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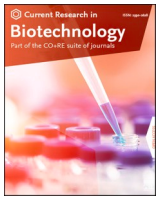

CRISPR/Cas9 genetic screens in hepatocellular carcinoma gene discovery

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ABSTRACT

The clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9) system is a powerful gene editing tool originated from prokaryotes. The modern CRISPR/Cas9 system allows high-throughput genetic screening to be carried out *in vitro* and *in vivo*. The high efficacy and flexibility of CRISPR/Cas9 system allows identification of hepatocellular carcinoma (HCC) related genes in the past decades. Numerous efforts have been applied in the past to improve this system, such as off-target improvement, gRNA efficiency enhancement, and modified Cas9 nuclease for performing visionary base editor screens and epigenetic screens. With these merits, the CRISPR/Cas9 offers tremendous opportunities in various biomedical research and clinical application. Recently, the use of the CRISPR/Cas9 system has also been combined with different technologies, including single-cell sequencing and machine learning, to further understand HCC pathogenesis and explore its utility in gene therapy. This review provides a summary of HCC carcinogenesis CRISPR/Cas9 screens conducted in recent years with different genetic contexts, epidemiological backgrounds, and progression of HCC. Furthermore, this review also provides insight in the CRISPR/Cas9 potentials, current obstacles, and improvement of this system for its future utility in cocktail therapies.

Introduction

Primary liver cancer is a life-threatening disease that is ranked as the fourth most fatal cancer globally (Llovet, 2021). It is defined as the pathogenesis of the hepatocytes originated from the liver, and more than 90% of the primary liver cancer cases identified globally are hepatocellular carcinoma (HCC) (Minciuna, 2022). The HCC incidence occurs across the globe, affecting most areas of Asia, Europe and Africa, as well as Northern and Central America (Sagnelli, 2020). Several risk factors have been shown to associate with HCC, including exposure to aflatoxin or mutagens, obesity condition, hepatitis B virus (HBV) or hepatitis C virus (HCV) infection, steatosis, cirrhosis, alcoholic fatty liver disease (AFLD), non-alcoholic fatty liver disease (NAFLD), nonalcoholic steatohepatitis (NASH) or other liver diseases (Yen, 2021; Suresh et al., 2020). Patients with the risk factors could lead to their hepatocyte genome become unstable resulting in mutations of vital proto-oncogenes or tumour suppressor genes and developing HCC.

Based on the mutational landscape, HCC can be classified into two main molecular subclasses known as the genetic mutational subclass and the epigenetic dysregulation subclass (Rebouissou and Nault, 2020). Numerous genes, including *CTNNB1*, *TP53*, *AXIN1*, *APC*, *PIK3CA*, *PTEN*, *VEGFR*, *KEAP1*, *RB1*, *CDKN2A*, *ZBTB20*, have been identified to contribute to HCC development via distinct molecular mechanisms (He and Tang, 2020; Yang, 2021; Scheiter, 2022; Xu, 2021; Akula, 2019; Shigeta, 2020; Tao, 2021; Chand, 2022; To, 2021). The complex interactions between genetic mutation and epigenetic alteration further generate the sophisticated background of HCC. Therefore, it is essential to utilize more powerful and prescient biological technologies to provide a comprehensive investigation of HCC and further advance the development of new and precision therapy.

In light of that, the clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9) system in cancer research has recently aroused extensive interest in the cancer molecular mechanism and precise therapy. CRISPR/Cas9 system is discovered by Professor Charpentier and Professor Doudna, who were awarded the Nobel prize Chemistry 2020 for their discovery (Westermann et al., 2021). Performing a genome-wide genetic screen using CRISPR/Cas9 system allows the identification of the gene(s) responsible for carcinogenesis (Kieckhaefer, 2019). Various systems have been utilized for genetic screens, such as RNA interference (RNAi) and transcription activator-like effector nucleases (TALENs). However, the CRISPR/Cas9 system provides higher flexibility and efficacy in gene editing for high throughput screens of different cancer types both *in vitro* and *in vivo* (Kieckhaefer, 2019; Zhuang, 2017; Chen, 2022; Song, 2017).

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The CRISPR/Cas9 offered promising results and novel discoveries through the CRISPR/Cas9-mediated *in vitro* high-throughput genome-wide screens in different types of cancers, including breast cancer, ovarian cancer, colon cancer, small cell lung cancer, acute myeloid leukaemia and more (Pettitt, 2018; Chen, 2021; Zhang, 2022; Yu, 2022; Grunblatt, 2020; Hou, 2017). In a breast cancer study, CRISPR/Cas9 screens identified that the inhibition of the *Histone H3 associated protein kinase* (*HASPIN)* leads to enhanced therapeutic efficacy of alisertib (Chen, 2021). For ovarian cancer, *Protein-L-isoaspartate (D-aspartate) O-methyltransferase* (*PCMT1*) was elucidated to promote carcinogenesis by activating FAK-Src signalling (Zhang, 2022). In addition, *Polo-like kinase-1* (*PLK1*) inhibition was confirmed to suppress the oncogenic RTK signalling pathway to prevent colon cancer progression (Yu, 2022).

The CRISPR/Cas9 screens in immune-competent mice strengthen our understanding of tumour microenvironment with immune response *in vivo*. The immune cells involved in the tumour microenvironment can either possess an anti-tumour or pro-tumour effect (Anderson and Simon, 2020). The *in vivo* screens utilizing CRISPR/Cas9 include, but are not limited to, glioblastoma, colorectal cancer, ovarian cancer, breast cancer, renal cell carcinoma and prostate cancer. Several novel genes have also been identified through the cancer research with CRISPR technique (Chow, 2017; Yau, 2017; Kodama, 2017; Dai, 2021; Ji, 2022; Zou, 2022; Dubrot, 2021; Rushworth, 2020). For instance, *NAD kinase* (*NADK*) or *Ketohexokinase* (*KHK*) suppression inhibits tumour growth and could be a novel target for the treatment of *KRAS Proto-Oncogene, GTPase* (*KRAS*) mutated colorectal cancer (Yau, 2017). The NADK involves in activating the pentose phosphate pathway, where this pathway plays an important role in cancer cell metabolism and survival (Yau, 2017; Patra and Hay, 2014). Moreover, the KHK involves in fructose metabolism, and the elevated fructose metabolite level can be observed when KHK is suppressed. This causes liver inflammation and leads to carcinogenesis (Cheng, 2022). Furthermore, the breast cancer CRISPR screen identified the immune escape-related gene where the *Galectin 2* (*LGALS2*) expression leads to immunosuppression (Ji, 2022). This discovery suggested that it can act as a target for potential immunotherapy.

The rapid development of CRISPR/Cas9 system has proven its important role in identifying cancer genes in experimental and clinical settings. Moreover, this system is capable of editing genome precisely which allows engineering of immune cells (T cell and B cell), hematopoietic stem cells and progenitor cells for precision therapy (Kim, 2021). With in-depth investigations and improvements, the CRISPR/Cas9 genetic screens for HCC-related genes had been performed which strengthens our understanding of HCC tumorigenesis. In this review, the recent application of CRISPR/Cas9 screens and genes associated with HCC development are summarized. In addition, the potential utility of CRISPR/Cas9 in HCC investigation and clinical applications is explored.

The CRISPR/Cas9 System

The CRISPR sequences were initially identified in prokaryotic organisms in 1987, but not until 2013 that the system was first applied to eukaryotes (Xu, 2020). The CRISPR acts as an adaptive immune response in the prokaryotes to demolish the genetic material of the pathogen (Wang et al., 2022). The CRISPR mechanism works by integrating the pathogen's DNA into the CRISPR array of the prokaryotes during the first infection. When the same infection occurs, the CRISPR RNA (crRNA) is reverse transcribed from the integrated sequence and combined with the trans-activating RNA (tracrRNA) to form a guide RNA (gRNA). The gRNA then cooperates with the Cas9 nuclease to induce a blunt-end double-strand break (DSB) to the target pathogen genome and thus, protecting the cell from the infection (Behler and Hess, 2020; Butiuc-Keul, 2022). The CRISPR/Cas9 system is a modified version of the original CRISPR immune defence response of the bacteria and archaea against pathogen infection (Wu, 2020).

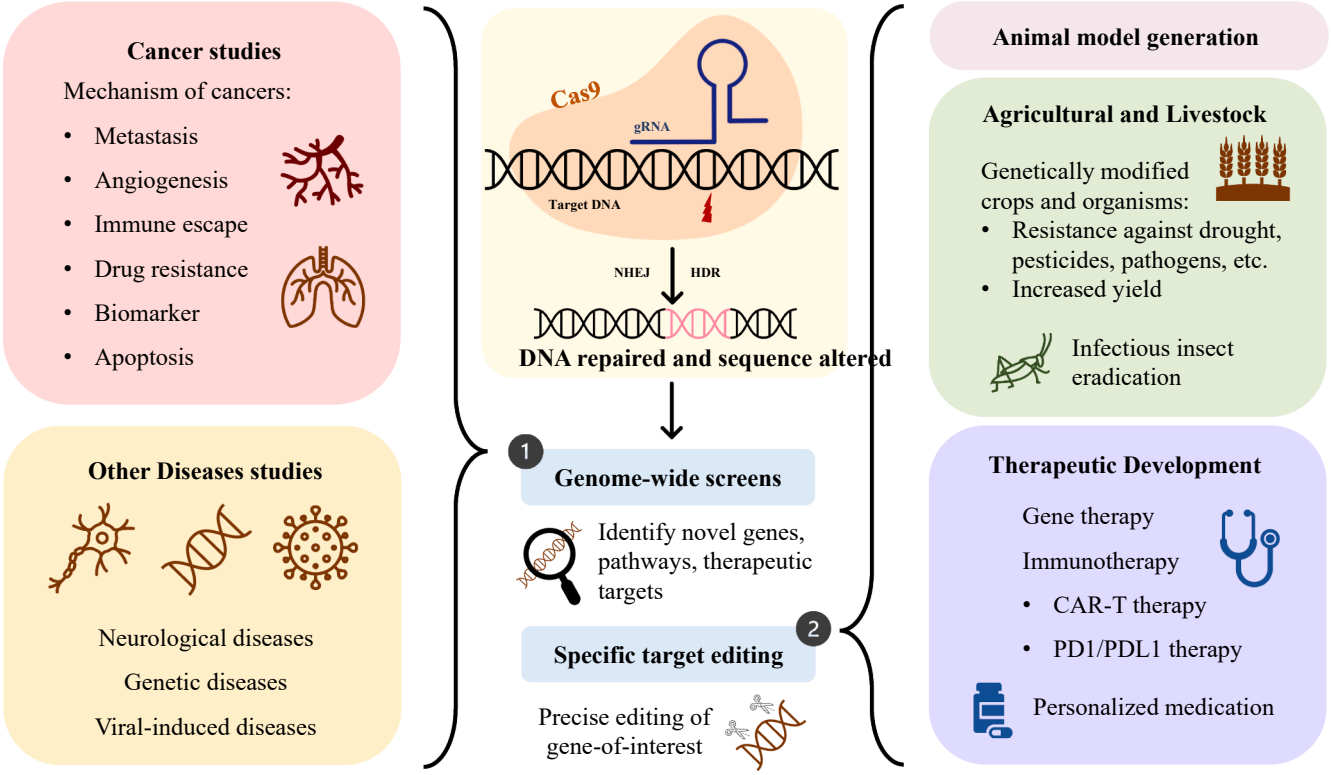

Figure 1. The modern CRISPR/Cas9 system and its applications. Upon binding of gRNA to its target DNA sequence, the Cas9 nuclease induces a DSB to the target. The cell repairs the damaged DNA by either NHEJ or HDR, and indels might be introduced to the repaired DNA. The CRISPR/Cas9 system can be utilized to perform genome-wide screens to reveal novel gene targets and mechanisms of different diseases. Its precise gene editing function also allows experimental animal model establishment, improves agricultural and livestock industries, and advances therapeutic developments.

