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Exploration of the influence of KHDC3L gene knock-out by CRISPR/Cas9 technology on PEG3 promoter

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Abstract

CRISPR-Cas9, enable precise DNA manipulation via RNA-guided breaks. KHDC3L and PEG3 genes are vital; CRISPR-Cas9 studies their roles in reproduction, development, and gene regulation. Focusing on KHDC3L's impact on PEG3 promoter in HCT116 cells, insights into gene functions and disease mechanisms emerge, informing potential therapies. This study aims to design sgRNAs for the KHDC3L gene using CRISPR tools involved ranking, off-target evaluation, and cloning. HCT116 cells were cultured, synchronized, and transfected with sgRNAs using lipofectamine. Successful transfections were confirmed by fluorescence microscopy. Clonal expansion followed, with DNA extracted and genotyped using PCR and Sanger sequencing. Bisulfite conversion analyzed DNA methylation, employing restriction enzymes for CpG site analysis. Statistical significance ($p \leq 0.05$) was assessed using SPSS software. The neighboring regions exhibited significant genomic changes. The designed sgRNAs were cloned into the PX458 plasmid, directing Cas9 to create double-strand breaks (DSBs) in KHDC3L exon 3. Transfected cells showed around 65% efficiency. Gap-PCR confirmed knock-out in 3 out of 17 clones. COBRA analysis revealed allele-specific CpG island methylation in PEG3, indicating the impact of KHDC3L knock-out on PEG3 promoter methylation and expression. The study demonstrates increased PEG3 promoter methylation upon KHDC3L deletion, indicating its role in modulation. Knockout correlates with reduced cell proliferation and colony formation, suggesting KHDC3L's role in promoting cell growth. The gene's relevance in PEG3 regulation and potential therapeutic implications are underscored, though further mechanistic insights are warranted.

Keywords: KHDC3L gene, PEG3 promoter, CRISPR/Cas9, gene knock-out

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1. Introduction

Genome editing has become a transformative scientific advance, offering enormous potential for decoding genetic diseases, developing innovative therapies, and unraveling the complex workings of life (1). This innovative technology is based on the CRISPR-Cas9 system, which is a

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remarkable molecular tool that allows for accurate and effective modification of genetic material (2). Originating from the adaptive immune system of bacteria, CRISPR-Cas9 consists of two essential components: CRISPR arrays, which include short DNA sequences called CRISPR repeats and spacers, and the Cas9 endnuclease protein (3). The CRISPR array acts as a genetic storage, while Cas9 acts as a molecular cutter. When directed by a single guide RNA (sgRNA), Cas9 combines with the target DNA, resulting in the creation of an RNA-DNA hybrid structure (4). Subsequently, Cas9's endonuclease activity starts a break in double-stranded DNA at a specific location, which then activates DNA repair mechanisms. This can lead to gene knockout through non-homologous end joining (NHEJ), which is prone to errors, or enable precise gene modifications through homology-directed repair (HDR) (1, 5).

The scientific community is highly interested in the KHDC3L gene because it may play a role in several biological processes and disease pathways (6). This gene produces a protein that contains KH domains, which are RNA-binding motifs responsible for regulating RNA metabolism (6). Although its exact function is not fully understood, studies suggest it plays an crucial role in reproductive biology, embryonic features, and stem cell pluripotency. Furtheremore, KHDC3L is involved in male germ cell development and spermatogenesis (7). Scientists can use the CRISPR-Cas9 system to specifically interfere with the KHDC3L gene and observe the resulting alterations in physical traits, which could provide valuable insight into Its importance in health and disease. This KHDC3L gene silencing study highlights the revolutionary potential of gene editing technology, especially CRISPR-Cas9, to advance our understanding of human biology and shed light on complex genetic interactions (8).

