

CRISPR-Cas9 Genome Editing in GBM

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Definition

Glioblastoma multiforme (GBM) is an aggressive malignancy of the brain and spinal cord with a poor life expectancy. The low survivability of GBM patients can be attributed, in part, to its heterogeneity and the presence of multiple genetic alterations causing rapid tumor growth and resistance to conventional therapy. The Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)-CRISPR associated (Cas) nuclease 9 (CRISPR-Cas9) system is a cost-effective and reliable gene editing technology, which is widely used in cancer research. It leads to novel discoveries of various oncogenes that regulate autophagy, angiogenesis, and invasion and play important role in pathogenesis of various malignancies, including GBM.

1. Introduction

Glioblastoma multiforme (GBM) is an aggressive primary tumor, which arises from the abnormal astroglial cells in the brain in most cases as well as in the spinal cord in less often cases ^{[1][2]}. The incidence of GBM is approximately less than 10 per 100,000 individuals worldwide, and a significant number of the GBM patients show a low survival rate of 14 months or less ^{[3][4]}. The heterogenous nature of the GBM contributes to its therapy-resistance and poor prognosis; hence, identifying genetic regulations of the hallmarks of this malignant disease may help device effective treatments ^{[5][6]}.

Almost all GBM patients are presented with the high-grade of the disease, which is characterized by rapid recurrence after surgery and resistance to radiotherapy and chemotherapy ^{[7][8]}. Resistance of GBM to therapies is a major challenge for treatment of the patients and it can result from several mechanisms, which collectively constitute the hallmarks of this malignancy ^{[7][8]}. The resistance mechanisms can either accelerate initial tumor growth or potentiate regrowth of the more resistant tumor after the treatment ^{[7][8]}. One of the major hallmarks of GBM is the presence of GBM stem cells (GSCs), which are genetically heterogeneous cells with more distinct properties than primary tumor cells, and they play critical roles in disease recurrence and therapy resistance ^{[9][10]}. Sustained proliferative signals is another major cause of resistance, which results from aberrant expression of the growth and trophic factors ^[11]. Escaping the programmed cell death or apoptosis and activating the pro-survival pathways are other major mechanisms of therapy resistance in GBM ^[12]. Autophagy or recycling of cellular building blocks is also an important survival mechanism that is activated in GBM and it is one of the major causes of therapy resistance ^[13]. Aberrant inflammation and immune response are correlated with rapid progression and resistance to therapy ^{[14][15]}. Angiogenesis or aberrant new blood vessel formation is also correlated with rapid progression and therapy resistance in GBM ^{[15][16]}. Finally, interaction between tumor cells and the surrounding microenvironment potentiates the migratory and invasive properties of the tumor cells, contributing to rapid relapse of GBM and poor outcome ^{[17][18][19]}.

Genetic knockout, knock in, and overexpression have been widely used to screen for the molecular pathways that govern the hallmarks of GBM and to study the function of various genes in pathogenesis and progression of this malignant disease ^{[20][21][22][23]}. In the past, scientists used 'gene targeting' for changing the genome in the specific places with addition or deletion of either entire genes or single bases. Although gene targeting has highly been useful in understanding the function of specific genes, this technology takes long time to make a mutant gene and it is expensive. Subsequently, several 'gene editing' technologies such as Transcription Activator-Like Effector Nucleases (TALENs) and Zinc-Finger

Nucleases (ZFNs) have been discovered to improve the gene targeting to a great extent. Still scientists were looking for a cheaper and quicker gene editing technology than TALENs and ZFNs. The Clustered Regularly Interspaced Short Palindromic Repeat (CRISPR)-CRISPR associated (Cas) nuclease 9 (CRISPR-Cas9) system is the latest gene editing technology [20], which stands out as the fastest, cheapest, highly versatile, and most reliable gene editing tool for using widely to discover genetic alterations, oncogenic targets, and epigenetic regulation. Currently, CRISPR-Cas9 system is the number one choice for editing genes or genome in various cancers including GBM [21][22][23][24].

2. Principle of CRISPR-Cas9 Genome Editing Technology

CRISPR-Cas9 is a naturally occurring protective immune system, which is found as the repeated DNA clusters of 21–47 bp in bacteria and Archaea [25][26][27][28]. In these prokaryotes, CRISPR-Cas9 provides an internal defense mechanism by recognizing and eliminating foreign viral DNA [25][26][27][28]. When the virus attacks the prokaryote for the first time, it introduces its DNA that triggers the immune system of the prokaryote to generate small fragments of DNA called CRISPR arrays [25][26][27][28]. The CRISPR arrays help the bacteria recognize subsequent viral invasion and transcribe guide RNA targeting the viral DNA, which is then degraded by an endonuclease enzyme called the Cas9 protein [25][26][27][28]. Using similar approach, CRISPR-Cas 9 gene editing technology has been developed and widely used to study the functions of various genes that are responsible for GBM development and progression both in vitro and in vivo.

