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REVIEW

Will CRISPR-Cas9 Have Cards to Play Against Cancer? An Update on its Applications

Precilla S. Daisy[1] · Kuduvalli S. Shreyas[1] · T. S. Anitha[1]

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Abstract

Genome editing employs targeted nucleases as powerful tools to precisely alter the genome of target cells and regulate functional genes. Various strategies have been risen so far as the molecular scissors-mediated genome editing that includes zinc finger nuclease, transcription activator-like effector nucleases, and clustered regularly interspaced short palindromic repeats—CRISPR-related protein 9. These tools allow researchers to understand the basics of manipulating the genome, create animal models to study human diseases, understand host–pathogen interactions and design disease targets. Targeted genome modification utilizing RNA-guided nucleases are of recent curiosity, as it is a fast and effective strategy that enables the researchers to manipulate the gene of interest, carry out functional studies, understand the molecular basis of the disease and design targeted therapies. CRISPR-Cas9, a bacterial defense system employed against viruses, consists of a single-strand RNA-guided Cas9 nuclease connected to the corresponding complementary target sequence. This powerful and versatile tool has gained tremendous attention among the researchers, owing to its ability to correct genetic disorders. To help illustrate the potential of this gene editor in unexplored corners of oncology, we describe the history of CRISPR-Cas9, its rapid progression in cancer research as well as future perspectives.

Keywords CRISPR-Cas9 · Genome editing · Glioblastoma multiforme · gRNA · Oncological research

Introduction

Genome/gene editing involves modification of genes in an organism using enzymes specific to the target gene sequence. This technique deals with grouping of advancements that enable researchers to change a living organism's DNA [1]. These innovations permit hereditary material to be either included, expelled, or changed at specific areas in the genome [2]. A few ways to deal with genome altering include zinc finger nuclease, transcription activator-like effector nucleases, etc. A recent interest of most of the researchers is CRISPR-Cas9 (clustered regularly interspaced short palindromic repeats and CRISPR associated protein-9), discovered in the year 2012 by Jennifer Doudna and Emmanuelle Charpentier, University of California Berkeley and University of Vienna [3]. The CRISPR-Cas9 framework has produced a great curiosity among researchers, since they are quicker, less expensive, increasingly precise, and more effective than other existing genome altering strategies [4].

As of now, based on the CRISPR database, CRISPR structures were found to be present in 202 (870 CRISPR sequences were found) out of 232 archaeal genomes analyzed and 3059 (8069 CRISPR sequences were found) out of 6782 bacterial genomes analyzed (https://crispr.i2bc.paris-saclay.fr/). Till date, CRISPR has provided a clear idea on how a gene product contributes to development as well as disease in an organism [5]. To demonstrate "what actually CRISPR is" and "the steps towards fulfilling the genome editor's potential in cancer treatment" we include in this review, the emerging oncology-based applications of CRISPR, their role in cancer diagnosis and the future impact of this tool in medicine and diagnostics.

✉ T. S. Anitha
tsanitha6@gmail.com

[1] Central Inter-Disciplinary Research Facility, Sri Balaji Vidyapeeth (Deemed To-Be University), Mahatma Gandhi Medical College and Research Institute Campus, Pillaiyarkuppam, Puducherry 607403, India

A Crispy History of CRISPR Technology

Initially, CRISPR was identified 30 years ago in *Escherichia coli* during the investigation of the gene responsible for isozyme conversion of alkaline phosphatase [6] (Fig. 1). At that point, it was not really conceivable to anticipate the function of these repeated sequences because of the absence of adequate DNA sequencing data. The genuine capacity of this sequence stayed mysterious until the mid-2000s. In 1993, CRISPRs were seen for the first time in Achaea, explicitly in *Haloferax mediterranei* [7], and, was further identified in an expanding number of bacterial and archaeal genomes. In the mid-2000s, the revelation of sequence similarity between the spacer areas of CRISPRs with the sequences of bacteriophages, archaea, and plasmids shed light on the exempting function of CRISPR as an immune system. Alongside, in 2002 certain genes that were proposed to encode DNA repair proteins for hyper thermophilic archaea were recognized to be closely associated with CRISPR and were assigned Cas (CRISPR-Associated) genes [8]. Relative genomic studies in this way recommended that CRISPR and Cas proteins work together, thereby comprising a putative acquired immunity framework to secure prokaryotic cells against attacking infections and plasmids, similar to the eukaryotic RNA interference (RNAi) framework [9].

The capacity of the CRISPR-Cas framework as a prokaryotic acquired immunity framework was at last tentatively demonstrated in 2007, employing the lactic acid bacterium *Streptococcus thermophilus* [10]. Inclusion of the phage sequence into the spacer portion of the CRISPR of *S. thermophilus* made this strain impervious to the corresponding phage. Nevertheless, this bacterial resistance to the phage vanished when the protospacer sequence was deleted from the phage genome. Furthermore, the CRISPR-Cas sequence of *S. thermophilus* expressed in *E. coli* demonstrated heterologous protection against plasmid transformation and provided immunity against phage infections [11]. The CRISPR-Cas9 sequence of *Streptococcus pyogenes* was then employed to carry out genome editing in human nerve cells and kidney cells of mouse [12, 13]. Consequently, CRISPR-Cas tool was widely referred to as an acquired immunity system in prokaryotes.

Process of CRISPR-Cas Mediated Acquired Immune System

CRISPR-Cas mediated-acquired immunity basically involves three main stages namely, adaptation, expression and interference [10]. During the adaptation stage, the invading DNA from a virus/phage will be recognized by the Cas proteins, followed by fragmentation and incorporation of the fragments into the spacer region of CRISPR. Transcription of the modified CRISPR region occurs at the expression stage resulting in pre-CRISPR RNA (pre-crRNA), which is further processed into smaller units, producing crRNA through the action of Cas- endonucleases. At the interference stage, by exploiting the homology of the spacer arrangement present in crRNA, the foreign DNA is caught, and a complex with Cas protein having nuclease activity degrades the exogenous DNA (Fig. 2) [14].

