Journal of Advances in Medical and Biomedical Research | ISSN:2676-6264

Targeting Positive Regulators of Sonic Hedgehog Signaling Pathway in Medulloblastoma by Designing CRISPR/Cas9 Single Guide RNAs

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Article Info

10.30699/jambs.31.149.594

Received: 2023/09/09;
Accepted: 2023/12/05;
Published Online: 29 Jan 2024;

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ABSTRACT

Background & Objective: Medulloblastoma formation is importantly related to granular cell proliferation and differentiation, processes which are under the influence of sonic hedgehog (SHH) signaling. Exons of genes encoding different components of this signaling pathway (e.g., ligands, co-receptor, transcription factors, and target genes) were investigated to identify the proper single guide RNAs for selective targeting of positive regulators of the SHH pathway.

Materials & Methods: The genomic DNA sequences of corresponding genes of several positive regulators of the SHH pathway (in Homo Sapiens), including *SHH*, *SMO*, *GLI1, GLI2, MYCN*, and *MYC*, were retrieved from the National Center for Biotechnology Information (NCBI) gene database. Next, the exon sequences of these genes were identified using protospacer adjacent motif (PAM) of NGG and Streptococcus pyogenes Cas9 nuclease and evaluated by CRISPOR software to select the best sgRNA for each target gene.

Results: The analyses revealed the best sgRNAs for the *SHH*, *SMO*, *GLI1*, *GLI2*, *MYCN*, and *MYC* genes targeted exon 1, -4, -4, -5, -2 and -2, respectively. Proper sgRNAs for targeting each exon in each gene were also identified.

Conclusion: This study revealed possible specific exonic targets of components of the SHH signaling pathway through designing proper sgRNAs using the CRISPR/Cas9 genome editing approach.

Keywords: CRISPR/Cas9, Sonic Hedgehog, Medulloblastoma, Guide RNA, Genome

Introduction

The developed cerebellar cortex consists of three layers: an outer molecular layer (comprised of axons/dendrites of cerebellar neurons), a layer of Purkinje cells, and a layer made up of granule cells (GCs) (1). Importantly contributing to the cerebellar cortex, GCs originate from their precursors, proliferate rapidly, and differentiate into mature GCs. The proliferation and differentiation of GCs are under the influence of the sonic hedgehog (SHH) signaling pathway (2). As such, aberrant activation of SHH signaling is critically associated with GC-related malignancies such as medulloblastoma.

Medulloblastoma has been classified into four principal subgroups: WNT, SHH, Group 3, and Group 4 (3). The WNT subgroup has the best prognosis and the pathophysiology is related to WNT signaling and somatic mutations of the Catenin Beta 1 (*CTNNB1*) gene (3). The SHH subgroup was defined after identification of SHH pathway aberrations, such as a mutation in the SHH receptor protein patched homolog 1 (PTCH1), and subsequent activation of SHH signaling (4). With metastatic features, Group 3 medulloblastoma exhibits high expression of MYCN and is therefore also referred to as the MYCN group (3, 5). Group 4 is the least molecularly characterized subgroup and shows an intermediate prognosis similar to the SHH group (3).

As a highly spatiotemporally regulated pathway, Hh signaling is conserved in vertebrates and crucially involved in embryonic development (6). Aberrations in this pathway can lead to craniofacial defects and various cancers (7). The HH/GLI cascade comprises several important components, including ligands (Sonic (SHH), Indian (IHH), and Desert Hh (DHH)), receptors (protein

patched homolog 1 and 2 (PTCH1 and -2)), co-receptors (*e.g.*, smoothened (SMO)), transcription factors (glioma-associated oncogene proteins (GLI1, GLI2, and GLI3)), and several target genes (*PTCH1, PTCH2, GLI1, MYCN*) (8). In addition, HH signaling can occur in response to canonical (ligand-dependent and/or receptor-induced signaling) and non-canonical activation (signaling triggered downstream of SMO) (7).

Clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated (Cas) systems represent an adaptive immune system in bacteria and archaea and are commonly used as a genomic engineering tool. Out of several CRISPR/Cas types, *Streptococcus pyogenes* (SpCas9)-derived CRISPR/Cas9 is the most commonly used system (9). Cas9 nuclease and a single guide RNA (sgRNA) form a complex that can detect a specific genomic site and generate double-stranded breaks producing insertions or deletions which may ultimately inactivate the target gene.

CRISPR selector (CRISPOR) is a free online tool for designing and selecting proper sgRNAs (10). CRISPOR offers various sgRNA ranking criteria, including specificity, efficiency, out-of-frame mutations, and off-targets, which facilitates selecting the sgRNA best suited for the desired purpose (11). Providing various options and comparing previous work, CRISPOR may be considered the best tool for sgRNA selection (12).

In this study, we aimed to identify the most efficient sgRNA exonic targets of various SHH signaling pathway components. Exons of the ligand producing gene *SHH*, co-receptor gene *SMO*, and various target genes (*GLI1, GLI2, MYCN,* and *MYC*) were evaluated using a SpCas9-derived CRISPR/Cas9 system; CRISPOR analysis led to the identification of proper sgRNAs to specifically and effectively target (exons of) these positive regulators of SHH signaling.

Materials and Methods

Genomic analysis

The genomic DNA sequences of corresponding genes of several positive regulators of the SHH signaling pathway (in Homo Sapiens) - i.e., *SHH*, *SMO*, *GLI1, GLI2, MYC*, and *MYCN* - were retrieved from the National Center for Biotechnology Information (NCBI) gene database and ensemble genome browser. Exons of each gene were investigated and the coding and non-coding sequences of each exon were demonstrated (Table 1, and Figure 1) since targeting the coding sequences of each exon is of priority.

Table 1. Location and number of exons of each target gene

Features		Genes					
		SHH	***SMO***	***GLI1***	***GLI2***	***MYCN***	***MYC***
Location	Map	7q36.3	7q32.1	12q13.3	2q14.2	2p24.3	8q24.21
	Source (bp)	1 - 19290	1 - 31677	1 - 19134	1 - 202363	1 - 13447	1-14518
	Gene (bp)	4811 - 17294	4762 - 29674	4651 - 17134	5001 - 200363	4990 - 11444	5798 - 12518
Number of Exons		3	12	12	14	3	3
Number of coding exons		3	12	11	13	2	3

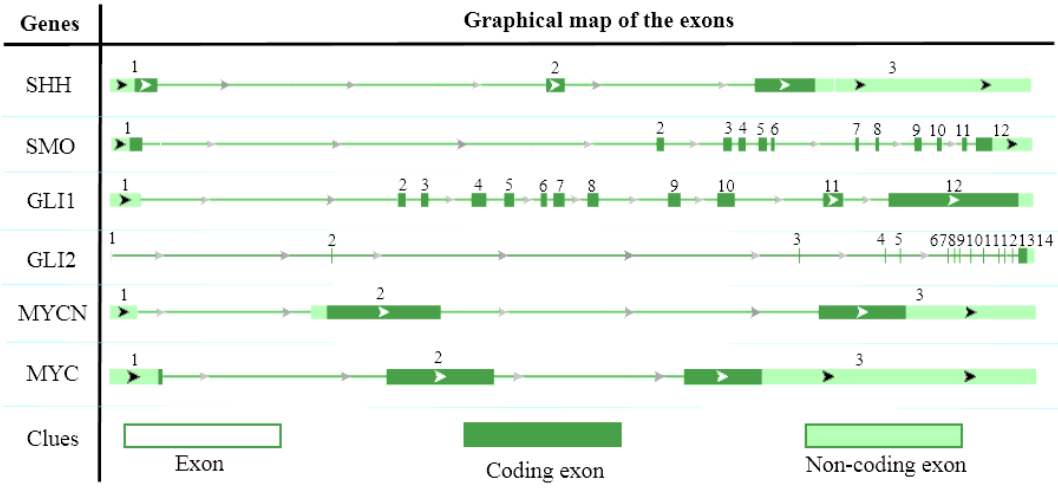

Figure 1. Graphical map of Coding and non-coding exons of the positive regulator genes of SHH signaling pathway. The figure is adopted from NCBI graphics of genes (https://www.ncbi.nlm.nih.gov/).