CRISPR-Cas9 gene editing technology is composed of two main elements: the guide RNA and the Cas9 endonuclease enzyme [29][30]. The guide RNA is a synthetic complex made by hybridization of two different RNAs: the CRISPR RNA (crRNA), which has a complementary nucleotide sequence to the target DNA; and the trans-activating CRISPR RNA (tracrRNA), which binds and activates the Cas9 nuclease [31][32]. On one side, the guide RNA binds to a complimentary sequence in the DNA; and on other side, it binds and directs Cas9 endonuclease enzyme to the target DNA segment to perform the genome editing [30][31][32]. In addition to the regular Cas9 nuclease that results in double-strand DNA break, Cas9 nickase has been developed via mutagenesis of the regular Cas9 nuclease, and Cas9 nickase enables genome editing via single-strand DNA break that permits more precise genome editing and minimizes the off-target effects of the Cas9 nuclease [31].

The Cas9 enzymes used in GBM research were obtained from different sources or expression vectors such as Cas9-expressing lentiviral vector, Cas9-expressing plasmid vector, or Cas9 synthetic protein [22][23][33]. Similarly, the guide RNA molecules were obtained from multiple sources such as lentiviral, plasmid, or synthetically derived guide single strand RNA [22][23][33]. CRISPR-Cas9 systems were delivered into GBM cells using viral (lentiviral mediated) or non-viral lipid-mediated (Lipofectamine 3000) methods [34][35]. After the delivery into the cells, the guide RNA that contains the targeting RNA sequence becomes complementary to the gene of interest to be edited [30][31][32]. Cas9 endonuclease then create a double-strand DNA break in the targeted region of the genome to edit the gene of interest [30][31][32].

Once the double-strand break is generated, a DNA repair machinery is activated to form either a non-homologous end joining (NHEJ) or a HDR [36][37][38]. NHEJ is 'non-homologous repair', in which the DNA break ends are directly ligated without requiring a homologous template, in contrast to HDR that requires a homologous sequence for guiding the DNA repair [36][37][38]. However, NHEJ results is imprecise joining of two ends of DNA, while HDR results in precise insertion due to involvement of a designed DNA template [36][37][38]. Puromycin or fluorescence-activated cell sorting (FACS) is commonly used for selection of the transfected cells [34][39][40] while further validation of gene editing is commonly performed using quantitative polymerase chain reaction (qPCR) or Western blotting [40][41][42].

3. Genome-Wide CRISPR-Cas9 Screens in GBM Research

CRISPR-Cas9 screens have been used in vitro and in vivo for identifying the novel biomarkers, oncogenic drivers, mechanisms of chemotherapy resistance, and genes that make tumor cells more responsive to standard or synergistic therapy. CRISPR-Cas9 genome-wide screening used in GBM research includes either knockouts or interference approaches; and they are performed mostly on GBM cell lines, GSCs, and less commonly are applied to cerebral organoid or in vivo in mice. CRISPR guide RNA library used in GBM

research are either coding or non-coding and are commonly transfected into GBM models using viral transduction (**Table 1**).

To identify new prognostic biomarkers and factors that sensitize tumor cells to chemotherapy, a group of investigators used CRISPR-Cas9 mediated genome-wide knockouts to identify ribosomal protein subunits 11, 16, and 18 as important biomarkers in GBM cell lines in response to treatment with topoisomerase II poisons [43]. Also, they identified that loss of ribosomal subunit 11 correlated with resistance to cell death in response to the common chemotherapeutic agents such as etoposide and doxorubicin [43]. Another group used CRISPR-Cas9 mediated screen to identify NF- κ B (nuclear factor kappa-light-chain-enhancer of activated B cells) and E2F6 (E2F transcription factor 6) genes as one of the major underlying molecular mechanisms of resistance to temozolomide (TMZ), an orally administered alkylating chemotherapeutic agent, in epidermal growth factor receptor (EGFR) variant III (EGFRvIII)-expressing U87MG cells [44]. Another study used in vivo CRISPR-Cas9 screen to identify GBM suppressor genes in mice [45]. In a different study, use of CRISPR-Cas9 mediated genome-wide knockout screen identified mitogen-activated protein kinase kinase kinase kinase-4 (MAP4K4) as an important regulator of invasion in U138MG cells [46]. Besides, use of an in vivo CRISPR-Cas9 knockout screen in mice identified genetic alterations in surface proteins of CD8+ T cells regulating T cell immunotherapy in GBM [47].

