


Understanding Gene Involvement in Hepatocellular Carcinoma: Implications for Gene Therapy and Personalized Medicine

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Abstract: Hepatocellular carcinoma (HCC) is the dominant type of liver cancers and is one of the deadliest health threats globally. The conventional therapeutic options for HCC are hampered by low efficiency and intolerable side effects. Gene therapy, however, now offers hope for the treatment of many disorders previously considered incurable, and gene therapy is beginning to address many of the shortcomings of conventional therapies. Herein, we summarize the involvement of genes in the pathogenesis and prognosis of HCC, with a special focus on dysregulated signaling pathways, genes involved in immune evasion, and non-coding RNAs as novel two-edged players, which collectively offer potential targets for the gene therapy of HCC. Herein, the opportunities and challenges of HCC gene therapy are discussed. These include innovative therapies such as genome editing and cell therapies. Moreover, advanced gene delivery technologies that recruit nanomedicines for use in gene therapy for HCC are highlighted. Finally, suggestions are offered for improved clinical translation and future directions in this area of endeavor.

Keywords: hepatocellular carcinoma, gene therapy, personalized medicine, nanomedicines, clinical translation

Introduction

Hepatocellular carcinoma (HCC), the most common type of liver cancers, is a serious life-terminating threat and a major cause of death across the globe. According to the American Cancer Society, more than 800,000 new cases are diagnosed annually, and the death toll exceeds 700,000 cases every year. The incidence rates have tripled over the past four decades, while the mortality rates have doubled.¹ According to the latest guidelines from both the American Association for the Study of Liver Diseases (AASLD) and the European Association for the Study of the Liver (EASL), surgical resection is adopted as the first choice in the treatment of localized HCC in the absence of liver cirrhosis. Neoadjuvant therapy using Atezolizumab and Bevacizumab as well as immune checkpoint inhibitors can be considered before and after surgery to reduce the risk of postoperative recurrence. For patients with non-resectable tumors, local ablative therapies such as thermal ablation or radiation segmentectomy are recommended. For ineligible patients, chemotherapy with multiple kinase inhibitors (eg Sorafenib) remains the available therapeutic option.^{2,3} Unfortunately, most HCC cases are diagnosed at advanced stages or along with viral hepatitis or liver cirrhosis, which collectively limit the applicability of the above choices. Moreover, systemic chemotherapy is hampered by serious side effects as well as the emergence of chemoresistance, leaving the survival rates not being improved significantly over the past decades.⁴ On the other hand, liver transplantation is not an affordable choice for a substantial proportion of patients, especially in the developing, low-income countries.⁵

Recent interest in innovative therapeutic approaches such as molecular medicine and cell therapies has grown thanks to breakthroughs in molecular biology and the relevant tools. Gene therapy has emerged to address the shortcomings of conventional therapies.⁶ Kymriah[®] was the first FDA-approved gene therapy for the treatment of blood tumors in 2017,⁷

followed by Luxturna[®] for the treatment of inherited blindness in the same year.⁸ Subsequently, Onpatro[®] appeared in 2018 as the first FDA-approved RNA drug for the treatment of hereditary transthyretin-mediated amyloidosis (hATTR).⁹ The gene-therapy revolution has continued with the evolution of messenger RNA (mRNA) vaccines in 2020,¹⁰ in parallel with significant advancements in genome editing technologies that resulted in the Nobel prize in chemistry for Jennifer Doudna and Emmanuelle Charpentier who pioneered the CRISPR-Cas9 modality.¹¹ Meanwhile, advancements in nucleic acid chemistry have enabled the design of chemically modified nucleosides that have promoted higher levels of stability and have lowered the innate immunogenicity of RNA therapeutics, which is an achievement that was acknowledged by the 2023 Nobel prize in physiology or medicine for Katalin Karikó and Drew Weissman.¹²

The present article sheds light on the involvement of genes in the development and progression of HCC and on the opportunities this offers for the treatment of HCC. In addition to dysregulation of the cellular signaling pathways, we highlight the roles of novel genetic pathways such as non-coding RNAs (ncRNAs) and genes that regulate tumor-specific immune responses. Then, we summarize the potential strategies that could be adopted for the gene therapy of HCC and the associated challenges. Subsequently, the advanced gene delivery technologies that recruit nanomedicines for HCC gene therapy are highlighted. Finally, the future direction of gene therapy for HCC are inspired.

Molecular Pathways in HCC Pathogenesis

Modern cell biology and bioinformatics have revealed a substantial involvement of multiple cellular signaling pathways in the initiation, survival, growth, invasion, migration, metastasis, and chemoresistance of HCC. An in-depth understanding of these pathways is a fundamental point in designing novel molecular and gene therapies against HCC.^{9,13} The precise determination of the differentially expressed genes associated with HCC progression in each individual patient is a promising tool in deciding which personalized medicines should be used.¹⁴ Moreover, some of these pathways offer characteristic molecular signatures that facilitate the early detection of HCC and/or predict the effectiveness of the proposed therapies. Furthermore, the assessment of certain molecular biomarkers is an emerging approach in evaluating the therapeutic outcomes of different treatments both in clinical practice and in clinical trials.¹⁵ In this section, we shed light on some of the recently discovered cellular pathways associated with HCC.

Tyrosine Kinases-Dependent Pathways

Tyrosine kinases comprise a family of enzymes that are capable of catalyzing the ATP-dependent phosphorylation of tyrosine residues in a wide variety of receptors that include Epidermal growth factor receptors (EGFRs), Vascular endothelial growth factor receptors (VEGFRs), Platelet-derived growth factor receptors (PDGFRs), Fibroblast growth factor receptors (FGFRs), Hepatocyte growth factor (HGF/ c-MET) receptors, and Stem cell growth factor (c-KIT) receptors. Therefore, tyrosine kinases act as a critical downstream signal for these receptors, which play a pivotal role in the activation of diverse cellular signaling pathways involved in the growth, survival, and invasion of cancer cells.^{13,16} Previous reports have revealed the overexpression of tyrosine kinases in various cancers, which includes HCC.^{17,18} Therefore, tyrosine kinases are interesting targets for several small molecular inhibitors that are used in the clinical management of HCC: Sorafenib (SOR), Lenvatinib, Cabozantinib, and Regorafenib.¹⁹ In the current section, we highlight some of the tyrosine kinase-dependent pathways and their role in HCC.

Vascular Endothelial Growth Factor (VEGF) Pathway

The abnormal and rapid growth of tumors requires an extensive blood supply to carry the essential nutrients and growth factors. Therefore, the majority of tumors are characterized by an uncontrolled formation of new blood vessels, a process that is referred to as “angiogenesis”.^{9,20} Furthermore, angiogenesis is a fundamental player in the metastasis of several types of tumors.²¹ Clinically, a widely used method for the management of hepatocellular carcinoma via the blockage of a tumor’s blood supply is a technique that is referred to as Transarterial chemoembolization (TACE).²² TACE, however, is limited by several challenges. These include the heterogeneity of HCC tumors that complicates the process of identifying patients who would benefit from TACE, the poor penetration of therapeutic drugs into HCC during the procedure, and the compensatory mechanisms arising from the complex crosstalk between the tumor microenvironment and adjacent tissues.²³ Therefore, alternative approaches should be considered.

Vascular endothelial growth factors (VEGFs) are a family of polypeptides that include VEGF, VEGF-B, VEGF-C, VEGF-D, VEGF-E, and placental growth factor (PlGF). VEGFs are responsible for regulating the recruitment of endothelial progenitors to form new blood vessels.²⁴ VEGFs act as ligands for subtypes of VEGFRs that mediate the subsequent cellular responses. Among the major subtypes of VEGFRs, VEGFR-2 is the most important in tumorous angiogenesis.²⁵ Clinical specimens have revealed that VEGF genes are highly expressed in HCC, which suggests a promising target for gene therapy.^{20,26} Moreover, the levels of VEGF could be used as a diagnostic biomarker to determine the stage of HCC and to predict the response to various treatments.²⁷

Epidermal Growth Factor (EGF) Pathway

EGF is an important factor in the regulation of growth, survival, proliferation, and differentiation of mammalian cells.²⁸ The receptor for this ligand, EGFR, is one of the most well-studied and characterized receptors in a wide variety of cancers, including liver, breast, and lung carcinomas.²⁹ With respect to HCC, interest in the EGF pathway is growing. Huang et al reported that the EGF pathway regulates the inflammatory environment of human HCC via the induction of the pro-inflammatory chemokines, CXCL5 and CXCL8.³⁰ Erlotinib and Vatalanib are small molecular inhibitors that were chemically designed to target EGFR, and these are currently undergoing Phase II clinical trials.^{31,32} Cetuximab is a monoclonal antibody against EGFR, which is also undergoing phase II clinical trials.³³ Despite the usefulness of such inhibitors to block the EGF pathway and induce apoptosis in HCC cells, mutations in EGFR could develop and lead to chemoresistance. Sueangoen and co-workers reported seven EGFR mutants in HCC cells (K757E, N808S, R831C, V897A, P937L, T940A, and M947T) in a fully functioning state, but all of them were resistant to Erlotinib.³⁴ The high rate of mutations in HCC necessitates the discovery of novel therapies beyond the classic drugs.

