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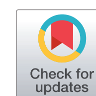
Review

Research progress and application of the CRISPR/Cas9 gene-editing technology based on hepatocellular carcinoma ☆



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ABSTRACT

Hepatocellular carcinoma (HCC) is now a common cause of cancer death, with no obvious change in patient survival over the past few years. Although the traditional therapeutic modalities for HCC patients mainly involved in surgery, chemotherapy, and radiotherapy, which have achieved admirable achievements, challenges are still existed, such as drug resistance and toxicity. The emerging gene therapy of clustered regularly interspaced short palindromic repeat/CRISPR-associated nuclease 9-based (CRISPR/Cas9), as an alternative to traditional treatment methods, has attracted considerable attention for eradicating resistant malignant tumors and regulating multiple crucial events of target gene-editing. Recently, advances in CRISPR/Cas9-based anti-drugs are presented at the intersection of science, such as chemistry, materials science, tumor biology, and genetics. In this review, the principle as well as statues of CRISPR/Cas9 technique were introduced first to show its feasibility. Additionally, the emphasis was placed on the applications of CRISPR/Cas9 technology in therapeutic HCC. Further, a broad overview of non-viral delivery systems for the CRISPR/Cas9-based anti-drugs in HCC treatment was summarized to delineate their design, action mechanisms, and anticancer applications. Finally, the limitations and prospects of current studies were also discussed, and we hope to provide comprehensively theoretical basis for the designing of anti-drugs.

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1. Introduction

One of the most common malignant tumors is liver cancer, which has a slow onset, a poor prognosis, and a high mortality rate. As of 2020, approximately 830,000 patients had died from liver cancer, with 900,000 new cases [1]. HCC, accounting for 75% to 85% of whole primary liver cancer, is the main kind of primary liver cancer [1]. At present, the preferred treatment modalities for HCC patients involves in surgery, radiotherapy, chemotherapy, interventional therapy, and radiofrequency ablation [2–4]. Due to the aggressive growth of HCC, advanced symptoms, high recurrence and metastasis [5,6], the majority of HCC patients are in the late stage of cancer at the time of diagnosis. Patients may be hardly withstood the trauma of surgical treatment. In summary, there is an urgent need to improve both the prognostic and predictive treatment of HCC.

Recently, researchers, combination of gene-editing for CRISPR/Cas9 method and molecular tools, have made remarkable achievements in the gene therapeutic field, such as more potential oncogenes and suppressor genes being found in cancer cases [7–10]. At the same time, quite similarities between animal models and humans in terms of physiology and molecules were generated via CRISPR/Cas9 technique, which could more accurately reflect the growth of tumors at various stages [11]. Currently, substantial results have also been achieved in different genetic experiments of HCC *in vivo* and *in vitro* [12]. The CRISPR/Cas9 technique is expected to break down the barriers of existing HCC therapy methods, provides more survival opportunities for cured HCC patients, and also gives theoretical foundation for the development of diverse cancer treatment methods including HCC.

CRISPR/Cas9 system is a self-protective mechanism in prokaryotes as well as an adaptive immune system used by bacteria to resist invasion of exogenous genetic material [13,14]. This gene technology has the advantages of simplicity, high efficiency, low design cost, and targeted editing of any gene, which has gradually replaced the zinc finger nuclease (ZFN) and the transcription activator-like effector nuclease (TALEN) gene-editing technology [14,15]. Currently, the CRISPR/Cas9 technology, which serves as universally applicable tool for gene modification, has captured extensive attention, and the first clinical trial of cancer treatment via this technology was conducted in 2016 at the West China Hospital of Sichuan University [16].

In view of the considerable potential of CRISPR/Cas9 in HCC treatment and the rapid development of CRISPR/Cas9-based anti-drugs in late years, the latest work and track the progress in this field were summarized as necessary. In this review, we established a CRISPR/Cas9-centered framework HCC-based treatment. The application cases and methods of the CRISPR/Cas9 technique in HCC treatment were overviewed, involving in the screening genes of drug resistance, generating tumor models, combination with other therapies, identifying biomarkers, and non-viral delivery strategies for HCC treatment. Furthermore, the directive role of CRISPR/Cas9 technique in current HCC therapy was also discussed. The effectiveness of the CRISPR/Cas9 technique for HCC treatment was demonstrated in detail, and it was expected to provide a wealth of theoretical foundations for preclinical research, HCC treatment, and the translation of research results into clinical application. This knowledge, we believe, can be used to guide and evaluate the development of additional drugs CRISPR/Cas9-based gene therapy.

2. Current status and principle of the CRISPR/Cas9 gene-editing technology

2.1. *Current status of the CRISPR/Cas9 gene-editing technology*

The CRISPR sequence was first discovered by researchers in 1987, but it received little attention at that time [13]. It took more than a decade for the sequence to be found in prokaryotes, confirming its existence [17]. Following a series of studies, the CRISPR/Cas9 technique was successfully kicked off in the domain of genetic research [18–24]. Nowadays, CRISPR/Cas9 technology has made prominent breakthroughs in the medical scope. For instance, this technology has been utilized to regulate a variety of crucial events, such as regulating T cell function, repairing related gene mutations, and a multitude of other critical occurrences [25]. Moreover, for the first time in clinical trial, the programmed cell death protein-1 (PD-1) was knocked out, and the safety assessment of the therapy for metastatic non-small cell lung cancer was conducted to demonstrate feasibility [16]. In addition, neutralizing PD-1 antibodies for lung cancer treatment had also achieved desirable results [26], indicating that targeting PD-1 using the CRISPR/Cas9 technology was potential. Concurrently, the PD-1 locus was knocked out using the CRISPR/Cas9 technique for the treatment of prostate cancer, kidney cancer, bladder cancer, and esophageal cancer, as well as the application for related experiments were completed [27]. Beyond that, the evidence indicated that the hypoxia inducible factor-1α (HIF-1α) may be connected to tumorigenesis in HCC, which the knockout of HIF-1α via CRISPR/Cas9 technique optimized the therapeutic effect of transarterial embolization in cell lines and prolonged the survival of HCC-bearing mice [28]. Chen et al. exploited CRISPR/Cas9 technology to knockdown the Kelch-like ECH-associated protein 1 (KEAP1) gene to determine whether the cross-talk between fibroblast growth factor 21 (FGF21) and NRF2 pathways was causing sorafenib resistance in HCC patients [29]. In addition to the above mentioned, its enormous potential for treatment of most human cancers was demonstrated by the fact that researchers used this technology to target the editing of genes associated with breast cancer [30], melanoma cancer [31], colorectal cancer [32], pancreatic cancer [33], cardiovascular disease [34], inflammatory bowel disease [35], and so on.

2.2. *Principle of the CRISPR/Cas9 gene-editing technology*

The external DNA or viral can be allowed to edit via CRISPR technology in three steps: (1) obtaining protospacers, (2) generating tracrRNA-crRNA complexes, and (3) interfering with exogenous genes (Fig. 1). The acquisition of protospacers initiates the adaptive phase. When exogenous virus or

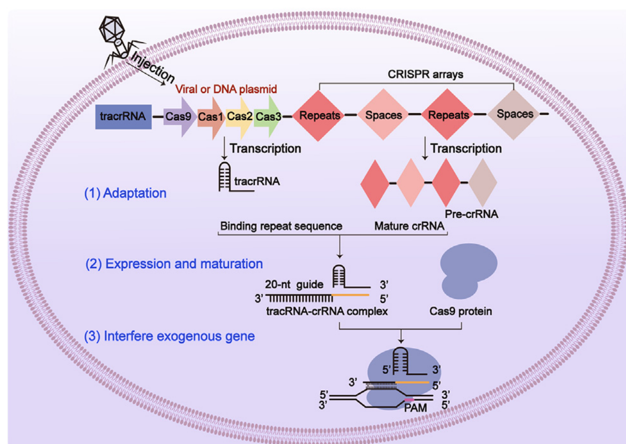

Fig. 1 – The structural schematic illustration of the three steps of CRISPR/Cas9 gene-editing.

