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Developing a CRISPR/Cas9 Plasmid Vector Containing Specific Single Guide RNAs Targeting *SMO* in the Sonic Hedgehog Signaling Pathway for SHH-Driven Cancers

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ABSTRACT

Background & Objective: Clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated (Cas) system is considered an efficient tool for genomic engineering in the field of cancer/gene therapy. Developing a plasmid vector to target the signaling pathway of sonic hedgehog (SHH), a key driver of malignancies such as medulloblastoma and basal cell carcinoma, is the aim of this study.

Materials & Methods: CRISPOR online platform was used to select three specific and efficient gRNAs (guide RNAs) to design and then synthesize a gRNA cassette in a plasmid vector to target SHH pathway. Following digestion of pCas-guide-EF1a-GFP, the Cas9 expressing vector, by BsrGI and BamHI restriction enzymes and ligation with T4 ligase, the gRNA cassette was cloned. After that, competent DH5α E. coli bacteria were used to amplificate the vector. Finally, after plasmid extraction, presence of the gRNA cassette was confirmed through DNA sequencing and polymerase chain reaction (PCR).

Results: To target SMO gene, as an upstream target of SHH pathway, three target-prone exons (2nd, 4th, and 6th) were used to synthesize efficient and specific gRNAs. The process of cloning and developing the interested vector was confirmed by sequencing and PCR.

Conclusion: Although this study lacks in vitro/in vivo validation of the candidate gRNAs within the plasmid vector, developing efficient, and specific CRISPR/Cas9 vectors may possibly be a promising approach in the field of cancer treatment through targeting neoplastic pathways at the level of positive regulators which may possibly lead to a better perception of different molecular pathways leading to cancer, and developing precise chemotherapeutic agents.

Keywords: CRISPR/Cas9, Plasmids/Genetics, Vector, Hedgehog Proteins/Genetics, Cancer

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1. Introduction

Considered as an adaptive immune system in bacteria and archaea, clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated (Cas) system is regarded an efficient tool for genomic engineering in the field of cancer/gene therapy, detection of pathogens, virus infections, developing cancer models, and agriculture (1, 2). Mechanism of action of CRISPR/Cas systems contains firstly, detection of the specific genomic site (target gene) by single guide RNAs (sgRNAs), which can be specifically designed, secondly, cleavage of the DNA by Cas endonucleases producing double strand breaks (DSBs), and finally repairment of the DSBs by cellular machinery which leads to gene deletions, sequence insertions/deletions, or desired mutations (1, 3). Developing cancer models for further investigations, detecting mutations, and direct manipulation of immune cells (in immunotherapy) by CRISPR/Cas system demonstrates the significance of this genome editing tool in the field of cancer therapy (4).

Sonic hedgehog (SHH) signaling is a highly complex pathway which significantly functions in proliferation, differentiation, and growth-regulation of cells and tissues (5, 6). Dysregulation of this important molecular pathway may lead to several types of cancers of which basal cell carcinoma and medulloblastoma are the most common cases (7, 8); other cases include malignant gliomas, leukemia, lung, breast, prostate, and pancreas neoplasms (9). Shh pathway has various components containing SHH protein as ligand, protein patched homolog 1 (PTCH1) as receptor, smoothened (SMO) as co-receptor, glioma-associated oncogene proteins 1/2/3 (Gli1/2/3) as transcription factors, and target genes (*GLI1, GLI2, MYCN*) (9). When SHH ligand binds to PTCH1 receptor, SMO co-receptor as an upstream effector of the pathway is activated and a cascade of events begins which lead to target genes expression (10).