EGF is highly expressed in HCC. Studies involving transgenic mice overexpressing EGF have documented a higher incidence of HCC.³⁵ In addition, clinical data suggest that the overexpression of EGF and EGFR are correlated with a high incidence of HCC and a poor prognosis in patients with Hepatitis B virus (HBV) or Hepatitis C virus (HCV) infections.^{36,37} EGF/EGFR knockdown induces cell-cycle arrest and apoptosis in HCC cells.³⁸ Therefore, it could be concluded that the EGF pathway is a good candidate for the gene therapy of HCC.

Fibroblast Growth Factor (FGF) Pathway

FGF is a family of more than 20 proteins that are responsible for a variety of cellular growth functions such as cell proliferation, metabolism, differentiation, and survival. Among them, FGF19 is the most predominant factor that is overexpressed in HCC patients and is associated with poor prognosis and survival.³⁹ In healthy conditions, FGF19 promotes liver regeneration after hepatic injuries.⁴⁰ Animal studies have revealed that transgenic mice with overexpressed FGF19 spontaneously develop HCC after 8–10 months of birth.⁴¹ Molecular studies have revealed that FGF19 induces several cellular signaling pathways in HCC, which include Wnt/ β -Catenin, EGF, Stat3/IL-6, and Ras-ERK-Mnk1.⁴² Moreover, FGF19 regulates endoplasmic reticulum (ER) stress, which promotes the immune cell evasion of HCC.⁴³ Furthermore, FGF19 affects chromatin organization and the expression of several transcription factors that promote hepatocarcinogenesis.⁴⁴ The multiple kinase inhibitor, Lenvatinib, has shown an ability to inhibit the FGF19 pathway and its relevant receptor, FGFR4.⁴⁵ Brivanib alaninate is an alanine ester prodrug that is hydrolyzed into its active form, BMS-540215, in vivo. It is also a multiple kinase inhibitor that can interfere with both VEGF and FGF pathways via its potent action on tyrosine kinase.⁴⁶ Despite its success in phase II clinical trials, Phase III clinical trials revealed no increase in the survival of HCC patients following its administration.⁴⁷ On the other hand, a novel molecular inhibitor to FGFR4, which is referred to as BLU9931, has shown positive therapeutic outcomes in one-third of HCC patients recruited in a clinical trial.⁴⁸

Platelet-Derived Growth Factor (PDGF)

PDGF is a tyrosine kinase-dependent growth factor that was originally recognized as a serum growth factor for fibroblasts. It has also been linked to cellular growth and tissue regeneration, particularly during the fetal stage as well as in wound-healing processes.⁴⁹ High serum levels of PDGF have been linked to several chronic liver diseases such as HCC, liver fibrosis, and cirrhosis.⁵⁰ Liver fibrosis, or desmoplasia, is a common complication of HCC that arises from the activation of hepatic stellate cells (HSCs) in the liver as a response to the inflammatory

environment of HCC. Severe desmoplasia is a fatal complication of HCC that leads to hepatic failure, ascites, portal hypertension, and death.⁵¹ PDGF plays a pivotal role in the activation of HSCs via the stimulation of multiple transcription factors that promote the conversion of HSCs from a quiescent phenotype (qHSCs) into an activated myofibroblast phenotype (aHSCs).⁵² Therefore, PDGF is an important therapeutic target for the attenuation of HCC-associated desmoplasia.⁵³ Moreover, the elevated serum levels of PDGF in the pathological status have been recently exploited by our group to achieve selective delivery of nanocarriers to aHSCs for the attenuation of liver fibrosis.^{54,55}

PDGF is known to be overexpressed in various cancerous cells with functions related to cell growth, differentiation, and migration. PDGF can promote tumorigenesis through the proliferative expansion of preneoplastic or genetically unstable cells.⁵⁶ Furthermore, PDGF contributes to the development of the tumor microenvironment via the recruitment of stromal cells.⁵⁷

There have been multiple pharmacological strategies to block PDGF and its receptors, PDGFRs. These include the use of neutralizing antibodies against PDGF, anti-PDGF aptamers, soluble receptor-like materials that bind PDGF in the serum, and blocking antibodies/ligands against PDGFRs. Tyrosine kinase inhibitors are also applied to block the synthesis of PDGF/PDGFRs.^{50,58,59} Moreover, small molecular inhibitors, such as TMPyP4, have been developed to knockdown PDGF at the genetic level via silencing PDGF-A promoter.⁶⁰

Stem Cell Growth Factor Receptor (c-KIT)

C-KIT is a tyrosine kinase-dependent receptor that binds to stem cell growth factor (SCF), a dimeric hematopoietic cytokine that basically induces the cell-cycle initiation and differentiation of hematopoietic stem and progenitor cells.⁶¹ In addition, SCF/c-KIT signaling plays vital roles in other physiological functions that include pigmentation, fertility, gut movement, and neurological signaling.⁶² In HCC, c-KIT is known to be overexpressed in addition to the presence of numerous gain-of-function mutations that mediate cell survival and migration.⁶³ Moreover, c-KIT signaling contributes to angiogenesis, which is a vital process in the progression of HCC. Furthermore, one of the mechanisms by which HBV induces hepatocarcinogenesis is known to be mediated through the HBV-associated protein PreS1, which activates the expression of c-KIT to promote the generation and growth of liver cancer stem cells.⁶⁴ A similar pathway has been discovered in the case of the HCV core protein, which activates c-KIT signaling to induce epithelial-mesenchymal transitions (EMT) and promote the survival of cells.⁶⁵ In a clinical follow-up model established by Cai et al, the overexpression of c-KIT correlated with the HCC recurrence in patients following hepatectomy.⁶⁶ Imatinib mesylate is a clinically used inhibitor that targets the c-KIT pathway. In addition, other multiple kinase inhibitors such as SOR and Sunitinib also impact c-KIT. Nevertheless, the use of such inhibitors should be carefully monitored since c-KIT is known to play a two-edged role in the liver, and if it is overly inhibited this could have a negative impact on the healing of liver injury and on the regeneration of hepatocytes.⁶³

Hepatocyte Growth Factor (HGF/ c-MET)

As its name suggests, HGF is responsible for regulating the regeneration and growth of hepatocytes under normal conditions. In HCC, the overexpression of HGF is associated with the proliferation, survival, and metastasis of the tumor cells via binding to c-MET receptors with a subsequent activation of various downstream signaling pathways that include PI3K, ERK, and STAT3.⁶⁷ Previous reports have detected an overexpression of c-MET in several human HCC cell lines such as MHCC97 and HCCLM3 cells.⁶⁸ Moreover, c-MET could be used as a prognostic marker for HCC patients, as well as an indicator for chemoresistance.⁶⁹ The HGF/c-MET axis is also associated with increases in angiogenesis and in the epithelial-mesenchymal transition in HCC, both of which promote tumor metastasis.⁷⁰ Owing to its roles in HCC progression, basic and clinical research has revealed the HGF/c-MET pathway is affected by small molecular inhibitors such as PHA665752 and AMG 337.^{71,72} Furthermore, c-MET has already been targeted by gene therapy. Zhang et al demonstrated that knocking down c-MET inhibits the growth of HCC both in vitro and in vivo.⁷³ In another recent study, knocking down c-MET suppressed tumor growth and reversed the resistance to cisplatin.⁷⁴

PI3K/AKT/mTOR Pathway

Phosphatidylinositol-3 kinase (PI3K)/protein kinase B (AKT)/mammalian target of rapamycin (mTOR) is considered the most critical signaling pathway in the progression of HCC, and has demonstrated hyperactivity in nearly 50% of patients. This signaling pathway regulates vital processes associated with the cycle, growth, metabolism, and survival of cells. In addition, this pathway provides a downstream signal for other cell-growth pathways that include EGF and insulin growth factor (IGF).⁷⁵ In a microarray transcriptome analysis, Boyault and co-workers reported an increase in AKT phosphorylation in about 23% of HCC patients, which was associated with early recurrence and poor prognosis.⁷⁶ In another bioinformatic analysis, Villanueva et al reported aberrant mTOR signaling in half of HCC cases.⁷⁷ Phosphatase and tensin homolog (PTEN) is an endogenous tumor suppressor that functions by inhibiting PI3K/AKT/mTOR signaling. It is noteworthy that either a mutation or a downregulation of the PTEN gene has been detected in about half of all HCC cases, which has led to the activation of PI3K/AKT/mTOR.⁷⁸ Horie et al reported a spontaneous formation of HCC in 66% of PTEN-knockout transgenic mice at the age of 80 weeks.⁷⁹