Additionally, PEG3 is a gene that is only expressed when inherited from the father and is located on chromosome 19q13.4. It is involved in various cellular functions and developmental pathways, including the regulation of gene expression, cellular proliferation, and apoptosis (9). As an imprinted gene, PEG3 exhibits haploinsufficient expression, where only one copy of the gene is expressed depending on its parental origin. Dysregulation of PEG3 is associated with various diseases, including cancer, suggesting its importance in maintaining cellular homeostasis (10). PEG3 has become a focus of research into the molecular basis of various physiological and pathological conditions due to its important role in gene regulation and its potential impact on disease states. Understanding how PEG3 functions in cellular processes is critical for understanding the complexity of regulation and potentially identifying new therapeutic targets for diseases such as cancer (11).

In this study, we aim to explore the influence of KHDC3L gene knockout using CRISPR-CAS9 system on the PEG3 promoter in HCT116 cell lines. This endeavor empowers researchers to unravel the complexities of gene function, unravel disease mechanisms, and pave the way for advanced therapeutic strategies at the molecular level.

2. Materials and methods

2.1. Ethics statement

This study conducted under the protocol approved by ethical commitment of Shiraz University of Medical Sciences (NO: IR.SUMS.REC.1399.787).

2.2. sgRNA design and CRISPR/Cas9 construction

Bioinformatic CRISPR design tools, namely Chopchop and CRISPR MIT, were utilized to design two sgRNAs targeting exon 3 of the KHDC3L gene (Table 1). These tools not only categorize sgRNAs but also assess their potential off-target effects through bioinformatics BLAST searches. Two pairs of designed sgRNAs were in-

Table 1. Two pair sgRNAs sequences for human KHDC3L gene knock-out and the potential off-target number.

sgRNAs	Sequences	Off target
sgRNA 1	F:CACCGCTCAGGAAAGGCCCTCGCC	0-0-0-6-78
	R:AAACGGCGAGGGCCTTTCCTGAGC	
sgRNA 2	F:CACCTGAATGGTTGCCAACACAAA	0-0-0-4-91
	R:AAACTTTGTGTTGGCAACCATTCA	

serted into pSpCas9(BB)-2A-GFP (PX458) (plasmid #48138; Addgene, Watertown, MA, USA) using a standard cloning protocol that includes a digestion/ligation step. In this approach, oligonucleotides encoding sgRNAs were annealed and inserted into the BbsI restriction site of plasmid PX458 (#ER1011; Thermo Scientific, Waltham, MA, USA). The cloned plasmid was then transformed into E. coli DH5α, and colony polymerase chain reaction (PCR) was employed to confirm the presence of positive colonies. Finally, Sanger sequencing was conducted to validate the results of the CRISPR/Cas9 construction.

2.3. Cell culture, transfection of CRISPR/Cas9 construcyion, and cell sorting using FACS

HCT116 cells were cultivated in a high glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS), 100 units penicillin/ml, and 100 µg streptomycin/ml. The cells were placed in an incubator at a temperature of 37C with 5% CO2 and were seeded onto 6-well plates at a density of 1 × 106 cells/well. Two pairs of sgRNAs were co-transfected into the cells using a total of 2 µg of plasmid DNA for each sgRNA and lipofectamine 2000. The efficiency of the transfection process was assessed using a fluorescence microscope after 18 and 36 hours. Cells that displayed enhanced green fluorescent protein (GFP) expression were individually isolated as single cells and distributed into three 96-well plates for clonal expansion using a FACSAria III flow cytometer.

2.4. Validation of clonal cell lines using PCR and Sanger sequencing

The genomic DNA of 17 expanded single cells was extracted using the DNA extraction kit (YektaTajhiz, Tehran, Iran). To detect the deletion mutation that caused the knock-out by CRISPR/CAS9 system, we performed gap PCR technique. Finally, sanger sequencing was done to validation of KHDC3L gene knock-out.