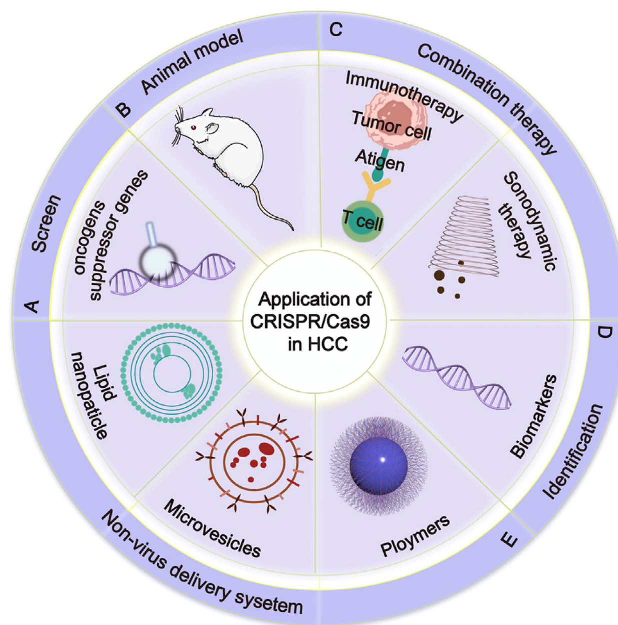

Fig. 2 – Overview of CRISPR/Cas9 gene-editing technology that participate in HCC treatment: (A) Using CRISPR/Cas9 screening oncogenes and suppressor genes for revealing mechanism of tumorigenesis, metastasis, and drug resistance. (B) Generating animal model with CRISPR/Cas9 gene-editing tools to study tumor pathogenesis. (C) The efficacy of immunotherapy or sonodynamic was improved via CRISPR/Cas9 technique to knock out relative genes of HCC. (D) The biomarkers were identified via CRISPR/Cas9 technique to predict and evaluate the process of treatment and overall outcomes in patients. (E) CRISPR/Cas9 non-viral delivery system was used to enhance HCC targeted therapy.

plasmid DNA is injected into the host [18,19], it is processed from invading DNA to form protospacers. The selection of the protospacers is based on the adjacent motifs (PAM) of the protospacer [36]. The protospacer is then integrated into a sequence that carries the invading plasmid or virus, which is a new spacer with specific memory. Finally, this new spacer is incorporated into the CRISPR arrays [37,38]. This tracrRNA-crRNA complex is formed in the second phase of expression by combining the pre-crRNA generated by the CRISPR array after transcription [39] with the repetitive sequence of the transcribed trans-activating CRISPR RNA (tracrRNA) [40]. In the ultimate phases of interference, the nuclease is activated by tracrRNA-crRNA complex binding to Cas9 protein [14,41]. when exogenous genes invade, sgRNA is used as a guide to specifically target PAM [42]. When PAM is recognized, Cas9 will cut at 3 nt upstream of PAM on the double-stranded DNA [43], which is a particular strategy that may protect the body from exogenous genome invasion and thus improving gene-editing targeting [12].

3. Application of CRISPR/Cas9 in HCC therapy

Recently, the CRISPR/Cas9 technique, with the merits of the short experimental periods and editing any gene, has been abundantly used in the field of HCC treatment, such as screening oncogenes and suppressor genes [44–46], generating HCC animal models [11,12,47,48], identifying HCC biomarkers [49–51], and so on. In particular, the combination of CRISPR/Cas9 technology with other therapies to regulate multiple crucial events and its role in combating oncogenes and suppressor genes offers superb promise [52–55]. Herein, the applications of CRISPR/Cas9 technology in HCC are summarized below (Fig. 2). Furthermore, the lack of effective delivery for CRISPR/Cas9-based anti-agents are the main obstacle for its popularization towards clinical application. As a result, the delivery system of CRISPR/Cas9-based anti-drugs in HCC treatment is also discussed. Detailed information will be presented in the following sections.

3.1. *Screening genes in HCC by the usage of the CRISPR/Cas9 technology*

The gene library screening technique is extensive utilized in the fields of biology and medicine, which considerably advances our understanding of the mechanism of HCC, drug resistance, and combination drugs. The CRISPR/Cas9 method of repairing double-strand breaks (DSB) by deleting, replacing, or adding gene sequences is illustrated, as is the molecular mechanism of its-mediated genome editing (Fig. 3). The CRISPR/Cas9 knockout library recognizes the target gene of the sgRNA and forms a complex with the endonuclease activity Cas9 protein. In the possession of guiding Cas9 protein to the target DNA sequence, if there is a PAM sequence downstream of the DNA double-strand, Cas9 protein will cleave the target DNA 3 nt upstream of the PAM to form a DSB [14,41,43,56]. Next, the cell begins repairing the DSB in two different ways: the DSB will be restored by the Non-Homologous End Joining (NHEJ) repair pathway in the absence of a homologous template and this process will entail base insertions and deletions. In addition to the foregoing, when there exists a homologous repair template, the Homology Direct Repair (HRD) pathway inserts the repair template into the broken position to accurately repair the DNA damage.

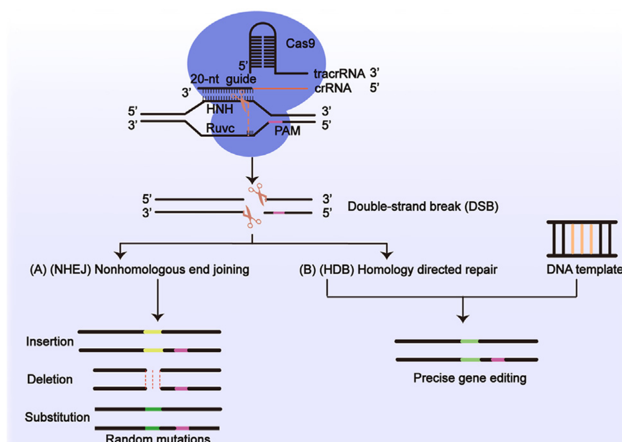

Fig. 3 – The schematic diagram of the molecular structure of CRISPR/Cas9 gene-editing. The Cas9 protein cleaves at 3 nt upstream of the PAM site, forming a DSB duplex and initiating DSB repair by HDR or NHEJ. When DSB repair is carried out by the NHEJ method, there are often three forms insertion, deletion, and replacement of genes. In addition, when DSB repair is performed by the HDR method, it requires DNA template assistance to repair DSB.

The CRISPR/Cas9 knockout library is extensively used in HCC research, and this method is mainly used to knock out cancer-related genes. Song et al. disscovered that the mitogen-activated protein kinase (MAPK) signaling pathway regulators could inhibit liver tumors by using CRISPR/Cas9 knockout library screening [57]. Owing to the traf3 gene in HepG2 cells could be knocked out via this technique, as well as the ability of proliferation, migration, and invasion of HepG2 cells could be heightened. In consequence, the function and mechanism of the Traf3 gene could be investigated in depth via this technique, which was a valid molecular tool [58]. This technology was applied to knock out the Bclaf1 gene (Bcl-2-related transcription factor 1) by Zhang et al. to verify that ginsenoside compounds could down-regulate the Bclaf1 expression for inhibition the HIF-1α-mediated glycolysis pathway under hypoxic conditions. Therefore, the genetic modification of Bel-7404 as well as Huh7 cells via CRISPR/Cas9 technique could inhibit the ability of their proliferation [59]. Different phenotypic changes caused by HCC cell-specific gene deletion in CRISPR/Cas9 knockout libraries are summarized (Table 1). Hence, the CRISPR/Cas9 technology could be proved that be an extremely powerful gene-editing tool for exploring the functions of specific genes in proliferation, metastasis, invasion, and phenotypic changes in HCC. The CRISPR/Cas9 technique plays specific biological functions in the humans by directionally targeting gene-editing and identifying potential therapeutic targets in order to provide satisfactory therapeutic strategies for HCC treatment.

3.1.1. *Identification of synthetic lethal interaction using CRISPR/Cas9 technology*

Although, there are hosts of treatment methods for HCC, these alternatives are not ideal for individuals with advanced HCC, and even the overall therapeutic effect for HCC patients is still at a lower level [74]. Single therapy of sorafenib and lenvatinib can only prolong the average survival time of patients by 3 to 6 months [75], and there is a serious drug resistance problem [76]. Accordingly, more and more research has begun to focus on the exploration of combination therapy to provide a better therapeutic effect for HCC. Using the CRISPR gene library to screen for vulnerabilities in HCC treatment by Qing et al. and determined that combined inhibition of CDC7 and mTOR could alleviate cell-autonomous or non-autonomous recurrence of liver tumors recurrence, in 2019 [77]. Qing et al. screened synthetic lethal genes again via CRISPR/Cas9 technique and discovered that the combination of drugs could block the EGFR-PAKI-ERK5 signaling axis activated by the fibroblast growth factor receptor (FGFR) inhibitors through the epidermal growth factor receptor (EGFR) inhibitors for HCC treatment. Therefore, the HCC therapeutic efficacy was improved under the synergistic action of the two inhibitors. The clinical therapeutic effect of HCC patients made this study move from "workbench to clinical", and it also confirmed that the screening of the CRISPR/Cas9 synthetic lethal genes was of great significance for clinical treatment combined with medication [74,78]. Additionally, Feng et al. discovered that DOCK1 was a synthetic lethal target of metformin in HCC, and that metformin could promote DOCK1 phosphorylation, which activated RAC1 to facilitate cell survival [79]. According to the preceding studies mentioned above, the identification of HCC synthetic lethal targets by CRISPR/Cas9 technology provides a novel treatment option for HCC patients with drug resistance.

3.1.2. *Screening of HCC drug resistance genes using the CRISPR/Cas9 gene library*

Currently, there are two conventionally used methods for screening drug resistance genes, namely, the strategies of interfering with gene expression are mainly divided into two types: functional deficiency screening and functional acquisition [80]. The high throughout loss-of-function genes were usually screened via RNAi [81,82]. Although RNAi library screening involves in the features of simplicity, high efficiency as well as high throughput, challenge is still existed, such as insufficient accuracy for drug resistance gene screening. Therefore, it is of great significance to find a new tool for tumor drug resistance gene screening. Lately, the behavioral traits such as survival, metastasis, drug resistance, and biomarker of cancer cells in various models were identified with the usage of CRISPR/Cas9 genome-wide screening [81,83]. CRISPR/Cas9 library, as a powerful gene screening tool, can identify genes associated with its biological activities, providing an accurate, efficient, and convenient gene-editing tool for tumors, as well as novel research methods for tumor drug resistance gene screening [81,84].