Sirolimus and Everolimus are selective molecular inhibitors of mTOR that have been investigated for use in therapy for HCC following the failure of SOR. In a recent meta-analysis by Yan et al, both drugs improved the survival of HCC patients post liver transplantation.⁸⁰ Grabinski et al reported that combining Everolimus and the pan AKT inhibitor MK-2206 synergistically inhibited the proliferation of three human HCC cell lines: Hep3B, HepG2 and Huh7.⁸¹ On the other hand, a randomized, double-blind, phase III clinical trial that recruited 546 patients with advanced SOR-intolerant HCC failed to show an increase in the survival rate of patients.⁸² In another phase II clinical trial, a combination of SOR and Temsirolimus, a mTOR inhibitor, failed to show the expected outcomes in patients with advanced HCC.⁸³ The contradicting clinical outcomes of PI3K/AKT/mTOR molecular inhibitors warrant the development of more innovative therapies against such a pathway, and gene therapy would be a promising choice.

Polo-Like Kinase I (PLK1) Pathway

PLK1, serine/threonine kinase, is a regulatory protein that plays multiple roles in cell-cycle progression, mostly through regulation of the spindle checkpoint in the M-phase. One of these roles is its overexpression in several types of tumors, where it has been associated with uncontrolled cell proliferation that tends to overwhelm mitotic checkpoints.⁸⁴ Mok et al reported that PLK1 was overexpressed 12-fold in HCC samples compared with the levels found in normal tissues. A knockdown of PLK1 has been used to reduce HCC viability in vitro and to suppress tumor growth in vivo in human HCC xenografts.⁸⁵ Arbutus Biopharma[®] corporation has developed TKM-080301, which is a gene-silencing therapy that targets PLK1, and has conducted phase I/II clinical trials to assess its safety, pharmacokinetics, and preliminary antitumor activity in a cohort with advanced HCC. While Phase I results showed a favorable biosafety profile, phase II results showed no improvement in the overall survival of patients compared with historical controls.⁸⁶

RAS/RAF/MEK/ERK Pathway

RAS/RAF/MEK/ERK is a complex pathway that acts as a downstream signal transduction mechanism that affects cell proliferation, differentiation, angiogenesis, and survival. This is accomplished via its components as well as its associated downstream proteins, which are referred to as mitogen-activated protein kinases (MAPKs).⁹ MEK1/2 phosphorylation is known to be 7-fold higher in HCC tissues compared with levels found in adjacent tissues.⁸⁷ In another study, Feng et al reported that abnormalities of RAS/RAF/MAPK tend to contribute to the malignant transformation of hepatocytes.⁸⁸ Ghousein et al reported that RAF1 protein was upregulated in almost 60% of samples derived from HCC patients.⁸⁹ In addition, previous reports have revealed that HBV is integrated into the host genome and expresses transcriptional factors that induce HCC via activation of the RAS/RAF/MEK/ERK pathway.⁹⁰

RAS/RAF/MEK/ERK signaling is a therapeutic target for multiple kinase inhibitors such as SOR and Lenvatinib. Even with progress such as this, clinical output has remained insufficient.⁹ CI-1040 is a small molecular inhibitor that selectively targets MEK/ERK. While phase I clinical trials have shown promising results, phase II results have been disappointing.⁹⁰ PD0325901 is a second-generation MEK/ERK inhibitor that has shown improved pharmacological properties and higher efficacy both in vitro and in vivo,⁹¹ but unfortunately it failed the phase II clinical trials.⁹² Such challenges have ushered gene therapy to the forefront as a potential solution. For example, Liu et al inhibited the growth

of a murine model of HCC via silencing the BRAF gene.⁹³ In another study, Bessard and co-workers demonstrated that ERK2 knockdown inhibited HCC growth both in vitro and in vivo.⁹⁴

Hedgehog (Hh) Pathway

The Hh pathway plays a pivotal role in hepatopathogenesis. The key components of this pathway are the Hh ligands: Sonic hedgehog (SHH), Indian hedgehog (IHH), and Desert hedgehog (DHH). Upon release, these ligands inactivate the membrane protein referred to as patched 1 (PTCH1), which is responsible for inhibiting smoothened (SMO) homolog under healthy conditions. Activation of SMO results in downstream events that activate glioma-associated transcription factors (GLI) to increase cell proliferation, differentiation, and invasion.⁵⁵ The Hh pathway is known to be hyperactivated in 50% of HCC cases and to correlate with tumor progression and invasion.⁹⁵ In addition, the Hh pathway is responsible for transforming the qHSCs in the liver into aHSCs that lead to the induction of desmoplasia.⁵⁵ On the other hand, the endogenous antagonist of Hh signaling, Hedgehog Interacting Protein (HHIP), is known to be downregulated in most HCC and liver fibrosis cases.^{9,54,96} Thus, the components of the Hh pathway are interesting targets of gene therapy for HCC.

Vismodegib is a small-molecular inhibitor of the Hh pathway, and has shown the ability to attenuate desmoplasia and HCC in several preclinical studies.^{97,98} Clinical data supporting its utility in the management of HCC, however, remains insufficient. Regarding gene therapy, Huang et al reported suppressed growth of the HCC cell line, SMMC-7721, through RNA interference (RNAi)-mediated knockdown of SMO.⁹⁹

JAK/STAT Pathway

Janus kinase/signal transducers and activators of transcription (JAK/STAT) signaling form a critical pathway that mediates the cellular responses to a wide variety of growth factors (eg EGF, PDGF, and VEGF) and cytokines (eg interleukin-6, IL-6). Upon activation, JAK/STAT regulates cell proliferation, differentiation, and apoptosis.¹⁰⁰ The activation of JAK/STAT is controlled by three families of inhibitory proteins: the protein inhibitors of activated STATS (PIAS), SH2-containing phosphatases (SHP), and the suppressors of cytokine signaling (SOCS). A dysregulation of JAK/STAT has been detected in HCC, either through gain-of-function mutations or via suppression of the endogenous modulators, which leads to uncontrolled cell growth, migration, and survival.¹⁰¹ Mohan and co-workers developed azaspirane-based 2-(1-(4-(2-cyanophenyl)1-benzyl-1H-indol-3-yl)-5-(4-methoxy-phenyl)-1-oxa-3-azaspiro(5,5) undecane (CIMO) as a potent small-molecular inhibitor of STAT3, and reported potent cytotoxicity to HCC cells (half-maximal inhibitory concentration, $IC_{50} = 7.3 \mu\text{M}$) in comparison with normal cells with an IC_{50} of more than 100 μM . Administration to mice bearing an orthotopic model of HCC led to an attenuation of tumor growth.¹⁰² Wang et al reported a gene knockdown of STAT3 that inhibited the cell growth and angiogenesis of HCC by affecting the signal transduction, telomerase, cell cycle, and apoptosis, and recommended its application to gene therapy as a treatment for HCC.¹⁰³ In addition, STAT3 signaling has been associated with the protumorous phenotype of tumor-associated macrophages (TAMs), which is referred to as the M2 phenotype, that are potential targets for anticancer immunotherapy.¹⁰⁴

Survival Pathways

In addition to the above-mentioned pathways, the following pathways play special roles in maintaining cellular survival and resistance to apoptosis in HCC. As a consequence, these pathways also have been associated with resistance to chemotherapy, which makes them interesting targets for gene therapy to resensitize HCC to chemotherapy.