Clinically, various therapeutic agents have been used to inhibit the SHH pathway at different signaling levels including ligand and receptor level such as Robotnikinin, co-receptor level (SMO inhibitors) such as Vismodegib (11), and Sonidegib, and target gene level (GLI inhibitors) like arsenic trioxide (12). However, tumors with active SHH pathway gradually develop resistance against therapeutic agents through secondary SMO mutations, and other signaling pathways intervention on target genes such as PI3K-AKT and aPKCτ/λ (12). Therefore, this study aims to develop a CRISPR/Cas9 vector containing specific gRNAs to target SMO co-receptor in order to inhibit the SHH signaling pathway as a non-chemotherapy anticancer approach in SHH-active neoplasms which are resistant to therapeutic agents.

2. Materials and Methods

2.1 Registration code and Ethical considerations

This study was registered in Iran university of Medical sciences as part of a PhD thesis research project (registration code: 17235) and was approved in the ethical committee of the Iran University of Medical Sciences (ethics code: IR.IUMS.FMD.REC.1399.543).

2.2 Single guide RNA and multiplex sgRNA cassette design

In order to design the best sgRNAs for knocking out SMO gene, the National Center for Biotechnology Information (NCBI) gene database was investigated. The exons' sequences (12 exons) were extracted from the reference sequence of *SMO*, and all of them were investigated in CRISPOR (13) online platform (http://crispor.tefor.net/) to design the most suitable sgRNAs. CRISPOR online platform represent possible sgRNAs for each exon and prioritize them according to specificity, efficiency, out-of-frame score, and intronic or exonic off-target mismatch counts (13, 14). Another sgRNA was selected according to Wu et al (15) study. Following selection of the suitable sgRNAs, a cassette was designed containing sequences for restriction enzymes, U6 promoter, sgRNAs, sgRNA scaffold, and termination signal for each of the three gRNAs (Figure 1).

2.3 Plasmid extraction and digestion

Two plasmid vectors were used in this study, pUC57 plasmid (Cat#: SD0171, Thermo Fisher Scientific) containing sgRNA cassette (Figure 4-A), and pCas9-Guide-EF1a-GFP (Cat#: GE100018, OriGene Technologies, Inc) (Figure 3-A) which is a mammalian vector for co-expressing sgRNA together with Cas9, TurboGFP, and nuclear localization signal (NLS) for genome editing using the S. pyogenes CRISPR/Cas9 system. Before digestion, plasmids needed to be extracted from bacteria. Bacteria (E-coli-DH-5α) containing plasmids are resistant to antibiotics, therefore in order to make sure there are no contamination, bacteria were cultured with ampicillin (100 µg/ml) while shaking (140 rpm) in Luria-Bertani (LB) broth medium at 37°C for 18 hr. Following confirmation of no contamination (using a control E.coli DH-5α without plasmid cultured with ampicillin), plasmids were extracted by plasmid extraction 2 prep Maxi-kit (FavorPrep™, FAVORGEN BIOTECH CORP, Ping Tung, Taiwan) according to the kit's manual. Following spectrophotometry (NanoDrop™ 2000/2000c, Thermo Fisher Scientific) for DNA concentration (250 to 400 ng/µl), proper plasmids were used for digestion. In order to digest the vectors, a mixture of 10 µl of vectors, 6 µl of universal buffer (Thermo Fisher Scientific), 2 µl of BamHI and 2 µl of BsrGI restriction enzymes (NEW ENGLAND BioLabs, Inc) were incubated at 37°C for 3 hr. Restriction sites in pUC57 plasmid and pCas9-Guide-EF1a-GFP plasmid are shown in figure 4 and 3, respectively.

2.4 Gel electrophoresis and DNA extraction

For detection of digested plasmids, gel electrophoresis was done (Figure 2). Agarose powder (Sigma-Aldrich^{R}, Darmstadt, Germany) was used to prepare 0.7% gel with TAE buffer. DNA safe stain (8X, Yekta Tajhiz Azma,

Tehran, Iran) was used for detections of DNA bands. Proper DNA marker, and digested plasmids were loaded by loading buffer (8X, Yekta Tajhiz Azma, Tehran, Iran) and connected to 100 V for 30 min. Proper DNA bands were extracted by gel purification kit (FavorPrep™, FAVORGEN BIOTECH CORP, Ping Tung, Taiwan) according to the kit's manual (Figure 2).

