Babol University of Medical Sciences
IJMCM
International Journal of Molecular and Cellular Medicine
p-ISSN: 2251-9637 e-ISSN: 2251-9645
Cellular and Molecular Biology Research Center

A Decrease in CD44 on Cell Surfaces (MKN-45 cell line) After RELA Knockout Using CRISPR/Cas9

Saeid Karimi[1], Sima Salmani[1], Akram Alizadeh[2], Leila Rezakhani[3], Zohreh Saltanatpour[4, 5], Sorayya Ghasemi[1*]

1. Cellular and Molecular Research Center, Basic Health Sciences Institute, Shahrekord University of Medical Sciences, Shahrekord, Iran.
2. Department of Tissue Engineering and Applied Cell Sciences, Faculty of Medicine, Semnan University of Medical Sciences, Semnan, Iran.
3. Fertility and Infertility Research Center, Health Technology Institute, Kermanshah University of Medical Sciences, Kermanshah, Iran.
4. Pediatric Cell and Gene Therapy Research Center, Gene, Cell & Tissue Research Institute, Tehran University of Medical Sciences, Tehran, Iran.
5. Stem Cell and Regenerative Medicine Center of Excellence, Tehran University of Medical Sciences, Tehran, Iran.

Article type:
Original Article

Received:
2022.01.16
Revised:
2023.01.14
Accepted:
2023.01.21
Pub Online:
2023.03.01

ABSTRACT

The NF-kB signaling pathway was introduced as a key pathway in carcinogenesis that is induced by inflammation in gastrointestinal malignancies. The RelA transcription factor is an important component of this signaling pathway. Furthermore, CD44 is implicated in the tumorigenesis and metastasis of gastric cancer. The aim of this study was to assay the effect of RELA knockout on CD44 expression in MKN45 cells. CRISPR/Cas9 was used to knock out RELA in MKN-45. The median fluorescence intensity (MFI) of CD44 before and after RELA knockout is analyzed in MKN45. The CRISPR/Cas9 vector pSpCas9 (BB)-2A-Puro (PX459) was used for gRNA cloning (two guides). The MKN-45 cell line was co-transfected. The purified co-transfected cells with puromycin were cultured and used for the RELA gene expression assay by real-time PCR. Flow cytometry was used for the analysis of the MFI of CD44+ in MKN45. The results showed that 180 nucleotide sequences between exon 2 and exon 3 of RELA were deleted in MKN45. RELA expression significantly ($P<0.001$) decreased after CRISPR/Cas9 knockout. Compared to the control group, the MFI of CD44 in transfected cells significantly decreased ($P <0.001$). Knockout of RELA significantly decreased CD44 expression in MKN45 cells. It can be concluded that the NF-kB signaling pathway via RELA is related to CD44 expression and consequently the tumorigenesis of gastric cancer. More studies about this relationship are recommended.

Keywords: RELA, knockout, CRISPR/Cas9, CD44, MKN-45 cell line

Cite this article: Karimi. S. A decrease in CD44 on cell surfaces (MKN-45 cell line) after RELA knockout using CRISPR/Cas9. *International Journal of Molecular and Cellular Medicine*. 2022; 11(2):117-126. **DOI:** 10.22088/IJMCM.BUMS.11.2.117

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***Corresponding Author:** Sorayya Ghasemi
Address: Cellular and Molecular Research Center, Basic Health Sciences Institute, Shahrekord University of Medical Sciences, Shahrekord, Iran. E-mail s.ghasemi@skums.ac.ir

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[DOI: 10.22088/IJMCM.BUMS.11.2.117]

Introduction

Gastric cancer (GC) is the fourth-leading cancer across the world and the second-leading cause of cancer deaths (1,2). The most important cause of GC is inflammation. Carcinogenicity occurs following chronic inflammation induced by various hosts and environmental factors such as *Helicobacter pylori* and lifestyle (3). Inflammatory responses play decisive roles at different stages of tumor development, including initiation, promotion, malignant conversion, invasion, and metastasis. The evidence suggests that inflammation causes inflammation-dependent carcinogenesis through inducing changes in cell proliferation, apoptosis, and some epigenetic alterations in cancer-related genes (4).

The studies show that the NF-κB signaling pathway is considered a prototypical proinflammatory signaling pathway. The abnormal changes in the NF-κB signaling pathway play a key role in inflammation-induced carcinogenesis in gastrointestinal malignancies (5–8). Also, NF-κB pathway is one of the important signaling pathways in cancer stem cells (CSCs). RelA/p65 is a main transcription factor in NF-κB signaling pathway (6,9,10). NF-κB signaling pathway controls the expression of various genes involved in cellular responses, such as cytokines and anti-apoptotic genes. Various functions are related to these genes, such as resistance to chemotherapy and increased proliferation and apoptosis inhibition (9).