Midkine (MK) is a heparin-binding growth cytokine that plays important roles in fetal life, particularly during the middle trimester of pregnancy, which is why it is referred to as “mid-kine”. The expression of MK diminishes following birth.¹⁰⁵ The resumption of MK expression in adulthood has been associated with several pathological disorders, which include cancers.^{9,106} In a recent study, we highlighted a novel mechanism by which MK mediates the resistance of HCC to SOR, which has no approved treatment thus far. By binding to anaplastic lymphoma kinase (ALK) receptors, MK activates PI3K/AKT, which subsequently activates MAPKs to upregulate STAT3 and downregulate the apoptosis-inducing gene, Caspase-3. Simultaneously, MK binds to Notch-2 receptors to activate the Notch cytoplasmic intracellular

domain (NCID) that further activates STAT3 and nuclear factor kappa (NF- κ B). The sum of these actions is to mechanistically oppose the pharmacodynamic effect of SOR on HCC cells and to impart resistance. The proposed mechanism is illustrated in Figure 1.¹⁰⁷ Knocking down MK expression via the use of small interfering RNA (siRNA) increases the sensitivity of HepG2 cells to SOR in vitro, which suggests a potential synergism between the two agents.⁴ Furthermore, MK gene silencing has been used to reverse the resistance to SOR in vivo and to enable the eradication of a SOR-resistant HCC model in mice.¹⁰⁷

Another member of the MAPKs, c-Jun-N-terminal-kinase (JNK), is a protein that mediates the anti-apoptotic activity that is essential for the survival of HCC. Previous reports have established that JNK mediates the cellular responses to various external stimuli and promotes proliferation, differentiation, migration, and invasion. Furthermore, JNK is involved in mediating inflammation and fibrosis via the TGF β /SMAD pathway.¹⁰⁸ Kuntzen et al showed that JNK knockdown by siRNA enhances CD95-mediated apoptosis and induces G₂/M Cell-Cycle arrest in HepG2 cells.¹⁰⁹

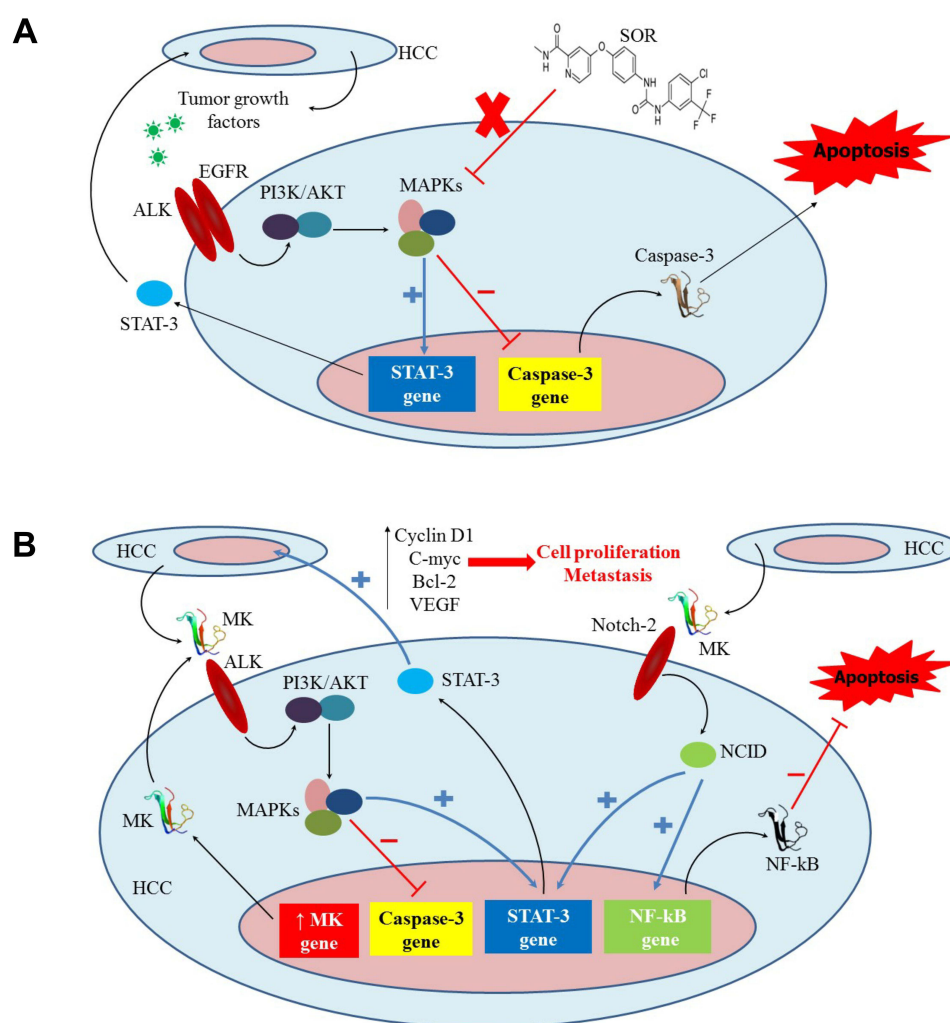


Figure 1 An illustration outlining the potential role of the MK signaling pathway in the acquired resistance to SOR by HCC cells. **(A)** The standard molecular mechanism of SOR when acting on HCC cells. **(B)** The overexpression of MK in HCC mechanistically opposes the therapeutic effects of SOR via upregulating STAT-3 and NF- κ B and downregulating Caspase-3. The figure is reprinted from *J Controlled Release*, Volume 331, Younis MA, Khalil IA, Elewa YHA, Kon Y, Harashima H. Ultra-small lipid nanoparticles encapsulating sorafenib and midkine-siRNA selectively-eradicate sorafenib-resistant hepatocellular carcinoma in vivo.335-349,¹⁰⁷ with permission from Elsevier (Copyright 2021, Elsevier).

Abbreviations: HCC, Hepatocellular carcinoma; ALK, Anaplastic lymphoma kinase; EGFR, Epidermal growth factor receptor; PI3K/AKT, Phosphatidylinositol-3 kinase/protein kinase B; MAPKs, Mitogen-activated protein kinases; STAT-3, Signal transducer and activator of transcription 3; C-myc, MYC proto-oncogene; Bcl-2, B-cell lymphoma 2; VEGF, Vascular endothelial growth factor; NCID, Notch cytoplasmic intracellular domain; NF- κ B, Nuclear factor kappa.

Myeloid cell leukemia-1 (Mcl-1) is an anti-apoptotic protein of the Bcl-2 family that plays an important role in the cell survival of various cancers including multiple myeloma, non-small cell lung cancer, B-cell non-Hodgkin's lymphomas, cervical carcinoma, oral cancer, and HCC.¹¹⁰ In one study, a knockdown of the Mcl-1 gene by siRNA increased the sensitivity of HCC to chemotherapy and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) treatments via enhanced Caspase-3-mediated apoptotic activity and the elimination of resistance.¹¹¹

B cell-specific Moloney murine leukemia virus integration site 1 (Bmi-1) is an oncogene that is overexpressed in HCC. It plays an important role, not only in carcinogenesis, but also in stem cell pluripotency, embryogenesis, and cell proliferation. Zhang et al reported that siRNA-mediated silencing of the Bmi-1 gene inhibits the proliferation and invasiveness of human HCC and increases their sensitivity to 5-Fluorouracil (5-FU) treatment.¹¹²

P-glycoprotein (P-gp) is a well-characterized membrane transporter that mediates the efflux of molecules out of cells. There is a cumulative evidence that cancer cells tend to upregulate P-gp, which amounts to an adaptive mechanism to escape chemotherapy. P-gp efflux is a fundamental mechanism for the development of chemoresistance against a wide variety of chemotherapeutic drugs including Doxorubicin (DOX), Paclitaxel (PTX), 5-FU, and Cisplatin. Therefore, P-gp is sometimes referred to as multidrug resistance 1 protein (MDR1).¹¹³ This is why the literature contains numerous reports of HCC studies targeting P-gp with gene therapy to reverse chemoresistance.^{114,115}

Inflammatory Pathways

Inflammation is an endogenous machinery the body uses to respond to various situations such as stress or cellular damage by recruiting diverse cytokines such as Tumor necrosis factor alpha (TNF- α) and IL-6 to increase the supply of blood and infiltrate the affected area with immune cells. Unfortunately, uncontrolled inflammatory responses sometimes lead to serious effects in tissues and can actually contribute to carcinogenesis. Moreover, HCC is known to secrete various proinflammatory signals that induce a persistent inflammatory microenvironment in the liver, which promotes the proliferation, migration, angiogenesis, and genomic instability of the tumor microenvironment.¹¹⁶

Lysophosphatidic acid (LPA) is an endogenous phospholipid the production of which is dependent mainly on Autotaxin (ATX) enzymes that also act as carriers of LPA to receptors. The LPA/ATX pathway is known to be highly associated with inflammation-related hepatocarcinogenesis through TNF α /NF κ B pathways. Mazzocca et al have reported that knocking down the LPA/ATX signaling pathway via the RNAi targeting of LPA receptor 6 (LPAR6) has impaired HCC tumorigenesis in a mouse xenograft model.¹¹⁷