2.5. Combined Bisulfite Restriction Analysis

The DNA from positive cells underwent bisulfite conversion based on Herman et al. protocol (12). To initiate the precipitation of the converted DNA, a mixture is prepared consisting of 800 µL of water, 20 µL of MgCl2 (1 M), and 800 µL of polyethylene glycol (PEG). This mixture is then incubated for a duration of 1 hour at a temperature of 0 °C. Following the incubation, the mixture is centrifuged for a period of 20 minutes at a speed of 15,000 g and a temperature of 4 °C. The resulting precipitated DNA is subsequently dissolved and desulfonated by the addition of 100 µL of NaOH (0.3 M) and incubating for 15 minutes at room temperature. To further process the DNA, it undergoes standard ethanol precipitation, after which it is resuspended in 50 µL of Tris-EDTA buffer (10 mM, pH 8.0). Finally, the DNA is stored at -80°C. The analysis of CpG sites in the PEG3 promoter region is conducted using bisulfite restriction analysis (COBRA).

The amplified PCR products are subjected to analysis through restriction enzyme digestion. Each PCR product is examined using two sets of restriction enzymes that can differentiate between unmethylated and methylated DNA. The methylation level of each tested region is then determined. TaqI, BstUI, and Cla1 are enzymes that have a CpG site in their recognition site. The first group of enzymes can only digest bisulfite-treated DNA when it is methylated. This is because methylation interferes with the conversion of cytosine to thymidine during bisulfite conversion. To ensure accuracy, each of these restriction digestion reactions is performed at least three times.

2.6. Statistical analysis

The statistical software utilized for conducting the statistical analyses was SPSS (Statistical Package for Social Sciences). Statistical significance was determined by p-values of ≤0.05.

3. Results

3.1. Evaluation of the CpG islands located in the PEG3 region

The PEG3 domain in mammals shows a high degree of conservation in its genomic organization. However, the surrounding regions exhibit significant genomic variation, such as the absence of the olfactory receptor cluster (OLFR) and vomeronasal organ receptor (VNO) cluster in the human genome. Since the boundaries of this imprinted domain are still unknown, we conducted an analysis on the regions surrounding the KHDC3L gene knockout in the human PEG3 domain. Within the human PEG3 region, we found 19 CpG

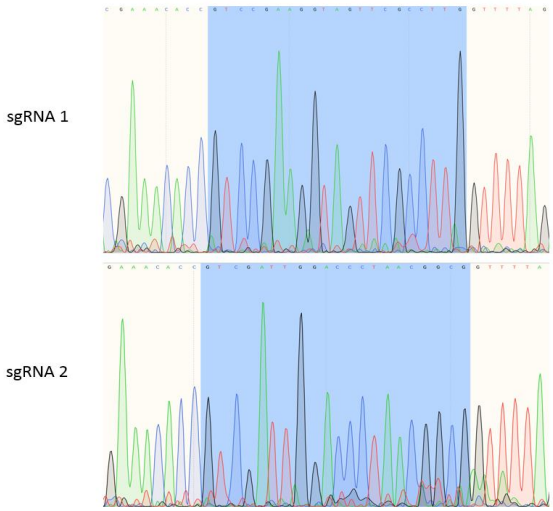

Figure 1. Sanger sequencing of two pair sgRNAs that cloned into the PX458 plasmid.

islands. Interestingly, the PEG3 region and its associated CpG islands exhibit some notable differences compared to the reference region. Firstly, although the overall GC content of the PEG3 region and the reference region is similar, the PEG3 region contains a higher number of CpG islands than expected. Secondly, both the PEG3 region and the CpG islands contain an abundance of tandem repeats that exceed the anticipated amount. These tandem repeats, ranging in size from approximately 15 bp to several hundred bp, were identified using the Tandem Repeat Finder program. Lastly, the repeat content within the PEG3 domain significantly differs from that of the reference region.

3.2 Construction of the CRISPR/Cas9 system for KHDC3L gene editing

The 20-bp sgRNAs that targets exon 3 of KHDC3L gene were inserted into the PX458 vector. This sgRNAs can direct the Cas9 enzyme to generate the double strand break in exon 3 of KHDC3L and causes the non homologous end joinig (NHEJ) repair system in this area and subsequently make a KHDC3L knock-out gene (Figure 1).

3.3 KHDC3L gene knock-out in HCT116 cells by the CRISPR/Cas9 technology

To analyze KHDC3L gene knockout, HCT116 cells were cotransfected with two pairs of pX458-KHDC3L sgRNA plasmids. Transfection efficiency was assessed by counting eGFP-expressing cells and showed that the efficiency was approximately 65% (36 hours post transfection) (Figure 2A).

