the use of carbon-based NPs as biosensors of miRNA is a relatively established technique [46–48].

In addition to the large surface area and stability for which nanoparticle synthesis materials are selected, mesoporous silica NPs have a novel advantage of tunable pore sizes, enabling easier encapsulation of the compounds, as well as easier duplexing of miRNA delivery with that of other drugs. These aspects were highlighted by Yu et al. to simultaneously deliver a small molecule inhibitor and an antagomiR to reduce miRNA 122 levels in the HCC cell line Huh7, as its inhibition reduces HCV infection [49]. A similar approach could be used to instead increase the level of miR-122, and therefore increase apoptosis and chemosensitivity in HCC [50]. Silica NPs have also been used to overcome multiple drug resistance by delivering miR-375 and doxorubicin to the human hepatoblastoma cell line HepG2, and to achieve antitumor

effects when used without DOX in mice models [51]. A similar approach was taken by P Zhao and colleagues, but they used lipid-coated doxorubicin-calcium carbonate NPs instead of mesoporous silica nanoparticles [52].

5. Organic nanocarriers for miRNA delivery in HCC

The unique aspect of organic NPs is that, in addition to the biocompatibility that inorganic NPs also possess, most of them are also biodegradable. However, this could present as both an advantage or disadvantage- while biodegradability means that the NPs will not accumulate in the body, and usually have lower immunogenicity [53], it also means that, if not designed carefully, the encapsulated miRNA might be released too early i.e. in the lysosome rather than the endosome, and therefore the miRNA conjugate might be destroyed. Or, due to heterogeneity of uptake by target cells, the effective concentration in the target cells may differ from the ideal dosage [54].

Organic nanoscale delivery systems for miRNA therapeutics can be subdivided into polymeric or lipid-based. The former can be categorized based on whether the polymer forming the NPs are synthetic or natural. Lipid-based NPs could be solid lipid nanoparticles or lipid nanocapsules, which can be further subdivided into nanosomes (single lipid monolayer) or liposomes (lipid bilayer). Liposomes of a biological origin are called exosomes, and they are already biofunctionalized, i.e. decorated with antibodies, ligands and proteins that enable it to reach the target cells.

5.1. Polymeric nanoparticles

Cationic polymer-based nanoparticles are arguably the most commonly used nonviral delivery systems for miRNA therapy, due to the ease of synthesis, relatively low toxicity, and amenability to tunable versatile physico-chemical modifications to suit therapeutic requirements. Polymeric NPs are made of cationic biocompatible and usually biodegradable polymers of synthetic or natural origin, such as PLA (polylactic acid), PEI (Polyethyleneimine) and PLGA (poly(lactic-co-glycolic acid), or alginate, albumin, and chitosan, respectively [55]. These polymer-based nanocarriers could be in the form of a nanocapsule or nanospheres, and the miRNA could be encapsulated inside, or adsorbed to the surface.

PLGA is the most commonly used polymer to create nanoparticle carriers of miRNA therapeutics. Its degradation products, lactic acid and glycolic acid, are biologically removed by surrounding cells through normal metabolic pathways without much problem. Though biodegradable, it is also hydrophobic (and therefore a poor candidate to carry negatively charged nucleic acid), and quickly removed from circulation by opsonization. Therefore, it is almost always used in conjugation with other polymers or peptides. PLGA NPs thusly modified have been used by three independent groups to suppress oncomiRs in human HCC cell lines [56–58]. A PEG-PLGA-poly(L-lysine)-lactobionic acid (PEG-PLGA-PLL-LA or PEAL-LA for short) nanocarrier was functionalized with an anti VEGF antibody (that would target tissue undergoing neoangiogenesis) in addition to the lactobionic acid already present to create a delivery system highly specific to HCC [59]. The system was successful in restoring the expression of miR-99a in a mouse xenograft model. PLGA nanoparticles have also been used to target miR-26a [58], *in vitro* in the human hepatoblastoma cell line HepG2.