Transforming growth factor beta 1 (TGF β 1) is an important proinflammatory signaling pathway in a wide variety of cancers, where its activation stimulates the intracellular effectors, SMADs, to translocate into the nucleus and activate several transcription factors that promote inflammation.¹¹⁸ Moreover, TGF β 1 is over-secreted by Kupffer cells in the liver in response to hepatic injuries, which results in a subsequent activation of HSCs that could initiate and propagate liver fibrosis to further proceed to cirrhosis and HCC. We have recently demonstrated that the simultaneous targeting of TGF β 1 and SMO in aHSCs by gene knockdown reprograms aHSCs into qHSCs and reverses liver fibrosis in mice.⁵⁵ In another patented finding, Evans and Lu reported that silencing TGF β 1 and cyclooxygenase-2 (COX-2) suppresses cancer growth — if combined with immunotherapy.¹¹⁹

Gene Involvement in Immune Responses to HCC

Gene dysregulation significantly alters the immune responses to HCC via affecting infiltration by immune cells, promoting immune evasion, or supporting a tumor-promoting microenvironment. HCC is known to possess a “cold” tumor microenvironment, in which there is a reduced infiltration of immune cells, a suppression of activity by cytotoxic T lymphocytes (CTL), and an increase in immune-suppressive regulatory T cells (Treg), which collectively create an immune-suppressive microenvironment that promotes tumor growth and invasion.¹²⁰ Shen and co-workers used bioinformatic analysis to establish an immune-related prognostic model for HCC patients. The results identified ten genes associated with immune suppression and poor prognosis: baculoviral IAP repeat containing 5 (BIRC5), cyclin-dependent kinase 4 (CDK4), deoxycytidine kinase (DCK), heat shock protein family A member 4 (HSPA4), heat shock protein 90 α family class A member 1 (HSP90AA1), Proteasome 26S Subunit Ubiquitin Receptor, Non-ATPase 2 (PSMD2), interleukin 1 receptor antagonist (IL1RN), placental growth factor (PGF), secreted phosphoprotein 1 (SPP1),

and stanniocalcin 2 (STC2). Therefore, the aforementioned genes all are promising targets for gene therapy to promote anticancer immunity.¹²¹

In addition, Stimulator of interferon genes (STING) pathway, which is an innate cytosolic sensor for DNA, is known to be important in anticancer immunity and is usually downregulated in several types of tumors, which includes HCC.¹²² Nakamura et al reported on the regaining of anticancer immunity upon the administration of a STING pathway agonist, cyclic di-GMP, to tumor-bearing mice, which was mediated via the activation of CD8⁺ T cells and natural-killer (NK) cells.¹²³ In a similar study, Khalifa et al reported a reduction in tumor growth as well as an upregulation of interferon gamma (IFN- γ) and NK cells post treatment with cyclic di-GMP.¹²⁴

Moreover, tumor-associated macrophages (TAMs) are known to favor the protumorous phenotype M2, which promotes an immune-suppressive microenvironment in tumors. In a recent study by our group, we reported the reprogramming of TAMs from the protumorous M2 phenotype into an anti-tumorous M1 phenotype via silencing STAT3 and hypoxia inducible factor 1 α (HIF-1 α) pathways, which helps promote anticancer immunotherapy.¹²⁵

Immune checkpoints form a natural mechanism to regulate the activity of T cells and prevent their reaction against auto-antigens. However, cancer cells often exploit such a mechanism to evade immune surveillance via the over-expression of Programmed cell death-ligand 1 (PD-L1). PD-L1 subsequently binds programmed cell death receptor-1 (PD-1) on the surface of T cells and inactivates them, thus inhibiting anticancer CTL activity. Immune checkpoint inhibitors have been developed to block such reactions via anti-PD-1 antibodies. Nivolumab (Opdivo[®]) was invented by the 2018 Nobel laureate Tasuku Honjo and is an FDA-approved immune checkpoint inhibitor that has been used to treat HCC since 2017. The FDA approval of Nivolumab was followed by approval for pembrolizumab in 2018.¹²⁶ Despite these breakthroughs, immune checkpoint inhibition therapy continues to face major obstacles in the clinical management of HCC: a low response rate, increased side effects, non-suitability for HBV and HCV-infected HCC patients, and the development of anti-PD-1-resistant tumors.¹²⁷ Therefore, gene therapy could provide a promising alternative. For example, Chen and co-workers used siRNA to silence PD-L1 genes and reported a subsequent inhibition of the tumor growth of an H22 HCC model in mice, which was mediated by an increase in the infiltration of T cells, an increase in the ratio of T cells in the spleen, an increase in the number of granzyme B⁺ T cells, as well as a synergistic interaction that potentiated the effects of the multiple kinase inhibitor, Lenvatinib.¹²⁸

Non-Coding RNAs (ncRNAs) and HCC

While gene therapy fundamentally focuses on protein-encoding genes, it is noteworthy that only 3% of the human genome encodes proteins, and the remainder comprises a non-coding genome. For several decades, non-coding genome was considered to be “junk”. However, recent research have shed the light on the tremendous importance of non-coding RNAs (ncRNAs) in regulating gene expression and for roles in various cellular responses. Functionally, ncRNAs can be classified into structural ncRNAs (eg, ribosomal RNA (rRNA) and transfer RNA (tRNA)) and regulatory ncRNAs (eg, microRNAs (miRNAs) and long ncRNAs (lncRNAs)). Regulatory ncRNAs are further classified based on their nucleotide length: the miRNAs have an average length comprised of 22 nucleotides whereas the lncRNAs have ≥ 200 nucleotides. Regulatory ncRNAs are responsible for modulating the gene expression processes by inhibiting the expression of certain genes (via miRNA that interferes with the corresponding mRNA of the target gene) or increasing the expression of others (via lncRNAs that absorb the inhibitory miRNAs, and thus allow expression of the target gene). In addition, other mechanisms such as epigenetic control, guidance, or acting as a scaffold could also be involved in gene regulation.^{129,130}

Numerous reports have revealed the critical roles that ncRNAs play in the initiation and progression of malignancies, which includes HCC.^{130–132} *HULC* is a well-characterized model of lncRNAs that are differentially upregulated in HCC compared with healthy liver tissue. *HULC* is thought to function by sequestering miR-107, which is a repressor of transcription factor E2F1, with a subsequent activation of E2F1 and sphingosine kinase 1 that leads to the promotion of angiogenesis.¹³³ Another mechanism was revealed by Li and co-workers, who demonstrated that *HULC* sequesters miR-200a-3p and subsequently promotes tumor metastasis by enhancing EMT via the ZEB1 signaling pathway.¹³⁴ *HOTAIR* is another example of lncRNAs that are upregulated in HCC and also are associated with a poor prognosis. *HOTAIR*

recruits the polycomb group complex 2 (PRC2) to epigenetically silence miR-218-2 and subsequently activates the P14 and P16 signaling pathways to promote tumor metastasis.¹³⁵

On the other hand, other ncRNAs are reported to play a tumor-suppressive role and are known to be downregulated in HCC. One of these is miR-17, that belongs to the miRNA family and has shown tumor-suppressing properties in HCC via the silencing of matrix metalloproteinase-3 (MMP-3), which leads to a reduction in cell migration and invasion. Therefore, miR-17 is a promising candidate for the treatment of metastatic HCC.¹³⁶ *DILC* is a model of lncRNAs that are downregulated in HCC, and has shown protective anticancer effects by suppressing STAT3 signaling in liver cancer stem cells, in addition to its ability to bind to IL-6 promoter and modulate the inflammatory status of the liver.¹³⁷ Table 1 lists additional examples of reported ncRNAs and their double-edged roles in HCC. For further information on ncRNAs in HCC, readers are directed to an intensive review article by Wong et al¹³⁸

Gene Therapy for HCC

As discussed in the previous sections, there are several interesting genetic pathways that could be targets of gene therapy for HCC. Nevertheless, there are also formidable obstacles that hamper the methods to accomplish this. In this section, we shed light on the possible opportunities as well as the challenges associated with gene therapy for HCC.

Opportunities for HCC Gene Therapy

There are multiple strategies that could accomplish gene therapy for HCC. Examples include knocking down the upregulated oncogenes, reactivation of the downregulated tumor-suppressor genes, correction of genetic mutations, development of genetically engineered anticancer viruses and cell therapies, application of anticancer vaccines, and recruitment of gene therapy to potentiate other conventional therapies. While the general approaches of gene therapy have been extensively reviewed elsewhere,^{9,155,156} herein we focused only on the applicability of such modalities in the treatment of HCC. Figure 2 outlines the possible gene therapy strategies for HCC.