The tyrosine kinase inhibitor (TKI) sorafenib, as a treatment for patients with advanced HCC, was approved in the United States [85–87]. However, these drugs could only provide limited therapeutic efficacy due to cellular resistance [76]. Consequently, researchers sought to determine the cause and mechanism of resistance for HCC drugs. In 2018, Wei et al. found that the phosphoglycerate dehydrogenase (PHGDH) was a critical driving factor of sorafenib resistance by using

Table 1 – Recent research on genes knockout for HCC cells via CRISPR/Cas9 gene-editing technique.

Condition	Target	Cell lines	Effect	Mechanism	Ref.
low oxygen	PTPMT1	MHCC97L	tumor volume and tumor weights sensitive to AD↑	ROS accumulation	[60]
	ALDOA	SMMC-7721 HepG2	proliferation migration cell cycle arrest	Depletion of lactate	[61]
	Bclaf1	Bel-7404, Huh7	proliferation	Inhibition the expression of HIF-1α	[59]
	HIF-1α	SMMC-7721	invasion migration apoptosis↑	Inhibition the expression of HIF-1α	[62]
	CircIPO11	Huh7, PLC	proliferation	Activation of Hedgehog signaling	[63]
	Wnt pathway gene Nf1	Hep3B, HepG2, Huh7	suppressor gene	Increasing levels of MAPK phosphorylation	[57]
	Traf3	HepG2	migration↑ proliferation↑ invasion↑		[58]
	ADAMTSL3 PTEN	SMMC-7721	proliferation↑ metastasis↑		[8]
	Cxcr2	Hepa1–6	PD-L1 expression	Suppression of c-Myc	[64]
	AXL	SNU475	phenotypic changes	DNA damage	[65]
	NSD1	HepG2, SK-Hep1	proliferation migration invasion	Inhibiting the expression of Wnt10b	[10]
	LMNA	HepG2	proliferation cell cycle arrest apoptosis↑ migration colony formation transmigration↑	The expression of MMP2 and MMP9 decreased	[66]
	SOX4	Hep3B	endothelial cell migration and tube formation angiogenesis reticular fiber	Decreasing the expression of CXCL12	[67]
	NCOA5	LM3	migration tumor microsphere formation	Suppress the epithelial-mesenchymal transition	[68]
	NCAPG	HepG2 HCCLM3	cell growth migration mitochondrial gene expression prognostic factor	Inhibiting the division of the cell	[9]
	Nogo-B	SMMC-7721 HCC-LM3	tumor growth migration invasion	Regulation of the IL-6 signaling pathway	[69]
	MDM2	HepG2 Huh7 MHCCLM3	cell growth invasion the sensitivity of Sorafenib↑	MDM2 degradation	[70]
	METTL6	SNU-423 SNU-475	colony formation proliferation migration invasion adhesion	Specific tRNA modifications and alter the expression of CAMs	[71]
	MiR-23a-3p	MHCC97L	tumor growth	The transcription activity of miR-23a-3p	[72]

(continued on next page)

Table 1 (*continued*)

Condition	Target	Cell lines	Effect	Mechanism	Ref.
	LAPTM5	HCC-LM3 MHCC97-H MHCC97-L Hepa1–6 Huh7 HepG2	tumor growth	LAPTM5 could promote intrinsic macroautophagic/autophagic flux via facilitating autolysosome formation to drive lenvatinib resistance	[46]
	TUBB4B	HKCI2 HKCI10	apoptosis pathway cell cycle arrest↑ cellular senescence↑	The induction of intrinsic and extrinsic apoptosis pathways to inhibit the growth of cancer cell	[73]

↑ Up-regulation.

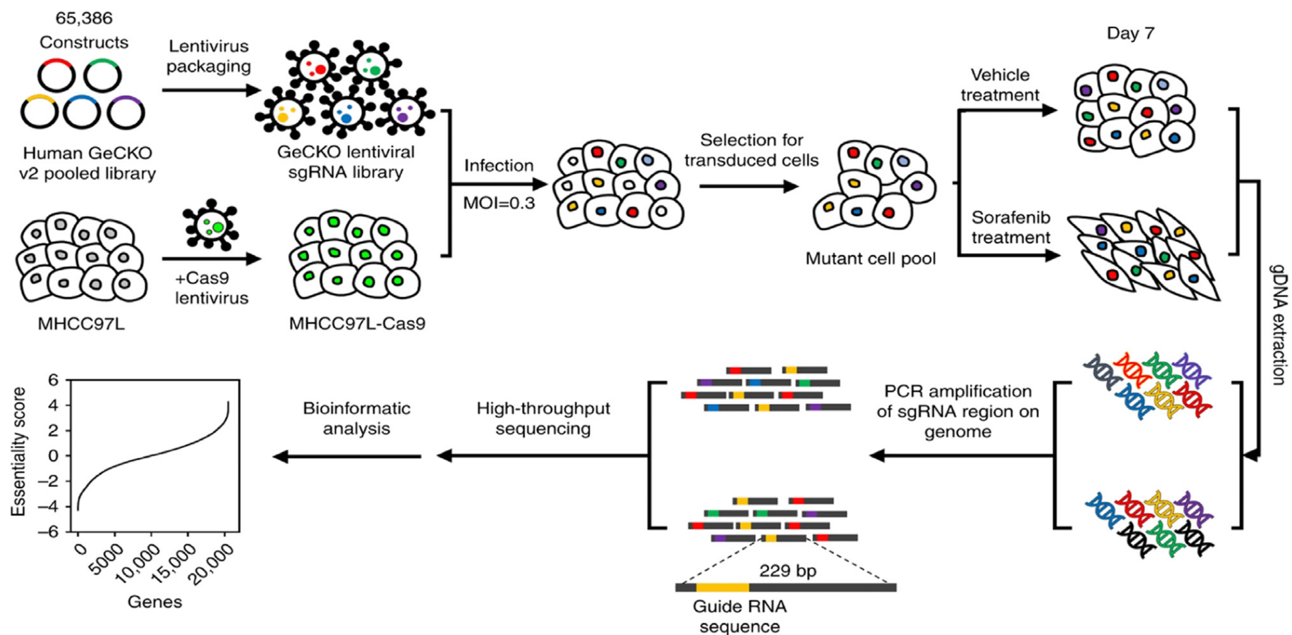

Fig. 4 – The workflow schematic of genome-wide CRISPR/Cas9 screening. Reprinted with permission from Ref. [88] Copyright 2019 Nature Communications.

the CRISPR/Cas9 knockout model and the administration with sorafenib led to the activation of the PHGDH in HCC patients (Fig. 4). Further, the level of the reactive oxygen species (ROS) could be increased as well as induced apoptosis. NCT-503, a PHGDH inhibitor, demonstrated a synergistic effect with sorafenib, which could suppress the HCC growth *in vivo*. (Fig. 5) Hence, targeting the PHGDH was an efficient way to reverse of multidrug resistance of HCC [88]. Cai et al. found that the LRP8 was a crucial factor in driving the acquisition of drug resistance in human Huh7 cells in 2020, and that the LRP8 increased resistance to sorafenib by activating β-catenin [89]. By the means of CRISPR screening library, Li et al. found that Metaxin 1 (MTA1) as a novel regulator of sorafenib resistance in HCC. Overexpression of the MTA1 could accelerate the rate of cell growth and reduce apoptosis posttreatment of sorafenib, and up-regulation of the MTA1 promoted the HCC resistance to sorafenib [90]. In addition, Sun et al. found that inhibiting the SGOL1 gene expression could reduce the activity of sorafenib after screening for the CRISPR knockout library [91]. Similarly, Crispra (CRISPR activation) was used to screen the second-line drug regorafenib for advanced HCC treatment to identify the genes associated with TKI resistance [92]. It showed that the hexokinase 1 (HK1), a key enzyme, was important for responsibility leading to the TKI resistance in Huh7 cells. Consequently, targeting HK1 may be a key factor in regulating the TKI resistance in HCC cells [93]. The above investigations have revealed that the CRISPR/Cas9 screening library could systematically analyze acquisition access and inhibitory mechanisms of drug resistance in cancer cells, which provides more and more theoretical guidance for clinical treatment.

3.2. CRISPR/Cas9 technology to generate animal models of hepatocarcinoma

The formation of tumors, as we all known, is the gathering of somatic mutations, but traditional cancer treatment