Selecting proper sgRNAs by CRISPOR online platform

The exon sequences of mentioned genes were identified using protospacer adjacent motif (PAM) of NGG and Streptococcus pyogenes Cas9 nuclease and evaluated by CRISPOR to select proper sgRNAs for each target gene. Proper sgRNAs for each exon of every gene were selected regarding the highest scores of specificity, efficiency, and off-target effects, simultaneously.

CRISPOR Guide list

The CRISPOR guide list (CRISPOR manual: http://crispor.tefor.net/manual/) shows several properties of guide sequences presented in various columns (10, 11):

Column 1: PAM position on the input and the strand; **Column 2**: guide sequence, the PAM, PCR, and cloning primers associated with that specific sequence, variants, high or low GC content leading to low target cleavage efficiency, inefficient guides, and overlapping restriction enzymes; **Column 3 and 4**: specificity scores of MIT and CFD. CRISPOR uses the MIT CRISPR Website but with a better and more sensitive engine. A specificity score of at least 50 is recommended; **Columns 5 and 6**: efficiency scores that predict how well the target may be cut by its RNA guide sequence. Two scoring methods are shown by default – the Doench (13) and Moreno-Mateos scores (14). The Doench score is the best score for guides expressed in cells from a U6 promoter, whereas the Moreno-Mateos score is most suitable when the guide is expressed *in vitro* with a T7 promoter; **Column 7**: out-of-frame score predicting the likeliness of a guide leading to out-of-frame deletions, which is relevant for gene knockouts with a single guide; **Column 8:** Lindel score predicts probability of a frameshift caused by any type of insertion or deletion; **Column 9:** off-target mismatch counts representing the number of possible off-targets in the genome for each number of mismatches. The second row (indicated in grey) shows the mismatches in the seed region – within 12 bp of the PAM. It has been reported that off-targets with mismatches in the seed region are very inefficiently cut; **Column 10**: off-target locations indicating whether the off-targets are introns or exons. Scores related to specificity, efficiency, and out of frame score, range from 0-100 with 100 being the best score (Figure 2).

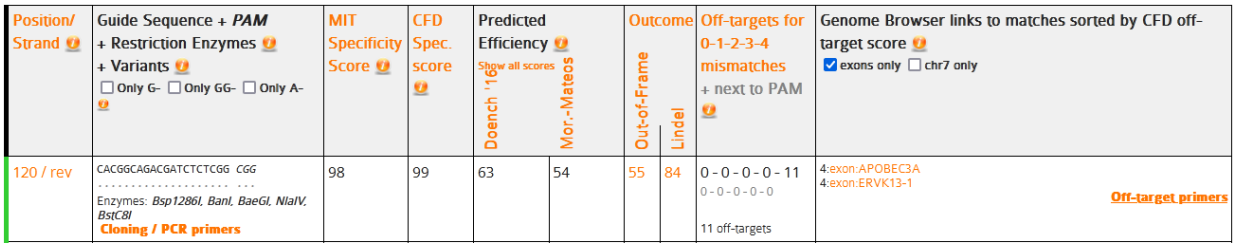

Figure 2. CRISPOR platform guide list. From left to right (column 1: PAM position on the input and the strand; column 2: guide sequence, the PAM, PCR and cloning primers; column 3 and 4: specificity score; column 5 and 6: efficiency score; column 7, 8: outcome scores; column 9: off-target mismatch counts; column 10: number of exonic or intronic off-targets).