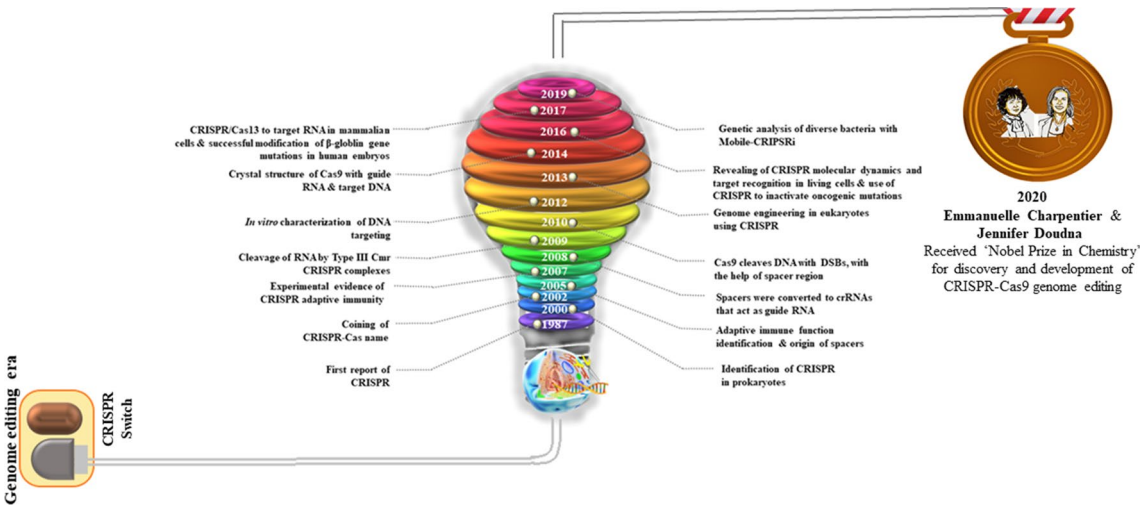

Fig. 1 Timeline of CRISPR-Cas9 technology

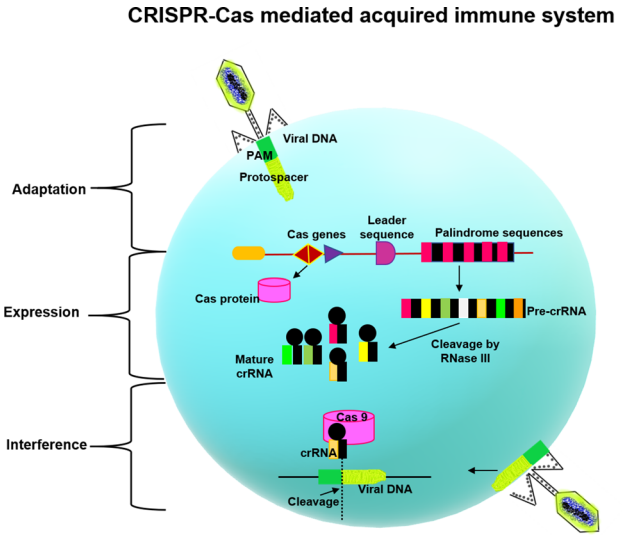

Fig. 2 CRISPR-Cas mediated acquired immune system

Components of CRISPR-Cas9

The CRISPR-Cas9 framework comprises two key components that introduces a change into the target DNA: The protein called Cas9, goes about as 'molecular scissors', that can cut the two strands of DNA at a particular region in the genome and a small sequence of RNA called guide RNA (gRNA), a pre-designed RNA succession (around 20 bases complementary to the target DNA) situated inside a long RNA framework [15]. The gRNA is intended to discover and bind to a particular sequence in the DNA. The enzyme, Cas9 follows the gRNA to the similar area and makes a cut across the two strands of the DNA [15]. The only prerequisite for proper binding of Cas-gRNA complex is the Protospacer adjacent motif (PAM). The PAM motif is recognized by the PAM binding domain of the Cas9 protein, thereby creating a double stranded break on both the DNA strands [16]. At this stage, the cell identifies the damaged DNA and attempts to fix it, either by non-homology end joining (NHEJ) or homology-directed repair (HDR) [16]. Among these two methods, NHEJ often leads to insertion or deletion mutations during repair and is often considered to be an error-prone repair mechanism [17]. In contrast to this strategy, HDR, during the process of reconstruction of cleaved DNA, makes use of the donor DNA template to introduce mutations [18]. Researchers therefore utilize the HDR mechanism to bring about changes within one or more genes in the genome of a cell of interest for precise editing. To design sgRNA libraries and to investigate pooled CRISPR-Cas9 screens, several web-based and bioinformatics resources have been created. Some of the frequently employed tools are listed (Table 1).

Table 1 Tools for designing sgRNA libraries

S.No	Name of the software	Nuclease	URL
1	CRISPR. mit [137]	SpCas9	http://crispr.mit.edu/
2	E-CRISP [138]	Custom PAM & Cas9	http://www.e-crisp.org/E-CRISP/
3	CRISPRscan [139]	SpCas9, AsCpf1, LbCpf1	http://www.crisprscan.org/
4	Protospacer workbench [140]	SpCaS9	www.protospacer.com
5	GuideScan [141]	SpCas9, AsCpf1, LbCpf1	http://www.guidescan.com/
6	CASPER [142]	Custom PAM	https://github.com/TrinhLab/CASPER
7	CLD [143]	SpCas9	https://github.com/boutroslab/cld
8	CROP-IT [144]	SpCas9	http://cheetah.bioch.virginia.edu/AdliLab/ CROP-IT/homepage.html
9	SgRNA Scorer 1.0 [145]	StCas9, SpCas9	https://crispr.med.harvard.edu/sgRNAScorerV1/
10	SgRNA Scorer 2.0 [146]	Custom PAM	https://crispr.med.harvard.edu/sgRNAScorerV2/
11	SSC [147]	SpCas9	http://cistrome.org/SSC/
12	WU-CRISPR [148]	SpCas9	http://crispr.wustl.edu
13	CHOPCHOP [149, 150]	Custom PAM	https://chopchop.rc.fas.harvard.edu/
14	Cas-OFFinder [151]	PAM	http://www.rgenome.net/cas-offinder/
15	CCTop [152]	PAM	https://crispr.cos.uniheidelberg.de/index.html
16	CRISPRdirect [153]	Custom PAM	http://crispr.dbcls.jp/
17	CRISPR MultiTargeter [154]	Custom PAM, SpCas9	http://www.multicrispr.net/
18	CRISPOR [155]	PAM	http://crispor.tefor.net/
19	sgRNA Designer [156]	SpCas9	https://portals.broadinstitute.org/gpp/public /analysis-tools/sgrna-design
20	CRISPRseek [157]	No	http://www.bioconductor.org/packages/rele ase/bioc/html/CRISPRseek.html (Local R Package)