2.5 Cloning of multiplex sgRNA cassette into the Cas9 plasmid vector

In order to clone our sgRNA cassette into the Cas9 plasmid vector, pUC57 plasmid (containing our sgRNA cassette) and the Cas9 plasmid (pCas-Guide-EF1a-GFP) were digested with the same restriction enzymes, so that proper ligation could be done. Following proper digestion and gel purification, desired DNA segments were ligated following incubation at room temperature (20-24ºC) for 2 hr, using a mixture of 2 µl of ligation buffer (10X), 2 µl of T4 DNA ligase (5U/UL, Bio Basic Inc, Markham, Canada), 2 µl of pCas9-Guide-EF1a-GFP vector, 6 µl of sgRNA cassette (extracted from pUC57 vector), and 8 µl of nuclease free ddH_2O. After ligation, Cas9 vector containing sgRNA cassette was transformed into E-coli-DH-5α bacteria. For transformation (16), fresh bacteria (E-coli-DH-5α) cultured for 4-6 hr while shaking (140 rpm) LB broth medium at 37ºC was used. Cultured bacteria were pelleted in 1.5 ml microcentrifuge tubes and put on ice, then 1 ml of calcium chloride dihydrate (99%, 0.1 M, Thermo Fisher Scientific) was added and mixed with bacterial pellet, and remained on ice for 1 hr, then centrifuged at 4000 g at 6ºC for 5 min. After that the surface fluid was removed and 200 µl of the prepared calcium chloride was added and mixed with the bacterial pellet. In another microtube, 5 µl of ligation mixture and 100 µl of prepared bacteria were mixed and put on ice for 45 min. Then, in order to heat shock the bacteria, the microtube containing the mixture was suspended on 42ºC water bath for 90 seconds, and after that immediately put on ice for 2 min. Then 300 to 500 µl of LB medium was added and the mixture was incubated at 37 ºC for 45 min. After that, 100 µl of the mixture was cultured on LB agar media (containing ampicillin) for 20 hr, then the colonies were checked and cultured for further use.

2.6 Quick analysis, PCR, and sequencing

Quick analysis of the colonies was done through phenol-chloroform DNA extraction and electrophoresis detection (17). Shortly, 10 ml of cultured colonies was pelleted, then a mixture containing 40 µl TE buffer, 50 µl of phenol-chloroform, and 5 µl loading buffer was added and vortexed for 4 min. After centrifugation at 5000 g for 10 min, 15 µl of the surface fluid was loaded on gel for electrophoresis detection of plasmids. PCR was done by primer pairs, designed by gene runner software (version 6.5.15) which were synthesized by metabion company (Steinkirchen, Germany) (Figure 3-B), and Pfu mastermix (Kiagene Fanavar, Tehran, Iran) according to the manufacturer protocol. PCR cycle included initial activation (95ºC, 2 min, 1 cycle), denaturation (95ºC, 30 secs, 35 cycles), annealing (55ºC, 30 secs, 35 cycles), extension (72ºC, 3 min, 35 cycles), final extension (72ºC, 5 min, 1 cycle). PCR product was sent to ShineGene Molecular Biotech, Inc for sequencing using ABI 3730 sequencer (Thermo Fisher Scientific).

3. Result

3.1 Selecting the most suitable sgRNAs for targeting SMO and synthesizing of multiplex sgRNA cassette

For all the exons of SMO (12 exons), sgRNAs were designed by CRISPOR online platform and two of the most suitable sgRNAs for each exon is represented in Table 1. Among the suggestions, the most suitable sgRNAs were for exons 4 (specificity: 98-99, Efficiency: 54-63, Exonic off-targets: 2), and 6 (specificity: 97-98, Efficiency: 52-73, Exonic off-targets: 2) with lowest off-targets and highest specificity and efficiency (Table 1). The cassette (1344 bp) containing three sgRNAs was synthesized by ShineGene Molecular Biotech, Inc, and delivered in pUC57 cloning vector (Figure 4).