Some of the most novel use of NPs in general are in sonodynamic therapy, where ultrasound-guided microbubbles are used to deliver the nanoparticles carrying one or more miRNA therapeutic molecules, and possibly other chemotherapeutic agents. PLGA nanoparticles were some of the first to be used in the *in vivo* testing of the efficacy of this form of treatment [60–63].

Non- PLGA peptide polymers are also used as nanocarriers for miRNA mimics or antagonists. A redox-sensitive, oligopeptide-guided, self-assembling, and efficiency-enhanced (ROSE) system was created by

Hu, Wang, and colleagues for miR-34a replacement therapy, and saw marked success in the in vitro and in vivo treatment of HCC [64]. A nanoparticle comprising a disulfide cross-linked, stearylated, and histidine-modified polyarginine peptide as the vector and a cell penetrating peptide-modified aptamer with specific binding to HCC cells was used to co-deliver miR-195 to suppress VEGF and the drug fasudil to suppress vasculogenic mimicry by blocking ROCK2. The resultant antiangiogenic treatment was successful in suppressing HCC both in vitro and in humanized mice models [65]. Another notable example was the RGD pentapeptide and apolipoprotein A-I (ApoA-I) nanoparticles loaded with Sorafenib and antimirRNA21, a chemogenetic therapy that could effectively treat HCC not just in vitro but also in vivo [66]. Self-assembling arginine α,β -dehydronaphthalene (R Δ F) nanoparticles that used efficiently targeted to HCC by conjugating with lactobionic acid, a ligand for the asialoglycoprotein receptor known to be overexpressed in HCC cell lines (Fig. 4). The treatment was successfully used to restore miR-199a-3p expression in HCC through the delivery of the aforementioned miRNA carried by NPs intravenously injected into mice xenograft models [67].

Notable instances of using other non-PLGA synthetic nanoparticles for miRNA-based therapy of HCC include the use of a small molecule upregulator of miR-34a expression, delivered via polyethylenimine-poly (epsilon-caprolactone) (PEI-PCL) nanoparticles [68]. Polyethylenimine (PEI)-based nanoparticles have been used for delivering siRNA molecules in HCC [69], however, PLGA remains the most commonly used primary polymer for the creation of NPs for miRNA-based therapies, since the transfection efficiency and cytotoxicity of PEI are positively correlated with its molecular weight. Cheng et al. found a way around this by introducing disulfide(-S-S-) linkage in low molecular weight PEI to form disulfide-containing polyethylenimine (SSBPEI), which, when combined with PEG and a peptide they call CC9, was able to suppress the growth and migration of the human hepatoma cell line HuH-7 [70].

Chitosan is a natural linear polycationic polysaccharide composed of β -(1-4)-linked D-glucosamine and N-acetyl-D-glucosamine, made by treating the chitinous shells of crustaceans with an alkali. As a nontoxic biocompatible polymer, it is a perfect candidate for carrying miRNA therapeutics. In combination with the anticancer drug 5-flourouracil,

galactosylated chitosan nanoparticles containing miRNA-122 could inhibit the proliferation of HCC in vitro, induce apoptosis, as well as downregulate the expression of two target genes of the miRNA, ADAM17 and Bcl-2 [71].

5.2. Lipid-based nanoparticles

Lipid-based nanoparticles are successful in delivering the conjugated miRNA due to their ability to easily cross the phospholipid bilayer that is the cell membrane. Given the role of hepatocytes in healthy tissue includes lipid metabolism, systemic delivery of miRNAs carried in lipid nanoparticles would eventually find their way to the liver, as would most other nanoparticle types. However, targeting NPs to oncogenically transformed hepatocytes helps reduce off-target side effects and increases efficacy (Fig. 5).

Apart from the lipopeptide nanoparticles, an example of which was discussed in 5.1, lipid nanoparticles can take the form of liposomes, or solid lipid nanoparticles, which contain lipophilic molecules dissolved in the solid lipid core stabilized by surfactants. Since nucleic acids are decidedly hydrophilic due to their polar nature, most lipid nanoparticles used in miRNA therapy are liposomes.