Unique Advantages of CRISPR in Cancer Therapy

It is well known that cancer is a complex disease and despites decades of research the statistics of cancer-related deaths is not spiking down. This unmet scientific need urges more effective and efficient therapeutic strategies. In addition to being a powerful research tool in cell and gene therapy, CRIPSR-Cas9 technology holds trump card in cancer treatment as well (Fig. 3). One possible attribute might be due to the regulation of CRIPSR-Cas9 on endogenous gene expression. As stated above, the gRNAs can recruit the inactive Cas9 protein to target specific DNA sites [19]. In the bound state, transcriptional inhibition or activation domains can be used to either inhibit or activate the expression of target genes in tumor cells [20]. Besides, in several cancers such as Ewing sarcoma, acute myeloblastic leukemia and tumors like Glioblastoma Multiforme (GBM), the epigenetic factors play a predominant role in cancer progression [21]. Hence by targeting the epigenetic machinery and its regulatory components, it would be plausible to deregulate cancer growth. This stratagem is also possible with CRISPR-Cas9, whereby the Cas9 protein can be tethered to either histone modifiers or other DNA methylation regulatory proteins to perform the epic 'epigenome editing' [22]. Nevertheless, CRISPR-Cas9 tools can also be employed to target the tumor makers in cancer cells directly, thereby offering an opportunity to eliminate tumor proliferation and invasion [23]. Also, in order to test the efficacy of drugs for optimizing the chemotherapy regimen in cancer treatment, in vitro and in vivo animal models that recapitulate the actual tumor microenvironment in patient setting is an obligatory requirement [24, 25]. Obviously, construction of such models would be expensive and time consuming. The advent of CRISPR-Cas9 forefronts has made this tedious job quite simpler and cheaper than the standard protocols. The best example to illustrate the essential role played by CRISPR-Cas9 technology in drug discovery process in oncology would be in ovarian cancer mouse model ID8, whereby CRIPSR-Cas9 mediated knockout of BRCA2 and TP53 genes, increased their sensitivity to the PARP inhibitor, rucaparib [26].

As such, this tool offers many advantages over the traditional TALENs and ZFNs. These include easy prediction of off-target sites, precise targeting of multiple genome sites simultaneously and easy design of genomic targets. Besides, CRISPR has shown promising results in targeting epigenetic changes occurring during carcinogenesis, which was not possible by the previously discovered ZFNs and TALENs [27].

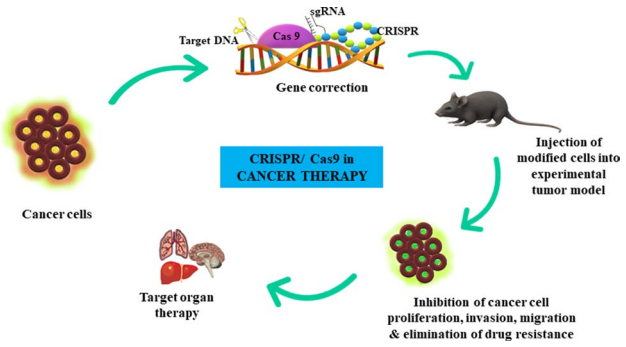

Fig. 3 CRISPR/Cas9 in cancer therapy

Applications of CRISPR in Cancer Research

CRISPR in Glioblastoma Multiforme (GBM)

GBM, a heterogenous primary brain tumor is often characterized by dismal prognosis emphasizing an unmet need for its treatment [28]. GBM is characterized by overexpression, dysregulation or activation of various membrane receptors such as epidermal growth factor receptor (EGFR), vascular endothelial growth factor (VEGF), mammalian target of rapamycin (mTOR), platelet derived growth factor receptor (PDGFR), protein kinase inhibitors, etc. [29]. Owing to the novel insights provided by CRISPR-Cas9 technology in various cancers, quest to determine its efficacy on alleviating GBM pathogenesis has now gained immense clinical importance. Huang et al. has recently employed CRISPR-Cas9 tool to knock out specifically EGFR exon 17 in glioma cells. Interestingly, this CRIPSR-mediated knockout increased the expression of UBX domain containing protein-1 (UBXN1), a suppressor of Nuclear Factor-ƙβ (NFƙβ) leading to the inhibition of glioma cell growth [30].

T-cell immune therapy often serves as an alternative to conventional treatment strategy, in the treatment of solid tumors [31]. Chimeric Antigen Receptor (CAR) is one of the most promising platforms in T-cell-based therapies [32]. However, till date CAR-T cells have not been that successful in GBM [33]. Surface expression of programmed cell death ligand 1 (PD-1) is one of the mechanisms, by which GBM cells hamper CAR-T cells. Hence, an attempt was made by Choi et al., employing CRISPR-Cas9 to knockout beta-2-microglobulin (B2M), PD1 and endogenous T-cell receptor (TRAC). The triple-gene edited version of CAR-T cells exhibited enhanced activity than their normal counterparts in a preclinical intracranial xenograft animal model [34]. It has been reported previously that in most of the GBM cases, the vascular basement protein called laminin-411(α4β1γ1) is over-expressed in grade IV gliomas, whereas their expression is comparatively lesser in the lower grades of gliomas [35]. Hence, laminin-411 is considered to be a progression related biomarker in glioma. Employing CRISPR-Cas9 tool,