3.2 Quick analysis, digestion, PCR and sequencing for ligation and transformation confirmation

After culturing 10 colonies of bacteria containing ligation product, quick analysis through phenol-chloroform DNA extraction and electrophoresis was done and compared with the pCas9-Guide-EF1a-GFP plasmid. All of the 10 colonies showed DNA bands a little heavier than the pCas9-Guide-EF1a-GFP plasmid showing ligation (Figure 5-A). Ligation was also confirmed by digesting the extracted plasmid from colonies with the aforementioned restriction enzymes having 1 specific restriction site in the resulted plasmid (Figure 5-B). PCR product demonstrated the expected plasmid band of 1633 (Figure 5-B). And finally sequencing of the PCR product reported the presence of sgRNA cassette (Figure 5-C).

Table 1. CRISPOR scoring.

Gene		CRISPOR scoring							
		Specificity		Efficiency		Outcome		Off-target	Number of Exonic off-targets
		MIT	CFD	D	MM	OF	MIT		
SMO Exons	4	98	99	63	54	55	84	0-0-0-0-11 0-0-0-0-0	2
		CACGGCAGACGATCTCTCGG *CGG* 120/rev							
	6	97	98	73	52	74	87	0-0-0-0-24 0-0-0-0-0	2
		CAAGAACTACCGATACCGTG *CGG* 62/fw							

MIT: MIT specificity score, CFD: CFD specificity score, D: Doench et al. study (46), MM: Moreno-Mateos et al. study (47), OF: out of frame, L: Lindel.



Figure 1. Multiplex gRNA cassette containing sequences for restriction enzymes (start: BamHI, end: BsrGI), U6 promoter, 3 sgRNAs, sgRNA scaffold, and termination signal (**Prepared by Authors, 2026**).

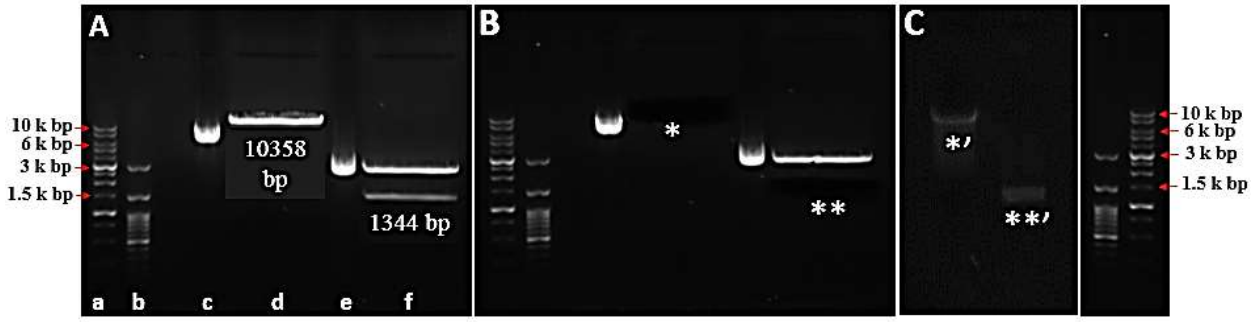


Figure 2. Digestion, gel extraction, and purification of digested plasmid vectors. (A) Digestion of plasmid vectors; (B) Extraction of the desired DNA bands; (C) Detection of extracted bands after gel purification; a: DNA marker (ladder) (1kbp), b: DNA marker (ladder) (100 bp), c: undigested pCas9-Guide-EF1a-GFP vector, d: digested (by BamHI, BsrGI) pCas9-Guide-EF1a-GFP vector, e: Undigested pUC57 vector, f: Digested (by BamHI, BsrGI) pUC57 vector (lower band is the sgRNA cassette); * Extracted digested pCas9-Guide-EF1a-GFP vector, ** Extracted sgRNA cassette; *' Detection of digested pCas9-Guide-EF1a-GFP vector after purification, **' Detection of sgRNA cassette after purification (**Prepared by Authors, 2026**).