Such an approach was taken in targeting miR-17 by Dhanasekaran and colleagues, who encapsulated antimir-17 into the cationic lipid RL01 in order to treat HCC in transgenic mice [72]. Soon after this study, Huang et al. delivered an antagonir against the entire miRNA-17 family to treat HCC in vitro and in vivo [73] using the same approach. Lactosylated gramicidin-containing lipid nanoparticles (Lac-GLN), targeted to HCC using N-lactobionyl-dioleoyl phosphatidylethanolamine, a ligand for the asialoglycoprotein receptor (ASGR) highly upregulated in hepatocellular carcinoma, were used to deliver anti-miR-155 which successfully suppressed the growth of HCC [74]. Lipid nanoparticles comprising cationic lipids as well as 'helper lipids' such as oleic acid were found to enhance the delivery of miRNA-122 and effectively treat HCC in both tumor cells and animals [75].

A synergistic combinatorial therapy of doxorubicin and miRNA-375, delivered through liposomes, significantly suppressed HCC hallmarks and in xenograft mouse models with only mild adverse effects, and

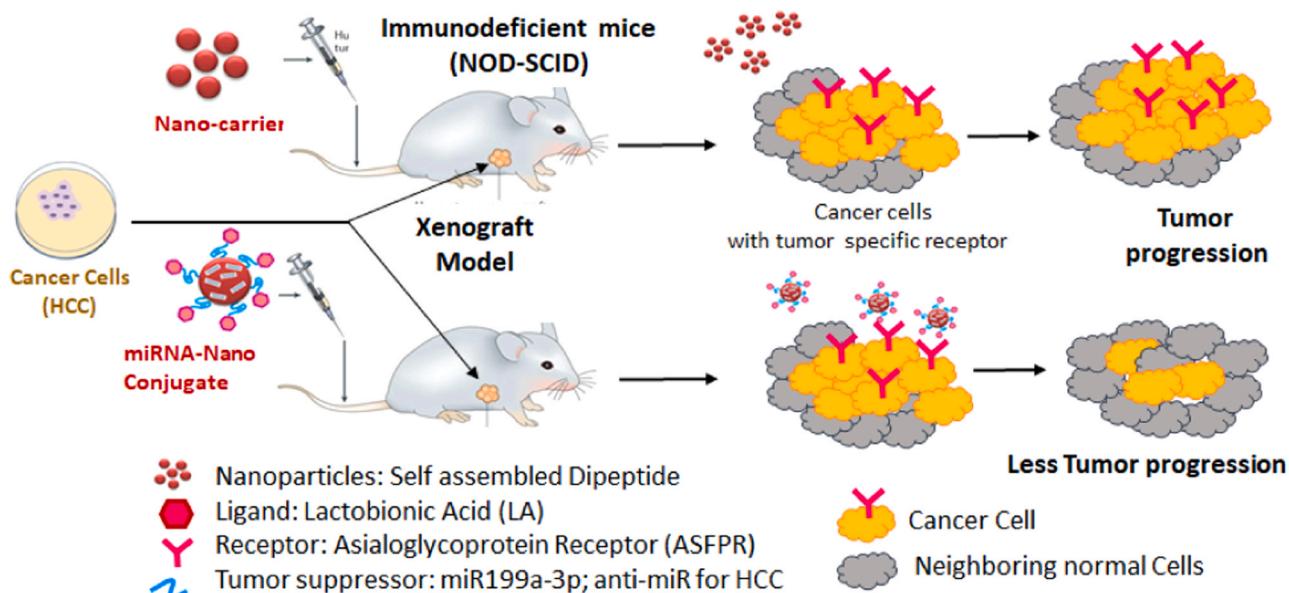


Fig. 4. In vivo strategy for antitumor efficacy of miRNA nano-conjugate using xenograft mouse model. During hepatocellular carcinoma (HCC) progression asialoglycoprotein receptor (ASGPR) is known to be primarily expressed on cancerous hepatocytes and minimally expressed on extra hepatic cells. The lactobionic acid (LA) is one of the known efficient ligands for ASGPR apart from galactose and asialofetuin. For selective targeting of NPs to tumor cells, LA conjugated self-assembling dipeptide arginine α,β -dehydronaphthalene nanoparticles were assembled with miR-199a-3p which is usually consistently downregulated in HCC. Nano-conjugate with or without miRNA were intravenously injected into HCC tumor bearing immunocompromised nude mice (NOD-SCID). And it was found that the miRNA nano-conjugate effectively reduced tumor progression with xenograft mouse model [57].