the α4 and β1 chains were knocked down in laminin-411 by Sun et al., in an intracranial mice model. The results showed a significant decrease in tumor growth and volume [35]. Besides, another study by MacLeod et al. showed that genome-wide CRISPR-Cas9 screening was able to assess the genes responsible for modulating the sensitivity of glioma stem cells to chemotherapy. Herein, it was found that the members of SOX family namely, SOCS3, DOT1L and USPS played a major role in GSCs growth and targeting them might improve GBM treatment chemo-resistance as well [36]. In a similar study, large-scale CRISPR-Cas9 loss of function screening of 4,574 genes responsible for membrane trafficking and invasion of GBM was performed. Among the genes studied, the strongest regulator of GBM invasion was found to be MAP4K4. Concomitant targeting of MAP4K4 employing sgRNAs in U138 human GBM resulted in decreased invasion and migration of the cells which insists that MAP4K4 would be a potential target candidate to limit GBM invasion [37]. The RecQ family of DNA helicases plays an important role in DNA replication and recombination [38]. Mutations in these genes result in pre-mature aging and cancer predisposition [39]. Previous studies on GBM pathogenesis have stated that RecQL4 is significantly upregulated both at mRNA and protein levels when compared with normal astrocytes [40]. CRISPR-Cas9 mediated knockout of RecQL4 in LN-18 and U87MG human glioma cells resulted in decreased proliferation and impaired cell viability thereby providing rationale for further elucidation of RecQL4 role in GBM pathogenesis [40]. Therefore, advancing CRISPR against GBM would possibly open up a new therapeutic avenue to combat glioma pathogenesis.

CRISPR in Lung Cancer

Lung cancer, with high morbidity and mortality rate, ranks first among all the malignancies in developed and developing countries such as India, Europe, China and the USA [41]. Various tumor suppressor genes associated with lung cancer include, tumor suppressor gene TP53 [42], Adenomatous polyposis coli (APC) [43], Bromodomain containing 7 (BRD7) [44], Cyclin Dependent Kinase Inhibitor 2A (CDKN2A) [45] and NM23 [46]. Proto-oncogenes constituting lung cancer are receptor tyrosine kinase (RTK) [47], MYC [48], JUN [49], FOS [50], RAF1 [51] and ERBB1&2 [52]. Mutations in tumor suppressor genes and overexpression of proto-oncogenes promoted the development of lung cancer [53]. The robust nature of CRIPSR-Cas9 in editing the genome of a cell was exploited in tumor gene therapy to knockout oncogenes which are mutated or overexpressed such as EGFR [54], NESTIN [55], Insulin-like growth factor 1 factor (IGF1R) [56] and remodeling and spacing factor 1 (RSF1) [57]. Knockout of the oncogenic mutant EGFR allele, using CRISPR-Cas9 repressed the proliferation and growth of lung cancer growth cell lines A549, H1975, and H1650 [58, 59]. In Kirsen Rat Sarcoma viral oncogene homolog (KRAS) mutant, non-small cell lung cancer cells (NSCLC), tenacious DNA damage and increased sensitivity to radio-therapy was observed after knockout of focal adhesion kinase (FAK) gene employing CRISPR-Cas9 tool [60]. Furthermore, it was reported by Liu et al., that knockout of NESTIN in A549 and H1299 lung cancer cells enhanced apoptosis, inhibiting the multiplication, colonization, and invasion of the tumor cells by suppressing their transition from epithelium to the mesenchymal area (EMT) [55]. Coming to its role on tumor suppressor genes, CRISPR-Cas9 gene editing technique is being widely employed to target, repair and restore the function of tumor suppressor genes thereby inhibiting tumorigenesis [61]. Among these, knockout of Keap1 in a KRAS- mouse model of lung adenocarcinoma utilizing CRISPR-Cas9 indicated overactivation of Nrf2 leading to glutaminolysis thereby inhibiting tumor progression [62]. Another study by Xu et al., affirmed that genetic knockout of the tumor-suppressor gene mitofusion 2 (MFN2) in the lung cancer cell line A549 promoted survival, cell viability, and cell growth, and invasion by upregulating the mTORC2/Akt signaling pathway [61]. Also, knockout of the tumor-silencer miR-1304 in human lung adenocarcinoma cells, A549 and NCI-H1975 employing CRISPR-Cas9 significantly increased heme oxygenase-1 (HO-1) expression, promoting the growth and survival of the cells [63].

So far, scarcely any investigations have analyzed tumor suppressor genes and proto-oncogenes in lung cancer utilizing the CRISPR-Cas9 tool, and this ought to be considered as a significant heading for future research. Targeted repair and restoration of the role of tumor-suppressors by utilizing CRISPR-Cas9 technology can possibly shed light on lung cancer treatment.

CRISPR in Breast Cancer

Breast cancer is the second most common type of cancer-related death in women, impacting 2.1 million women each year and 1.2 million cases diagnosed in 2018 [64]. Based on the expression of receptors such as Ki-67, estrogen receptor, ERBB2 and progesterone receptor, it can be sub-divided into triple negative breast cancer, luminal A, luminal B and HER2 enriched [65]. Among these, the estrogen positive breast cancers are very common accounting for 70% of breast cancer cases [66]. To improve prognosis and patient's outcome, CRISPR-mediated genome editing was employed to elucidate the molecular markers as targets for breast cancer treatment. Treuren et al., used CRISPR-Cas9 to study the effect of Migration and Invasion Enhancer 1(MIEN 1) knockout in breast cancer cell lines namely MDA-MB-231 and its three metastatic variants MDA-MB-831, 1833 and 4175 [67]. However,