The CRISPR-mediated KHDC3L gene knockout were evaluated at the genome level by gap PCR. According to the gap PCR results, 3 out of 17 expanded clones contained the desired deletion in the KHDC3L gene. Sanger sequencing of this clone showed that his KHDC3L gene at exon 3 was silenced (Figure 2B).

3.4 COBRA analyses of the PEG3 domain after KHDC3L gene knock-out

A set of comparable analyses was conducted using DNA extracted from both wild and mutant cells. The investigation focused on CpG islands linked to PEG3. The results obtained from PEG3 promoter COBRA revealed that the PEG3 promoter was digested by Bstu1, Taq1, and Cla1 enzymes, indicating that the CpG island was solely methylated in the wild-type KHDC3L cell line. As anticipated, the CpG island adjacent to the PEG3 promoter exhibited allele-specific methylation (Figure 3).

4. Discussion

The CRISPR-CAS9 system has been utilized to knock out the KHDC3L gene, and recent studies have revealed that this can have a significant impact on the expression of the PEG3 promoter. The PEG3 promoter is a crucial factor in the regulation of cell growth and differentiation, and its dysregulation has been linked to various diseases, including cancer (8). The results of this study demonstrate that knockdown of the KHDC3L gene using the CRISPR-CAS9 system leads to increased PEG3 promoter methylation in HCT116 cell lines.

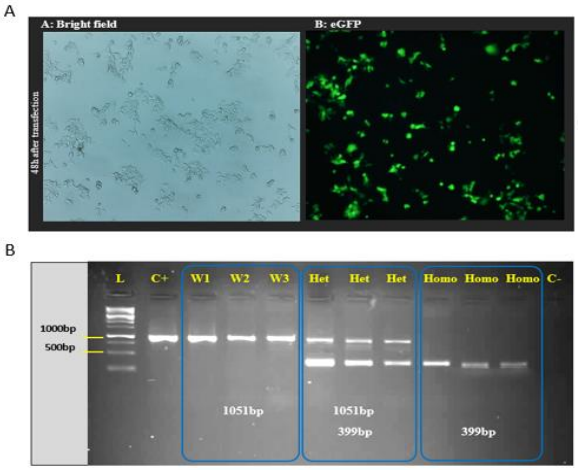

Figure 2. (A) Fluorescence microscopy showing that the GFP-positive HCT116 cells were transfected with two pairs of pX458-KHDC3L sgRNAs, followed by an 36-hour incubation period. (BGap-PCR was performed on the DNA of individual clones. The agarose gel electrophoresis analysis of the gap-PCR products revealed the presence of both mutant and wild-type alleles in certain clones. The wild-type-specific PCR products were observed in the W1/2/3 rows, while the Het rows displayed heterozygous mutant-specific products. The Homo rows exhibited PCR products containing both the wild and mutant alleles. The lanes labeled 'L' represented the 1 kb plus ladder DNA marker.

This suggests that KHDC3L plays an important role in regulating PEG3 promoter activity.

Familial recurrent hydatidiform mole (RHM) affects around 70% of women with recessive mutations of the NLRP7 gene. In contrast, genetic abnormalities of the KHDC3L gene are less common, occurring in only approximately 10% of patients without NLRP7 involvement. In both cases, these mutations cause RHM through the maternal-effect (13).

The analysis conducted in this research focused on the CpG islands linked to the PEG3 promoter. The methylation status of each CpG island was evaluated in both wild and mutant cells. Furthermore, the study demonstrated that the removal of the KHDC3L gene through the CRISPR/Cas9 system had a notable impact on cell proliferation and colony formation in the HCT116 cell line. These findings indicate that KHDC3L potentially plays a crucial role in facilitating cell growth and proliferation (14, 15). The KHDC3L gene is known as a regulation factor of gene expression, and researchers have been investigating the effects of knocking out this gene using the CRISPR-CAS9 system. Specifically, they are interested in how this knockout affects the PEG3 promoter (16).