The current CRISPR/Cas9 system works in a similar mechanism as the native immune CRISPR system in the microbes (Figure 1). Researchers design the gRNA sequences to target the gene-of-interest (GOI) in the target cell genome. The Cas9 nuclease expressed from the cells together with the gRNA induces a DSB to the target after the recognition of the protospacer adjacent motif (PAM) sequence of the GOI. After the induction of DSB, the genome is repaired by either non-homologous end joining (NHEJ) or homology directed repair (HDR) mechanisms (Wang, 2022). This would lead to base insertion/deletion (indels) of the DNA sequence, which results in gene mutation and gene loss-of-function (Wang, 2022). The rapid development of CRISPR/Cas9 mediated genome-wide screens and specific genome editing have expanded its application scope including mechanistic study of various cancers and other diseases while also promoting personalized CRISPR-based gene therapies (Figure 1). The CRISPR/Cas9 system can also be applied in agricultural and livestock industries to engineer genetically modified organisms with enhanced disease resistance, extreme weather tolerance, and improved product yields (Figure 1).

To perform a genetic screen for potential HCC-related genes discovery, gRNAs are firstly designed to target the GOI in the mammalian genome and are constructed into a library of vectors. Multiple CRISPR gRNA libraries have been developed in the past and the total number of gRNA in these libraries ranges from 17,032 to 188,509, with at least 2 gRNAs designed to target each gene (Table 1). Amongst the CRISPR gRNA libraries targeting the mouse genome, the Mouse CRISPR Knockout Pooled Library (Gouda) targets the highest number of genes and includes both positive and negative control gRNA sequences (Table 2). Moreover, it provides an optimized protocol for gRNA library amplification and PCR of gRNAs for next-generation sequencing (NGS). Therefore, this library is recommended for researchers who aim at performing *in vivo* screens with a higher number of genes. For human genome CRISPR gRNA libraries, the Human genome-wide library v1 targets the highest number of genes. However, the number of control gRNA is unspecified (Table 2). Alternatively, the Human CRISPR Knockout Pooled Library (Gattinara), targets slightly lesser genes than the Human genome-wide library v1 but the control gRNAs and protocols for library amplification and gRNA PCR are readily available (Table 2). Thus, this library serves as a valuable tool for general research purposes. Nevertheless, researchers should select the library based on their specific needs.

For CRISPR/Cas9-mediated gene targeting, different tools are available online for researchers to design their own gRNA sequences (Table 3). Most of these tools can be employed to design mouse and human gRNAs and provide the on- and off-target effect of the suggested gRNAs. These design platforms can be used together with gene databases, such as the Cancer dependency Map (DepMap) portal (https://depmap.org/portal/) to predict the knockout effect of the gene (Tsherniak, 2017). To facilitate user experience, a flexible input format of the GOI was utilized in these gRNA design platforms with some tools that enable designing gRNAs for multiple genes simultaneously (Table 4). After inputting the required data, the platform generates and suggests gRNAs in a table format or graphical illustration that reveals its targeted location in the genome (Table 5). Furthermore, these platforms may also assist in the gRNA selection process. For instance, the CRISPRon generates gRNA design with gRNA targeting domain information, while the Guides provides non-targeting gRNA sequences for easy experimental setup as well (Table 5). In general, CCTop and CHOPCHOP would be recommended. The CCTOP provides numerous choices of target genome and Cas nuclease as well as allows multiple gene query at the same time. The CHOPCHOP also adopts a convenient input strategy for users by allowing different target input methods. Both the table and graphical view of results can be downloaded from CHOPCHOP, and it can also generate SnapGene format of the gRNAs for users. Nevertheless, researchers may select the appropriate platform according to their experimental needs.

Table 1
Summary of the currently available CRISPR libraries.

Library	Developer	Total gRNAs	gRNA per gene	Species	Reference
Mouse CRISPR Knockout Pooled Library (Gouda)	Doench	44,155	2	Mouse	(DeWeirdt, 2020)
Broad GPP genome-wide Brie	Doench and Root	78,637	4	Mouse	(Doench, 2016)
Mouse CRISPR Knockout Library in lentiCRISPR v2-Blast	Liu	185,836	10	Mouse	(Gu, 2021)
Mouse Toronto KnockOut (mTKO) CRISPR Library	Moffat	94,528	Around 5	Mouse	(Lawson, 2020)
Two plasmid mouse activity-optimized genome-wide library	Sabatini and Lander	188,509	10	Mouse	(Wang, 2017)
Retroviral Mouse Genome-wide CRISPR Knockout Library	Teichmann	90,230	5	Mouse	(Henriksson, 2019)
Mouse Cherry Brie Pooled Library	Tolar	78,637	Around 4	Mouse	(Newman and Tolar, 2021)
Mouse improved genome-wide library v2	Yusa	90,230	5	Mouse	(Tzelepis, 2016)
Mouse GeCKO v2	Zhang	130,209	6	Mouse	(Sanjana et al., 2014)
Human CRISPR Knockout Pooled Library (Gattinara)	Doench	40,964	2	Human	(DeWeirdt, 2020)
Broad GPP genome-wide Brunello	Doench and Root	76,441	4	Human	(Doench, 2016)
Broad GPP Humagne Set C and Humagne Set D	Doench and Root	40,710	2	Human	(DeWeirdt, 2021)
Mini-human AsCpf1-based Human Genome-wide Knockout Library	Draetta	17,032	3 to 4	Human	(Liu, 2019)
MYC-CRISPR Library	Dzikiewicz-Krawczyk	46,354	1 to 7	Human	(Kazimierska, et al., 2021)
Garnett Lab MinLibCas9 Library	Garnett	37,722	2	Human	(Gonçalves, 2021)
Toronto KnockOut - Version 1	Moffat	176,500	12	Human	(Hart, 2015)

(*continued on next page*)

Table 1 (*continued*)

Library	Developer	Total gRNAs	gRNA per gene	Species	Reference
Toronto KnockOut - Version 3	Moffat	70,948	4	Human	(Mair, 2019)
Human Genome-Wide Reduced Double-gRNA Library	Parts	59,576	3	Human	(Peets, 2019)
Activity-optimized genome-wide library	Sabatini and Lander	187,535	10	Human	(Wang, 2015)
Two plasmid human activity-optimized genome-wide library	Sabatini and Lander	187,536	10	Human	(Park, 2017)
BARBEKO sgRNA Library	Wei	53,502	3	Human	(Xu, 2021)
Human Whole Genome sgRNA iBAR Library	Wei	58,630	3	Human	(Zhu, 2019)
Human genome-wide library v1	Wu	77,406	4	Human	(Ma, 2015)
Human improved genome-wide library v1	Yusa	90,709	5	Human	(Tzelepis, 2016)
Human GeCKO v2	Zhang	123,411	6	Human	(Sanjana et al., 2014)

CRISPR/Cas9 Screen Library Delivery and Experimental Workflow

Delivery of gRNA Library

To perform CRISPR/Cas9 screens, gRNA library delivery is a critical process in the early stage of an experiment. The CRISPR/Cas9 screen can be conducted *in vitro* and *in vivo* manner, where the *in vivo* screen can be further subdivided into direct or indirect screens. The direct *in vivo* screen involves direct injection of naked gRNA library into the animals whereas the indirect *in vivo* screen required transplantation of gRNA mutated cells into the animals. Various gRNA library delivery approaches have been employed in the direct *in vivo* screen such as hydrodynamic tail vein injection and subcutaneous injection (Weber, 2015; Chow and Chen, 2018; Manguso, 2017). For *in vitro* screens, the gRNA library needs to be delivered into the cells. Common delivery approaches include viral-based transfection (lentivirus transduction and adeno-associated virus (AAV) transduction), non-viral-based transfection (lipofection and nanoparticle-based transfection reagent), and electroporation.

Viral-based transfection is commonly utilized for gRNA library delivery in CRISPR/Cas9 screens (Keys and Knouse, 2022; Elegheert, 2018). The gRNA library is first packaged into the lentivirus or AAV, followed by infecting the target cell lines. Lentiviral transduction is one of the most common methods for gene delivery (Wollebo et al., 2013). It allows long-term, stable expression of the GOI as well as capable of infecting different cell types (Wollebo et al., 2013; Balak, 2019). Unlike lentivirus, the AAV-delivered plasmids remain in the cytosol and do not integrate into the host genome (Chow and Chen, 2018). Therefore, AAV is considered a non-pathogenic method of gRNA delivery and is rarely immunogenic (Zhao, 2021). However, due to the non-integrative nature of the AAV plasmid, the conventional sequencing method to reveal gRNA hit is not applicable. To overcome this obstacle, the molecular inversion probe can be utilized to perform targeted capture sequencing to reveal the gRNA hit (Wang, 2021; Cantsilieris, 2017).

Apart from the viral-based transduction, lipofection is another technique for delivering the gRNA library. This method utilizes the lipid structure to deliver gRNA library into the cells via endocytosis or direct fusion (Carter et al., 2015). The use of nanoparticles is also a feasible way to deliver gRNA libraries. These nanoparticles include but are not limited to DNA nanoclews, lipid nanoparticles and gold-based nanoparticles (Sun, 2015; Kazemian, 2022; Wei, 2020). Among the nanoparticle-based delivery, the charge-reversal nano complexes-based system shows great potential in clinical application (Nie, 2021). Finally, electroporation is another approach to deliver the gRNA library, which involves the application of high voltage to the target cell (Potter, 2003). This method is applicable to most cell types and could result in a transient or stable transformation (Potter, 2003; Thomsen, 2022).