it was reported that CRISPR-mediated gene deletion did not have any effect on the proliferation and survival of breast cancer cells. Mixed lineage kinase-3 (MLK3), a member of MAP3K pathway mediates signal transduction and activation of the ERK pathway [68]. Downregulation of MLK3 in highly metastatic TNBC-4T1 cells using CRISPR-Cas9 technology led to repression of cellular migration and invasion [69]. Liao et al., has reported that deletion of Ubr5, in a murine triple negative breast cancer model suppressed tumor growth and metastasis and promoted apoptosis in those cells [70]. Several functional proteins are also being investigated for their therapeutic potential against breast cancer. One such study focused on the microtubule associated serine/threonine like (MASTL) protein, which was over-expressed in estrogen receptor positive breast cancer, and contributed to poor prognosis and adverse clinical outcomes [71]. CRISPR-mediated knockout of MASTL in breast cancer cell lines reduced the proliferation of the cells and some therapeutic effects were observed in vivo as well [72]. The SRC family kinases, protein tyrosine phosphatase N23 (PTPN23) and FYN also plays a major role in breast cancer [73, 74]. The effect on CRISPR-Cas9 mediated knockout of both FYN and PTPN23 in vitro in Cal-51 (breast cancer cell line) cells and in vivo in an orthotopic xenograft mice model was performed. It was found that CRISPR-mediated knockout attenuated the growth of cancerous cell in both the models. Interestingly, the authors found that FYN was associated with chemoresistance, as it was over-expressed in the resistant cells when compared with their normal counterparts [74].

With regard to RNA-binding proteins, Zheng et al., employed CRISPR technology to screen PHD finger protein 5A (PHF5A), which is ought to be a key factor in breast cancer proliferation and migration [75]. Another similar report by Yang et al., denoted that PHF5A is a novel oncoprotein in lung adenocarcinoma [76]. Also, Hubert et al., has shown that suppression of PHF5A resulted in a significant decrease in GBM tumor growth in a xenograft animal model [77]. Thus, PHF5A would serve as potent therapeutic target, not only in breast cancer, but in other tumors like lung adenocarcinoma and GBM as well.

Recently, non-coding RNAs (ncRNAs) have gained interest in cancer research, and their dysregulation is associated with carcinogenesis [78]. One such study employing ncRNAs was carried out by Singh et al., in MCF-7 cell line and xenograft-induced animal model. It was observed that abrogation of BC-200, a ncRNA which regulates protein synthesis in estrogen receptor positive breast cancer cells, reduced cancer cell growth by over-expression of Bcl-2 [79]. Adding to this, abrogation of another ncRNA, miR-10b in MDA-MB-231 breast cancer cells, suppressed cancer cell migration as well [80]. These data provide insights on the efficacy of targeting ncRNAs for breast cancer treatment.

CRISPR in Liver Cancer

Liver cancer, which is more common in men, is the fourth main cause of cancer related death worldwide, with a 5-year survival rate of 19% [64]. Hepatocellular carcinoma (HCC) and intrahepatic cholangiocarcinoma are the two molecular subtypes of primary liver cancer [81]. Patients with liver cancer are characterized by poor prognosis, frequent recurrence and limited therapeutic options are available currently [82]. Hence, employing CRISPR technology to sort out potential targets would be a promising approach for liver cancer treatment. The oncogenic role of CXCR-4 a chemokine receptor, in promoting HCC proliferation, survival and migration via activation of P13K/Akt and Ras-MAPK is well-known [83]. CRISPR-mediated knockout of CXCR-4 in HepG2 cells resulted in suppression of cancer cell proliferation and migration. Also, CXCR-4 attenuation resulted in reduced tumor size in vivo [84]. Nuclear receptor coactivator (NCOA5) was also studied as a target for liver cancer in an in vitro study employing HCC cells. It was found that knockout of NCOA5 inhibited the migration of cells and suppressed the epithelial to mesenchymal transition (EMT) as well [85]. These aforementioned targets can be considered for elucidation of their potential in vivo or in clinical trials for liver cancer treatment.

CRISPR in Colorectal Cancer

Colorectal cancer (CRC) develops through a series selection of genetic changes. The initiating or gate-keeping mutation is primarily 2-hit mutations in APC, which occur in > 80% of the cases [86]. Subsequent activating mutations in KRAS occur in the early to intermediate adenoma stage, while loss-of-function mutations in SMAD4 and TP53 occur in the intermediate to late adenoma and late adenoma to adenocarcinoma stages, respectively [87, 88]. In addition to the commonly mutated APC, KRAS, mothers against decapentaplegic homolog 4 (SMAD4), and tumor protein 53 (TP53) genes, large-scale genome sequencing studies have identified numerous other genes that are infrequently mutated in CRC [89], revealing extensive inter and intra tumor genetic heterogeneity in cancer tissues. These complications in CRC raise the need for new technologies which can overcome problems of genetic factors. In the recent years, CRISPR gene editing has been employed to overcome the problem of genetic mutation that enhance chemoresistance and tumor progression in CRCs.

Takeda et al., has performed CRISPR-Cas9 screening and validated in organoids derived from intestinal tumors that developed in mice carrying a heterozygous loss-of-function

mutation in APC and an activating mutation in KRAS. They have reported that co-occurrent mutations in receptors for activin and transforming growth factor-β (TGF-β) synergistically promote tumorigenesis, and shed light on the role of activin receptors in CRC which can be targeted for CRC treatment [90]. A study by Li et al. has reported the role of CD133 in colon cancer, established an *in-vitro* model by knocking out CD133 in colon cancer (LoVo) cell line using CRISPR-Cas9 gene editing system. The authors have suggested that knocking out of CD133 contributed to attenuate the abilities of colon cancer cells, including colony formation, cell proliferation, migration, and invasion. Surprisingly, CD133-knockout cells showed remarkable suppression of tumorigenicity of CRC, including cell proliferation and colony formation capacities, migration, and invasion. Therefore, modulation CD133 expression could be an effective treatment for the CD133 + group of patients [91]. Recently, Matano et al. has used the CRISPR-Cas9 genome-editing system to introduce multiple mutations into organoids derived from normal human intestinal epithelium. By modulating the culture conditions to mimic that of the intestinal niche, they selected isogenic organoids harboring mutations in the tumor suppressor genes APC, SMAD4 and TP53, and in the oncogenes KRAS and/or PIK3CA [92]. Their results suggest that multiple driver gene mutations contribute to niche-independent stem cell maintenance but not to metastatic progression of CRC tumors in mice. However, further studies will be required to elucidate the functional contribution of other molecular abrasions, such as copy number variations or epigenetic alterations and/ or aneuploidy in the malignant progression of human CRC in this system.