PEG3 expression is suppressed in various types of tumors, such as gliomas, choriocarcinomas, and ovarian tumors. This inhibition of PEG3 in humans is a result of DNA methylation (17). Our findings also validate the presence of cytosine hypermethylation at PEG3 in DNA derived from the KHDC3L gene. The PEG3 promoter is responsible for regulating the expression of the PEG3 gene,

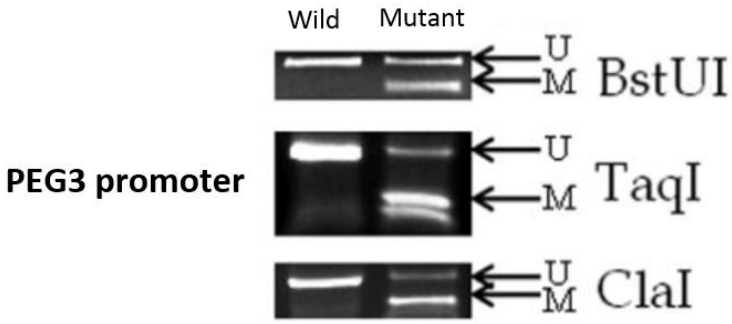

Figure 3. Wild-type and mutant cell lines were used for DNA sampling. These samples were then treated with sodium bisulfite and used in the PCR reaction. The resulting DNA was subjected to COBRA analysis, where the U arrow represents undigested and unmethylated DNA, and the M arrow represents digested and methylated DNA. Consistent results were obtained through repeated analyzes and representative images were chosen to present the results.Based on these results, it can be concluded that inactivation of the KHDC3L gene leads to methylation of the PEG3 promoter, which then reduces its expression.

which plays a crucial role in multiple cellular processes, including cell growth and differentiation (18). Previous research suggests that the KHDC3L gene may be involved in controlling the activity of this promoter. Recent experiments utilizing CRISPR-CAS9 technology have demonstrated that inactivation of the KHDC3L gene can indeed lead to alterations in PEG3 catalytic activity (6, 19). Delgado et al. conducted a study investigating the relationship between hydatidiform moles in women with NLRP7 and KHDC3L maternal-effect mutations. They revealed that HYMA1, PEG10, and PEG3, which experience hypomethylation and are affected by NLRP7 and KHDC3L mutations, exhibit biallelic expression (13).

El-Maarri et al. found that PEG3 and SNRPN genes, which are maternally methylated, were hypomethylated in molar pregnancies. The reason why paternally imprinted genes, which should not be methylated, are hypermethylated is still unclear. It is conceivable that the imprinting marks remain partially intact within the Primordial Germ Cells (PGCs) of affected individuals, resulting in the presence of hypermethylation on the mentioned loci in the oocyte shared by these patients (20). Kou et al. also examined the methylation of PEG3 and KCNQ1OT1, which showed hypomethylation consistent with a previous study (21).

The decrease in PEG3 expression was observed by the researchers following the inactivation of KHDC3L. Furthermore, recent investigations have uncovered that mutations in multiple genes (NLRP7, KHDC3L) may underlie the molecular and genetic causes of recurrent miscarriage, potentially playing a crucial role in preserving the genetic imprinting (22, 23).

In their research, Demond et al demonstrated that a mutation in the KHDC3L gene can lead to a widespread deficiency in DNA methylation when compared to individuals without this mutation. Notably, this mutation also affected germline differentially methylated regions (gDMRs) of imprinted genes. Various levels of methylation loss were observed in different genomic features, including abnormalities in certain promoter regions, suggesting a potential impact on PEG promoter (8).

5. Conclusion

In conclusion, the results indicate that the KHDC3L gene might have a significant impact on controlling PEG3 expression. Utilizing CRISPR-CAS9 to manipulate this gene could offer a hopeful signs for investigating the functionality of the PEG3 promoter and its related gene. Nevertheless, additional investigation is required to comprehensively evaluated the underlying mechanisms of these effects and ascertain the therapeutic possibilities of this approach for specific diseases.

Conflict of Interest

The authors declare no conflict of interest.

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