Experimental workflow

The classic CRISPR/Cas9 screen approach in mammalian cells normally involves the generation of a Cas9-expressing cell line by antibiotic selective pressure before introducing the gRNA library. This allows the optimization of the Cas9 activity which increases the efficacy of the CRISPR screens (Tzelepis, 2016; Li, 2018). Once the gRNA library is delivered into the cells via different approaches, the cells will undergo enrichment to select for the mutants. The gRNA library-expressing cells can be transplanted into an animal model to perform indirect *in vivo* screens or can be directly used as an *in vitro* screen model (Figure 2). Additionally, the naked gRNA library can be injected into an animal model to perform direct *in vivo* screens (Figure 2). After disease progression and tumour development, the DNA can be extracted from gRNA-targeted tissues and undergo PCR amplification of the inserted gRNA regions (Figure 2). The PCR product is made into a sequencing library by adding barcodes for performing the NGS reaction (Figure 2). The NGS raw reads are generated as data output, which can be aligned to the reference gRNA sequence to calculate the gRNA count. Once the gRNA sequence has been elucidated, the gRNA can be mapped to the genome to identify the targeted locus. Candidate genes can then be shortlisted according to the gRNA hit rate. After analyzing the candidate genes by comparison to online omics data, literature and clinical relevance, target candidate(s) can be selected for in-depth study (Figure 2). Downstream validation and analysis can be performed when the validation gRNA plasmids are available (Figure 2).

CRISPR/Cas9 enabled screens for HCC-associated genes

Various CRISPR/Cas9 screens have been performed to amplify our understanding of HCC. These include drug resistant screens, metastasis screens, tumour microenvironment screens, and general screens for HCC-related gene identification.

CRISPR/Cas9 screens with model pre-exposed to different HCC risk factors

It is well known that different risk factors could lead to HCC occurrence via specific genetic alterations. Previously, the CRISPR/Cas9 system was utilized in validating the function of certain genes that could lead to liver impairment, such as *Membrane bound O-acyltransferase domain containing 7* (*MBOAT7*) in NAFLD and *Bone morphogenetic protein 9* (*BMP9*) in liver steatosis (Meroni, 2020; Yang, 2020). With the maturation of the CRISPR technology, HCC carcinogenesis mechanism was investigated through high throughput CRISPR/Cas9 screen studies with predisposed backgrounds of HCC, such as hepatitis virus infection

Table 2
Comparison of the CRISPR libraries.

Library	Transduction method	No. of gene targeted	No. of control gRNA	PCR protocol	Library amplification protocol
Mouse CRISPR Knockout Pooled Library (Gouda)	Lentivirus	21,601	500 non-targeting, 500 one-intergenic site targeting	Available	Available
Broad GPP genome-wide Brie	Lentivirus	19,674	1,000	Available	Available
Mouse CRISPR Knockout Library in lentiCRISPR v2-Blast	Lentivirus	18,741	341	Available	Available
Mouse Toronto KnockOut (mTKO) CRISPR Library	Lentivirus	19,463	418 targeting EGFP, LacZ and Luciferase	Available	Available
Two plasmid mouse activity-optimized genome-wide library	Lentivirus	18,986	199	Available	Unavailable
Retroviral Mouse Genome-wide CRISPR Knockout Library	Retrovirus	18,424	Unspecified	Unavailable	Available
Mouse Cherry Brie Pooled Library	Lentivirus	19,674	1,000	Available	Available
Mouse improved genome-wide library v2	Lentivirus	18,424	Unspecified	Unavailable	Available
Mouse GeCKO v2	Lentivirus	20,611	2000	Unavailable	Available
Human CRISPR Knockout Pooled Library (Gattinara)	Lentivirus	19,993	500 non-targeting, 500 one-intergenic site targeting	Available	Available
Broad GPP genome-wide Brunello	Lentivirus	19,114	1,000	Available	Available
Broad GPP Humagne Set C and Humagne Set D	Lentivirus	19,755	100 non-targeting pairs and 500 one-intergenic-site pairs in each library	Available	Available
Mini-human AsCpf1-based Human Genome-wide Knockout Library	Lentivirus	16,977	55 non-targeting	Available	Available
MYC-CRISPR Library	Lentivirus	24,981E-boxes	4 targeting MYC, 1,000 non-targeting	Unavailable	Available
Garnett Lab MinLibCas9 Library	Lentivirus	18,761	200 non-targeting	Available	Available
Toronto KnockOut - Version 1	Lentivirus	17,661	Targets LacZ, EGFP, luciferase and random loci on chromosome 10, number unspecificed	Unavailable	Unavailable
Toronto KnockOut - Version 3	Lentivirus	18,053	142 targeting EGFP, LacZ and Luciferase	Available	Available
Human Genome-Wide Reduced Double-gRNA Library	Lentivirus	19,657	398 non-targeting	Available	Available
Activity-optimized genome-wide library	Lentivirus	18,663	1,000 non-targeting, 500 intergenic	Available	Available
Two plasmid human activity-optimized genome-wide library	Lentivirus	18,543	1504	Available	Unavailable
BARBEKO sgRNA Library	Lentivirus	17,501	500 non-targeting, 499 targeting safe-harbor regions	Available	Available
Human Whole Genome sgRNA iBAR Library	Lentivirus	19,210	1,000 non-targeting	Available	Unavailable
Human genome-wide library v1	Lentivirus	20,121	Unspecified	Unavailable	Unavailable
Human improved genome-wide library v1	Lentivirus	18,010	Unspecified	Unavailable	Available
Human GeCKO v2	Lentivirus	19,050	2,000	Unavailable	Available

or liver diseases (Table 6). These screens were performed with background of liver fibrosis, cirrhosis, NAFLD, and NASH. The results confirmed the role of T*ransforming growth factor beta receptor 1* (*TGFBR1*), *SMAD family member 4* (*SMAD4*), *Actin alpha 2, smooth muscle* (*ACTA2*), *Cytochrome P450 family 46 subfamily A member 1* (*CYP46A1*), *Polycystin 1, transient receptor potential channel interacting* (*PKD1*), *Lysine methyltransferase 2D* (*KMT2D*), and *AT-rich interaction domain 1A* (*ARID1A*) in leading to liver diseases (Yu, 2022; Si, 2020; Zhu, 2019). For instance, TGF-β signalling activation in hepatic stellate cells (HSC) by *TGFBR1* and *SMAD4* results in HSC activation and fibrogenesis in the liver, which promotes HCC development (Yu, 2022). Interestingly, the activation of the TGF-β signalling is known to be involved in the process of HCC development and to facilitate the immune escape of cancer cells (Gonzalez-Sanchez, 2021). Therefore, TGF-β signalling is an important target to prevent HCC development at the fibrosis stage. Statistic data revealed that more than 50% of HCC patients are diagnosed with late-stage HCC which is nearly impossible to treat (Patel et al., 2015). Therefore, these discoveries would be useful in HCC prevention and facilitate treatment through identification of early-stage liver pathological transformations.

CRISPR/Cas9 screens have also been performed with HBV or HCV infection background, where these infections account for more than 50% of HCC cases (D'Souza, 2020). The genes identified from these screenings are usually related to viral replication or viral invasions, such as *Zinc finger CCHC-Type containing 14* (*ZCCHC14*), *Terminal Nucleotidyltransferase 4A/B (TENT4A/B*), *Claudin 1* (*CLDN1*), or *Tripartite motif containing 26* (*TRIM26*) (Hyrina, 2019; Ren, 2015; Liang, 2021). The synthesis of HBV viral surface antigen production is one of the most important steps for HBV replication and proliferation in the hepatocytes, as it is involved in the production of new viruses for further infections. Hyrina *et al.* identified the host protein, ZCCHC14 and TENT4A/B, are involved in regulating HBV antigen expression by binding to the antigen RNA and stabilizing them via RNA tailing (Hyrina, 2019). Therefore, the downregulation of ZCCHC14 and TENT4A/B could suppress HBV antigen production. Interestingly, *ZCCHC14* was also screened out from the HAV background, where the infection could lead to acute liver inflammation and result in relapsing hepatitis in the long-term (Abutaleb and Kottilil, 2020; Kulsuptrakul, 2021). Thus, CRISPR/Cas9 screen with a hepatitis virus infection background may offer insight into the mechanism for other similar viruses.

As a multi-risk factor-induced cancer, the number of CRISPR/Cas9 screens with different backgrounds is still rather limited. The involvement of other HCC risk factors (AFLD, liver steatosis, obesity, and aflatoxin/other mutagen exposure) shall be thoroughly investigated in the future to consolidate the HCC mechanism.

Drug resistance CRISPR/Cas9 screens

CRISPR/Cas9 screens for HCC also allow for in-depth investigation of drug resistance mechanisms (Table 7). These screens aim at identifying the specific gene(s) that induce the drug resistance mechanisms observed in HCC patients. The drug-of-interest usually includes sorafenib, regorafenib and lenvatinib. Drug resistance is of interest in HCC as it poses difficult challenges to physicians and late-stage HCC patients. This obstacle diminishes the therapeutic efficacy while reducing the lifespan of HCC patients and causing severe side effects. Moreover, this

Table 3
Available online platforms for gRNA design. Most of the tools enable human and mouse gRNA design. The Cas enzymes used in these platforms mostly include SpCas9.

Platform	Species	Enzyme	Weblink	Reference
CCTop	Human Mouse Etc	Multiple*	https://cctop.cos.uni-heidelberg.de:8043/	(Stemmer, 2015)
CHOPCHOP	Human Mouse Etc	Multiple*	http://chopchop.cbu.uib.no/	(Labun, 2019)
CRISPOR	Human Mouse Etc	Multiple*	http://crispor.tefor.net/	(Concordet and Haeussler, 2018)
CRISPick	Human Mouse Etc	Multiple*	https://portals.broadinstitute.org/gppx/crispick/public	(Doench, 2016; Sanson, 2018)
CRISPRdirect	Human Mouse Etc	Multiple*	https://crispr.dbcls.jp/	(Naito, 2015)
CRISPRml	Human Mouse Etc	Unspecified#	https://crispr.ml/	(Doench, 2016; Listgarten, 2018)
CRISPRon	Human Mouse Etc	SpCas9	https://rth.dk/resources/crispr/crispron/	(Xiang, 2021)
CRISPRRgen	Human Mouse Etc	Multiple*	http://www.rgenome.net/cas-designer/	(Park et al., 2015; Bae et al., 2014)
CRISPRScan	Human Mouse Etc	Multiple*	https://www.crisprscan.org/gene/	(Moreno-Mateos, 2015)
E-CRISP	Human Mouse Etc	Multiple*	http://www.e-crisp.org/E-CRISP/	(Heigwer et al., 2014)
Guides	Human Mouse Only	Unspecified#	http://guides.sanjanalab.org/#/	(Meier et al., 2017)
GuideScan	Human Mouse Etc	SpCas9, Cpf1	https://guidescan.com/	(Perez, 2017)
Off-Spotter	Human Mouse and Yeast	Multiple*	https://cm.jefferson.edu/Off-Spotter/	(Pliatsika and Rigoutsos, 2015)
SSC	Unspecified	Unspecified	http://crispr.dfci.harvard.edu/SSC/	(Xu, 2015)
SYNTHEGO	Human Mouse Etc	SpCas9	https://design.synthego.com/#/	(Conant, 2022)
VBC-Score	Human Mouse Etc	Unspecified#	https://www.vbc-score.org/	(Michlits, 2020)
WU-CRISPR	Human Mouse Only	Unspecified	https://crisprdb.org/wu-crispr/	(Wong et al., 2015; Hiranniramol, 2020)

* Includes SpCas9
\# PAM sequence specified: NGG

Table 4
Comparison of the properties and features of different gRNA design platforms.