Results

Genomic analysis

The location and number of exons for each target gene are listed in Table 1. *SHH* contains 3 exons each of which is transcribed; *SMO* includes 12 exons which are all transcribed; *GLI1* has 11 exons from which exon 1 is not transcribed; *GLI2* contains 14 exons from which exon 14 is not transcribed; *MYCN* has 3 genes from which exon 1 is not transcribed; *MYC* has 3 genes which are all transcribed.

Proper sgRNAs to target SHH signaling pathway

The exons of each gene were investigated for proper sgRNAs by CRISPOR (Table 2). The following pairs describe the gene and the exon best targeted by the designed (proper) sgRNA: *SHH* - exon 1, *SMO* - exon 4, *GLI1* - exon 2, *GLI2* - exon 5, *MYCN* - exon 2, and *MYC* - exon 2.

Table 2. Proper sgRNAs for targeting each individual gene.

Gene and exons		CRISPOR scoring							
		Specificity		Efficiency		Outcome		Off-target	Number of Exonic off-targets
		MIT	CFD	D	MM	OF	L		
SHH	1	99	99	60	60	72	74	0-0-0-0-16 0-0-0-0-0	None
		First sgRNA: CTATATAACCTTGCCCGCCG *CGG* (157/rev)							

Gene and exons		CRISPOR scoring							
		Specificity		Efficiency		Outcome		Off-target	Number of Exonic off-targets
		MIT	CFD	D	MM	OF	L		
		98	99	42	45	64	69	0-0-0-0-7 0-0-0-0-1	1
		Second sgRNA: CGCGGCGGGCAAGGTTATAT *AGG* (178/fw)							
		98	99	63	54	55	84	0-0-0-0-11 0-0-0-0-0	2
SMO	4	sgRNA: CACGGCAGACGATCTCTCGG *CGG* 120/rev							
		97	99	61	66	58	73	0-0-0-2-10 0-0-0-0-0	2
		sgRNA: ACGGCAGACGATCTCTCGGC *GGG* 119/rev							
		94	97	73	37	65	88	0-0-0-3-23 0-0-0-1-0	2
		sgRNA: GCGAGTTGATGAAAGCTACG *AGG* 122/rev							
Gli1	4	94	98	53	27	65	82	0-0-0-1-20 0-0-0-1-0	2
		sgRNA: AACTCGCGATGCACATCTCC *AGG* 158/fw							
		98	98	54	45	57	73	0-0-0-0-11 0-0-0-0-0	0
		sgRNA: GGTGTCGCATGTCAATCGGT *AGG* 35/rev							
Gli2	2	98	99	54	45	57	84	0-0-0-0-11 0-0-0-0-0	0
		sgRNA: ACCGATTGACATGCGACACC *AGG* 58/fw							
		99	98	71	73	67	82	0-0-0-0-11 0-0-0-0-0	2
		sgRNA: CGGTATTAAAACGAACGGGG CGG 79/fw							
MYCN	2	98	97	41	29	67	77	0-0-0-3-6 0-0-0-0-0	3
		sgRNA: CCCCCGGTATTAAAACGAAC GGG 75/fw							
		98	100	53	69	65	73	0-0-0-3-15 0-0-0-0-0	1
		sgRNA: ACGTTGAGGGGCATCGTCGC *GGG* 7/rev							
MYC	2	99	99	57	51	66	51	0-0-0-1-12 0-0-0-0-0	1
		sgRNA: AACGTTGAGGGGCATCGTCG *CGG* 8/rev							

MIT: MIT specificity score, CFD: CFD specificity score, D: Doench score (13), MM: Moreno-Mateos score (14), OF: out of frame, L: Lindel.