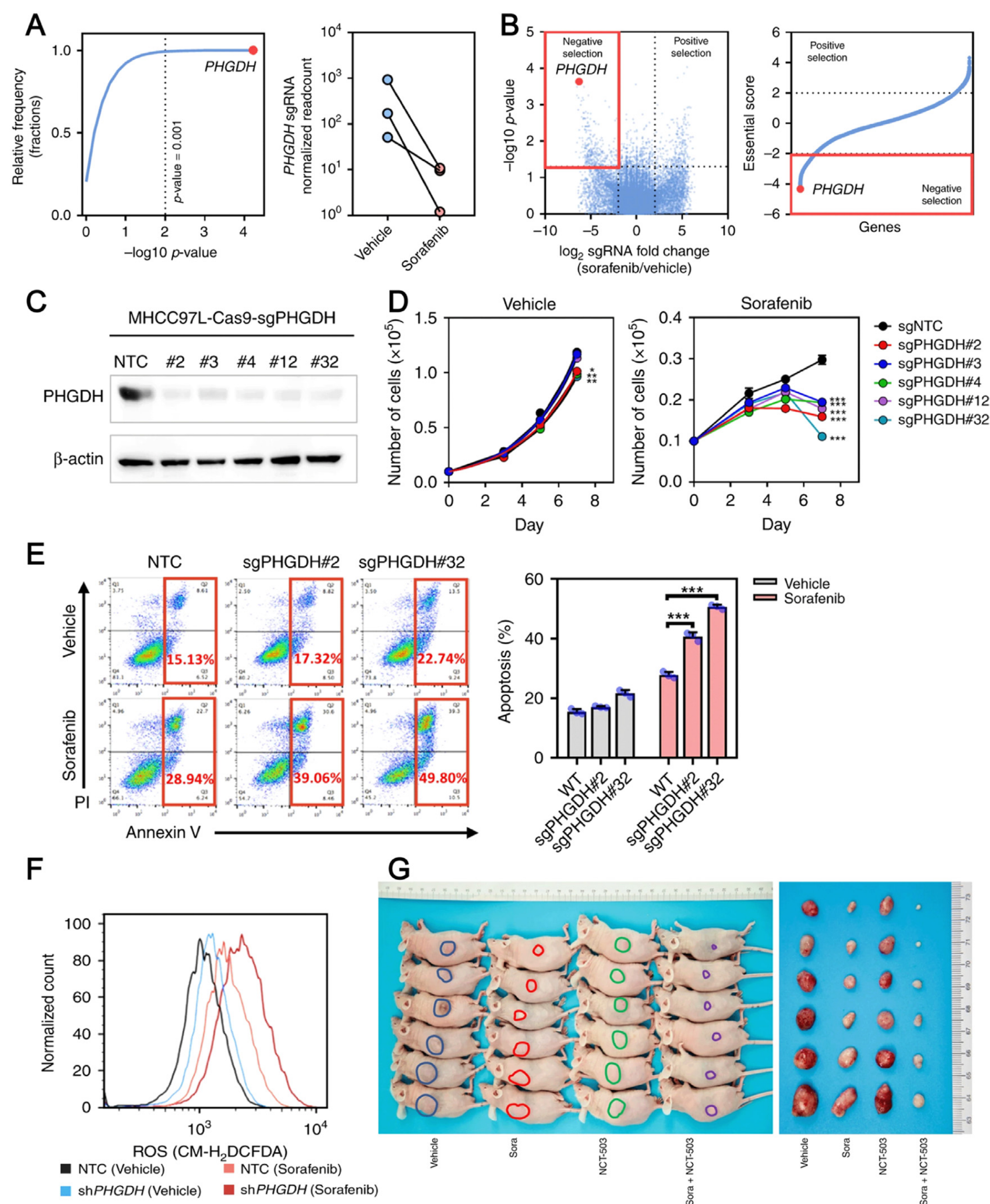

Fig. 5 – Screening of sorafenib resistance genes by CRISPR/Cas9 and revealing the role of this gene: (A) As shown in the red dot position, PHGDH was considered to be the most noteworthy gene via screening. sgRNAs targeting PHGDH could be persistently depleted in cells treated with sorafenib. (B) The volcano plots indicated that PHGDH was a crucial gene for HCC cells to survive after Sorafenib treatment. (C) WB analyzed the expression of PHGDH gene in MHCC97L cells. (D) Without sorafenib treatment, knocking out PHGDH had little effect on cell proliferation, and in cells treated with sorafenib, knocking out PHGDH significantly inhibited cell proliferation. (E) In cells treated with sorafenib, knockout of PHGDH induced significantly cell apoptosis. (F) Knockdown of PHGDH via CRISPR/Cas9 could augment sorafenib-induced ROS in HCC cells. (G) Combination of PHGDH inhibitor and sorafenib markedly inhibited tumor growth. Reprinted with permission from Ref. [88] Copyright 2019 Nature Communications.

has limited efficacy and a high recurrence rate [94]. As an alternative to traditional therapies, CRISPR/Cas9 gene-editing technology can generate indels, point mutations, knock-outs and knock-ins, and chromosomal rearrangements [12,47,48], to activate tumor suppressor genes and inactivate oncogenes [95]. Due to animal models being physiologically and molecularly similar to human biology, it best reflects the situation of tumor growth [94]. The CRISPR/Cas9 technique can be contribute to help researchers create specific gene-edited animal models [12,96] for HCC treatmennt by targeting gene knockout [97–100] or knock-in [101].

Xue et al. first reported a successful tumor model application of DNA plasmids of Cas9 and sgRNA via hydrodynamic injection into the liver in 2014 [102,103]. The CRISPR/Cas9 technique, compared with the Cre-Loxp-mediated model of deleted *Pten* and *p53*, was indicated feasibly to generate tumor models of suppressor genes and oncogene in efficient and convenient manner, which provided an innovative experimental scheme for HCC models and functional genomics [102]. Moreover, the rat CYP2E1 target was knocked out through CRISPR/Cas9 technique to obtain rodent models, providing a novel approach for studying the pharmacokinetics, toxicity, carcinogenicity, and core mechanisms in drug interactions of the CYP2E1 gene [97]. In addition, a dual adeno-associated virus (AAV) vector system model was built, which enabled CRISPR/Cas9-mediated gene therapy to be applicable to ornithine transcarbamylase deficiency for all patients, regardless of mutation or clinical state [96]. In a nutshell, these studies demonstrated that the CRISPR/Cas9 technique could be regarded as a method to achieve tumor models. On the other hand, it was also showed that this gene-editing technology could be helpful to design a more scientific cancer model *in vivo* that better reflects the complexity of human diseases.

The features of tumor cells can be stably maintained by the usage of the CRISPR/Cas9 technique to construct animal models and, preferably, imitate the characteristics of human cancer. This technology is conducive to further study of the tumor growth microenvironment as well as the mechanism of tumor occurrence and metastasis. As a result, the mechanism of gene mutation in cancer cells can be preferably explored, and further targeted drugs can be developed or screened on that basis.

3.3. *The effect of the CRISPR/Cas9 technology in combination therapy for HCC*

3.3.1. *Combined tumor immunotherapy to synergistically enhance the therapeutic effect of HCC*

At present, immunotherapy has shown magnificent application prospects, especially in cancer. This main process in this therapy is that patient-derived immune cells are genetically modified *in vitro* before being reinfused into the patient to mediate specific recognition and killing of the tumor cells. The CRISPR/Cas9 technique, which serves as emerging of an innovative tool for modifying genes, has successfully acquired increasing attention in tumor immunotherapy in past few years. For instance, the PD-1 target of T cells was first knocked out via the CRISPR/Cas9 for clinically therapeutic metastatic non-small cell carcinoma in 2016, as some certain therapeutic results were achieved in clinical research [16,27].

The chimeric antigen receptor T (CAR T) cells, expressed by CAR-expressing T cells, can specifically recognize and activate tumor cells as well as stimulate specific immune response of tumor cells to achieve tumor elimination immunotherapy [104]. PD-1 is involved in both activated and regulatory T cells, and in multiple tumor cells can be found its ligand, PD-L1 [105–107]. The destruction of endogenous PD-1 locus was demonstrated to propitious to improve the ability to kill tumor cells for CAR T therapy [108,109]. In a recent study, researchers edited PD-1 locus at the immune checkpoint to enhance the ability of kill cancer cells for CAR T therapy against HCC by the CRISPR/Cas9 technique [53,110], which further showed the feasibility of the CRISPR/Cas9 in tumor immunotherapy efficacy. This method could effectively alleviate the issue of inferior function of CAR T therapy caused through the expression of PD-L1 on tumor cells. Simultaneously, it demonstrated that targeting directional PD-1 locus of modification could augment the role of CAR T cells in HCC treatment. Aside from the above-mentioned finding that modifying the PD-1 site with CRISPR/Cas9 could reinforce the antitumor effect of CAR T treatment [53,107,110], Huang et al. found that the cytokine-induced killing (CIK) immunotherapy could also be used to treat HCC. Therefore, research knocked out the PD-1 locus with the CRISPR/Cas9 technique and combined it with the human telomerase reverse transcriptase (hTERT) to modify the CIK cells. The method not only prolonged the life span of the CIK cells but also increased their anti-hepatoma ability, and provided a novel avenue of immunotherapy for patients with HCC [55].

In addition, other immunobiological targets related to HCC treatment have been probed to improve the efficacy of immunotherapy. Zhou et al. knocked out the cell cycle-related kinase (CCRK) with usage CRISPR/Cas9 technology, which strengthened the blocking effect of the PD-L1 and inhibited the accumulation of the myeloid-derived suppressor cells (MDSC). Thereby, this means could reduce the MDSC-mediated immunosuppression and thus weaken the tumorigenicity of HCC [111]. Furthermore, Wang et al. explored studies indicating that the lysine-specific demethylase 1A (KDM1A) could promote PD-L1 expression in patients with HCC [112].

3.3.2. *Combined sonodynamic therapy to synergistically enhance the effect of HCC treatment*

Sonodynamic therapy (SDT) is an emerging non-invasive method of development based on ultrasound technology. In this method, low-intensity ultrasound is used to activate the sonosensitizers and induces them to produce cytotoxic singlet oxygen, which captures electrons from the tumor cells and destroys the organelles of the tumor cells, resulting in tumor cells death [113–115]. The self-defense system of the reactive oxygen species (ROS) in tumor cells can hinder the application of SDT in the clinical transformation of tumor therapy. This gene locus was targeted and edited via CRISPR/Cas9 technique, which boosted the SDT therapeutic effect and promoted SDT to clinical transformation by destroying of the oxidative stress and antioxidant defense system for tumor cells.