Platform	No. of species available	No. of Cas nuclease available	Input format for gRNA design	Inquire multiple gene at the same time
CCTop	Numerous, search function available	17	Sequence < 500 bp, FASTA file	Y
CHOPCHOP	Numerous, search function available	7 or custom request PAM sequence	Gene name, Gene ID, RefSeq ID, ENSEMBL ID, Genetic coordinates, FASTA file	N
CRISPOR	Numerous, search function available	38	Sequence < 2300 bp	N
CRISPick	3	4	Sequence < 20,000 bp, gene name, gene ID, transcript ID, FASTA file, range coordinates	Y
CRISPRdirect	Numerous, search genome available	Custom request PAM sequence	Sequence < 10,000 bp, NCBI accession number, genome location, FASTA file	N
CRISPRml	NA	1	ENSEMBL ID, sequence (bp limit unspecified)	N
CRISPRon	6	1	Sequence < 10,000 bp, gene name, ENSEMBL ID	N
CRISPRRgen	73	33	Sequence < 1,000 bp, FASTA file	N
CRISPRScan	20	7	Sequence (bp limit unspecified), gene name, FASTA file	N
E-CRISP	55	>2*	Sequence (bp limit unspecified), gene symble, ENSEMBL ID, FASTA	N
Guides	2	1	Gene name	Y
GuideScan	7	2	Gene name, gene ID, genomic coordinates, Enterz gene ID	Y
Off-Spotter	4	4	Sequence < 1,500 bp	N
SSC	NA	NA	Sequence < 10,000 bp, FASTA	N
SYNTHEGO	Numerous, search function available	1	Gene name, gene ID	N
VBC-Score	6	1	Sequence (bp limit unspecified), gene name	Y
WU-CRISPR	2	NA	Sequence < 30,000 bp, gene name, gene ID, Genbank accession number	N

* PAM sequence can be NGG, NAG (with off-targets tolerating mismatch bases), or NAG (with off-targets tolerating full-length perfect match).

also increases the medical financial burden on HCC patients (Shlomai et al., 2018). Therefore, elucidating the drug resistance mechanisms could help improve the therapeutic efficacy and would be favorable for patients with late-stage HCC. The sorafenib and lenvatinib, first-line receptor tyrosine kinase inhibitors, are commonly employed in CRISPR/Cas9 screens while fewer studies utilize regorafenib (second-line inhibitor) (Rimini, 2021; Llovet, 2021). To our best knowledge, no CRISPR/Cas9 drug resistance screens have been performed on other systemic HCC therapeutic drugs (cabozantinib, ramucirumab, atezolizumab, and bevacizumab). Therefore, further studies on drug resistance are warranted to enrich our knowledge and improve therapeutic outcomes for late-stage HCC patients.

Nevertheless, CRISPR/Cas9 screens enlighten the current understanding of drug resistance mechanisms in HCC. Several genes have been identified for sorafenib drug resistance mechanism, which undergoes distinct signalling axis such as oncogenic WNT signalling pathway. These include *Kelch-like ECH-associated protein 1* (KEAP1), *Phosphoglycerate dehydrogenase* (*PHGDH*), *N-methyl-D-aspartate receptor* (*NMDAR1*), *Cyclin-dependent kinase 12* (*CDK12*) and *Shugoshin-like 1* (*SGOL1*) (Zheng, 2019; Wei, 2019; Xu, 2021; Wang, 2020; Sun, 2018; Chen,

Table 5
Comparison of the results generated by different gRNA design platforms.

Platform	Result in table view/ Download table view results	Result in graphical view/Download results in SnapGene format with gRNA locations	Other output features
CCTop	Y/Y	Y/N	/
CHOPCHOP	Y/Y	Y/Y	View results in UCSC Genome Browser
CRISPOR	Y/Y	Y/Y	/
CRISPick	N/Y	N/N	/
CRISPRdirect	N/Y	N/N	Highlight highly specific gRNAs
CRISPRml	N/Y	N/N	Require register and login
CRISPRon	N/Y	Y/N	gRNA targeted domain information, view results in UCSC Genome Browser
CRISPRRgen	N/Y	N/N	/
CRISPRScan	N/Y	Y/Y	/
E-CRISP	N/Y	Y/N	gRNA mismatch base pair information
Guides	N/Y	N/N	gRNA designed per gene can be selected, target protein domain option, download non-targeting control sequences, expression of exon in diff tissues
GuideScan	Y/Y	Y/N	/
Off-Spotter	N/Y	N/N	/
SSC	N/Y	N/N	/
SYNTHEGO	N/N	Y/N	Directly order suggested gRNAs
VBC-Score	N/Y	N/N	Amino acid cut site information available
WU-CRISPR	Y/N	N/N	No result download function

2022). However, the majority of genes responsible for sorafenib drug resistance are not applicable to lenvatinib resistance (Table 7). These findings suggest that drug resistance varies across different drug candidates despite having similar modes of action. Although differences can be observed, the *KEAP1* appears to involve in drug resistance mechanism of sorafenib, lenvatinib and regorafenib. These tyrosine kinase inhibitors abolish cancer cells through the generation of reactive oxygen species (ROS) (Coriat, 2012; Tan, 2022; Cucarull, 2021). The loss-of-function of *KEAP1* up-regulates *Nuclear factor erythroid 2–related factor 2* (*NRF2*), which results in ROS level reduction (Zheng, 2019). Therefore, this decreases the potency of these tyrosine kinase inhibitors. Interestingly, these studies demonstrated that sorafenib coupled with an additional inhibitor, such as PHGDH inhibitor, NMDAR inhibitor, THZ531 or NRF2 inhibitor, could reverse the drug resistance (Wei, 2019; Xu, 2021; Wang, 2020; Chen, 2022). Since systemic therapy is the only treatment option for late-stage HCC patients, it is crucial to improve treatment outcomes and develop personalized therapy through the identification of resistance genes.

Furthermore, different cancer cell lines were used in the drug resistance screens (Table 7). These cell lines can be classified into early well-differentiated HCC (Huh7, Hep3B, HepG2), late-stage poorly-differentiated HCC (SNU449, HLF), metastatic HCC (MHCC97L) and Hela cell derivatives (SMMC7721) (Nwosu, 2018; Lin and Li, 2020; Lin, 2014; Rebouissou, 2017). Given that receptor tyrosine kinase inhibitors target advanced HCC, late-stage or metastatic HCC cell lines would offer a more representative and accurate screening results for clinical observation. However, the use of SMMC7721 should be avoided due to concerns about whether the cell line originated from the liver (Rebouissou, 2017). It is widely accepted that the choice of cell line has a sufficient impact on the screening results. Thus, several factors (cell line background, origin and contamination status) must be carefully considered before performing CRISPR/Cas9 screen.

Apart from the multi-receptor tyrosine kinase inhibitors, immunotherapy, such as immune checkpoint inhibitors, cytokine-based therapy, and engineering T cell, is another regimen for HCC patients (Llovet, 2022). Immunotherapies have been proven effective in solid tumour treatment (Liu, 2021). However, resistance to immunotherapy often occurs in HCC patients due to the complex immune microenvironment and the high degree of tumour heterogeneity (Zhong, 2021). Currently, few CRISPR/Cas9 studies have revealed certain genes that are related to immune response, such as *Janus kinase 1* (*JAK1*) in antigen presentation and *RAS P21 protein activator 2* (*RASA2*) in T cell stimulation (Shifrut, 2018; Freeman, 2019). However, to our best knowledge, CRISPR/Cas9 screen applied to study immunotherapy resistance is rather limited. It is important and essential that more CRISPR/Cas9 screens for HCC immunotherapy resistance be performed to enhance treatment efficacy and develop personalized medication.

CRISPR/Cas9 screens related to HCC metastasis, tumour microenvironment, and genetic alteration

With the success of CRISPR/Cas9 in various studies, the utility of CRISPR/Cas9 was further expanded to investigate the HCC tumour development and metastasis (Table 8). Through CRISPR/Cas9 screens, it has been revealed that the cellular functions (proliferation, migration and invasion) were altered to promote metastasis in cancer (Table 8). Genes responsible for metastasis involve the *A disintegrin-like and metalloprotease domain with thrombospondin type I motifs-like 3* (*ADAMTSL3*), *Phosphatase and tensin homolog* (*PTEN*), *WW domain containing oxidoreductase* (*WWOX*) and *MicroRNA 4310* (*miR-4310*) (Zhou, 2020; Yang, 2021; Li, 2021). The *ADAMTSL3* and *PTEN* were screened out to examine their role in tumour metastasis. The results indicated their capability in elevating cell proliferation and metastasis of HCC cells respectively (Zhou, 2020). Yang *et al.* and Luo *et al.* identified the anti-metastasis effectiveness of toosendanin (TSN) depends on the *WWOX* (Yang, 2021; Luo, 2018). The *WWOX* is a tumour suppressor where low expression was found in tumour samples (Zhou, 2018). Furthermore, Yang *et al.* also discovered that the binding of TSN to WWOX activates and upregulates WWOX to exert its anti-metastatic function via inhibition of WNT and JAK2/STAT3 signalling.

Different models were used for the above metastasis screens, including nude mice, HepG2 (early-stage HCC), and MHCC97L (metastatic HCC). The rationale for the selection of different models varies amongst different studies. For instance, screening with an early-stage HCC cell line allowed recognition of metastasis genes that is responsible for cell epithelial-to-mesenchymal (EMT) changes at the initial stage whereas the metastatic HCC cell line enables the identification of genes that further facilitate the EMT. Apart from *in vitro* studies, metastasis screening *in vivo* provides a realistic and comprehensive environment for tumour progression. These include diversified cell type interactions and sophisticated immune responses involved in tumour development (Muranen, 2017; Miller, 2017). This is also applicable for studying genes related to the tumour microenvironment that involve the interplay of biological systems (Bao, 2021). Despite *in vitro* screens enable metastasis-related gene discovery, validation with *in vivo* models shall be conducted to consolidate and verify the findings (Table 8).