According to the substantial role of inflammation in the production of gastric cancer stem cells (GCSCs), the inflammatory NF-κB pathway appears to be involved in CD44 expression (9). CD44 is the surface marker of CSCs in GC (10). Inflammation seems to induce CD44 expression in GC. Then, CD44 expression may be influenced by suppression of the NF-κB pathway (11). In fact, evidence suggests that CD44 is a specific marker of CSCs and a key regulator of cancer cell-based properties such as metastasis, invasion, and self-renewal. In fact, CD44 can be used alone to isolate CSCs. Thus, targeting this marker can improve cancer treatment (12). Moreover, this marker can be used as a biological prognostic factor for GC (13). The NF-κB pathway is permanently active in CSCs and plays an important role in their proliferation, survival, expansion, and maintenance of their founding characteristics (14). The NF-κB pathway is one of the important pathways for causing inflammation and maintaining the fundamental function of GCSCs (15,16).

CRISPR / Cas9 gene-editing technique is able to make stable, fast, and accurate changes in the cell genome (17,18). By using this technique, we knock out the RELA gene and thus turn off the NF-κB pathway in GCSC cells to assay the effect of RELA knockout on CD44 marker expression (MFI).

Materials and Methods

CRISPR-Cas9 mediated RELA knockout

gRNAs design and plasmid construction

The two sgRNA oligonucleotides for the RELA gene were designed with online related tools (E-CRISPR, Deskgen, and DNA 2.0 CRISPR gRNA Design Tool). Each gRNA cloning was separately done in a knockout CRISPR-Cas9 vector with a puromycin resistance gene, [pSpCas9-2A-Puro (PX459) (Addgene plasmid # (62988)]. For this purpose, 1 µL of BbsI (Thermo Fisher Scientific) and its buffer were used for pX459 digestion according to the company instructions. After digestion, sgRNAs insertion in the

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BbsI-digested site was done in downstream U6 according to the Addgene protocol (cloning guidelines, PX459 vector).

The plasmid transformation into Stbl3 was prepared, and the extraction of plasmids was done using mini (THERMO #K0502) and maxi (Favorgen #FAPDE000) prep plasmid extraction kits. The quantity and quality of extracted plasmids were evaluated using nanodrop and agarose gel. Colony PCR was done to verify the accuracy of insertion. Finally, positive cloning was selected by plasmid sequencing with the U6 F primer.

Cell culture and transfection

MKN45 cell line (RRID: CVCL_0434) was purchased from the Pasteur Institute of Iran and was cultured in Dulbecco's Modified Eagle's medium (RPMI) (Gibco, USA) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin in polystyrene T-25 flasks and incubated at 37 °C in 5% CO_2 and 90% humidity. The minimum concentration of puromycin (Sigma) that can kill the whole MKN45 cells was calculated. Then, after 24-hour cultivation of MKN45 cell line in 24-well plates (5000 cells per well), they were co-transfected with 250ng of each vector (with each gRNA cloning) and 0.75 µL Lipofectamine 3000 (Invitrogen #L3000001). The transfected cells were then selected 48 hours after co-transfection and media change using the optimal puromycin concentration. Two groups of MKN45 cells were cultured. One group included MKN45 cell line without RELA knockout, and another group had RELA knockout.

Single-cell expansion

For selection of transfected cells, the remaining cells were used after adding the optimal concentration of puromycin (during 72 h) so that single cells could be obtained with serial dilutions of single cells.

Each single cell was cultured in one well of a 96-well plate. After an adequate increase in the number of cells, the cultured cells from a single cell were passaged and transfected into a 24-well plate. These cells were colonized in two separate wells for DNA extraction and back-up.

DNA deletion detection: PCR and gel electrophoresis

To design primers for this gene, we first obtained the RELA gene sequence from the NCBI site. And the sequence was entered in the primer blast site, and primers were obtained using this site, the primer design criteria for sequencing were reviewed, and finally, the sequence was blasted to ensure its accuracy and specificity. Genomic DNA was isolated from the transfected cells in each well and from the control group (non-transfected cells). DNA extraction was done with the Mini Kit (Yekta Tajhiz Azma, Iran) according to the manufacturer's instructions. To screen knockout clones, PCR primers were designed (Table 1) for the sites next to knockout regions that were targeted by gRNAs. PCR was performed with 10 ng of DNA in 35 cycles in an automated thermocycler (MWG Biotech Primus, Germany) at 95 °C to denature double-strand DNA, annealing at 61 °C for 90 s, and temperature extension at 72 °C for 45 s. For analysis of the PCR product, 10 µL of each of the cell groups was electrophoresed on a 2% agarose gel.