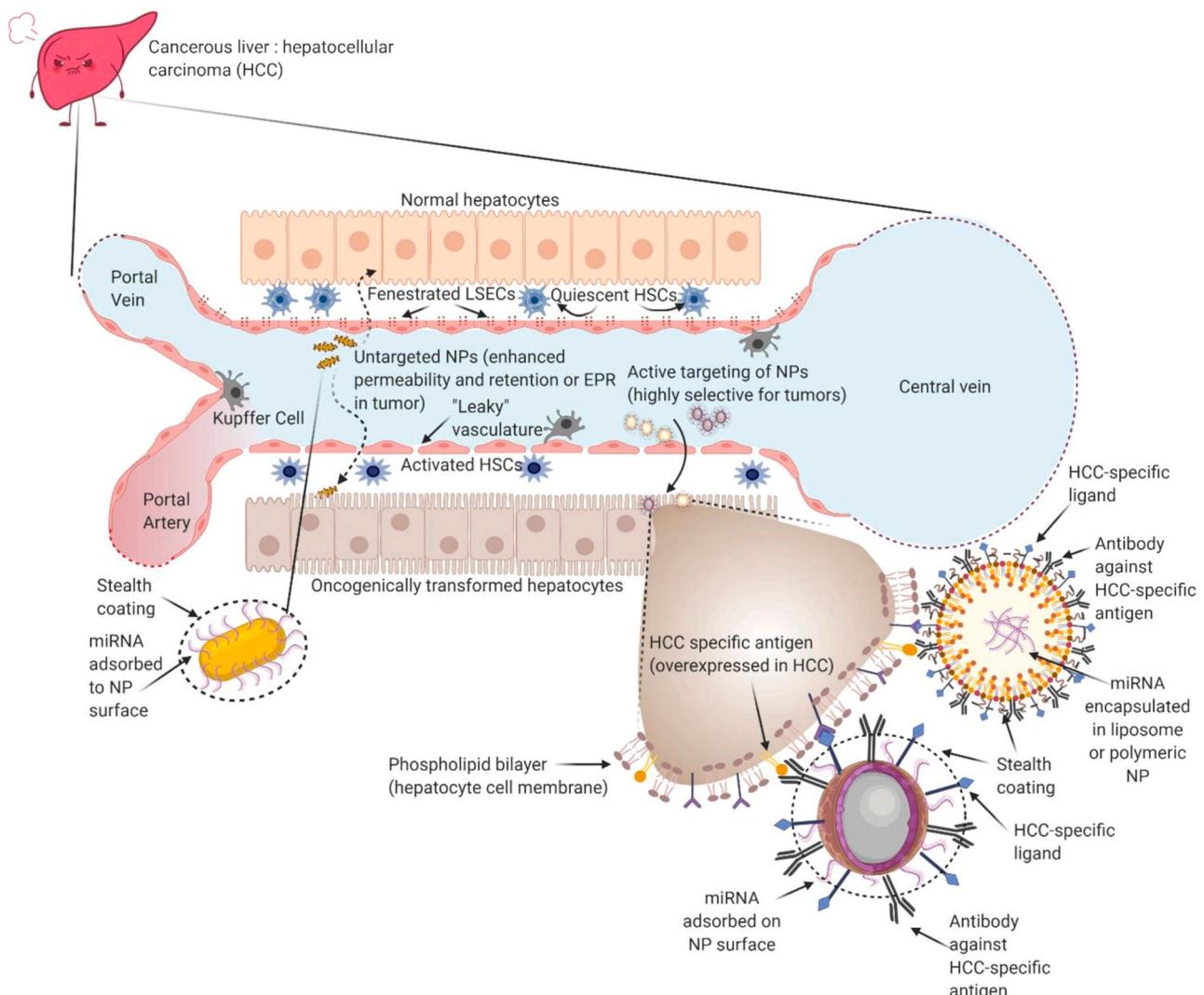


Fig. 5. Schematic illustration of targeted nanocarrier-based delivery for miRNA in liver tissue against HCC. In the liver blood flows from the hepatic artery and portal vein and leaves the liver through the hepatic vein. The portal vein and hepatic artery are located in portal triads in close proximity to the bile ducts. Oxygenated incoming blood with nutrients flows from the portal zone through radial sinusoids and into the central vein. Non-parenchymal cells associated with the sinusoid include fenestrated liver sinusoidal endothelial cells (LSECs), resident macrophage Kupffer cells and hepatic stellate cells (HSCs) which reside in the space of Disse. Leaky vasculature during HCC helps untargeted nanocarriers to accumulate in tumor tissue combined with the EPR (enhanced permeability and retention) effect. However, some amount of these nanoparticles may affect healthy cells as well, resulting in side effects and a higher dose to treat the cancer. On the other hand, active targeting of nanocarriers by decorating the nanoparticle surface with antibodies against or ligands for HCC-specific antigens or receptors achieve high specificity and therefore fewer off-target effects. In the second case of targeted delivery, only oncogenetically transformed hepatocyte interact with liposome or nanoparticle (NP) with antibody against HCC specific antigen where miRNA can be either absorbed in NP surface or encapsulated within liposome or polymeric NPs.