Discussion

Numerous studies have meticulously investigated SHH signaling (6-8). Considering this pathway is crucial for both proliferation and differentiation during embryogenesis, tumorigenesis can be expected when dysregulation or mutation occurs (15). For example, germline mutations of the tumor suppressor gene

PTCH, a receptor for SHH, can lead to Gorlin syndrome, which is associated with several cancers, including basal cell carcinoma (BCC), medulloblastoma, and rhabdomyosarcoma **(16)**. In addition, aberrant activation of the HH pathway through overexpression of ligands also promotes the development of various cancers, including prostate cancer, pancreatic cancer, gastric and upper gastrointestinal tract cancer, and small cell lung cancer **(17)**.

HH signaling comprises events driven on different levels by ligands, receptors, co-receptors, transcription factors, and target genes **(6-8, 15, 16)**. In Drosophila, only one gene (*HH*) is associated with HH ligand secretion, whereas three different *HH* gene homologs (i.e., *SHH, IHH, and DHH*) are spatiotemporally expressed in vertebrates **(15, 18)**. The receptor of these ligands is PTCH; in the absence of a ligand, PTCH inhibits the activity of the co-receptor, SMO **(15)**, rendering the HH pathway inactive. Ligand binding to PTCH results in SMO activation and transduction of the HH signal from the cytoplasm to the nucleus via the GLI zinc-finger protein family of transcription factors **(6)**. To date, three GLI transcription factors have been identified, of which GLI1 and GLI2 serve as transcriptional activators and GLI3 as an activator of repressor depending on its post-translational modification **(19)**. HH pathway activation results in the expression of HH target genes, such as *GLI1*, *PTCH*, *MYC*, and *BCL-2* **(6, 15)**.

MYC and *MYCN* genes are of potential significance in medulloblastoma **(3, 20)**. Regarding disease subtypes (*see* Introduction), a high expression level of *MYCN* has been reported in the SHH group, while *MYCN* is highly expressed in WNT and Group 3 medulloblastoma; conversely, Group 4 medulloblastoma shows minimal expression of either gene **(3, 5, 20)**. *MYCN* (located on chromosome 12) is a GLI target gene that promotes proliferation of cerebellar GC precursors **(21)**, and is directly induced by SHH **(22)**. *MYCN* not only regulates the proliferation of GC precursors but also affects GC differentiation when its levels are decreased **(2, 21)**. As such, *MYCN* plays a significant role in proliferation and tumorigenesis mediated by SHH **(22)**.

Based on current literature, targeting SHH signaling at the level of SMO is a common approach, and several SMO inhibitors have been identified/developed and utilized **(23)**. Cyclopamine – a natural product of corn lilies – was the first identified SMO inhibitor but exhibited poor efficiency and oral solubility **(24)**. Several other SMO inhibitors, including vismodegib, sonidegib, and saridegib, have since been introduced with variable efficiency **(25-28)**. Among these inhibitors, which function through the prevention of cilial translocation of SMO **(23)**, vismodegib and sonidegib demonstrated promising results in the treatment of BCC and medulloblastoma **(25-28)**. Similarly, itraconazole – an SMO inhibitor effective against SMO-resistant mutations – has shown inhibitory effects on BCC and medulloblastoma growth as well **(29)**. Shh signaling can also be targeted at the level of GLI, considering the pathway can be activated non-canonically. GLI inhibitors include the small molecules GANT58 and GANT61, HPI 1-4, and arsenic trioxide (ATO) **(30)**.

Although the use of SMO inhibitors is the most commonly applied targeted therapy for inhibition of SHH signaling, the problem is that SHH-activated-derived tumors (BBB and medulloblastoma) frequently develop resistance to SMO inhibitors **(31, 32)**. This may occur through several mechanisms, including resistance mutations in SMO **(31)**, amplification of downstream SHH target genes (such as GLI2 **(32)** or cyclin D), activation of non-canonical GLI signaling through PI3K-AKT pathway **(32)**, and atypical protein kinase C (PKCzeta/lambda) activation **(23, 33, 34)**. This emphasizes the need for alternative targeted strategies to effectively inactivate SHH signaling.