Gene Silencing

As alluded to above, there is a wide variety of upregulated oncogenes that are involved in the vital processes associated with hepatocarcinogenesis. These processes include cell proliferation, differentiation, migration, and angiogenesis. These genes are excellent candidates for use in gene silencing therapy. RNA interference (RNAi) is the most common strategy for this approach. Upon intracellular delivery, synthetic short (18–25 nucleotide length) RNA molecules (eg, siRNA,

Table 1 Examples of ncRNAs That Have Been Reported to Play Roles in the Progression/Suppression of HCC

Functional Classification	ncRNA name	Category	Mechanism of Action	References
Tumor promoter	miR-21	miRNA	Induced by HBV to activate STAT3	[139]
	miR-222		Silences PPP2R2A to activate AKT	[140]
	miR-151		Silences RhoGDI A to promote metastasis	[141]
	miR-429		Silences RBBP4 to activate T-ICs	[142]
	<i>ANRIL</i>	lncRNA	Silences KLF2 to resist apoptosis	[143]
	<i>DBH-AS1</i>		Activates MAPK signaling	[144]
	<i>lnc-β-Catm</i>		Stabilizes β-catenin to promote LCSCs	[145]
	<i>linc01225</i>		Stabilizes EGFR	[146]
Tumor suppressor	let-7	miRNA	Silences PBX3 to inhibit T-ICs	[147]
	miR-195		Silences VEGF to inhibit angiogenesis	[148]
	miR-199a-3p		Silences PAK4 to inhibit RAF–MEK–ERK	[149]
	miR-142-3p	lncRNA	Silences CD133 to inhibit LCSCs	[150]
	<i>AOC4P</i>		Degrades of vimentin to inhibit EMT	[151]
	<i>FTX</i>		Sequesters miR-374 to repress Wnt/β-catenin	[152]
	<i>PTENP1</i>		Sequesters miR-17, miR-19b, miR-20a to rescue PTEN	[153]
	<i>MEG3</i>		Activates p53-mediated transcriptional activity	[154]

Abbreviations: PPP2R2A, Protein phosphatase 2A subunit B; RhoGDI A, Rho GDP-dissociation inhibitor A; RBBP4, Rb binding protein 4; T-ICs, Tumor-initiating cells; KLF2, Kruppel-like factor 2; LCSCs, Liver cancer stem cells; PBX3, PBX Homeobox 3.

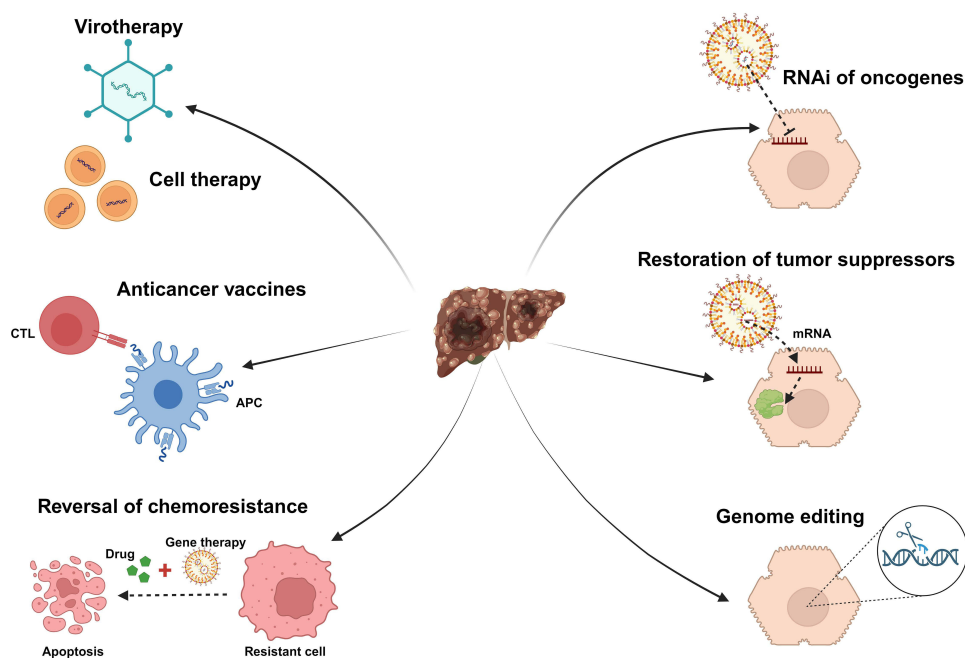


Figure 2 An outline for some of the potential strategies that could be applied in the gene therapy of HCC. The figure was created with BioRender.com, with a publication license.

miRNA, or small hairpin RNA (shRNA)), are assembled with a certain family of cytosolic proteins (eg, Argonaute-2, Ago-2) to generate a RNA-induced silencing complex (RISC). Subsequently, the tailored RNA molecules guide the RISC to the complementary nucleotide sequences of the target mRNA in order to facilitate cleavage that silences the translation of the target gene into an unwanted protein.¹⁵⁷ RNAi has been widely adopted over the past decade thanks to the ease of synthesis of short RNAs with a high degree of specificity, in addition to their high potency compared with previously used modalities such as antisense oligonucleotides (ASO).¹⁵⁸ Examples in the literature for the target genes of RNAi in HCC therapy include ERK2,⁹⁴ STAT3,¹⁰³ c-MET,⁷³ and VEGF.¹⁵⁹

Gene Introduction

Gene introduction is the opposite of gene silencing, and introduction enables the reactivation of downregulated tumor-suppressor pathways in HCC (ie, protein replacement therapy), which could amount to either the introduction of suicide genes or the introduction of modulatory proteins to regulate the cellular processes.⁹ Plasmid DNA (pDNA) and messenger RNA (mRNA) are two vectors that are commonly used to achieve gene introduction. Recently, the paradigm has shifted towards mRNA over pDNA thanks to its ease of production on a large scale (particularly after the establishment of manufacturing plants during the COVID-19 pandemic), higher encapsulation efficiency, and direct functioning in the cytosol that eliminates the necessity for nuclear translocation.¹⁰ An example of gene introduction was accomplished by Liu and co-workers who introduced a pDNA encoding TRAIL into an orthotopic murine model of HCC. TRAIL induced apoptosis into HCC and suppressed tumor growth, in addition to attenuating the desmoplasia associated with HCC via the inactivation of aHSCs.⁵¹ Deng et al delivered a mRNA encoding co-stimulator, Oxford 40 ligand (OX40L), to HCC in order to activate anticancer immunity via the induction of CTL activity.¹⁶⁰ Additional approaches include the introduction of anti-angiogenic factors that suppress tumor growth and metastasis such as endostatin, pigment epithelium-derived factor (PEDF), and NK4.¹⁶¹

Genome Editing

Genome editing is a strategy that enables the knock-out of oncogenes, the knock-in of missing genes, or the correction of faulty genes. This can be accomplished via a variety of modalities that possess the capability of cleaving DNA such as zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and clustered regularly

interspaced short palindromic repeats (CRISPR) with CRISPR-associated systems (Cas). The key characteristics of these tools are further elucidated in our previously published article.⁹ Genome editing has superior advantages over the other gene therapy modalities, particularly in its capability to induce permanent therapeutic effects that are inherited by subsequent daughter cells, thus eliminating the need for multiple dosing. Jennifer Doudna and Emmanuelle Charpentier won the 2020 Nobel prize in chemistry in an acknowledgement of their tremendous efforts in the development of CRISPR-Cas9 technology.¹¹ Genome editing has revolutionized cancer research owing to the capability of generating diverse knockout animal models for genetic research.¹⁶² In addition, genome editing has enabled the targeting of genetic pathways involved in hepatocarcinogenesis. Niu et al demonstrated that the knockout of aldolase A (ALDOA) in HCC cells by CRISPR-cas9 demonstrated reductions in cell proliferation and migration, as well as inducing cell-cycle arrest via lactate depletion.¹⁶³ Wan et al reported the downregulation of PD-L1 and a subsequent sensitivity to anticancer immunity that was achieved with the gene knockout of C-X-C chemokine receptor 2 (Cxcr2).¹⁶⁴ Further examples are described in a focused review article by Yu et al.¹⁶⁵ Nonetheless, the in vivo application of genome editing technology continues to be a challenge owing to multiple technical problems, which include difficulties in the delivery of high molecular weight proteins (eg, Cas9) and concerns about the side mutations that could be induced in the host genome. To mitigate these problems, the delivery of Cas9 in the form of mRNA could be the solution.¹⁶⁶