dramatically overcame drug resistance [76]. A similar success was achieved when DOX was combined with miR-101 and co-delivered by liposomes [77]. Liposomes containing the pH-sensitive lipid YSK05 proved to be an effective form of miRNA suppression therapy for the miRNA-122 in mice models of MAFLD, and therefore a similar strategy could be used to instead supplement miRNA-122 in HCC [78]. A cationic liposome comprising hydrogenated soybean phospholipid (HSPC), cholesterol (CHOL), and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine (DSPE) along with a methoxy (polyethylene glycol) (mPEG) stealth layer was successful in similarly inhibiting miRNA-221 [9]. Liposomes combining the chemotherapeutic agent Cisplatin and miRNA-375 enhanced apoptosis and induced cell cycle arrest in HCC cells in vitro, and significantly inhibited tumor growth and delayed the tumor relapse in a mouse model of HCC [79]. Most notable among these liposome-based miRNA therapies, of course, is MRX34, a liposomal miR-34a mimic [80] which was the first of its kind to go to clinical trial [21,22].

5.3. miRNA delivery through exosomes as a therapeutic option for Hepatocellular carcinoma

Exosomes are extracellular vesicles used in cell-to-cell communication. Created from the cell membrane (phospholipid bilayer) of the cell of origin, they are already decorated with biofunctionalization groups that help them receive a warm welcome from the target cell. Moreover, miRNAs are amongst the molecules carried as “messages” to the target cell, and these miRNAs are processed just as endogenous miRNAs would be. Dysregulation of exosomal miRNA expression has the potential to accelerate disease progression through the modulation of genome-wide signaling networks. Exosomal miRNAs are involved in tumor development, immunomodulation, chemoresistance, and metastasis. Therefore, exosomes used as drug delivery vehicles could provide a novel way to target therapeutic agents to cancer cells, including HCC.