DNA deletion detection: DNA sequencing

For RELA gene sequencing, 500 ng of amplified DNA of transfected cells (a colony from a single cell with a deletion detected on a 2% agarose gel) was sequenced. Then the analysis and observation of the

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results were performed with Version 3.2.1 SnapGene software. The original gene was compared at the NCBI.

Table 1. The used primers for cell knockout screening: genomic DNA screening RELA gene.

Primer		Primer sequence from 5′ to 3′	Annealing temperature (°C)	Length (bp)
RELA	F	CTATGTGGAGATCATTGAGCAG	61	573
RELA	R	CTGGAACTCATCTGCTAGAG	61	

qRT-PCR

RNX (Cinna Gen, Inc., Iran) is based on the manufacturer's protocol for total RNA extraction from cell groups. After RNA extraction, cDNA was synthesized from 1µg of the total RNA (Thermo Fisher Scientific, Inc., MA, USA) as instructed by the company. Using electrophoresis and spectrophotometry, the quality and quantity of the extracted RNA were measured. The extracted RNAs were treated with DNase I (Thermo Fisher Scientific, Inc., MA, USA). The primers for qPCR analysis for RELA expression were designed by Primer3 and Integrated DNA Technologies (IDT) (Coralville, IA). Their specificity was checked with Primer-BLAST. In qPCR, the expression of RELA was measured in transfected and non-transfected cells (as the control group). GAPDH was used as an internal control. The PCR conditions were as follows: after initial denaturation at 98 °C for 30 seconds, 40 cycles were performed, each consisting of denaturation at 95 °C for 15 seconds, primer annealing at 60 °C for 30 seconds, and extension at 72 °C for 30 seconds, followed by a final extension at 72 °C for 10 minutes. $2^{-\Delta\Delta Ct}$ was used for data analysis. Table 2 shows the primer sequences used in qRT-PCR.

Table 2. The primer sequences used in qRT-PCR.

Primer		Primer sequence: 5′ to 3′	Annealing temperatures (°C)	Length (bp)
RELA	F	CTATGTGGAGATCATTGAGCAG	60	119
RELA	R	GGTGGGTCTTGGTGGTAT	60	
GAPDH	F	CTCTCTGCTCCTCCTGTTCG	60	114
GAPDH	R	ACGACCAAATCCGTTGACTC	60	

Analysis of CD44 expression in MKN45 cell line before and after RELA knockout by flow cytometry

The presence, frequency (%), and median fluorescence intensity (MFI) of $CD44^+$ cells were investigated before and after RELA knockout by flow cytometry.

For this purpose, 1 µL of the CD44 antibody (Cat# 14-0441-82) was added to 200,000 cells of each group in 100 µL of phosphate buffer solution (PBS) and left at 4 °C for 30 min. Then, centrifugation was performed at 1500 rpm for 5 min, approximately 50 µL of PBS was removed, and then pipetting was performed to isolate the cells. The cells were incubated at 4 °C for 20 minutes in the dark. Flow cytometry (Accuri, BD Biosciences, CA, USA) was done in both groups.

Statistical analysis

All data were presented as mean±SD. All statistical analyses were performed using SPSS version 18.0 (SPSS Inc., Chicago, IL, USA). Intergroup comparisons were made using Student's t-test. A $P<0.05$ was considered a significance level. All tests were performed in triplicate.

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Results

Targeting RELA gene with CRISPR-Cas9

Using the above-mentioned online tools, two gRNAs targeting coding exons 2 and 3 of RELA were designed and synthesized (Figure 1A). Each of gRNAs nucleotides was cloned in a PX459 vector (Figures 1B, 1C) and after colony PCR, positive clones were selected. We used two primer pairs to perform colony PCR. The PX459 vector contains a puromycin selection marker. The minimum concentration of puromycin for the whole MKN45 cell line was 2 µg/mL for 48 hours.