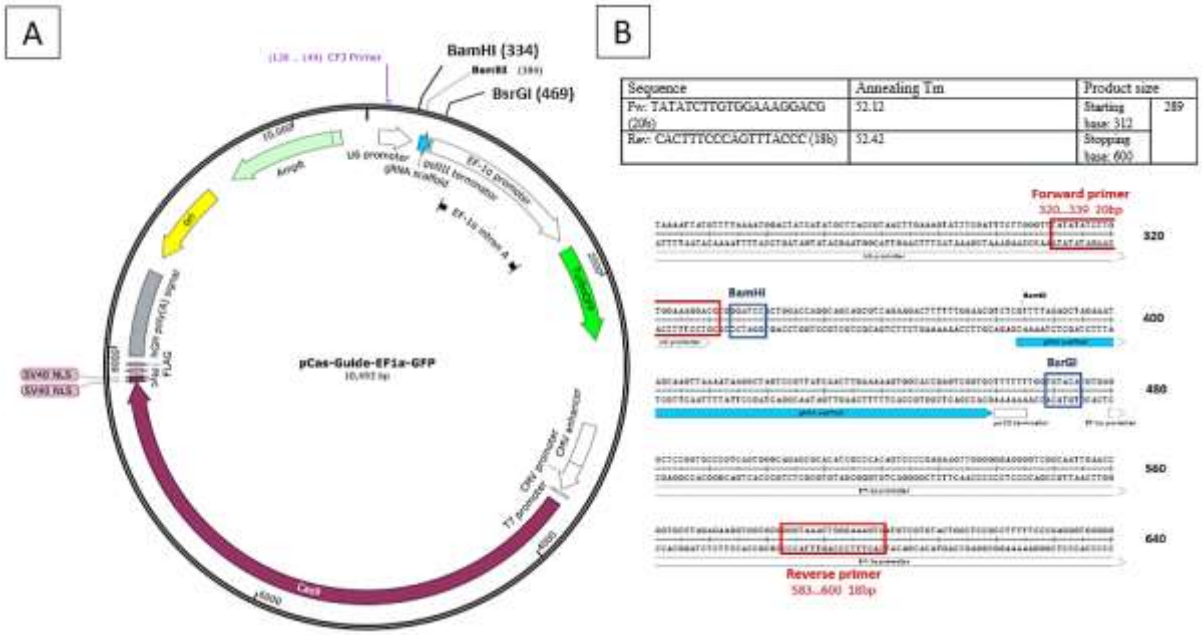


Figure 3. pCas9-Guide-EF1a-GFP vector and PCR primer-pair designed for detection of sgRNA cassette. (A) pCas9-Guide-EF1a-GFP vector contents including turboGFP, Cas9, NLS regions, and specific restriction sites; (B) PCR primer-pair features (**Prepared by Authors, 2026**).