Genetically Engineered Viruses

Virotherapy is an approach by which cytopathic viruses are recruited to kill target cells (eg, cancer cells). Advances in genetic engineering have enabled the creation of modified viruses that can replicate into the cancer cells either preferentially or exclusively, which is why these are referred to as “oncolytic” viruses. The principle of such a technology relies on introducing mutations in the genes that encode critical proteins for viral replication that will be activated only in cancer cells, which usually demonstrate alterations in certain molecular pathways (eg, p53), thus enabling a tumor-specific viral replication. Another strategy is to introduce tumor-specific promoters into such genes to accomplish the same goal.¹⁶¹ ONYX-015 was the first oncolytic virus to appear in 1996, and it featured a deletion in the E1B 55K gene to favor tumor-specific replication.¹⁶⁷ Despite promising results shown in basic research, its clinical significance as a monotherapy was insufficient during phase II clinical trials.¹⁶⁸ Takahashi et al developed an adenovirus with an alpha fetoprotein (AFP) promoter/enhancer in the E1A-13S gene to achieve specific viral replication and subsequent oncolysis in HCC cells that differentially express AFP.¹⁶⁹ Broad anticancer activity was achieved by another adenovirus engineered with a human telomerase reverse transcriptase (hTERT) promoter, as revealed by Wirth et al.¹⁷⁰ Studies in the literature describe viruses that are engineered to express either suicide proteins (eg, TRAIL)¹⁷¹ or cytokines (eg, IL-12) to promote anticancer immunity.¹⁷²

Genetically Engineered Cell Therapies

Genetic engineering has also enabled the modification of human cells to enhance their ability to recognize tumors, or to acquire extra anticancer properties prior to being transferred to cancer patients in a process that is referred to as adoptive cell transfer.¹⁷³ CAR-T cells are a typical model of cell therapy by which human CTLs are transfected ex vivo with chimeric antigen receptors (CARs) to enhance their tumor-recognition ability in a process that is referred to as expansion. Subsequently, the modified cells are administered to cancer patients to exert their CTL activity in vivo. Kymriah[®] was the first FDA-approved therapy of this class for the treatment of lymphoblastic leukemia and large B-cell lymphoma. Such a strategy has been investigated in solid tumors as well, which includes HCC.⁹ For example, CAR-T cells that recognize epithelial cell-adhesion molecule (EpCAM) have been developed and entered into phase II clinical trials.¹⁷⁴ In another study, Finn and co-workers transfected autologous T cells with T cell receptors (TCRs) that recognize AFP in HCC and the treatment is currently undergoing phase I trials.¹⁷⁵ Moreover, other kinds of cells can be genetically engineered to serve as anticancer fighters. Peron et al modified murine fibroblasts or MM45T-Li HCC cells to express IL-12 prior to adoptive transfer to mice bearing an orthotopic model of HCC. The results showed enhanced tumor suppression with a high degree of biosafety.¹⁷⁶ Furthermore, Zhu et al enhanced T cell activation via the modification of human dendritic cells (DCs) with three co-stimulatory molecules, namely B7-1, ICAM-1, and LFA-3.¹⁷⁷ Another case was presented by Vollmer, Jr., and co-workers who developed engineered DCs that express AFP to serve as an anti-HCC vaccine.¹⁷⁸

Anticancer Vaccines

The evolution of nucleic acid delivery technology has enriched the area of anticancer immunotherapy and revived hope in the venerable dream of establishing efficient and realistic anticancer vaccines. The principle depends on the delivery of a mRNA/DNA encoding a certain neoantigen to antigen-presenting cells (APCs) such as DCs, so as to enhance the ability of the host immunity to detect and develop a CTL-based immune response against a target tumor.^{10,179} An early trial has been reported by Grimm et al who transfected APCs with AFP DNA to elicit CTL activity in mice bearing a syngeneic Hepa 1–6 HCC model.¹⁸⁰ Other recent studies in the recruiting of neoantigen-based mRNA vaccines have demonstrated promising results in clinical trials. An example of these vaccines is FixVac (BNT111), which was developed by BioNTech[®], and showed good results in phase I clinical trials against melanoma. Such a performance encourages the possibility for use against other tumors including HCC.¹⁸¹ One limitation that hampers the use of neoantigen vaccines is the high variability in expressing such neoantigens from a patient to another. In this respect, personalized medicine can be the best choice to tackle this challenge. Through biomarker and genetic screening, the neoantigens differentially expressed by each individual patient could be recognized. Subsequently, best-fit personalized neoantigen vaccines could be selected.¹⁸²

Gene Therapy Augmenting Conventional Therapies

In addition to its individual applicability as an anticancer therapy, several studies have applied gene therapy to potentiate the therapeutic effects of other conventional therapeutics. A common model of such an approach is silencing P-gp to reverse the resistance to DOX.¹¹⁵ We previously demonstrated that knocking down the expression of MK with siRNA (MK-siRNA) reverses the resistance of HCC to SOR both in vitro and in vivo.¹⁰⁷ Wang et al combined gene therapy and thermal therapy of HCC using magnetic mesoporous silica nanoparticles (MSNs) that co-delivered ganciclovir (GCV) and a pDNA encoding Herpes Simplex Virus thymidine kinase (HSV/Tk) enzyme. HSV/Tk activates the prodrug, GCV, in situ to induce cytotoxicity. Furthermore, an external magnetic field was applied to guide the nanoparticles to the tumor region and induce hyperthermia that synergizes with the cytotoxic effects of the drug.¹⁸³

Challenges Associated with Gene Delivery to HCC

Despite promise, there are several obstacles that hamper the gene therapy of HCC. While the general technical challenges associated with nucleic acid delivery have been extensively discussed elsewhere,^{9,179} herein, we focus on the additional challenges rendered by the unique features of HCC tumors.

The Reticulo-endothelial system (RES) is the first barrier that encounters the systemically-administered gene therapies to HCC, ending up with the majority of the administered dose being cleared by Kupffer cells, splenocytes, and peripheral monocytes. Therefore, the administered therapeutics should be shielded from such recognition and have a sufficient retention time in the blood to reach tumor tissues. In addition, the inflammatory hepatic microenvironment activates Kupffer cells with increasing possibilities of engulfing the administered cargo before it reaches the target cells. Moreover, the associated desmoplasia in the liver narrows the fenestrations of the liver sinusoids and capillaries, thus hampering the extravasation of the administered medicines. Furthermore, HCC has a stroma-rich tumor microenvironment that arrests the administered therapeutics and reduces their ability to access the affected hepatocytes.^{9,54} These challenges are illustrated in [Figure 3](#).⁹

Nanomedicines Enabling the Gene Delivery to HCC

Viral vectors, particularly adeno-associated viruses (AAV) and lentiviruses, have been extensively recruited as gene delivery vectors owing to their intrinsic capabilities of transfecting cells and delivering their genome with high efficiency. Luxturna[®] and Vaxzevria[®] are representative examples of approved nucleic acid therapeutics recruiting viral delivery vectors.^{8,184} Nevertheless, concerns are increasing regarding the safety of viral vectors due to their immunogenicity and unpredictable side effects.¹⁵⁵ Non-viral vectors, particularly nanoparticles, offer a better alternative to viral vectors, with acceptable biosafety, flexibility, and scalability. Lipid nanoparticles (LNPs) are the most well-established and clinically successful non-viral platform for gene delivery, particularly following the FDA approval of two LNP-based vaccines for COVID-19, namely Comirnaty[®] and Moderna[®] COVID-19 vaccine. The outstanding in vivo performance of LNPs could be attributed to their high tolerability as well as to their similarity to endogenous cellular membranes.¹⁰ Polymeric, metallic, protein, and hybrid nanoparticles have also been

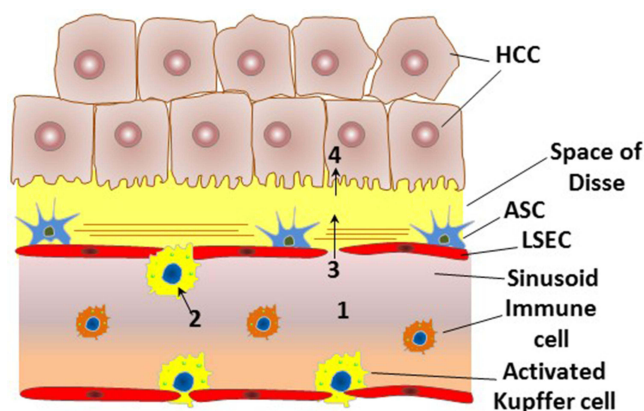


Figure 3 An outline of the major challenges that are encountered in gene delivery to HCC. (1) The systemically administered vectors must escape from the host immunity and from the reticulo-endothelial system and possess a sufficient residence time in blood circulation in order to reach the tumor region. (2) The administered vectors must escape the phagocytosis promoted by the activated Kupffer cells in the tumor microenvironment. (3) The vectors must be small enough to extravasate from the narrowed fenestrations and to penetrate the stroma-rich tumor microenvironment to access HCC cells. (4) The vectors must be taken up efficiently and selectively by HCC cells, with a subsequent escape of the nucleic acid cargos from lysosomal degradation to exert their intracellular action. The figure is reprinted from Younis MA, Khalil IA, Harashima H. Gene Therapy for Hepatocellular Carcinoma: highlighting the Journey from Theory to Clinical Applications. *Advanced Therapeutics*. 2020;3(11):2000087. © 2020 Wiley-VCH GmbH.⁹