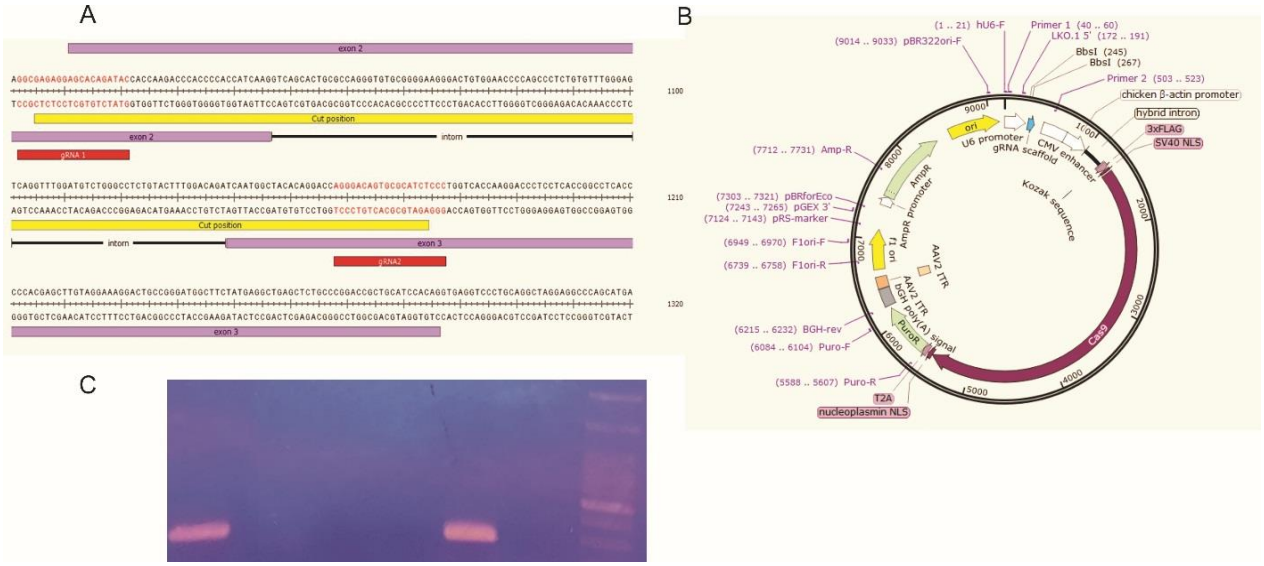


Fig.1. Preparation and validation of the knocked-out MKN45 cell line. A) The sequences and positions of the guides on the RELA gene (red sequences). gRNA1: GGCGAGAGGAGCACAGATAC, and gRNA2: AGGGAC AGTGCGCATCTCCC. Yellow region: The sequences that are removed from the genome by knockout. Purple Zone: Parts of Exons 2 and 3. B) A schematic picture of plasmid PX459 cloned with gRNAs separately. C) Prove the cloning of guides in two vectors separately. A forward primer on the U6 and complementary sequence of guides as reversal primers; Primer sgRNA1 reverse: GGCGAGAGGAGCACAGATAC, and Primer sgRNA2 reverse: AGGGACAG TGCGCATCTCCC, and U6-F: GAGGGCCTATTTCCCATGATT Product length is 267 bp.

DNA deletion detection: PCR and sequencing

After DNA extraction from the MKN45 cell line (control group) and obtaining of colonies from single (knockout) cells, an oligonucleotide deletion in genomic DNA was detected with PCR in a single colony cell. Oligonucleotide deletion in targeting sites was done with two simultaneous concurrent DSBs by both gRNAs. Without DNA deletion, by this primer set, PCR product is 573 bp. The length of PCR product in single cell colony in which deletions occurred was 393 bp. The results of sequencing also confirmed the deletion of 180 sequence lengths (Figure 2A-B).

qRT-PCR

The transfected and non-transfected cells were used for qRT-PCR. The expression levels of RELA significantly decreased (-11.39-fold), (P = 0.01) (Figure 3).

Analysis of CD44 expression before and after RELA knockout in GC by flow cytometry (MFI)

Analysis of CD44 expression before and after RELA knockout in GC by Flow cytometry showed that MFI significantly decreased in the transfected cells (Figure 4).