CRISPR in Pancreatic Cancer

Pancreatic ductal adenocarcinoma (PDAC) accounts for > 85% of all cases of pancreatic cancer. For all the stages combined, the first and fifth years relative survival rates are 28% and 7%, respectively [93]. Though there have been substantial advances in the development of novel treatment strategies such as targeted therapies directed against molecular alterations arising in cancer cells, PDAC still remains a lethal disease [94]. In this context, a study by Christina et al., revealed that, a novel microRNA factor, fork-head box protein A2 (FOXA2), acts as a tumor suppressor gene in pancreatic cancer (PC), affecting PC proliferation and invasiveness. CRISPR-Cas9 mediated knockout of FOXA2 resulted in enhanced tumor growth in a PC xenograft model, thereby highlighting the role of FOXA2 in alleviating PC pathogenesis [95]. Another study by Watanabe et al., clarified the prognostic impact of Lysine-specific demethylase 6A (KDM6A) deficiency on PDAC and the therapeutic potential of this subtype by knocking out KDM6A gene by CRISPR-Cas9. The study demonstrated that KDM6A deficiency is a characteristic of the malignant subtype of PDAC, which contributes to epigenetic inactivation of CDKN1A through the modulation of histone acetylation. Thus, KDM6A expression can be used as a prognostic biomarker in PDACs [96].

CRISPR in Ovarian Cancer

Ovarian cancer (OC) is the fifth most lethal cancer affecting women worldwide with a high incidence of 10–15 out of 100,000 females being affected worldwide [97]. Though most of the patients are diagnosed at an early stage, epithelial ovarian cancer (EOC) remains the most lethal gynaecologic cancer [98]. This is mainly attributed to the fact that nearly 70% of the patients relapse even after receiving optimal cytoreductive surgery (CRS) along with chemotherapy. This leads to a low 5-year survival rate among OC patients [99].

B-cell-specific Moloney murine leukemia virus integration site 1 (BMI1), a main constituent of Polycomb group proteins (PcG), is an important mediator of epigenetic modulation in vital cellular events and has been reported to be present in many human malignancies [100]. In a study by Zhao. Q et. al., the BMI1 gene was knocked out by CRISPR mediated gene editing to identify the molecular traits in EOC in both *in-vitro* ($SKOV_3$) and *in-vivo* (BALB/c mice) models. They reported that the knockout of BMI1, responsible for the regulation of PI3K/ AKT pathway could be a novel prognostic biomarker for EOC [101].

Recent studies have used the CRISPR-Cas9 tool to elucidate synergistic gene knockouts, to overcome drug resistance in various cancers. A study in 2012 by Alan et. al., employed CombiGEM-CRISPR to create a library of 23,409 barcoded g-RNA combinations for EOC. Using these combinations, high-throughput pooled screening was performed to identify the gene pairs that inhibited proliferation of EOC. The study showed that the knockout of dual genes, using CRISPR-cas9 reduced the proliferation in OVCAR8 human PC cells. [102]. This dual knockout of genes can be used as a treatment strategy in targeted gene therapy.

Applications of CRISPR in Other Fields of Medicine

Even before elucidation of their use in genome editing, CRISPR loci was employed as genetic markers for genotyping, species identification, introducing transcriptional and epigenetic modifications [5]. For example, to trace out the microevolution of *Yersinia pestis,* the causative agent of plaque, CRISPR tool was used for genotyping [103]. Liu et al., reported that CRISPR tool was able to accurately differentiate the strains of the same serotype of *Salmonella*, which was not possible by the conventional serotyping

techniques such as Amplified Fragment Length Polymorphism (AFLP), Multiple locus variable number tandem repeat analysis (MLVA), etc. [104]. This novel tool was employed to produce sequence-specific antimicrobial agents that can cleave genomes of pathogenic bacterial strains. For instance, a mouse skin colonization model was created by Bikard et al., so as to show that CRISPR-Cas treatment can be utilized to specifically inhibit *Staphylococci.* Undoubtedly, a significant decrease in bacterial growth was observed, highlighting their reliability as a valuable remedy for the rising tide of antibiotic resistant strains such as β-lactam and vancomycin resistant *Staphylococci* [105]. Nevertheless, Cas9 variants have emerged as toolboxes to induce specific single nucleotide changes within the genome. These variants rely on Cas9- nickases coupled to the enzyme, cytidine deaminase to bring about single nucleotide transitions such as G to A or C to T. One such work was reported by Komor et al., wherein the target base, cytidine was directly converted to uridine without any double stranded breaks in the DNA by fusing Cas9 protein with cytidine deaminase domain thereby acting as a key to open door for correction of variety of point mutations in human diseases [106].

CRISPR to Create Animal Models for Human Diseases

Disease modeling was one of the early uses of CRISPR-Cas9 in medical research. CRISPR-Cas9 can also be used to represent multi-genic disorders more easily than traditional transgenic systems [107]. Cancer, for instance, is a disorder resulting in various hereditary changes. Establishment of cancer models through the conventional transgenic procedures regularly is laborious and tedious. CRISPR-Cas9, in contrast, can legitimately induce somatic mutations in mice more efficiently than the transgenic systems [108, 109]. Duchenne muscular dystrophy (DMD), for instance, is brought about by mutation in the gene encoding for dystrophin. By focusing on two exons at the same time in the rat DMD gene with CRISPR-Cas9, Nakamura et al., over-ruled the expression of dystrophin in rats, thereby establishing a rat model of DMD [110]. Similar work was carried out by Xue et al., wherein CRISPR-Cas9 segments inserted into the mice through tail vein hydrodynamic infusion, effectively prompted both gain-of-function and loss-of-function (LOF) mutations in mouse liver cells which were responsible for hepatocarcinogenesis [108].

CRISPR Altered T-cells for Cancer Therapy

The most inspiring area in the utilizations of CRISPR-Cas9 is its potential for gene therapy. Only a while after its introduction into mammalian cells [12], CRISPR-Cas9 exhibited its potential in gene therapy by inducing mutations in HIV-1 virus so as to reduce its expression in human T-cells [111]. From that point forward, many efforts have been made to investigate the efficacy of CRISPR-Cas9 in battling infectious diseases and to induce protective mutations in the host cells as well [112].