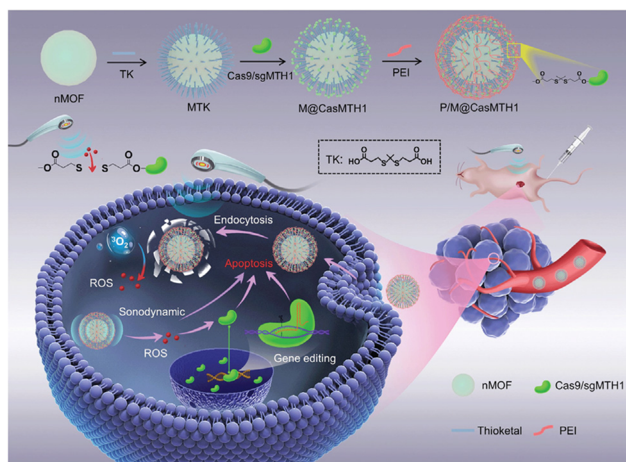

Fig. 6 – The schematic diagram of P/M@CasMTH1 NPs preparation: Sonosensitizers-triggered gene-editing-augmented SDT for tumor therapeutic effects. Reprinted with permission from ref. [54] Copyright 2021 Advanced materials.

Human MutT homolog 1 (MTH1) could hydrolyze and remove the oxidized nucleotides in cancer cells, rendering them unable to participate in the process of DNA synthesis, so that tumor cells could not be induced to kill by a high level of ROS, thereby affecting the therapeutic effect of SDT tumors [116–118]. For the first time, Pu et al. first applied CRISPR/Cas9 gene-editing technology in combination with SDT to treat tumors [54]. Ultrasound energy was first collected to generate a deal of singlet oxygen to induce SDT, which subsequently triggered cleavage of ROS-responsive thioether bonds, resulting in the controlled release of CRISPR/Cas9 components to target the knockout of MTH1 gene (Fig. 6). This method showed that the ROS-defensive system of tumor cells was destroyed, which could promote tumor cell apoptosis and inhibit tumor growth. Thus, this way could conspicuously augment the therapeutic efficacy of SDT [54] (Fig. 7). Moreover, it was found that the nuclear factor erythroid 2-related factor (NFE2L2), a transcription factor, had an anti-oxidative stress effect and could maintain the redox homeostasis of cancer cells. Meanwhile, it also hindered ROS to regulate the redox homeostasis in tumor cells and decreased the ability of ROS on killing tumor cells [119,120]. Based on this, the NFE2L2 target was knocked down by Yin et al. via CRISPR/Cas9 technique to disrupt the oxidative stress system as well as trigger apoptosis of HCC cell lines, thereby alleviating the adverse reactions of SDT, and enhancing its efficacy of SDT in HCC therapy [52]. These studies opened up a new path for solid tumor therapy and laid the foundation for the combination of SDT and gene therapy in the synergistic therapeutic HCC.

On the one hand, the CRISPR/Cas9 technique, when combined with SDT in the synergistic therapeutic cancer, circumvented the adverse reactions of traditional SDT. On the other hand, this technology realizes the targeted release of these system components in cells and accurate gene-editing. In a nutshell, the CRISPR/Cas9 technique could reduce the likelihood of tumor metastasis and recurrence while also providing a feasibility scheme for other cancer treatments.

3.4. *Identification of prognostic and predictive biomarkers using the CRISPR/Cas9 for HCC*

Recently, with the rapid development of research tools in the molecular field, a host of researchers have taken an intense interest in identifying and studying cancer biomarkers. Biomarkers are mainly divided into three types: predictive, prognostic, and pharmacodynamic. Pharmacodynamic biomarkers are generally applied to detect the effect of recent tumor medication. Prognostic and predictive biomarkers mainly refer to biological features, which can objectively detect and evaluate indicators of biological status or process, and further can be used to predict the natural course of cancer and assess the benefit level of patients for certain treatments. Recent studies have shown that, due to the peculiarity of precision for CRISPR/Cas9 technique, it can be used to anticipate and analyze cancer biomarkers associated with the process and overall outcomes in patients, as well as gives potential treatments for patients [49–51]. In the following, we will go over several biomarkers of predictive and prognostic identified by CRISPR/Cas9 technique.

The Shugoshin-like 1 (SGOL1) and human serine/threonine proteins phosphatase 2A (PP2A) protein co-regulate the phosphate group of the cohesion and prevent the sister chromatids at centromere from being separated before meiosis [121]. Overexpression of the SGOL1 was discovered to cause treatment resistance in non-small cell lung cancer to docetaxel and paclitaxel [122]. Sun et al. knocked the drug-resistant SGOL1 gene via CRISPR/Cas9 technology, which could significantly reduce sorafenib toxicity *in vivo* and prolong life cycle of patients. This study showed that the SGOL1 expression might be able to take for a prognostic indicator of overall survival rate and chemotherapy response in patients with HCC [91].

Wnt3a is one of the Wnt signaling pathway ligands, and the Wnt signaling pathway plays a crucial role in embryonic development, cell growth, differentiation, as well as tumorigenesis [123,124]. It has been found that blockage the Wnt3a signaling pathway in glioblastoma could reduce cell proliferation, migration, and drug resistance [125]. HCC patients with high expression of the Wnt3a shown poorer survival rate. The oncogene Wnt3a was silenced by Zheng et al. utilizing CRISPR/Cas9, which drastically suppressed cell proliferation and tumor growth. The study also found that higher serum Wnt3a levels in HCC patients could increase the sensitivity of serological alpha-fetoprotein detection. It demonstrated that the Wnt3a could be a novel therapeutic target for HCC patients. Meanwhile, as a predictive index, Wnt3a can be probable target for reversing drug-resistant in therapeutic HCC patients [126].

Cytochrome P450 (CYP450) is a monooxygenase that takes an important role in the early diagnosis as well as prognosis evaluation of HCC patients. The CYP1A2, CYP2W1, CYP3A5, and CYP17A1 are the metabolic enzymes of CYP450, which provide the basis for the prediction of biomarkers for HCC recurrence, prognostic indicators for HCC treatment, inhibitory genes regulating signaling

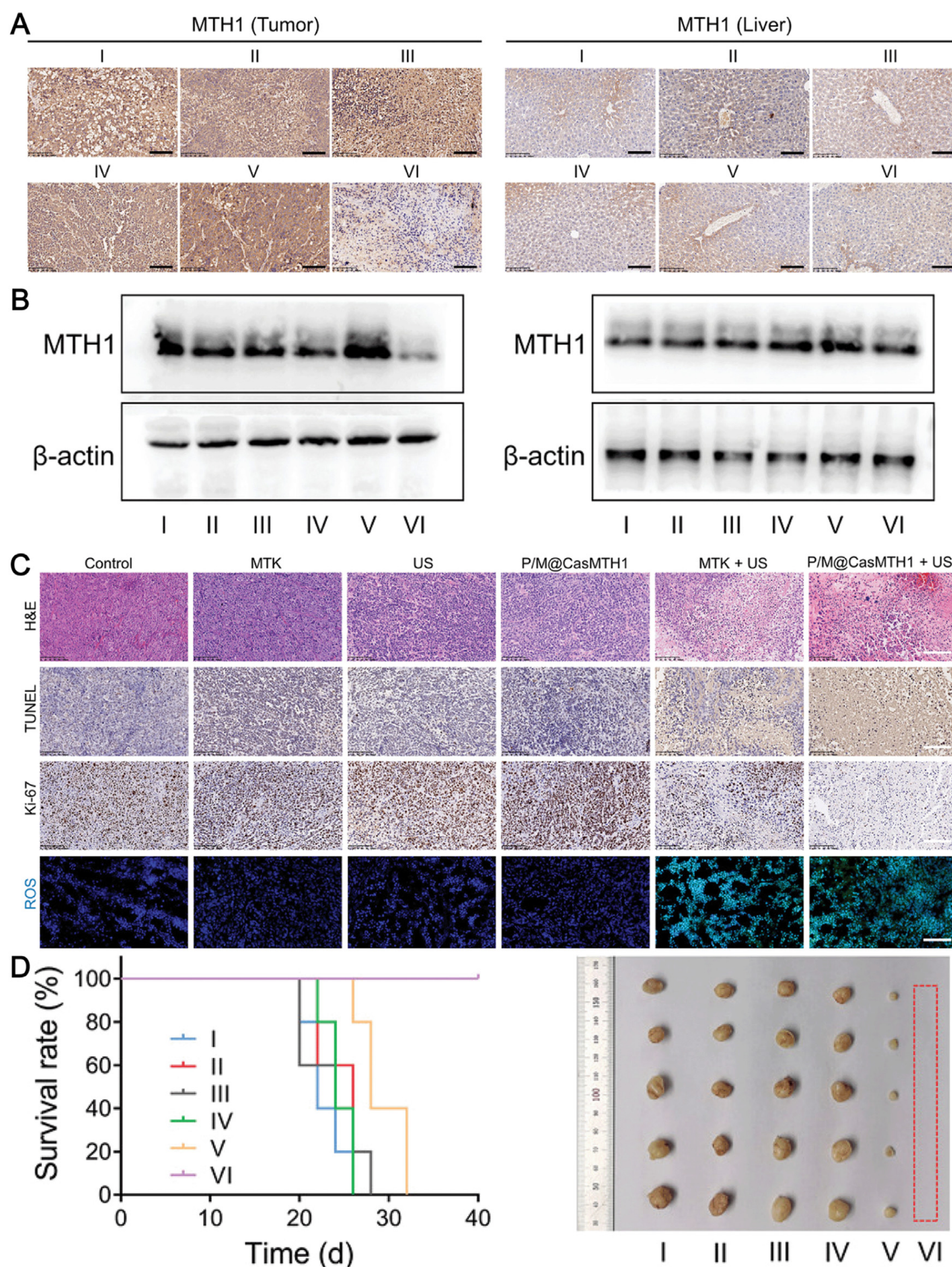

Fig. 7 – CRISPR/Cas9 technology combined with sonodynamic therapy to synergistically enhance therapeutic effect of HCC treatment and the potential mechanism was analyzed in detail: (A) IHC staining images of different groups of MTH1 protein. (B) WB analysis the protein expression of MTH1 in tumor and liver tissues. (C) H&E, TUNE and Ki-67 staining were used to verify the apoptosis and proliferation of tumor tissues. (D) Survival rate and tumor size of digital photographs. Reprinted with permission from Ref. [54] Copyright 2021 Advanced materials.