The alteration of gene expression level leads to the clonal evolution of tumour cells is an important cancer event (Craig, 2020). Various CRISPR/Cas9 screens were conducted to investigate whether alteration in gene expression could initial or promote HCC development (Table 8). Most of the screens were first performed *in vitro* followed by validation *in vitro* and *in vivo*. The *Non-SMC condensin I complex subunit G* (*NCAPG*), *Transformation/transcription domain associated protein* (*TRRAP*) and *Aryl hydrocarbon receptor* (*AHR*) were revealed to promote HCC development via distinct mechanisms (Wang, 2019; Kwan, 2020; Zhu, 2021). It is elucidated that TRRAP and its cofactor Histone acetyltransferase (KAT5)

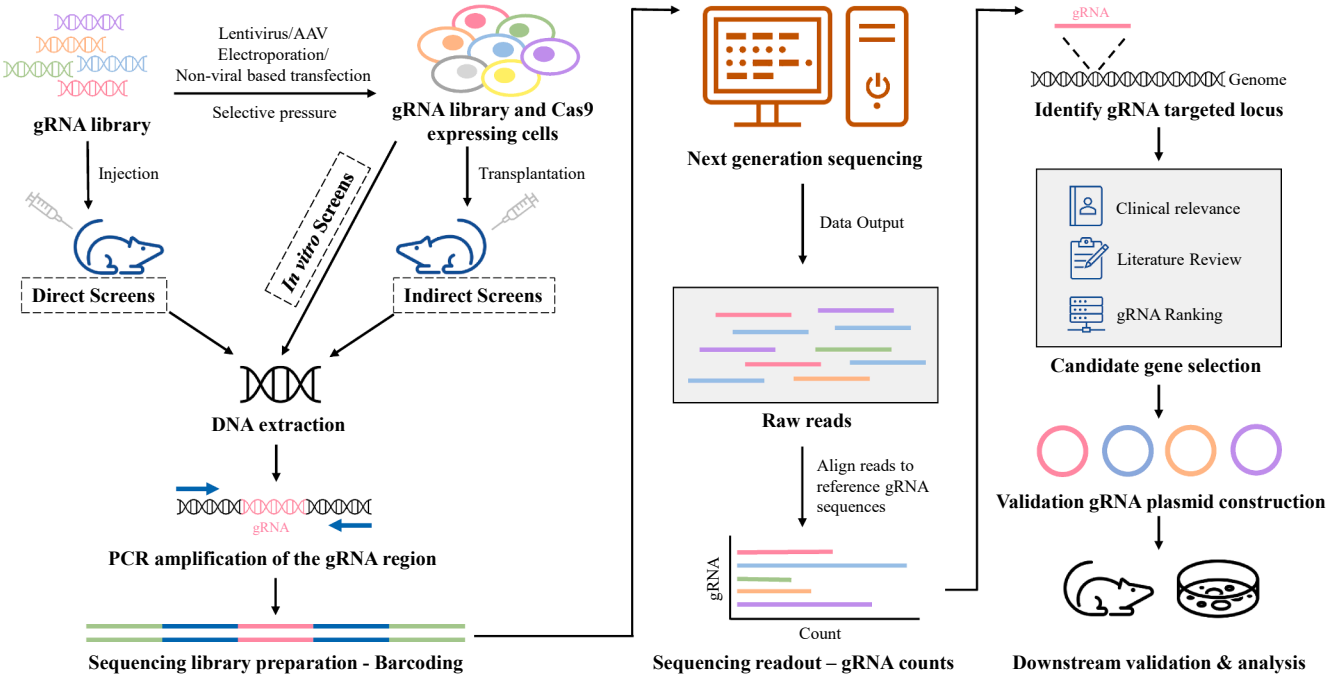

Figure 2. Schematic illustration of *in vitro* and *in vivo* CRISPR/Cas9 screen approaches and the experimental workflow. Direct *in vivo* screen involves the injection of naked gRNA library into the animal whereas indirect *in vivo* screen requires transplantation of gRNA library and Cas9-expressing cells into the animal. The gRNA library and Cas9-expressing cells can also be used to perform *in vitro* screens. DNA can be extracted from the cells or tissue samples and undergoes PCR amplification of the gRNA region. The PCR product will be subject to next generation sequencing (NGS) after barcoding into a sequencing library. The NGS sequencing results are aligned to the reference gRNA sequence to generate gRNA counts. After mapping the gRNA to its targeted locus, candidate gene(s) can be selected and proceed to downstream validation experiments.

Table 6
Summary of CRISPR/Cas9 screens performed with different HCC backgrounds.

Induced background of screening model	Screening model	CRISPR Library	Gene identified	Validation model	Mechanism	Reference
Fibrosis/NASH	Hepatic Stellate Cells (HSC)	In House	*TGFBR1, SMAD4, ACTA2* etc.	HSC with macrophages and hepatocytes	HSC activation via TGF-β pathway	(Yu, 2022)
NAFLD	L02	GeCKO	*CYP46A1*	L02	Lipid droplet metabolism pathway	(Si, 2020)
Cirrhosis	Fah -/- mouse	In House	*PKD1, KMT2D,* and *ARID1A*	*Arid1a* or *Kmt2d* or *Pkd1* floxed mice	clonal expansion	(Zhu, 2019)
HAV	Huh7.5.1	GeCKO v2	*RPL26, PAPD5/7* and *ZCCHC14*	Huh7.5.1	Liver acute inflammation, viral translation	(Kulsuptrakul, 2021)
HBV	HepG2	In House	*ZCCHC14* and *TENT4A/B*	HepG2-HBV, HepG2-hNTCP, and HBV reporter cell lines	Viral Surface Antigen Production	(Hyrina, 2019)
HCV	Huh7.5 (NIrD)	In House	*CLDN1, OCLN* and *CD81*	Huh 7.5 (NIrD)	HCV cell-free entry and cell-to-cell transmission	(Ren, 2015)
HCV	Huh7.5	In House	*TRIM26*	Huh7	HCV replication	(Liang, 2021)

Table 7
Summary of CRISPR/Cas9 screens performed with drug resistance backgrounds.

Drug	Library	Resistance gene	Cell line	Mechanism	Reference
Sorafenib, Lenvatinib, regorafenib	GeCKO v2	*KEAP1*	Huh7	KEAP1/Nrf2 signalling	(Zheng, 2019)
Sorafenib	GeCKO v2	*PHGDH*	MHCC97L	Serine synthesis signalling	(Wei, 2019)
Sorafenib	CombiGEM-CRISPR v2.0	*NMDAR1*	MHCC97L	ER stress, stemness, and WNT signalling	(Xu, 2021)
Sorafenib	In house CRISPR kinome	*CDK12*	Hep3B, Huh7	CDK12 expression	(Wang, 2020)
Sorafenib	GeCKO v2	*SGOL1*	Huh7, SMMC-7721	Apoptosis, cytotoxicity	(Sun, 2018)
Sorafenib	GeCKO v1	*KEAP1*	HepG2	KEAP1/NRF2/FGF2 signalling	(Chen, 2022)
Lenvatinib	In house CRISPR kinome	*EGFR*	SNU449	EGFR–PAK2–ERK5 signalling	(Jin, 2021)
Lenvatinib	GeCKO v2	*NF1, DUSP9*	Huh7	PI3K/AKT and MAPK/ERK signalling	(Lu, 2021)
Lenvatinib	GeCKO v2	*DUSP4*	HepG2	MAPK/ERK signalling	(Huang, 2022)
Lenvatinib	GeCKO v2	*LAPTM5*	Huh7	Autophagy	(Pan, 2022)
Regorafenib	Brunello CRISPR kinome	*LATS2*	HLF	Hippo signalling	(Suemura, 2019)

Table 8
Summary of CRISPR/Cas9 screens studying HCC metastasis, tumour microenvironment or genetic alterations.

Study	Library	Screening Model	Gene	Validation model	Cellular function affected	Reference
Metastasis	hGeCKOa	Nude mice	*ADAMTSL3 PTEN*	Hep3B, Nude mice	Proliferation, Migration, Invasion	(Zhou, 2020)
Metastasis	CRISPR-Pool TMKOUT	MHCC97L	*WWOX*	SK-Hep-1, MHCC97L, SMMC8821, Huh7, Nude mice	Proliferation, Migration	(Yang, 2021)
Metastasis	GeCKO v2	HepG2	*miR-4310*	HepG2, SMMC7721, Nude mice	Lipid metabolism, Proliferation, Migration, Invasion	(Li, 2021)
Hypoxia	GeCKO v2	MHCC97L	*PTPMT1*	MHCC97L, Nude mice	Electron trapping at mitochondria, Proliferation	(Bao, 2021)
Transcriptional changes	GeCKO v2	HCCLM3, SNU449	*NCAPG*	HepG2, HCCLM3, Mice (species unspecified)	Proliferation, Migration, Cell cycle	(Wang, 2019)
Transcriptional changes	mGeCKOa	Nude mice	*Nf1, Plxnb1, Flrt2, B9d1*	Hep3B, Huh7, $p53^{flox/flox}$;Alb-Cre mice	Tumour formation	(Song, 2017)
Transcriptional changes	kinome CRISPR	Huh7, Hep3B, HepG2	*TRRAP*	Huh7, Hep3B, SNU475, Nude mice	Proliferation	(Kwan, 2020)
Transcriptional changes	Brunello CRISPR	PLC/PRF/5	*AHR*	PLC/PRF/5, Huh7, C57BL/6 mice	Cell metabolism, Differentiation, Immunity	(Zhu, 2021)

together activate the transcription of mitotic genes, such as *DNA topoisomerase II alpha* (*TOP2A*) to promote HCC cell proliferation (Kwan, 2020). When TRRAP/KAT5 is downregulated, cell arrest occurs at the G2/M phase and thus proliferation is inhibited (Kwan, 2020).

For the CRISPR/Cas9 *in vivo* screen that is performed in mice using mouse gRNA library, *Neurofibromin 1* (*Nf1*) was identified for its role in tumour formation, which was also validated in human liver immortalized/cancer cell lines for its functions (Song, 2017). The *NF1* knockout activates the RAS signalling pathway via MAPK, resulting in the upregulation of *High-mobility group AT-hook 2* (*HMGA2*) and *Transcription factor SOX-9* (*SOX9*) to promote tumour formation (Song, 2017). Screening performed in mice can be correlated to humans as the gene functions of both species are highly conserved with around 80% of amino acid similarity (Monaco, 2015). The degree of conservation was also true at the epigenetic level (Zhou, 2017). With the solid grounding on the highly conserved protein sequence in both species, the *in vivo* screening could provide more comprehensive information regarding the cellular and systemic interactions that lead to HCC development. Therefore, direct screening with mice could be a reliable approach to study HCC.

AAV-based CRISPR/Cas9 screens

The majority of gRNA library delivery methods utilized lentivirus transduction in most of the above-mentioned studies, with few exceptions employed electroporation of ribonucleoprotein complex (Yu, 2022), hydrodynamic tail vein injection of transposon-based gRNA (Zhu, 2019), and the transplantation of lentivirus transduced gRNA library expressing cells (Song, 2017). The introduction of gRNA library with AAV, however, is rather limited when compared with other studies (Table 9). Newman *et al.* and Tzelepis *et al.* utilized Cas9-expressing mice for screening to compensate for the low cargo size of the AAV (Table 9). One study successfully identified genes that are capable of accelerating tumour development in liver under a Trp53-deficient background. These genes including *Capicua transcriptional repressor* (*Cic*), *Phosphatidylinositol 3-kinase regulatory subunit alpha* (*Pik3r1*), *Pten*, *Serine/threonine kinase 11* (*Stk11*), *AT-rich interactive domain-containing protein 2* (*Arid2*) and *Lysine-specific demethylase 5C* (*Kdm5c*), are able to accelerate the tumour development in the liver under a Trp53-deficient background (Wang, 2018). The co-occurrence and correlation analysis performed in this study also identified the absence of *Beta-2-Microglobulin* (*B2m*) and *KAT8 Regulatory NSL Complex Subunit 1* (*Kansl1*) would promote HCC synergistically (Wang, 2018). The second study identified *lysine*

Table 9
Summary of CRISPR/Cas9 screens in HCC using AAV-based method.