Theoretically, exosomes or extracellular vesicles (EVs) of any cell of a non-hepatocellular origin could be extracted through density gradient

ultracentrifugation to be used as a liposome “loaded” with a miRNA or antagomir. This would apply for all non-parenchymatous liver cells, or any other cells that communicate with hepatocytes- the sinusoidal endothelial cells, stellate cells, adipocytes, Kupffer cells which are the resident macrophages, or any immune cells recruited to the liver. Based on these facts, ongoing research across the globe is attempting to exploit exosomes as a therapeutic option for treating HCC. Some examples include the work by Rao et al., who showed that tumor cell-derived exosomes were capable of suppressing hepatocellular carcinoma by immunomodulation of dendritic cells [81]. This concept has significant translational value, in the form of personalized dendritic cell vaccines [82]. In fact, it is possible to engineer an exosome displaying both tumor antigens and adjuvants [83], or they deliver a chemotherapeutic agent [84,85] in addition with conjugated miRNA.

Mesenchymal stem cells can be recruited from the bone marrow to the tumor microenvironment, making them another candidate for the secretion of exosomes with tumor-suppressive properties. Indeed, EVs from adipocyte-derived MSCs were capable of suppressing the growth of HCC in vivo [86,87], or increasing chemosensitivity [50]. Such methods could be classified as a “preloading” technique, wherein the donor cell is modified or selected to secrete exosomes containing the desired conjugated miRNA.

Since a significant proportion of HCC cases arise in a background of liver fibrosis or cirrhosis, Wang et al. demonstrated that stellate-cell derived exosomes “actively loaded” with miR-335–5p using Lipofectamine could inhibit HCC cell proliferation and invasion in vitro as well as in vivo [88]. Liang and colleagues engineered HEK293T cells to secrete exosomes targeted to hepatocytes, which they electroporated to load with miR-26a. These exosomes were capable of decreasing cell migration and proliferation in vitro [89]. A similar approach was taken by Pomatto et al., with the only difference that they used renal carcinoma endothelial cells as the exosome donor that they electroporated to load with the tumor suppressors miR-31 and the chemosensitivity-inducing miR-451a [90].

6. Conclusion

MicroRNAs are a powerful tool to manipulate entire gene expression networks, and at present are underutilized in the treatment of hepatocellular carcinoma. Emerging techniques such as sonodynamic therapy to deliver miRNA-carrying nanoparticles, or engineering exosomes to deliver the miRNAs of choice need to be further explored as they are relatively safe and efficient treatment options. Future research may go in a few different directions. Multiplexing miRNAs on the same nanoparticle in order to personalize the therapy based on biomarkers found in patients is an exciting offshoot of personalized medicine. There exists an intimate interaction between Kupffer cells and hepatocytes. It is relatively easy to polarize macrophages in vitro to mimic tumor associated macrophage types 1 and 2 (M1 & M2), of which type 1 has antitumor properties. Therefore, the avenue of engineering M1 macrophages to secrete exosomes preloaded with desired miRNA therapeutics must be explored.

Lifestyle diseases are on the rise, and these include steatohepatitis, metabolism-related or otherwise. Infection by hepatotropic viruses are still very common in some of the most densely populated parts of the world, such as South Asia. These chronic conditions being the two major risk factors leading to the development of HCC means that hepatocellular carcinoma is going to affect more and more people as time goes on. Moreover, initial diagnosis commonly occurs at an advanced stage of the disease, leading to poor outcomes. Since one cure cannot suit all, as proven by resistance to first line chemotherapeutics such as Sorafenib, increasing treatment options may prove helpful in the battle against HCC. This would hold especially true in the case of miRNA-mediated therapies, because, in the near future, it could become a customizable treatment, with individual components such as the nanoparticles and oligonucleotides being used as per choice, availability, and cost.

Authors' contributions

B.R. has formulated and written the review. S. G. critically revised the MS. S.B. executed the supervision of the entire writeup including drafting of the manuscript; critically revised for intellectual content.

Competing interests

Authors declare no conflict of interest.

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Authors' information

Correspondence and request for materials should be addressed to S. B. (sbiswas2@amity.edu).

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