pathways, and molecular markers for HCC diagnosis [127–130]. Research showed that the CYP39A1 was linked to the clinicopathological features and prognosis of HCC patients. As a result, Li et al. used CRISPR/Cas9 to knock out the CYP39A1 gene, which could enhance the survival ability of HCC cell lines and accelerate their growth while simultaneously decreasing the overall survival rate of HCC patients. This study found that down-regulation of the CYP39A1 was related to carcinogenesis, tumor differentiation, as well as reduced overall survival rate of HCC, and proposed that CYP39A1 was an effective therapeutic target and a novel biomarker for HCC detection [131].

In addition, researchers also revealed by CRISPR/Cas9 tool that eukaryotic elongation factor 2, chromosome condensin I complex subunit G (NCAPG), zinc finger protein 384 (ZNF384), the enzyme phenylalanine hydroxylase (PAH), epigenetic-related genes (ERG), telomerase reverse transcriptase (TERT), and hexokinase 1 (HK1) could be applied to serve as predictive and prognostic biomarkers for HCC. Consequently, these biomarkers could provide a personalized and timely scheme for early diagnosis and prognosis therapy of HCC, as well as offered potential therapeutic targets for HCC patients and potential pathways for drug development [9,93,132–136].

3.5. *Delivery strategies on the CRISPR/Cas9 system for HCC*

The CRISPR/Cas9 technique provides a biological tool for biotechnology development that relies on the sgRNA and Cas9 protein co-editing DNA. However, the insufficiency of delivery system is also a major concern, restricting its clinical translation; therefore, suitable and accurate delivery systems for cancer treatment should be developed immediately. Although viral vectors have exhibited excellent effectiveness in delivering proteins, such as the plasmid-based CRISPR/Cas9 and RNP (Cas9 protein and sgRNA), the off-target effects as well as safety remain major problems in clinical treatment for viral vectors. In this regard, adenovirus had been successfully used to target and edit *Pten* genes in a mouse model of nonalcoholic steatohepatitis [137] due to its ability to carry substantial genetic cargoes and provide extra nuclear localization signals [138]. Although the fact that adenovirus-mediated CRISPR/Cas9 could effectively edit *Pten* gene and provide a novel delivery method for simulating human liver disease in mice, the introduction of adenovirus vector could induce inflammatory response *in vivo*, leading to the development of hepatomegaly after 4 months [137]. Additionally, even while adeno-associated virus could reduce the host immune response and improve the delivery effectiveness [139], the ongoing expression of Cas9 nuclease may result in severe off-target consequences [140]. As such, it is urgent to look for novel vectors for that can substitute traditional viral carriers for delivery of CRISPR/Cas9 components. In general, the perfect delivery system for CRISPR/Cas9-based anti-drugs should be meet the following conditions: (1) the vectors should be safe, such as avoid off-target effects, (2) the components of carriers should have the merit of biocompatibility, low toxicity and immunogenicity, (3) the vectors should protect the cargos from undesired leakage and be precisely released at target site. Non-virus delivery systems involve in liposomes, cationic polymers, inorganic nanoparticles, and microvesicles (MV) with excellent modifiable, biocompatible, as well as biodegradable properties that exhibit tremendous promise for accurately delivering and controlling release of CRISPR/Cas9 components. The commonly non-viral delivery vectors of CRISPR/Cas9-based anti-agents for HCC treatment are summarized in Table 2.

3.5.1. *Biomimetic nanotechnology*

The development of nanotechnology provides the possibility to construct a safe, efficient, accurate and controllable drug delivery system. Although, organic or inorganic synthetic nanocarriers have been extensively reported and used for the delivery of tumor therapeutic agents, partial carriers have following issues: easy rapidly clearance *in vivo* by the immune system, cumbersome preparation process, and poor safety *in vivo* [151]. In recent years, with the developing of biomedicine, nanodrug delivery system with immune cells-derived membranous layers based on biomimetic technology has brought novel hope for tumor targeted therapy due to its organic integration of the low immunogenicity, tumor targeting of natural biofilms, the controllability, and versatility of intelligent nanocarrier design [152]. Currently, the majority of delivery strategies for advanced anti-drugs are inclined to place emphasis on active targeting in order to achieve more efficient delivery drugs, genes components, and theranostics to the interestedly targeted sites, so that anti-drugs can reach the target cells as much as possible [151,153,154]. The emergence of biomimetic nanotechnology provides the possibility for the design of active targeting anti-drugs, so which is favored by numerous researchers. In a recent study, a vector of naturally safe tumor cell-derived microvesicles targeted was constructed by He et al. to deliver CRISPR/Cas9 components, which could be rapidly absorbed by recipient cancer cells, allowing CRISPR/Cas9 components to selectively accumulate in targeted tumor cells [141]. In the field of therapeutic cancer, biomimetic nanotechnology, such as the carrier surface being modified or camouflaged by cell membranes or sever as a vector, endowing nanomedicines with biological properties so that enable carriers to effectively deliver drugs, can provide a fantastic solution for the problems of immunogenicity and targeting of vectors or carrier-free.

3.5.2. *Cationic polymer nanoparticles*

Cationic polymer nanoparticles are easy to preferable consideration to be a potential effectively delivery platform for various types of gene components, such as plasmid DNA, RNA, and mRNA [155]. For instances, common cationic polymer vectors include polyethyleneimine (PEI), polyaminoamines (PAAs), polyaminoesters (PAEs), positively charged ethanolamine (EA), and natural biocompatible polymer materials, such as lactose-derived branched cationic biopolymer (LBP) [156]. Cationic polymers have been widely used as anti-drugs carriers due to their long circulation, high encapsulation efficiency, excellent biocompatibility, and ease of functionalization [157]. Moreover, the positive charged of cationic polymers can protect plasmids or proteins from degradation, accelerate endosomal escape, as well as promote

Table 2 – The non-viral delivery vectors for HCC treatment are introduced in detail below.

Carrier types	Delivery Vectors	Types CRISPR/Cas9	Target sites	Results	Ref.
Biomimetic nanotechnology	Cancer-derived microvesicles	plasmid-based CRISPR/Cas9	IQGAP1	MV efficiently delivered CRISPR/Cas9 components.	[141]
	Exosome	Cas9 protein and sgRNA	P53	Exosome efficiently delivered Cas9 protein and sgRNA into the nucleus and targeted the editing of p53 gene.	[142]
Cationic polymer	LBP	plasmid-based CRISPR/Cas9	SURVIVIN	Delivery of CRISPR/Cas9 components based on LBP can treat HCC.	[143]
	Ethanolamine-based cationic polymer	plasmid-based CRISPR/Cas9	SURVIVIN	The system can induce apoptosis and inhibit cell proliferation, migration, and invasion.	[144]
	Cationic polymer-coated gold nanorods	plasmid-based CRISPR/Cas9	*Fas*	The efficient transport of CRISPR/Cas9 plasmids into various cell types and the mediation of genomic disruption and transcription activation were made possible by the CRISPR/Cas9 delivery system.	[145]
Lipid nanoparticles	Ionizable cationic lipid	Cas9 mRNA and sgRNA, Cas9 protein and sgRNA	PCSK9	SORT allows nanoparticles to deliver the CRISPR/Cas9 system to specific organs.	[146]
	Permanent cationic nanoparticles	cas9 protein and sgRNA	PCSK9	Modified lipid nanoparticles to efficiently deliver cas9 protein and sgRNA.	[147]
	Ionizable phospholipids-based	cas9 mRNA and sgRNA	tdTomato protein	iPLNPs enable tissue-selective delivery of CRISPR/Cas9 components.	[148]
	Ionizable lipid-based	plasmid-based CRISPR/Cas9	PLK1	LNP can effectively deliver CRISPR/Cas9 components and provide a promising cancer treatment regimen base on editing of oncogenes.	[149]
Inorganic nanoparticles	Hollow silica nanoparticles based on polyamide aptamer	cas9 protein and sgRNA	EGFR-PI3K-AKt	Achieving efficient EGFR gene therapy and enhancing tumor sensitivity to chemotherapy.	[150]

cellular uptake of them, with regardless of sizes [158,159]. In a recent study, a charge-reversal delivery carrier was constructed by Nie et al., resulting in the delivery of plasmid-based CRISPR/Cas9 targeting to oncogene *survivin* for HCC treatment (Fig. 8) [144]. CRISPR/Cas9 targeted drug delivery system (Hep@PGEA/pCas9) was constructed by negative charged heparin core and positive charged cyclodextrin-PGEA shell. The plasmid was loaded via charge adsorption, and the GSH response enhanced the plasmid's release in tumor cells. The Hep@PGEA/pCas9 NPs established by cationic polymer as a vector could induce apoptosis, inhibit the proliferation, migration, and invasion for HCC cells, and exhibited a high anti-tumor efficiency (Fig. 9). Recently, such as redox, temperature, photothermal, PH, etc. that stimulate the carrier polymer can achieve responsive reactions, which have also been extensively developed [160] (Table 2). Importantly, the design of novel polymers has been used for imaging and drug development due to their ease of functionalization, providing an effective strategy for the design of anticancer drugs.