Study	Library	Screening model	Gene identified	Validation model	Cellular function affected	Reference
Tumour suppressor genes	AAV-mTSG library	Rosa-LSL-Cas9-EGFP knock-in mice	*Cic, Pik3r1, Pten, Stk11,Arid2, Kdm5c B2m, Kansl1*	Rosa-LSL-Cas9-EGFP knock-in mice (with Trp53 knockdown background)	Accelerated liver tumorigenesis Synergistic effect in accelerating liver tumor development	(Wang, 2018)
Tumour suppressor genes	AAV-mTSG library	LSL-Cas9 LSL-Fluc mice	*KMT2D*	LSL-Cas9 LSL-Fluc mice	Enhanced immune infiltration, elevated DNA damage. activation of transposable elements	(Wang, 2020)

methyltransferase 2D (*Kmt2d*) as an important gene that would promote immune infiltration, increase DNA damage and activate transposable elements during HCC development (Wang, 2020). Currently, AAV-based CRISPR/Cas9 library is rather limited as public resources, and the library used in the above studies only targets tumour suppressor genes and house-keeping genes. Given that AAV is non-pathogenic and non-integrating (Thomsen, 2022; Loesch et al., 2019), further development of AAV-based gRNA library is warranted (such as AAV library targeting all known and/or unknown genes). With the merits of AAV, this could potentially translate into clinical therapy in the future.

Advantages, concerns and improvements of CRISPR/Cas9 screens

The CRISPR/Cas9 system is a revolutionary tool for gene targeting due to its ability in modifying the genome with high specificity and flexibility (Wu, 2020). The CRISPR/Cas9 system requires only the 20-nucleotide gRNA sequence to precisely target the GOI, where this drastically reduces the workload and time when compared to TALENs or zinc finger nucleases (ZFNs) (Gupta, 2019). Owing to its high efficiency, the CRISPR/Cas9 system can simultaneously target multiple gene loci by various gRNAs which enables high-throughput screening. Therefore, the CRISPR system is a time-efficient system and becoming the major trend for exploring cancer genes for HCC tumorigenesis process (Figure 3).

Despite being a major breakthrough in gene editing, certain limitations must not be neglected. The most obvious drawback is the off-target effect of the gRNAs (Janik, 2020). The off-targeting of the gRNAs could result from (a) the number and location of mismatch between the gRNA and its on- or off-target sites, (b) the GC content of the target site, and (c) Cas9 nuclease activity (Modrzejewski, 2020). It is suggested that the gRNA designed should possess as minimal mismatches as possible and the mismatched bases should be confined in the eight closest bases to the PAM sequence to reduce the off-target effect to the largest extent (Hahn and Nekrasov, 2019). Furthermore, it is observed that the target sequence with low GC content could reduce the off-target effect in most cases, thus the gRNA with low GC content is recommended (Yu, 2017). The Cas9 nuclease originated from the *Streptococcus pyogenes* (SpCas9), it has been used more extensively in screening studies when compared to other Cas proteins (Modrzejewski, 2020). The SpCas9 nuclease activity could affect the occurrence of the off-target effect and therefore, an improved version of SpCas9, SpCas9-high fidelity, would be recommended (Zhang, 2017).

The off-target effect of CRISPR/Cas9 is regarded as the most undesirable obstacle in clinical practice (Chen, 2020). With the improvements in gRNA designs, studies have been conducted to evaluate the potentials of this system to treat different diseases including liver cancer (Table 10). Several genes were also identified to be the potential targets for HCC treatments, such as *WEE1 G2 checkpoint kinase* (*WEE1*), *Cell division cycle 7* (*CDC7*), *Wnt family member 3A* (*Wnt3a*), *Canopy FGF signaling regulator 2* (*CNPY2*), *Aspartate beta-hydroxylase* (*ASPH*), *Eukaryotic translation elongation factor 2* (*EEF2*), *Fibroblast growth factor receptor 4* (*FGFR4*) and *FGF19* (Liang, 2020; Guo, 2021; Zheng, 2019; Wang, 2018; Iwagami, 2016; Pott, 2017; Gao, 2017; Ardelt, 2019). In 2021, the first clinical trial for CRISPR/Cas9 (Clinical trial no. NCT04774536) has been approved by the U.S. Food and Drug Administration to treat sickle cell diseases. Recently, the CRISPR/Cas9 system was used to reinforce the treatment efficacy of sonodynamic therapy of HCC (Yin, 2021). These promising progressions of the CRISPR/Cas9 system further suggest its potential as a clinical treatment.

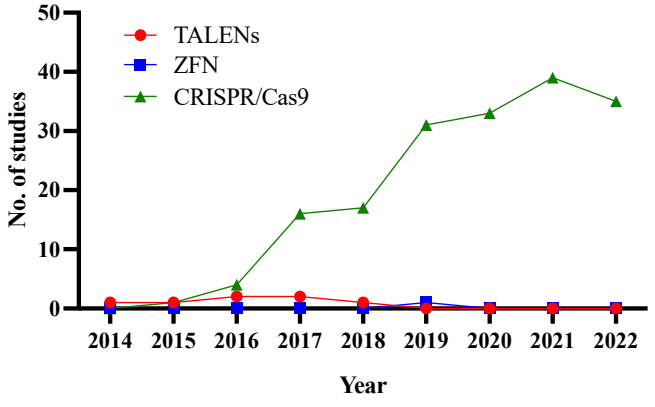

Figure 3. The number of HCC studies with TALENs, ZFN, and CRISPR/Cas9 in PubMed of the National Center for Biotechnology Information. The number of HCC studies using TALENs or ZFN remains low whereas HCC studies with CRISPR/Cas9 exhibited a general increasing trend between 2014-2022.

Apart the gRNA off-target problem, the efficiency of the gRNA also needs to be evaluated when choosing the gRNAs. Several criteria are commonly considered, such as gRNA targeting strand, the base location in the gRNA and GC content of the gRNA. It has been revealed that the gRNA efficiency would be higher if it targets the DNA coding strand (Wang, 2014). Furthermore, it has also been demonstrated that the gRNA efficiency would increase with the last four nucleotides being adenine or guanine (position 17-20) in the gRNA (Wang, 2014). Alternatively, gRNA with absence of cytosine (at position 3), guanine (at position 20) and cytosine (at position 16) would also improve the efficiency (Doench, 2014). A study has also suggested that 30-65% of GC content of gRNA would be the optimal concentration to increase the efficiency (Ren, 2019). It is worth to note that the off-target effect also increases along with the increased GC content (Yu, 2017). Thus, preselection and evaluation of gRNA are critical factors for the gRNAs design. In addition, the GC content also needs to be carefully considered to yield optimal experimental performance and outcome.

The CRISPR/Cas9 system function requires the induction of DSB followed by NHEJ or HDR. Thus, another proposed drawback of this system is the introduction of unpredictable DSB = into the genome regardless of whether it is on- or off-target (Antoniou et al., 2021). This

Table 10
Studies evaluating the potential of CRISPR/Cas9 system for disease treatment. Summary of the CRISPR/Cas9 system for treating different diseases including liver cancer.

Disease	CRISPR Therapy	*In vitro*	*In vivo*	Reference
α1-antitrypsin deficiency	*hSERPINA1* mutation		Y	(Bjursell, 2018)
Bladder cancer	*ATG5* knockout	Y		(Chen, 2018)
Breast cancer	*PARP1* or *APOBEC3G* knockout	Y		(Mintz, 2020; Mendes de Almeida, 2019)
Cataracts	*Crygc* correction	Y		(Wu, 2015)
Cervical cancer	*E6* and *E7* knockout	Y	Y	(Zhen, 2014)
Colorectal cancer	*KRAS* knockout	Y		(Wan, 2020)
Cystic fibrosis	*CFTR* point mutation	Y		(Firth, 2015)
Genetic Deafness	*Tmc1* mutation		Y	(György, 2019)
Human immunodeficiency virus infection	*CXCR4* and *CCR5* knockout	Y		(Hou, 2015; Liu, 2017; Yu, 2018)
Huntington disease	*HTT* deletion	Y	Y	(Monteys, 2017)
Liver cancer	*Pten* and *p53* knockout		Y	(Xue, 2014)
Lung cancer	*PTEN* or *EGFR* knockout	Y		(Perumal, 2019; Cheung, 2018)
Non-small cell lung cancer	*NPM1* knockout	Y	Y	(Li, 2020)
Prostate cancer	*p53* knockout	Y		(Batır et al., 2019)
Sickle cell disease	*BCL11A* enhancer targeting	Y		(Wu, 2019)
WPW syndrome	*PRKAG2* mutation		Y	(Xie, 2016)

could result in a mutation that leads to carcinogenesis. Therefore, a modified CRISPR/Cas9 system has been developed to perform base editing. The modified system involves a linkage of catalytically inactivated Cas9 (dCas9) to a nucleoside deaminase enzyme (editor) to perform base editing via the gRNA (Tan, 2022). The cytidine base editor converts base C to T, while the adenine base editor converts base A to G (Antoniou et al., 2021). Thus, base editing allows the precise and predictable modification of the genome without DSB introduction (Tan, 2022). With this technique, undesirable mutation can be prevented while maintaining the gene editing function of CRISPR. It has been suggested that more than half of genetic diseases are caused by single point mutation, and restoring these mutations would potentially reverse the pathogenic effects and cure the disease (Rees and Liu, 2018). Given the importance of base editing screens for identifying and fixing mutations, this serves as an important area for further exploration. Currently, base editing screens have been performed in bacteria, yeast and mammalian cells (Liu, 2022; Schubert, 2022; Kweon, 2020). Certain base editing screens have identified single nucleotide variants of genes that are related to drug sensitivity or related to DNA damage response in multiple normal and cancerous cell lines (Hanna, 2021; Cuella-Martin, 2021). Furthermore, it has also been elucidated that base editor screens of *BRCA1 DNA repair associated* (*BRCA1*) and *BRCA2* (breast cancer associated genes) revealed more than 900 loss-of-function variants that can contribute to clinical diagnosis and treatment (Huang, 2021). Another base editor screen of *Janus kinase 1* (*JAK1*) in colorectal cancer cell lines also identified variants that affect the IFNγ pathway (Coelho, et al., 2022). The base editor screen allows for studying single nucleotide variants, thus it can be used as a follow-up screen for a particular gene that is identified in a CRISPR/Cas9 screen. The combination of these two techniques offers the whole picture of the gene function and allows in-depth study of the unknown variants observed in the clinic.