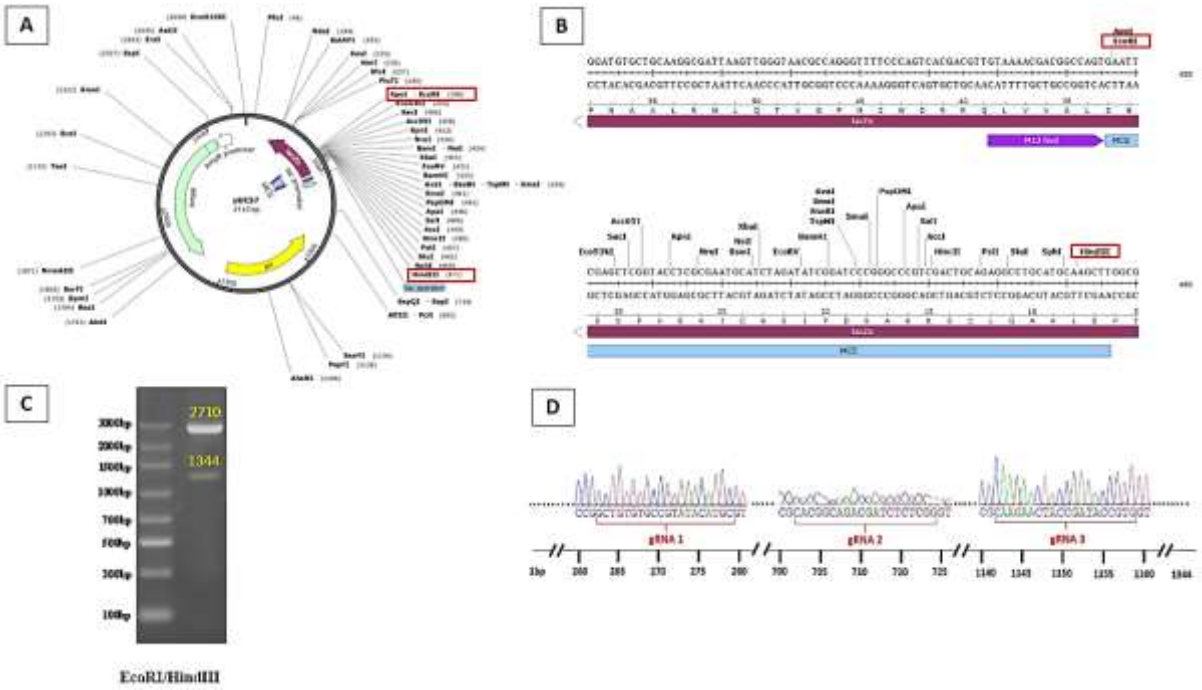


Figure 4. pUC57 vector containing gRNA cassette. (A) pUC57 plasmid vector, (B) linear sequence of the vector with restriction sites, (C) Digestion with EcorI/HindIII, (D) Sequence of three gRNAs in gRNA cassette (**Prepared by Authors, 2026**).