Recent advances in gene therapy have led to the utilization of specific nucleases for gene editing such as zinc finger nucleases (ZFNs), transcription activator-like effector nuclease (TALENs), and CRISPR-associated (Cas) proteins (35). The CRISPR/Cas system is an eminent genomic engineering tool which can be simply applied, is less toxic compared to other approaches, and its accuracy in targeting is high, although some off-target effects are inevitable.

Cancers are typically not the result of a single gene or single pathway abnormality. Thus, drugs interfering upstream of a signaling pathway may be ineffective in controlling the disease (e.g., SMO inhibitors in the treatment of medulloblastoma), because the expression of target genes can be induced through various signaling pathways converging at the transcriptional level **(32-34)**. Therefore, targeting efficiently, specifically, and precisely (reducing off-targets) is of crucial importance and can be achieved by designing optimal sgRNAs using the CRISPR/Cas system **(11)**.

Among several online platforms for designing sgRNAs, three comprehensive tools including CHOPCHOP, CRISPR RGEN tools, and CRISPOR are more common to be used **(12)**. CRISPOR is an ensemble of multiple tools, so it is very comprehensive and meets the necessities of designing proper gRNAs **(10)**. For instance, for the efficiency score, the result of 10 different designing tools is considered, so CRISPOR functions as a great database for designing sgRNAs **(13)**. When the results of CHOPCHOP and CRISPOR designing platforms are compared, at least one of the top three suggested sgRNA sequences are the same, but CRISPOR results are more comprehensive since it represents a clear view of intronic and exonic off-targets and possible polymerase chain reaction primers to identify the off-target sequences **(11)**.

Several CRISPR/Cas9 intervention studies have been done, Li *et al.* **(36)** edited a TERT gene promoter-activating mutation using a single guide RNA and reported proliferation arrest in a glioblastoma cell line, and growth inhibition of gliomas when adeno-associated viruses expressing specific sgRNAs were locally injected. A recent attempt by Salimi-Jeda *et al.* **(37)** to inhibit HIV-1 replication demonstrated decreased specific antigen levels and viral RNA load. They transfected HEK-293T cells infected by the HIV virus with a CRISPR/Cas9 multiplex cassette containing three different sgRNAs designed by CRISPOR online platform.

This is a bioinformatics study aiming to identify ways to target the SHH signaling pathway through designing proper sgRNAs using the CRISPR/Cas9 genome editing tool; none of the sgRNAs proposed here are tested in a laboratory by the authors.

Conclusion

The results of this study demonstrate an effective CRISPR/CRISPOR approach identifying proficient sgRNAs that specifically target susceptible exons of various genes involved in the activation of SHH signaling (an important pathway in cancer development and progression) **(2, 7, 8, 15, 16, 18, 22, 23, 26, 30, 32)**. Our findings suggest further exploration of sgRNAs as a direct and targeted approach to inhibit significant cancer-related molecular pathways, such as SHH signaling, at various levels is promising and might aid the development of novel therapeutic strategies for the treatment of medulloblastoma and other cancers.

Acknowledgments

This study was supported by the Iran University of Medical Sciences, Tehran, Iran (ethics code: IR.IUMS.FMD.REC.1399.543).

Authors' Contribution

This work was carried out in collaboration among all authors. M. Gh., H. M., and M. M. designed the study, MGH performed the bioinformatics study, M. Gh. wrote the first draft of the manuscript. H. M. and M. M. supervised and commented on the study, H. M. edited and added corrections. All authors read and approved the final manuscript.

Funding

This study was funded by Iran University of Medical Sciences, Tehran, Iran.

Conflict of Interest

No conflict of interest is declared.

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How to Cite This Article:

Ghorbanlou M, Marzban H, Mehdizadeh M. Targeting Positive Regulators of Sonic Hedgehog Signaling Pathway in Medulloblastoma by Designing CRISPR/Cas9 Single Guide RNAs. J Adv Med Biomed Res. 2023; 31(139):594-601.

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