To identify genetic regulation of GBM stemness, an investigation used CRISPR-Cas9 screen to identify key regulators or transcription factors that controlled growth, stemness, and TMZ resistance in GSCs [48]. Employment of CRISPR-Cas9 mediated genome-wide screen identified new molecular regulator of cancer stem cells in three-dimensional bioprinted complex systems, which conferred the interaction between GBM cells and the surrounding microenvironment [49]. Interestingly, another group has used CRISPR-Cas9 knockout screen to identify the loss of redundancy between PKMYT1 (protein kinase, membrane associated tyrosine/threonine 1) and WEE1 ('wee phenotype' 1 protein kinase), which are major regulators of mitosis, in GSCs when compared with neural stem cells (NSCs), enhancing growth in GSCs [50].

Apart from transcriptional screen, CRISPR-Cas9 systems have been used to explore the role of non-coding regions in pathogenesis of GBM. For example, use of CRISPR-Cas9 interference screen identified genetic alterations in long non-coding RNAs (lncRNAs) that could control growth in U87MG cells and sensitize them to therapeutic doses of ionizing radiations [51]. Also, use of CRISPR-Cas9 interference screen identified amplification of non-coding region in the DNA that could regulate the co-amplified oncogenes in GBM [52]. All these revolutionized and provided insights into functional correlation among heterogenous GBM mutations, which could be potential therapeutic targets.

Table 1. CRISPR-Cas9 genome-wide screens used in GBM.

Tumor Model	Type of Screen	References
SNB19	Genome-scale CRISPR knockout screen	[43]
U138MG	Large-scale CRISPR-Cas9 mediated loss of function screen	[46]
GSCs	Whole-genome CRISPR screening	[49]
U87MG	CRISPR interference (CRISPRi) screen	[51]
GBM3565, GSC23	CRISPRi screen	[52]
Patient-derived GSCs	Genome-wide CRISPR-Cas9 screens	[48]
Mice	In vivo CRISPR screen	[45]
Patient-derived GSCs and human NSCs	Genome-wide CRISPR-Cas9 screen	[50]
U87MG and U87MG-EGFRvIII cells	Pooled genome wide CRISPR screening	[44]

4. Application of CRISPR-Cas9 to Identifying Genetic Regulators of Autophagy in GBM

Autophagy is a catabolic mechanism of recycling intracellular components and organelles by normal and tumor cells [53]. Autophagy includes a sequence of events from autophagosome formation to fusion with lysosome and finally lysis of the engulfed materials [53]. In GBM, autophagy plays controversial roles in developing and advancing this disease; however, several studies have correlated autophagy activation in GBM with aggressive disease and therapy resistance [13][54]. CRISPR-Cas9 system has been used in GBM research to identify transcriptional regulation, biological function, and interactions of genes that control autophagy activation and autophagy flux in GBM (Table 2).

A study has shown inhibition of autophagy induction by CRISPR-Cas9-mediated ATG5 gene knockout in TGS01 and TGS04 cells in conjunction with a calcium mobilization agent (nigericin) that works together to increase mitochondrial reactive oxygen species for cell death [55]. Another study showed that CRISPR-Cas9 mediated knockout of TSC2 (Tuberous Sclerosis 2) gene, an autophagy promoting molecule, in GBM LN18 cells rendering them to be more susceptible to cell death in response to photodynamic therapy (PDT) [56]. In contrast, another study reports that ATG5 and ATG7, both of which are autophagy related genes, are important for cell death in GBM while CRISPR-Cas9 mediated knockout of ATG5 and ATG7 in GBM MZ-54 cells protect the cells from cell death when compared with control cells in response to various autophagy inducers (loperamide, pimozide, and STF-62247) [57]. The difference in the results obtained from these studies could be attributed to the heterogenous nature of GBM and the different effect of autophagy regulatory gene knockout in different cell lines used. Also, it could be due to synergetic or antagonistic effect of different combination therapies that were used along with ATG5 knockout, resulting in different effects on GBM cell death. This also could explain why autophagy induction or activation remained controversial in GBM treatment. Similarly, CRISPR-Cas9 mediated knockout of ataxia-telangiectasia mutated (ATM) gene, a tumor suppressor, in two human GBM cell lines such as LN18 and LN229 potentiated autophagy and increased their responsiveness to platinum treatment [58].

Table 2. CRISPR-Cas9 mediated knockouts of autophagy genes in GBM.

Target Gene	Type of CRISPR-Cas9 Mediated Genome Editing	GBM Model	References
ATM	Knockout	LN18, LN229	[58]
ATG5 and ATG7	Knockout	MZ-54 GBM cells	[57]
ATG5	Knockout	TGS01 or TGS04	[55]
TSC2	Knockout	LN18 cells	[56]

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