Abbreviations: LSEC, liver sinusoidal endothelial cell; ASC, activated stellate cell; HCC, hepatocellular carcinoma cells.

investigated as non-viral gene-delivery vectors.^{179,185} Examples of the applicability of these systems in the gene therapy of HCC are described in our previously published article.⁹

Nanoparticles are able to recruit either one or a combination of two main mechanisms to accomplish the delivery of their cargos to HCC. The first mechanism exploits the anatomical abnormalities in the tumor vasculature to accumulate the injected nanoparticles into the tumor region, which is referred to as passive targeting. This is usually achieved by the design of long-circulating nanoparticles that can be shielded from RES and can accumulate in tumors over a prolonged period of time. Coating with polyethylene glycol (PEG), simply known as PEGylation, has been widely adopted to achieve this goal. However, PEGylation is also associated with reduced gene delivery efficiency that is a result of impairments in cellular uptake and endosomal escape capabilities, which is described as the “PEG dilemma”.¹⁵⁵ The second mechanism is to equip the nanoparticles with targeting moieties (eg, ligands, antibodies, or chemical groups) that could be recognized by certain differentially expressed receptors on HCC cells; alternatively, nanoparticles can be equipped with the ability to respond to certain stimuli (eg, pH, redox status, or enzymes) in the tumor region to achieve selective delivery of payloads to HCC cells. The latter approach is referred to as active targeting.¹⁵⁶

Although the active targeting of nanoparticles has demonstrated high potential for use in vitro, the in vivo efficiency in HCC has usually been limited. Addressing this issue will require intense focus on the design and features of the nanocarrier itself rather than focusing only on the targeting ligand that can exert its full performance at the target cells only after overcoming the successive in vivo biological barriers that precede the cellular uptake process. As a case study, we modified LNPs with a HCC-targeting peptide, SP94, for the co-delivery of SOR and MK-siRNA to HCC cells. Although the SP94 peptide has demonstrated outstanding selectivity for HCC cells in vitro,⁴ the initially prepared LNPs failed to access HCC or induce gene silencing in vivo. Subsequently, we designed an integrative strategy that was focused on tweaking the composition and physico-chemical properties of LNPs to control their in vivo performance and tackle the successive challenges associated with gene delivery to HCC. The composition of the LNPs was carefully optimized to improve their pharmacokinetic profile. Microfluidics technology was applied to prepare ultra-small LNPs (usLNPs) with an average particle diameter ~60 nm to maximize their ability to penetrate the stroma barrier in the tumor microenvironment and reach HCC cells. The combination of an ionizable lipid, YSK05, and a helper phospholipid, 1,2-Dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE), was formulated to improve the capability of the siRNA payload to escape from endosome following the cellular uptake process. Subsequently, the optimized LNPs succeeded in demonstrating potent gene silencing in an HCC tumor, with a median effective siRNA dose (ED₅₀) of 0.1 mg/Kg, the lowest reported thus far, post intravenous administration. Such powerful gene silencing enabled nearly full knockdown (~80%) of the MK gene, which synergized with SOR to eradicate the tumor at a low dose of 2.5 mg/Kg compared with the 10 mg/Kg in previous reports. Furthermore, the novel combination reversed the chemoresistance and enabled eradication of a SOR-resistant HCC model

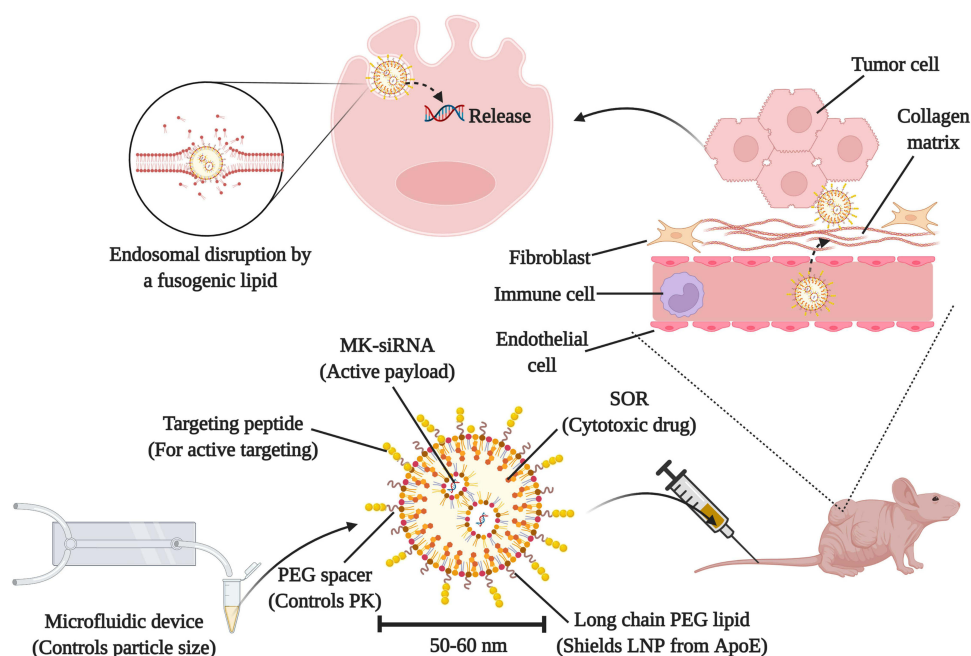


Figure 4 A graphical summary of a comprehensive strategy to achieve efficient drug and gene delivery to HCC using microfluidics and targeted lipid nanoparticles technologies. The figure is reprinted from *Adv. Drug Delivery Rev.*, Volume 188, Nakamura T, Sato Y, Yamada Y, et al. Extrahepatic targeting of lipid nanoparticles in vivo with intracellular targeting for future nanomedicines. 114417,¹⁵⁶ with permission from Elsevier (Copyright 2022, Elsevier).

Abbreviations: LNP, lipid nanoparticle; PK, pharmacokinetics; siRNA, small interfering RNA; SOR, sorafenib; PEG, polyethylene glycol; ApoE, apolipoprotein E.

in vivo.¹⁰⁷ This integrative strategy scheme is outlined in Figure 4.¹⁵⁶ In addition to classic active targeting, a recent approach emerged whereby the composition and properties of the nanocarrier were manipulated to allow recruitment of the endogenous transport mechanisms in order to achieve ligand-free targeting. This approach can be described as protein corona-based targeting, wherein the composition of the protein corona that is formed around nanoparticles in the blood, the key determinant of their in vivo fate, could be manipulated by tailoring the nanocarrier itself.^{54,55}

Concluding Remarks and Future Outlook

Gene therapy is rekindling a measure of hope in our ability to treat incurable life-terminating diseases such as HCC. Advances in molecular analyses have enabled the identification of multiple genetic pathways that play pivotal roles in the initiation and progression of HCC. These pathways are ideal targets for gene therapy. Nanoparticle-based delivery systems enable highly efficient and selective delivery of nucleic acid cargoes to HCC cells. An increasing number of published studies are describing basic research into HCC gene therapy. Moreover, a growing number of studies are currently undergoing various stages of clinical trials.⁹ Nevertheless, the clinical success rates of these therapies remain low. In addition to the technical challenges alluded to above, there are issues related to the clinical application of HCC gene therapies. First, the high incidences of inter-patient variability complicates the selection of genetic pathways to be targeted, which is a situation that brings the pursuit of personalized medicine to the forefront. Second, basic studies that rely on simple cell culture or animal models reflect a low level of clinical relevance because they do not address the actual features of human HCC. This could be overcome by recruiting representative animal models, such as patient-derived xenografts (PDX) or non-human primates.^{179,186} Third, the scale-up of most sophisticated delivery systems reported in basic research is not economically feasible, and this factor discourages industries from adopting these ideas in spite of the novelty and promising results. In this area, protein corona-based targeting offers a direction by which the scale-up process could be simplified and the production cost could be reduced. In addition, the adoption of scalable production technologies such as microfluidic mixing could facilitate an economical large-scale production of nucleic acid-loaded nanoparticles with high levels of uniformity.¹⁷⁹ Fourth, in most clinical trials that involve HCC patients, the clinical outcomes are judged using classic tools such as radiological examinations that do not precisely reflect the impact

of gene therapy. The implementation of biomarkers in the clinical assessment of outcomes would reflect a more accurate image of the benefits of gene therapy. We believe that the future holds great promise for this area of endeavor, and that personalized medicine and gene therapy will shape the future of medicine in this century.

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Disclosure

The authors report no conflicts of interest in this work.

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