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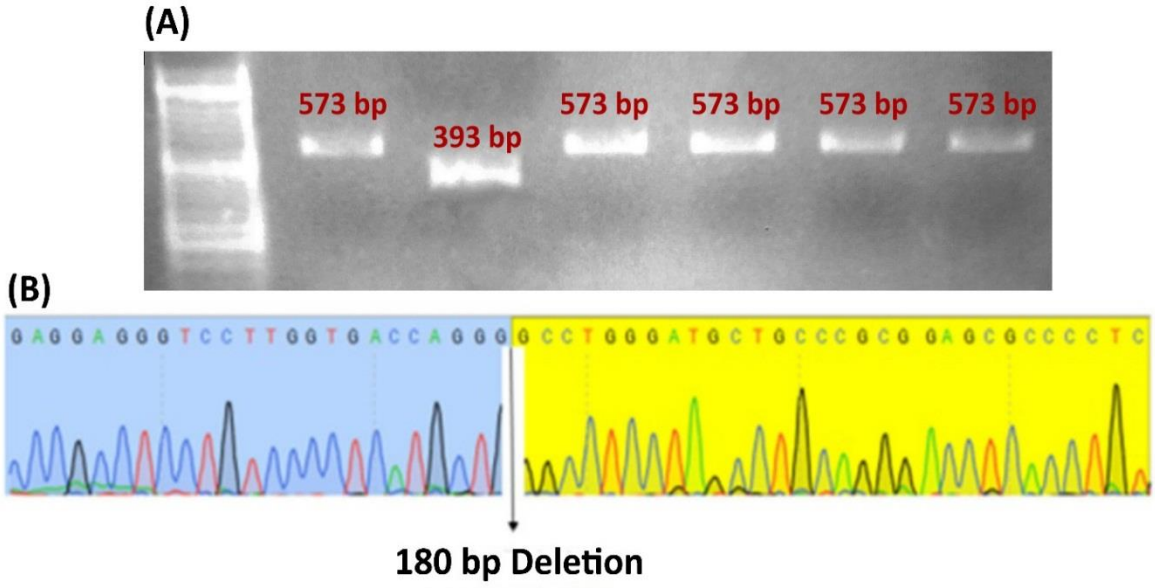


Fig.2. Positive cell confirmation results. A) PCR results in six clones originating from a single amplified cell show that both gRNAs acted in only one cell clone, deleting about 180 bp in the RELA gene. Knock out colony cells with a 180 bp deletion of the RELA gene sequence on the agarose gel. (Marker M: 100 bp ladder). B) Positive cells sequencing result confirms 180 bp deletion. The arrow indicates the location of the deletion.

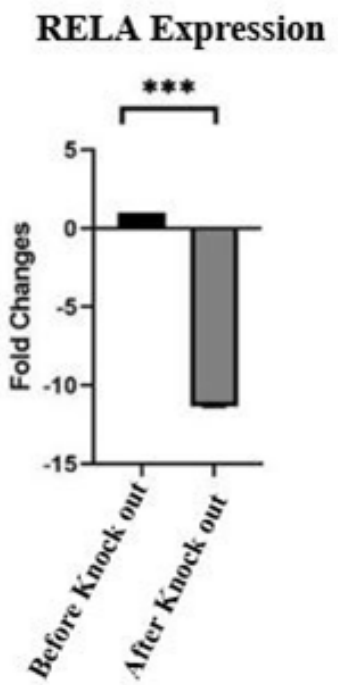


Fig.3. RELA gene expression. After transfection, expression RELA was examined in triplicate, and the results are presented as mean ± SD. Expression levels RELA were normalized by GAPDH as an internal control. A decrease in RELA expression was observed in the transfected cells group (MKN-45 Knock out) compared to the control group (*** = P<0.001).

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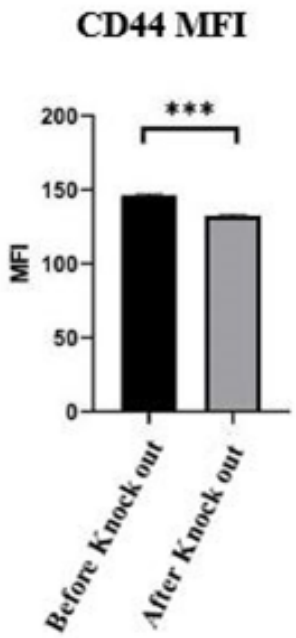


Fig.4. MFI averages in the control (MKN-45) and treatment (MKN-45 knockout) groups. MFI was decreased significantly in the transfected cells (*** = P<0.001).