3.5.3. *Lipid nanoparticles*

Lipid nanoparticles (LNPs) are one of the most familiarly used deliver systems for multiple decades [161]. LNPs have the unique advantages of superior biocompatibility, non-toxicity, degradability, and low immunogenicity, which can improve the stability of encapsulated drugs and effectively control drug release. Therefore, LNPs are often carefully modified and structurally functionalized by researchers to achieve drugs targeted delivery and precise controlled

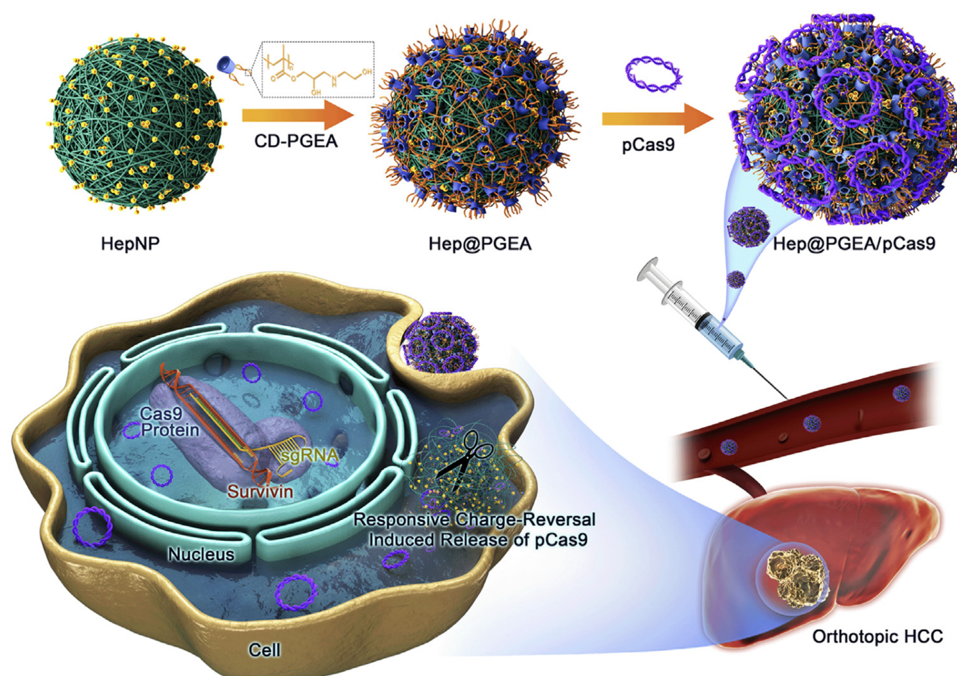

Fig. 8 – The schematic diagram illustration of Hep@PGEA targeting survivin gene for the treatment of HCC. Reprinted with permission from Ref. [144] Copyright 2021 Journal control release.

release. Furthermore, positively charged lipids combined with negatively charged nucleic acids, including proteins, plasmids, mRNA or RNP (sgRNA and Cas9) to form LNPs through electrostatic interactions, which could protect components from degradation as well as increase cellular uptake efficiency [162–164]. Early, siRNA, mRNA, and cre mRNA were usually delivered by LNPs, which were prevailingly constructed via natural phospholipids or their derivatives, cholesterol, cationic, ionizable lipids or polyethylene glycol lipids, involving in 1,2-di-O-octadecenyl-3-trimethylammonium propane (DOTMA), 1,2-dioleoyl-3-trimethylammonium propane (DOTAP) and dimethyldioctadecylammonium (DDAB) [165]. With the continuous development of gene therapy, delivery vectors for nucleic acids were also suitable for the delivery of CRISPR/Cas9 components. In this process, lipid nano-delivery systems were also evolving, as permanent cationic lipid nanoparticles are gradually replaced by ionizable cationic lipid nanoparticles with high transfection effectiveness and low toxicity, such as iPLNPs and iLP181 (Table 2) [148,149]. Recently, an effective selective organ targeting (SORT) LNP with adding a fifth lipid component to an established LNP formulation had been reported by Siegwart et al., and the delivery system could opsonize and capture the reticuloendothelial system or cause charge changes on the surface of the carrier to selectively accumulate different tissues depending on the additive component of the delivery system [146]. The discovery of a selective targeted delivery system revealed a highly selective effect on certain cells positions, allowing SORT LNPs to treat different target organs with highly precision, providing an innovative scheme for the design of anticancer drugs vectors based on gene therapy.

3.5.4. *Inorganic nanoparticles*

Hollow mesoporous silica nanospheres (HMSNs) are porous particles with pore channels that can be used to accommodate drug molecules as carriers for drug delivery [166]. It has some attractive advantages, such as excellent biocompatibility, adjustable particle size, thermostability, hollow cavities, larger surface area, and satisfactory water dispersion [167,168]. Since HMSNs are composed of a hollow network encapsulated by mesoporous shells and its interior is surrounded by some small mesoporous silica, they have a larger hollow inside that can load more therapeutic drugs, such as fluorescent molecules, chemotherapy drugs, DNA, proteins, and so on [169–171]. During transmembrane transport, HMSNs can protect the load drugs from degradation by various biological substances in cells. In addition, active targeting of HMSNs can be improved through targeting as well as surface functionalization, preventing excessive leakage of drugs from HMSNs. An effective delivery carrier based on the core-shell nanostructure of HMSN coated with PAMAM-Apt could control the release of components for HCC treatment by Zhang et al. (Fig. 10) [150]. The CRISPR/Cas9 targeted delivery system was constructed by non-covalently bonding adsorbing anti-EpCAM Apt and PAMAM conjugates onto the surface of HMSN nanocarrier, which was able to enable more drugs to reach the inside of the tumor and release the related components by the means of controlled manner in tumor microenvironment. In this nano-delivery system, chemotherapy drug and CRISPR/Cas9 gene-editing could synergistically inhibit the PI3K-AKT signaling pathway, and this delivery system could significantly improve the off-target problem under the action