In the conventional CRISPR/Cas9 screens, the gRNA library itself also possesses a limitation. As summarized in Table 1 and Table 2, these libraries target only a limited number of genes in the genome, with a maximum of 21,601 and 20,121 in mouse and human respectively. Currently, nearly 50,000 genes have been identified in mouse and more than 60,000 genes are present in the human genome (Bult, 2019; Nurk, 2022). In light of this, the current libraries are unable to target all genes, and such omissions are inadequate to uncover the gene functions. It is worth to note that screening with a given library containing a high number of targeted genes might potentially lower the screen accuracy due to a reduction in gRNA coverage. Thus, a secondary screen may be required to further narrow down the high hit genes from the first screen and produce high-confidence results. Moreover, the non-coding DNA parts of the genome should also be evaluated as they can be transcribed into non-coding RNAs (ncRNAs) which affects different cellular functions and carcinogenesis. For instance, ncRNAs (miR-21, miR-115, miR-29) have been shown to involve in regulating cell proliferation and apoptosis in liver cancer and other cancers (Le, 2021). There are currently a few CRISPR/Cas9 studies that screen for long non-coding RNAs (lncRNAs) in various human cancer cell types (Zhu, 2016; Liu, 2018; Arnan, 2022), and several studies utilized libraries that target genes controlling epigenetic regulation to study cardiomyocyte maturation, lung cancer or breast cancer (Li, 2020; VanDusen, 2021; Li, 2020; Yedier-Bayram, et al., 2021). Considering the vast number of studies associated with protein-coding genes, the lncRNAs screen is rather insufficient. Therefore, it is important to construct more library that targets non-coding DNA as well. Additionally, a truncated gRNA library can also be constructed to achieve epigenetic regulation with wild-type Cas9. It is reported that a truncated gRNA of 14-nucleotide long can target the promoter or the downstream locations of the GOI to activate or block the gene transcription respectively (Vora, 2016). Other than using the conventional Cas9 for screening, researchers can also employ dCas9 to accomplish epigenetic CRISPR screens. When the dCas9 is fused with *Kruppel-associated box* (*KRAB*), *Lysine-specific demethylase 1* (*LSD1*) or *DNA methyltransferase* (*DNMT3a*), this CRISPRi system would repress gene expression (Vora, 2016; Lo and Qi, 2017). In contrast, fusion with *E1A-associated protein p300* (*EP300*) or *Tet methylcytosine dioxygenase 1* (*TET1*) would transform the dCas9 system functions to CRISPRa system which functions as enhancing gene expression (Vora, 2016; Lo and Qi, 2017). Therefore, with appropriate modification of the CRISPR/Cas9 system, the epigenetic regulation study is also feasible.

Another concern about the CRISPR/Cas9 screens is the choice of the validation model. The candidate genes identified from the screens can be validated *in vitro* and/or *in vivo*, but there are concerns about whether mouse models are suitable for validating human genes. Indeed, most of the time the mouse serves as an appropriate validation model of human genes due to the genetic conservation between the two species, and the similar cancer development process. Studies have shown that human and mice have approximately 30,000 protein-coding genes, with gene functions highly conserved and the amino acid sequences of orthologous genes being highly similar (Monaco, 2015; Emes, 2003). It has also been confirmed that the orthologous genes show very similar expression patterns within the two species, especially in the liver, muscle, and nerve cells (Monaco, 2015). Based on these similarities, the gene functions validated in mice are usually translational to humans. For studies that exclusively use mice as the screening and validation model, 91.7% of the validated candidate genes can correlate to human HCC development (Table 11). Amongst these genes, *AHR* and *PI3KR1* were also identified in human with therapeutic drugs available for disease treatment (O'Donnell, 2010; Ye, 2019). Therefore, genes that were identified and validated in mice could also be translated to human. It is worth to note that some screens performed with human cell lines also utilized mice as the validation model (Table 11). This is because the development of cancer is closely related to the tumour microenvironment and immune interactions which can only be provided by an *in vivo* model. Nevertheless, validation in mice is only suitable for gene function confirmation. For the single nucleotide variants found by the base editor screens, *in vitro* validation would be more appropriate to identify the difference between each variant.

The screening model plays an important role in the CRISPR/Cas9 screens and should be chosen with cautions. CRISPR/Cas9 screens can be performed with immortalized primary hepatocytes or liver cancer cell lines. Example of immortalized primary hepatocytes include Fa2N-4, HepLi5, HepLL, HepZ, HHE6E7T-1/2, HHL-5/7/16, IHH-A5, THLE and TPH1. The HHLs are one of the commonly used cell lines that express CYP450 and allow the binding with HCV (Ramboer, 2015). Therefore, it would be useful for screens related to drug response and hepatitis virus infection. In contrast, the Fa2N-4 displays a lower expression of constitutive androstane receptors and drug-metabolizing enzymes, thus it may not be suitable for drug-related studies (Ramboer, 2015). The genetic backgrounds of the liver cancer cell lines are more complicated than the immortalized primary hepatocytes. Liver cancer cells are categorized into distinctive differentiation statuses: hepatoblast-like, mixed epithelial-mesenchymal-like, and mesenchymal-like cells (Caruso, 2019). More than 30 common liver cancer cell lines present with a unique combination of genetic mutations. The well-differentiated liver cancer cell lines, such as HepG2, Hep3B and Huh7, express *Alpha fetoprotein* (*AFP*), *E-cadherin* (*CDH1*) and *HNF1 Homeobox A* (*HNF-1α*) with low metastatic properties (Yuzugullu, 2009). For the poorly-differentiated liver cancer cell lines, such as the SNUs, express different mesenchymal markers and thus, are suitable for studying late-stage HCC and metastasis (Yuzugullu, 2009). It has been revealed that more than 70% of the liver cancer cell lines show TP53 and/or TERT mutation, including the majority of the JHHs and SNUs (Caruso, 2019). Other frequent mutated genes in liver cancer cell lines include *Axin 1* (*AXIN1*), *Cyclin D1* (*CCND1*), *KMT2D*, *ARID1A*, *Cyclin dependent kinase inhibitor 2D* (*CDKN2D*) (Caruso, 2019). The choice of the cancer cell line as a screening model is mainly based on the aim of the study and should be selecting the cell lines with desired genetic backgrounds. For instance, the Huh 7.5 and Huh 7.5.1 are cell lines

Table 11
Studies that validate the candidate genes in mice.

Study	Screening model	Validation model	Gene identified	Related to human HCC
(Zhu, 2019)	Mouse	Mouse	*Pkd1, Arid1a, Kmt2d*	Y (Ge, 2021; Xiao, 2021; Valiante et al., 2023)
(Zhou, 2020)	Mouse	Human, Mouse	*ADAMTSL3, PTEN*	Y (Zhang, 2021; Shearn and Petersen, 2015)
(Yang, 2021)	Human	Human, Mouse	*WWOX*	Y (Abu-Remaileh, 2018)
(Li, 2021)	Human	Human, Mouse	*MIR-4310*	Y (Tao, 2022)
(Bao, 2021)	Human	Human, Mouse	*PTPMT1*	Unknown
(Wang, 2019)	Human	Human, Mouse	*NCAPG*	Y (Zhang, 2022)
(Song, 2017)	Mouse	Human, Mouse	*NF1, PLXNB1, FLRT2*	Y (Song, 2017)
			B9D1	Unknown
(Kwan, 2020)	Human	Human, Mouse	*TRRAP*	Y (Kwan, 2020)
(Zhu, 2021)	Human	Human, Mouse	*AHR*	Y (Hsu, 2017)
(Wang, 2018)	Mouse	Mouse	*Cic, Pik3r1, Pten, Stk11, Arid2, Kdm5c, B2m*	Y (Shearn and Petersen, 2015; Kim, 2018; Liu, 2022; Kim, 2004; Jiang, et al., 2020; Ji, 2015; Malaguarnera, 2000)
			Kansl1	Unknown
(Wang, 2020)	Mouse	Mouse	*Kmt2d*	Y (Valiante et al., 2023)

that allow hepatitis viral replication. Therefore, few research groups have chosen these cell lines to study the pathogenesis of HCC caused by hepatitis viral infection (Ren, 2015; Liang, 2021; Kulsuptrakul, 2021; Kawamoto, 2020). As a late-stage HCC metastatic cell line, the MHCC97L has been utilized to investigate the metastasis and mechanism of drug resistance (Wei, 2019; Xu, 2021; Yang, 2021). Therefore, screening should be conducted with the most appropriate cell line based on the cell background and research needs.

Future directions of CRISPR/Cas9 screens in HCC carcinogenesis mechanism and therapy application

From the discovery of CRISPR in prokaryotes to the application of the CRISPR/Cas9 system for cancer-related gene screening, encouraging and promising results were observed in unravelling the mechanism of HCC development. The gRNA readout identified from the CRISPR/Cas9 screens mainly resulted from bulk samples instead of single cells (Shalem, 2014). Those results demonstrate certain cellular functions with respect to a specific gene, it is however unable to unmask the difference among individual cells, which is important in understanding HCC carcinogenesis thoroughly.

To provide a comprehensive picture on HCC mechanisms, CRISPR/Cas9 screen can be combined with single-cell sequencing. Instead of the conventional DNA Illumina sequencing on bulk samples, the combination of those two techniques could provide an analysis with higher resolution (Kuhn et al., 2021). Studies have attempted to overcome the obstacles of the combination technique, such as the mRNA/gRNA isolation and analyzation methods (Kuhn et al., 2021; Replogle, 2020). Single-cell sequencing enables the identification of genotype-to-phenotype relationships and gene-gene interactions in cancer cells or between cancer cells and normal cells (Brunello, 2022; Li, 2022; Hoffmann, 2011). As a result, the combination of the two techniques could simultaneously analyze the genomic alteration at DNA level and gene expression at RNA level (Jaitin, 2016). The combination of the CRISPR/Cas9 screen and single-cell sequencing is also capable of acting as a molecular labeling of individual cancer cells, allowing the identification of cell-cell communication and investigation of immune surveillance escape mechanisms. At present, only a few studies utilized this combination method, including proof-of-concept studies (Replogle, 2020; Datlinger, 2017), immune system function examinations (Shifrut, 2018; Jaitin, 2016; Dixit, 2016), cancer researches (Pierce et al., 2021; Yang, 2021), enhancer study (Xie, 2017) and functional genomic research (Adamson, 2016). The feasibility of CRISPR/Cas9 screen analyzed with single-cell sequencing in cancer research has offered promising results, further deepening our understanding of cancer cell epigenetics and transcription regulation (Pierce et al., 2021; Yang, 2021). Furthermore, this combined technique demonstrated excellent compatibility with both mouse and human cell lines (ten Hacken, 2020; Kim, 2021). In terms of *in vivo* applicability, to our best knowledge, it has only been applied in an autism spectrum disorder study to understand the changes in cellular pathways (Jin, 2020). With this regard, additional efforts are warranted to investigate the technological capability of the combined technique in HCC studies.