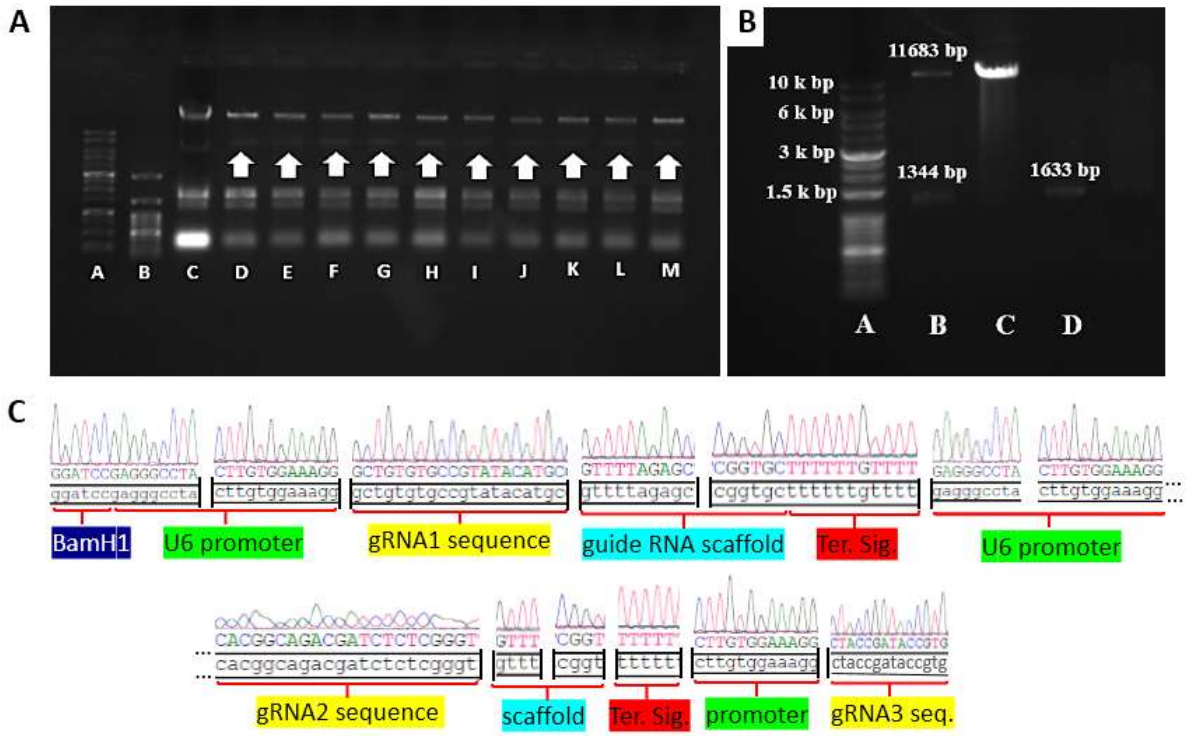


Figure 5. Quick analysis, confirmation of ligation by digestion, PCR product, and sequencing. **(A)** Quick analysis (by phenol-chloroform) of plasmids extracted from colonies (10 ml of cultured colonies) showing higher bands (white arrows) compared to pCas9-Guide-EF1a-GFP vector (C), (A: 1kb DNA marker, B: 100 bp DNA, D-M: plasmids extracted from colonies). **(B)** Ligation confirmation by digestion with same restriction enzymes (B), and PCR product (D). **(C)** Sequencing revealed the gRNA cassette sequence in the PCR product (**Prepared by Authors, 2026**).

4. Discussion

As one of the most leading causes of mortality, cancer, is a complex condition which is difficult to cure or prevent **(18)**. Several anti-cancer approaches have been suggested including surgery, chemotherapy and radiotherapy, immunotherapy, and gene therapy **(19)**. Every approach involves complications (such as toxicity, tumor regression, and resistance development) or limitations which necessitate further investigations for anti-cancer methods **(20, 21)**. Gene therapy through CRISPR/Cas9 editing tool is simple, practical, and widely used for tumor and immune cells manipulation through gene knockout, targeted insertion, chromosome translocation, and base edition **(19)**. Various applications of CRISPR/Cas9 gene editing tool renders it as a unique anti-cancer approach.

In this study, a CRISPR/Cas9 plasmid vector containing specific sgRNAs to target an upstream gene of SHH signaling pathway was developed. Specific and efficient sgRNAs were carefully selected **(22)** according to CRISPOR online platform for targeting 2nd, 4th, and 6th exons of SMO gene. Following synthesizing a cassette containing aforementioned three sgRNAs, cloning into a Cas9 vector was performed. And finally, the presence of sgRNA cassette was confirmed by PCR, enzymatic digestion, and sequencing.

SHH signaling pathway may have a significant role in various cancers, specifically basal cell carcinoma and medulloblastoma **(7, 8)**. Several pharmaceutical interventions have triggered SHH signaling pathways at different levels **(23)** among which SMO inhibitors are the most common **(12)**. Vismodegib, sonidegib, and itraconazole are SMO-inhibitors which have demonstrated promising results **(24-28)**. However, resistance to SMO inhibitors through different mechanism including resistance mutations in SMO **(29)**, and amplification of downstream SHH target genes (such as GLI2) **(30)**, renders their application less efficient. Furthermore, cancers are typically not the consequence of a single abnormal gene or pathway. Therefore, targeting an upstream gene by drug intervention may not result in efficient treatment. Gene editing through CRISPR/Cas9 is an approach which enables specific, efficient, and precise targeting, although some off-target functions depending on the specific target is inevitable **(31)**.