Discussion

RELA as a prognostic parameter (6) is an important component of the NF-κB signaling pathway that plays a role as an oncogene in different cancers. Also, due to the important role of cancer stem cells in the tumorigenicity of cancer, in this study, we investigated the effect of RELA on CSCs in the MKN45 cells (19,20). We used the MKN45 cell line because it contains a high percentage (about 70-80%) of CSCs (21) and it has been shown that using only the CD44 marker can be sufficient to detect CSCs in this cell line (22). CRISPR-Cas9 technology was used in our research. CRISPR-Cas9 is the most remarkable technology in genome editing. Through RNA-directed Cas9 nucleases, the CRISPR-Cas9 system can modify DNA with greater precision than available technologies such as TALEN and ZFN. Besides that, this system is preferred over other mutagenic techniques due to the relative simplicity of its plasmid design and its construction (23). We specifically design sgRNAs to achieve the highest on-target and the lowest off-target activities. In addition, CRISPR-Cas9 has the potential to generate gene knockouts in different cancer cell lines (24). In our study, for the first time, the effect of RELA knockout on CD44 expression was studied using the CRISPR-Cas9 gene editing system.

Our results showed the successful knockout of RELA in MKN45 cells. We reduced RelA expression by using two gRNAs for the 180-bp RELA gene deletion. Since in the current study, transient gene silencing methods such as small interfering RNA-mediated knockdown were not used, the problems with transient silencing methods and external (ectopic) RNA consequences were not observed. Therefore, CRISPR-Cas9-mediated knockout techniques can more realistically show the relation between RELA gene expression and CD44 as an important marker of CSCs.

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To better examine the relationship between RelA and CD44 expression, an MFI assay was performed. The results from MFI indicated that CRISPR-Cas9 mediated knockout of RELA decreased CD44 average expression in the MKN45 cell line, which was statistically significant. These findings indicate that RelA/p65 is associated with the expression of CD44, an important CSC marker. As RelA/p65 is one of the NF-κB families and participates in the inflammatory NF-κB pathway, it seems that the NF-κB pathway is associated with CD44 expression and MKN45 stemness. It can be concluded that RELA knockout decreased CD44 expression in MKN45 cells and led to a decrease in the average expression of CD44 per cell. However, protein-level studies, such as western blotting, may complement this study, and further studies are needed to elucidate the regulatory mechanism by which RELA regulates CD44 expression in MKN45 and other cancer cell lines.

Yet, a direct link between the CD44 marker and the NF-κB signaling pathway has not been established, however, the NF-kB signaling pathway has been shown to be one of the most important pathways in CSCs (25). Since this signaling pathway is permanently active in some types of CSCs, it may be an important pathway in the maintenance of CSCs (14). Targeting RELA has been shown to be a new strategy for inhibiting the invasion, migration, proliferation, and growth of cancer cells, including skin cancer (26). One of the best ways to remove CSCs is to target the signaling pathways of these cells. Since CSCs play important roles in resistance to conventional therapies and recurrence of cancer, signaling pathways of these cells may be a worthy choice for targeting these cells (27). A study shows that in bone marrow cancer cells, the expression of both NF-kB and CD44 increases simultaneously (28). Evidence suggests that the STAT3 protein binds to NF-κB factor in the cell nucleus to form a complex that binds to the promoter of the hTERT gene to increase the expression of this gene, which increases the expression of the CD44 marker (29). Sasaki et al. (2001) studied 64 tissues of patients with GC and found that transcription factor P65 (RELA) is permanently expressed in GC tissues and showed that the activation of the NF-κB pathway plays a key role in cell division, apoptosis, the production of cytokines, and carcinogenicity (30).

According to data, inhibiting NF-κB activation has an impact on the proliferation and invasiveness of triple-negative breast cancer cells by decreasing the expression of its target genes, such as CD44 (31). Some natural compounds that are known to have anti-inflammatory properties have also been found to have anti-CSC effects (32). Therefore, it seems that the main pathways of inflammation and CSCs overlap. One of these connections could be our findings in this article.

It would be beneficial to compare the function of RELA knockout cells in various cancer lines to the function of its non-knockout cells in the other cancer lines to do more study on how RELA influences other biological processes, such as proliferation.

Conclusion

According to our results, it seems that NF-κB signaling pathway is involved through the RELA gene in the CD44 expression in the human GC cell line MKN45, which showed the role of this pathway in CSCs' persistence. However, further studies are needed to clarify the RelA/p65-mediated relationship between CD44 gene expression and NF-κB signaling pathway.

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Acknowledgments

The authors declare no conflicts of interest. The study was funded by Shahrekord University of Medical Sciences (Grant No. 2705). Compliance with ethical standards: Informed consent Written informed consent was obtained from the participants. The study protocol was approved by the Ethics Committee of Shahrekord University of Medical Sciences (approval code: IR.SKUMS.REC.1397.3).

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