At the point when it was revealed in 2012, researchers had incredible expectations that this gene editing tool could treat or fix hundreds to thousands of hereditary disorders. As a step towards it, from last year, specialists in the USA started testing its efficacy in individuals, a significant initial phase in deciding if the innovation can satisfy its medicinal potentiality. These clinical trials are trying to elucidate the safety and efficiency of CRISPR-Cas9 against blood disorders [113], cancer and patients with visual impairment [114]. Many such preliminaries are awaited upon to start soon.

Results from the specialists at the University of Pennsylvania, about the completed clinical trials, highlighted that "CRISPR treatment intended to enhance the efficacy of T-cells, the cancer-fighting immune cells was safe". The outcomes are from three patients -two with myeloma and one with sarcoma—whose T-cells were removed and modified in the lab. CRISPR crippled genes in the T-cells, and equipped the cells with a "warhead"—a gene intended to guide the T-cells to tumor cells that have a particular protein on their surfaces [115]. Previously, genetically engineered cells called CAR T-cells, have been utilized in patients for quite a long time to fight cancer [116]; enhancing T-cells' cancer fighting ability with the assistance of CRISPR is another interesting development. These findings were presented on December 7, 2019 at the American Society of Hematology meeting in Orlando, Fla. It was demonstrated that CRISPR-altered T-cells grabbed hold and was reproduced in the patients employed for the study. None of the three individuals had any side effects related with the altered cells [115]. This indeed is an uplifting news since the T-cell-based modifications to fight cancer, have caused high fevers, low pulse, seizures and adverse side effects. Though the treatment didn't have a significant impact on reducing the development of the patients' tumors, their safety and feasibility has been demonstrated through this study. Scientists in the mere future will be substantially more centered around the effectiveness in employing CRISPR-altered T-cells for therapy.

Adding on to the soup, similar trial on CRISPR-altered T-cells is under progress in China. Furthermore, CRISPR Therapeutics, a Cambridge, Mass-based organization, hopes to start three clinical trials in which altered T-cells target blood and kidney cancers [113].

CRISPR-Cas Systems in Diagnostics

CRISPR-Cas systems have been utilized by various researchers working on disease diagnostics as well. Pardee et al., combined CRISPR-Cas9 with nucleic acid sequence-based

amplification (NASBA), an isothermal enhancement method, to precisely differentiate the closely related Zika infection strains in vitro and in a macaque model. The team attached a synthetic trigger sequence to NASBA-amplified viral RNA and utilized a sgRNA-Cas9 complex to create a strand break in the subsequent dsDNA. The presence or absence of a strain-specific PAM brought about either truncated or full-length DNA segments upon Cas9 cleavage. Full-length strands, enacted the trigger switch, which prompted a change in color on a paper-based sensor, enabling strain differentiation in vitro [117]. In another study, Muller et al., compiled CRISPR-Cas9 with optical DNA mapping to detect antibiotic resistance genes in bacteria. In this, guide RNA (gRNA)-Cas9 complex binds and cleaves nucleic acid sequences of plasmids containing genes conferring resistance and a fluorescent dye (YOYO-1) and netropsin freely binds to the DNA at AT-rich areas specifically, bringing about an emission intensity which is unique to every DNA fragment. Through this assay, researchers can recognize plasmids that confer an extended-spectrum β-lactamases (ESBLs), including cefotaxime 15 (CTX-M-15) and CTX-M-14 and carbapenemases including *Klebsiella pneumoniae* carbapenemase (KPC) and New Delhi metallo-β-lactamase 1 (NDM-1) [118]. Guk et al., coupled CRISPR-Cas9 with DNA fluorescent in situ hybridization (FISH) to identify methicillin-resistant *Staphylococcus aureus* (MRSA). This strategy uses a dead CRISPR associated protein (dCas9) framework wherein a sgRNA-dCas9 complex combined with a SYBR green I fluorescent test identifies the gene mecA of MRSA. While the complex detects the target DNA sequence, dCas9 does not induce DNA cleavage, subsequently making it reasonable for detection by FISH. This test can recognize MRSA at a concentration of 10 CFU/ml and can specifically distinguish *S. aureus* isolated with and without the mecA gene [119]. Further, CRISPR-Cas12 entered into genome editing era wherein, it was employed in a technique called DNA-endonuclease targeted CRISPR trans reporter (DETECTR) for detection of human papillomavirus from patients [120]. Another high-throughput technique combining CRISPR-Cas12 with a fluorescent-based point of care technology for accurate detection of African Swine Fever Virus (ASFV) has been developed. In this system, once the cas12a/crRNA detects and precisely binds to the target DNA, the cas12a/crRNA/DNA complex gets activated, degrading the fluorescent reporter [121]. Currently, CRIPSR-Cas13-based platforms, which integrates an isothermal- RPA (recombinase polymerase amplification) or a reverse transcription- RPA with the nuclease cas13a, termed as SHERLOCK (specific high-sensitivity enzyme reporter unlocking) has been developed. This cas13/crRNA complex, binds and targets the nucleic acids of pathogens along with the cleavage of a nontarget RNA-coupled fluorescence reporter, resulting in the emission of fluorescent signal which can be detected real-time. Employing SHERLOCK technique, Gootenberg has identified *Escherichia coli*, *Pseudomonas aeruginosa,* and has differentiated these strains from *Mycobacterium tuberculosis*, *Staphylococcus aureus* and *Klebsiella pneumoniae* [122]. The SHERLOCK technique along with heating unextracted diagnostic samples to obliterate nucleases (HUDSON) was employed by Myhrvold et al., which allows rapid detection of dengue virus, within 2 h from the body fluids of patients such as saliva, serum and whole blood [123]. COVID-19, which outbroke in November 2019, has become a pandemic and has spread globally. RT-PCR is the usual diagnostic method; however, inadequate access to reagents and increasing count of patients has put forth a vital need for easily affordable and highly sensitive techniques. One such protocol employing SHERLOCK to quickly diagnose SARS-CoV-2 was put forth by Feng Zhang et al. This technique involves isolation of RNA from patient sample followed by isothermal amplification, cas13 detection of target RNA sequence and visual reading of the reporter signal [124]. Besides, Broughton et al., has introduced a quick and accurate CRISPR-Cas12-based lateral flow assay to detect SARS-CoV-2 employing primers targeting the nucleoprotein and envelope of the virus [125]. Much more recently, a paper-based strip test known as Feluda, to detect and target SARS-CoV-2 within 30 min has been developed by CSIR-IGIB (Institute of Genomics and Integrative Biology) and Tata group. This Feluda test deploys CRISPR-Cas9-based forefront to detect the virus with about 98% specificity and 96% sensitivity. When compared with the qRT-PCR test and antigen-based tests, this CRISPR-Cas9-based paper-strips would be a "game changer" as these are of affordable cost (Rs. 500/kit), more rapid (results would return in an hour), highly specific and accurate, which would rule-out the false positive/negative results as well [126].