CRISPOR as the most comprehensive tool for designing gRNAs **(32)**, has been applied in several recent studies of gene manipulation through CRISPR system **(33, 34)**. Designing at least two gRNAs for targeting the most suitable exon with highest specificity and efficiency, and lowest off-target score is encouraged in several gene manipulation studies through CRISPR system **(15, 33, 34)**.

One previous knock-out experiment of SMO gene in embryonic stem cells by CRISPR/Cas9 has shown a successful 40bp deletion in exon 2 of SMO causing early termination **(15)**. In this study two sgRNAs were designed to target exon 2 of SMO, one of them could identify the

target sequence and Cas9 nuclease helped to break and delete the sequence, as a result of cell repair of the double strand breaks, leading to SMO knock out (15). In our study this unique sgRNA is also considered as one of the three sgRNAs developed as a cassette and cloned into the pCas-guide-EF1a-GFP.

In another study Salimi-Jeda et al (35), same approaches of cloning a vector from gRNA design, gRNA cassette development, digestion of vectors, and ligation was applied to develop a CRISPR/Cas9 vector to inhibit HIV-1 replication. In the mentioned study, authors reported disruption of HIV-1 DNA, and significant decreased viral RNA load in transfected cells, although further experiments for increasing the efficiency of this system is suggested.

Although CRISPR/Cas9 system has revolutionized the realm of genetic engineering, it faces several challenges such as off-target performance and delivery methods (36, 37). In silico tools such as CRISPOR can predict and reduce the off-target effects (38), although off-targets effects independent of gRNAs could also exist (39). Delivery methods can be categorized into different approaches such as physical, viral vector, and non-viral vector (36). Physical delivery methods such as microinjection, and electroporation can be easily operated without cargo restrictions, but they are time consuming, and mostly limited to in vitro studies (40, 41). Viral vectors such as adeno-associated virus or adenovirus and lentivirus may have high delivery efficiency and provide persistent gene transfer, but may cause high immunogenicity or insertional mutation (42). Non-viral methods such as nanoparticles can be easily operated, and show stability and histocompatibility, but they may have variable efficiency and cytotoxicity (43).

5. Conclusion

Developing specific, and efficient CRISPR/Cas9 vectors to target neoplastic pathways may possibly be a promising approach in the field of cancer treatment which could inform future therapeutic strategies leading to a better perception of different molecular pathways causing neoplasms, and developing precise chemotherapeutic agents. This study introduced a CRISPR/Cas9 vector containing gRNAs for targeting SMO to knock-out SHH signaling pathway. Further in vitro studies on relatable cell lines (such as DAOY medulloblastoma) and in vivo studies (such as medulloblastoma animal models) are required to test the efficiency of this vector.

6. Declarations

6.1 Acknowledgments

None.

6.2 Ethical Considerations

This study was registered in Iran university of Medical sciences as part of a PhD thesis research project (registration code: 17235) and was approved in the ethical committee of the Iran University of Medical Sciences (ethics code: IR.IUMS.FMD.REC.1399.543).

6.3 Authors' Contributions

M.G., A.S.J., R.S., F.M., M.M.,; Conceptualization, Supervision, Founding acquisition. M.G., A.S.J., M.R.R.,; Methodology, Software, Validation. M.G., H.M.,; Writing – original draft, Writing – Review and editing. All authors read and approved the final manuscript.

6.4 Conflict of Interest

The authors declare no conflict of interest.

6.5 Fund or Financial Support

This study was supported by Iran University of medical sciences, Tehran, Iran.

6.6 Using Artificial Intelligence Tools (AI Tools)

The authors did not utilize AI tools.

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