Recently, machine learning (ML) is an emerging field of biological study, where algorithms with artificial intelligence are trained to analyze big data (including sequencing data, and patient data) to assist downstream validation and develop personalized medication (Greener, 2022; Reel, 2021). Instead of modifying the cells with the CRISPR/Cas9 system, the ML algorithm simply requires the input of the HCC clinical data and CRISPR gRNA library to achieve screening. Given the simplicity of this approach, the ML has identified *Phenylalanyl-TRNA synthetase subunit beta* (*FARSB*), *NOP58 ribonucleoprotein* (*NOP58*), *Chaperonin containing TCP1 subunit 4* (*CCT4*), *DEAH-Box helicase 37* (*DHX37*) and *Tyrosyl-TRNA synthetase 1* (*YARS*) as genes that associated with drug sensitivity and proliferation (Liu, 2022).

Currently, ML in CRISPR/Cas9 system mainly focuses on utilizing ML power to identify the on- and off-target of the gRNAs. The ML algorithms have been employed for the prediction of on- and off-target effects of the gRNA include CRISTA, CRISPRater, CRISPRpred, Cripr2vec, FlashFry and more (Abadi, 2017; Labuhn, 2018; Rahman and Rahman, 2017; Trivedi, et al., 2020; McKenna and Shendure, 2018). To our best knowledge, there is no study utilizing ML power to analyze the CRISPR/Cas9 screen sequencing results for validation. However, ML has already been used in assisting the analysis of single-cell sequencing and RNA sequencing results. One study inputted the RNA sequencing results from a GSE dataset into the ML algorithm “Weka 3” for differentially expressed genes (DEGs) analysis (Wang, 2018). The algorithm was able to identify more accurate DEGs, and the results were successfully validated using real-time quantitative PCR (Wang, 2018). This study demonstrated that ML could improve the accuracy and sensitivity of DEG analysis compared to conventional analysis methods. Conventional analysis methods rely on fold change, false discovery rate and p-value, which involve transcript quantification, normalization, and statistical methods during the analysis process. However, these methods could lead to false-positive or false-negative results, and these unwanted outcomes can be avoided by using ML (Wang, 2018). Another study demonstrated that novel cells can be identified from the single-cell RNA sequencing data by the ML algorithm (Li, 2022). Furthermore, marker

gene expression combination and protein expression can also be predicted from the single-cell RNA sequencing results by NS-Forest v2.0 and sciPENN respectively (Aevermann, 2021; Lakkis, 2022). In light of this, we believe that the CRISPR/Cas9 screen sequencing data can also be inputted into a well-trained ML algorithm for analysis to save time and increase accuracy by minimizing human error. To develop a high-quality ML algorithm for CRISPR/Cas9 screen analysis, a large amount of raw sequencing data and metadata (data of analysis performed by ML on the raw sequencing data) can be fed into the ML algorithm to train and improve its accuracy. Raw sequencing data to be fed into the ML algorithm for training can be obtained from online databases and publicly available datasets, such as the Gene Expression Omnibus (GEO), The Cancer Genome Atlas (TCGA), Genomics Evidence Neoplasia Information Exchange (GENIE) and Mendeley repository (Creighton, 2018; Kaur, 2019; Rukhsar, 2022). Indeed, applying ML to single-cell sequencing data analysis would be a major trend in the future. Cell type identification is the fundamental purpose of single-cell sequencing for studying tissue functions and pathogenesis mechanisms. ML enables cell type identification in a highly efficient approach compared to using manpower alone, where manual analysis of individual cells is extremely difficult and time-consuming (Liu, 2021). The clustering or neural networks ML algorithms are capable to identify the similarity between cell clusters for cell type annotation (Liu, 2021). With adequate training and improvement, the ML could potentially offer a high degree of accuracy for CRISPR/Cas9 screen analysis in HCC pathogenesis and disease progression.

The recent pandemic of COVID-19 also negatively impacted the health status of HCC patients due to the ability of the COVID-19 virus to derange the gastrointestinal systems (Sharma, 2021). The pandemic not only hampers the early HCC screening process and treatment opportunity, but the infection also could worsen the health status of HCC patients in the long term (Mehta, 2021; Amaddeo, 2021). Moreover, it is also unknown whether long-COVID could further complexify the genetic context of HCC patients. Based on the statistical data, 15%-55% of infected person shows liver enzyme abnormities (Sharma, 2021). As a result, there is a need to examine the HCC patient's status and the molecular change in HCC cells in order to determine the impact of COVID-19 on HCC patients. The CRISPR/Cas9 technology can be applied to screen for the involved genes that further impair the genetic integrity of the HCC cells. Comparison can be made between the sequencing profiles of the COVID-19 infected and non-infected HCC cells to study its mechanism in HCC progression.

Apart from mutations in tumour cells, the immune landscape of HCC patients also affects the treatment outcome. There is a complex interaction between the tumour mutations and the immune environment, such as *Catenin beta 1* (*CTNNB1*) mutation is observed with lower levels of leukocytes (Woller, 2021). With the recent development in immunotherapy to battle cancer cells, the ability of the immune cells to precisely target tumour cells is of research interest. Therefore, the chimeric antigen receptor (CAR) T-cell (CAR-T) therapy become one of the popular immunotherapies involving gene editing of T cells for HCC treatment. Before such targeting can be achieved, the mechanism of T cell activation for recognizing tumour HCC cells must be thoroughly reviewed. Currently, the CRISPR/Cas9 was mainly applied as direct gene editing in T cell engineering (Rupp, 2017; Choi, 2019; Ren, 2017; Zhang, 2017; Jung, 2018; Tang, 2020). The high throughput CRISPR/Cas9 screen can be applied to mutate the T cells and screen out activated T cell genes with enhanced recognition ability for HCC treatment. This technique demonstrated its potential as a tool for T cells gene editing to enhance the efficacy of CAR-T therapy in HCC treatment. However, studies in this area are rather inadequate with only one study associated with glioblastoma can be found (Wang, 2021). Therefore, this area warrants further investigation.

The application of the CRISPR/Cas9 system in the current clinical trial for cancer treatment involves indirect therapy of T cells/immune cells genome editing. The direct utility of CRISPR in clinical settings is still under evaluation as the long-term side effects remain unclear (Janik, 2020). Moreover, the different genomic content of individual tumour cells contributes significantly to tumour heterogeneity, which serves as another barrier to CRISPR/Cas9 application in gene therapy (Chen, 2019). The true potential of CRISPR/Cas9 system in gene therapy can be unleashed only when the above-mentioned problems have been fully addressed. Nevertheless, with continuous improvement of the CRISPR/Cas9 system and an in-depth understanding of HCC development, the application of CRISPR/Cas9 system in HCC gene therapy of HCC shall be expected in due time.

Currently, systemic therapy with multi-receptor tyrosine kinase inhibitor or immune checkpoint inhibitor is the only treatment strategy for late-stage HCC (Zhang, 2022). However, lenvatinib, the most effective multi-receptor tyrosine kinase inhibitor for late-stage HCC, is capable of extending patients' survival for only 1 year with response rate of 24.1% (Kudo, 2018). Therefore, a combination treatment of lenvatinib and EGFR inhibitor was adopted to enhance the response rate up to 50% (Jin, 2021). Other studies also showed that combined therapy of Nivolumab with Ipilimumab or Atezolizumab with Bevacizumab could enhance treatment outcomes by increasing the overall response rate and survival rate (El-Khoueiry, 2021; Lee, 2020). Moreover, the continuous advancement in immunotherapy had identified the *B7 homolog 3 protein* (*B7-H3*) as a target for various cancer types including HCC (Kontos, 2021). Despite high expression of B7-H3 in most HCC cases with effective B7-H3 immuno-targeting outcomes, some patients still showed limited response due to the differences in tumour heterogeneity (Kang, 2015). The advancement in combined therapy offers insights into the future therapeutic strategy for cancer treatment. Cancer treatment with cocktail therapy could be the major trend for HCC due to the complex heterogeneity of tumour in HCC patients. Existing drug resistance screens examine either mono-treatment or receptor tyrosine kinase-oriented mechanism, which only partially reveals the resistance mechanism. With the merit and capability of CRISPR/Cas9, drug resistance screens with cocktail therapy (e.g. receptor tyrosine kinase coupled with other therapeutic agents) would better reflect and mimic the clinical conditions. Thus, this could further assist in developing personalized cocktail therapy for HCC patients. However, these studies are rather very limited in HCC. Currently, a study utilized the CRISPR/Cas9 system to screen for genes that are responsible for the non-respondent of the B7-H3 immunotherapy in ovarian cancer. They compared the sequencing profiles between the unsorted CRISPR library mutant cell pool, sorted high B7-H3 expressing (as therapy respondent) cell pool and sorted low B7-H3 expressing (as therapy non-respondent) cell pool (Zhao, 2022). The group identified the difference in expression of *B7-H3* regulators, the *SPT20 Homolog, SAGA Complex Component* (*SUPT20H*) negative regulator, and *eukaryotic translation initiation factor 4E* (*eIF4E*) positive regulator are accountable for the diverse treatment outcome (Zhao, 2022). With this encouraging result, similar studies can also be conducted for HCC.

In light of this, downstream personalized cocktail therapy can be customized for individual HCC patients. Personalized cocktail therapy is a major trend and strategy in to maximize treatment outcomes in future cancer treatment. It allows the targeting of complex genetic background of individual tumour cells that hampers the treatment efficacy. In HCC, microRNA cocktail therapy has been proposed as a treatment approach based on the specific mutation that the patients uniquely possessed (Shao, 2020). Likewise, cocktail therapy for individual HCC patients can be developed by in-depth study of the genomic content in each patient with CRISPR/Cas9. The ML can also be incorporated into the analysis to aid the investigation process for choosing the most appropriate cocktail therapy for the patients.

Conclusion

This review consolidated the recent CRISPR/Cas9 studies related to HCC development and compared the existing gRNA libraries and gRNA

design platforms. The CRISPR/Cas9 system is a versatile and effective system for gene editing and has been used extensively in genetic screens. It allows identification and validation of the HCC gene functions and carcinogenesis mechanisms. Over the years, improvement has been made to this system to extend its application to a wider range of screens, such as base editor screens and epigenetic screens. The discovery of molecular mechanisms and genes involved in HCC through CRISPR/Cas9 screen would enable alternative paths for HCC treatment in clinical settings. The maturation of CRISPR/Cas9 system has shed light on the possibility in gene therapy to combat various cancer types. Furthermore, the capability of CRISPR/Cas9 can also be magnified with other powerful techniques, such as single-cell sequencing and ML, to investigate and treat HCC. Overall, the CRISPR/Cas9 system stands out as a promising tool for effective gene therapies in the future given that the off-target and other drawbacks have been fully addressed.

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The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

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