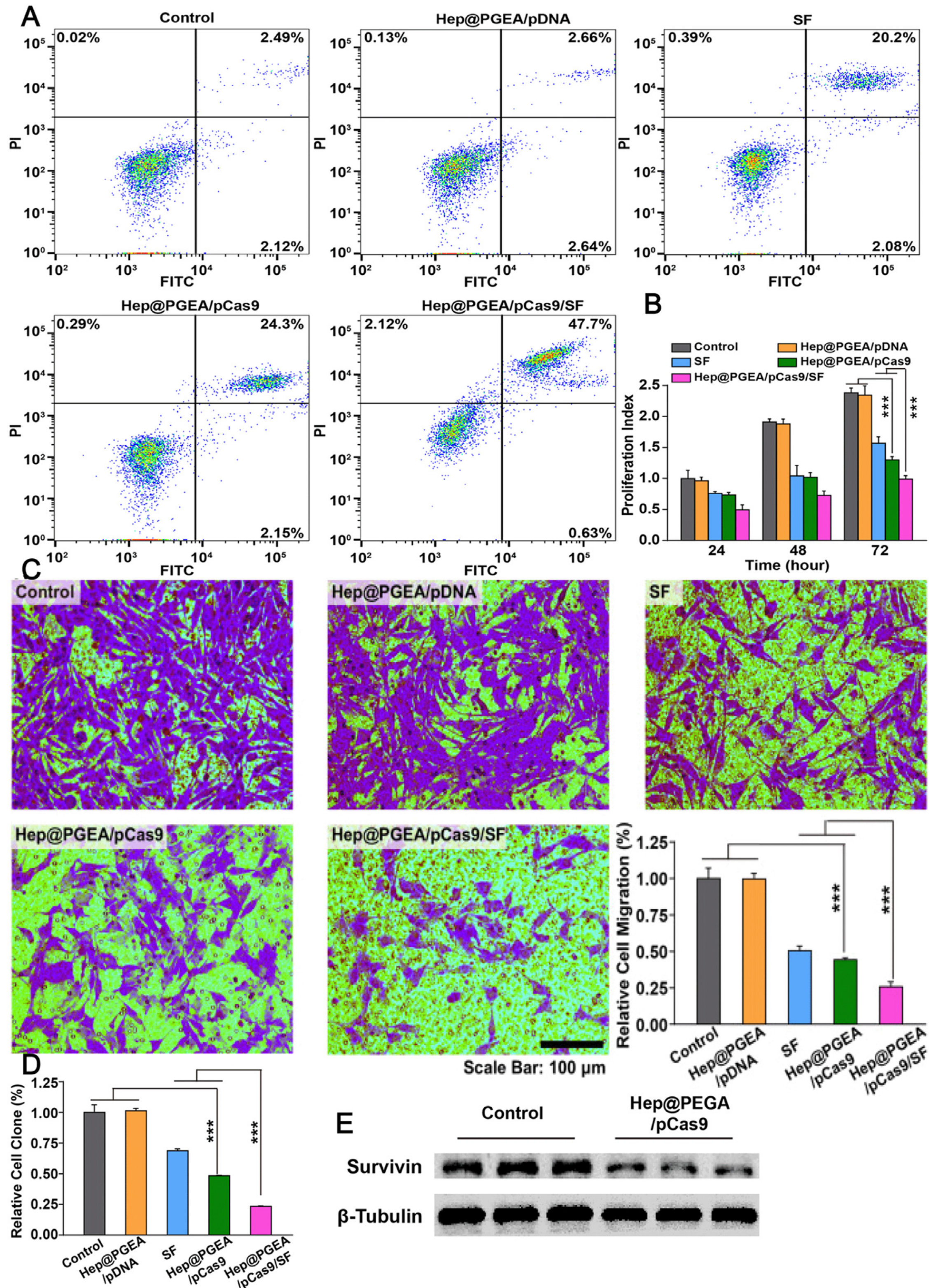

Fig. 9 – The CRISPR/Cas9 delivery system of targeted survivin gene for HCC treatment: (A) Inducing cell apoptosis after treatment with different groups of drugs. (B) Relative cell proliferation index after drug treatment. (C) Cell migration experiments after different groups of drugs treated cells. (D) Proliferation index of BEL7402 cells treated with different groups of drugs. (E) The expression of protein in BEL7402 cells was analyzed via WB. Reprinted with permission from Ref. [144] Copyright 2021 Journal control release.

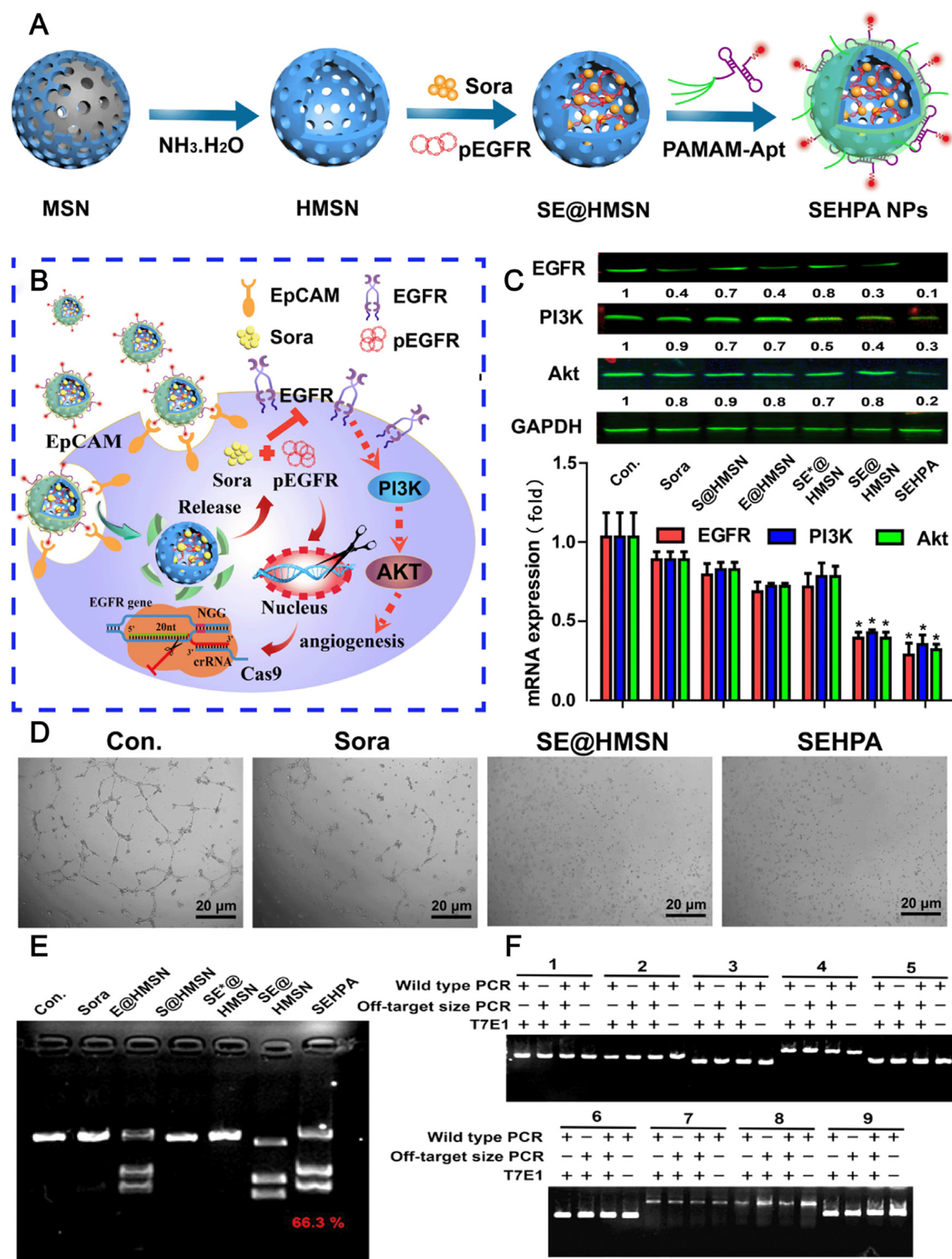

Fig. 10 – Targeted EGFR gene for HCC treatment via CRISPR/Cas9 delivery system: (A) The schematic diagram of SEHPA NPs preparation. (B) Schematic diagram of the synergistic inhibitory effect of the chemotherapeutic drug of sorafenib and the gene editing group of pEGFR on the EGFR-PI3K-Akt pathway in SEHPA NPs. (C) WB analysis the expression of protein and mRNA in HepG2 cells. (D) The anti-angiogenic activity of different drugs was studied by tube formation assay of HUVEC cells in Matrigel experiment. (E) T7E1 was used to assay the efficiency of genome editing EGFR. (F) Detection of off-target effect of SEHPA NPs in HepG2 cells. Reprinted with permission from Ref. [150] Copyright 2020 ACS Applied Materials & Interfaces.

of HMSN, with higher safety. This HMSN-based drug carrier for synergetically targeted gene-chemotherapy may have the enormous potential for future application due to its unique characteristics.

4. Conclusion and prospect

With the sharpening of our understanding of CRISPR/Cas9 technology as well as its relationship with other biological processes, cancer therapeutic outcome would be improved by combination therapy simultaneously [52,172]. Nanomaterial, an excellent platform, can be carefully designed and modified with various functionalized ligand for bioimaging and drug delivery. Although research on improving bioavailability methods based on CRISPR/Cas9 nanodrug delivery systems is currently insufficient, improving the targeted delivery of CRISPR/Cas9-based anti-drugs on the whole has been obtained admirable progress. Through being supported with nanomedical technology, CRISPR/Cas9 has overcame, to some extent, the undesirable properties of traditional small molecule drugs, including poor solubility and drug resistance. Among the nanodrugs construction strategy for CRISPR/Cas9 delivery system, the safety of nanocarrier is considered the most crucial factor for clinical application. In this aspect, delivery systems based on carrier-free nanodrug are expected to be another alternative for improving safety due to their higher drug loading capacity, favorable safety, and without any exotic organic or inorganic non-therapeutic carriers [173].

Although the current research on CRISPR/Cas9 technology is comparatively deep, there are still several critical challenges remain to be addressed in this field. For instance, some gene expression makers associated with signaling pathways may not be observed in all cell lines. How to find this marker, CRISPR/Cas9 screening technology may be helpful to solve this class of issues. In addition, it is critical to study the sensitivity of different tumor cells to CRISPR/Cas9-based anti-drugs. Whether a tumor cell is sensitive to CRISPR/Cas9-based anti-drugs is determined by numerous genes. Consequently, bioinformatics analysis can be used to forecast if a tumor cell is sensitive to anti-drugs and as a basis on this for the selection of experimental cells in order to increase the feasibility of experiment. Furthermore, the majority of CRISPR/Cas9-based anti-drugs were designed based on a single signaling pathway, and screening tumor oncogenes or suppressor genes using CRISPR/Cas9 technology and synergizing them with other therapeutic signaling pathways might be a novel approach to obtaining preferential therapeutic effects [150,172,173].

This review comprehensively summarized the development process of the CRISPR/Cas9 technique from its original inception to its specific application and analyzed its principle, application methods, as well as effect on cancer treatment. The biomarkers of HCC were quickly identified and thoroughly analyzed using the CRISPR/Cas9 technique, allowing the effectiveness of HCC treatment methods to be improved. This technology has a satisfactory targeting effect, it can enable quick and accurate to perform knockout function, as well as modification of cancer cells to provide precisely targeted gene-editing. When combined with other related HCC treatment methods, this technology could be used to inhibit tumor growth and improve overall survival in HCC patients. With the further developing of the CRISPR/Cas9 technique, it is wished that there will be more research results and clinical treatment cases in the future, which will not only open up novel cases for the research of CRISPR/Cas9 technique, but also can further promote this technology to better benefit mankind.

Declaration of Competing Interest

The authors declare that they have no conflict of interest.

Acknowledgments

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