Challenges in Employing CRISPR/Cas Technology in cancer Treatment

As the saying "Every advantage has its own disadvantage" goes by, though CRISPR-based systems have laid a profound podium for cancer treatment, there are a lot of factors yet to be defined to increase its efficacy, especially if it is intended to treat cancer patients. First and foremost, the off-target effect of CRIPSR system makes it difficult for the researchers to target a specific genomic locus [127, 128]. This is because, CRISPR-based editing usually creates indels at undesired loci of the genome [125, 126]. Continuation of genetic modification at that point would obviously raise the risk of toxicity in adjacent normal cells and unwanted mutations. Hence, while designing CRISPR-based technology in oncology research, it is detrimental to identify and take

control over the off-target events. In this regard, Kleinstiver et al., has formulated the ways by which Cas9 protein can be modified to enhance the specific recognition of target DNA and maintain specificity of CRISPR-based genome editing, which are under evaluation currently [129, 130]. Many such studies concerning ways to minimize the off-target effects are warranted in future. Besides, the editing efficiency is yet another hurdle which has to be sorted out before implementing CRISPR-based therapeutics for oncological therapy. The efficiency of NHEJ and HDR in repair of double stranded DNA break is one among the important determining factors for overall gene editing efficiency. The efficacy of NHEJ and HDR in DNA repair varies for different cell types. Broadly speaking, NHEJ is an error-prone mechanism, which incorporates indels at the site of cleavage; HDR, on the other side, replaces the target gene with a recombinant alternative sequence. However, HDR template requires a viral or non-viral vector for translocation into the nucleus [131]. Hence, strategies such as nanocarriers, to enhance the productivity of HDR template delivery into the nucleus along with co-delivery of CRISPR components would increase the specificity and efficiency of gene editing or gene correction in tumors. Though the off-target effect and efficient delivery is resolved, 'how fit the edited cells are?' is another unanswered question [132]. If the CRISPR- edited cells possess great adaptability and proliferation rate than the unedited counterparts, then these edited ones can reach the therapeutic threshold essential for fruitful treatment upshots. However, if the editing efficiency is low, or the edited cells are not able to adapt themselves like the unedited ones, this pitfall will have a huge effect on the expected therapeutic outcome. Hence, modification of the genome of the edited cells in vitro, followed by reinfusion of the cells back to the patients would help to overcome this obstacle at least partially. Last but not least, the immune response provoked by the Cas9 protein upon entry into patient's body is another limiting factor. This is due to the presence of short peptides on the surface of Cas9 which acts as epitopes that can bind to the MHC molecules. In fact, previous literature has reported that the immunogenicity caused by CRISPR-Cas9 technique was the prime factor for destabilization of the host cell [133]. Hence, strategies to minimize the host response following delivery of CRISPR-Cas system has to be framed before carrying this system to patient setting.

Future Perspectives

CRISPR-based forefronts have turned out be crucial for advancements in science and cancer research. This is because of their wide implementation in basic research to understand the queries concerning how genes function and in the designing of therapies against diseases like cancer. In any case, there are yet a few difficulties related with this tool that should be addressed too. For instance, adeno-associated viral vectors (AVV) are commonly employed to deliver Cas9 components into the host cell. Though these vectors are serotype-associated target specific and show reduced carcinogenic risk, they have a maximum carrying capacity of 4.7 kB only, whereas, the size of Cas9 gene is 4–7 kB [134]. This huge size of Cas9 makes it hard to pack the protein in low immunogenic AVV utilized in vivo and in vitro gene delivery. Additionally, Cas9 from *S. aureus* and *S. pyogenes* has been appeared to cause irresistible and infectious ailments in clinical trials [135]. One potential technique to surpass this issue is to update Cas9 or utilize an alternate bacterial protein that can get away from the host immune reactions. Intellia Therapeutics has built up a lipid-nanoparticle-based CRISPR-Cas9 framework, to modify genes in rodents and non-human primates. With the birth of the "CRISPR babies" in November 2018 [136], it is the responsibility of the researchers to respond to the challenges of CRISPR in this era of genetic inequality and its long-term implications. Despite the fact that these difficulties endure, CRISPR-based advancements hold huge potential and are an incredible expansion to the genome altering tool compartment for the improvement of biotherapies and betterment of humanity.

Conclusion

When compared with the past genome altering techniques, this short RNA-guided Cas9 nuclease framework has a few points of interest namely, wide genome availability, capacity to target multiple sites in a single step, and simplicity in designing of the targets. Regardless of the challenges in utilization of the CRISPR-Cas9 framework for in vivo investigations, scientists have already started to work on this framework to build up animal models for various diseases and to figure out the function of genes. To address their contributions in oncological field, the CRISPR-Cas9 framework will probably offer a novel and less tedious methodology for gene therapy, regulation of gene expression, and new wave of drug targets. In the mere future, CRISPR will slash to identify life-threatening diseases and provide roadmap to develop vaccines, thereby "nearing the beginning of the end of cancers, infectious diseases and hereditary disorders".

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