Iranian Journal of Immunology (IJI)
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ORIGINAL ARTICLE

Effects of CD133 Silencing on Survival and Migration of HT-29 Colorectal Cancer Cells

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ABSTRACT

Background: Colorectal cancer (CRC) is attributed as one of the most common malignancies worldwide. CD133 molecule, as a pentaspan transmembrane glycoprotein, confers stem cell-related characteristics, including self-renewal and multi-directional differentiation capability. CD133 plays important roles in the progression of CRC by conferring apoptotic resistance and migration ability. **Objective:** To investigate the anti-apoptotic and anti-angiogenic effect of CD-133 targeted siRNA in a colorectal cancer cell line. **Methods:** In this study, CD133-targeted siRNA transfection was conducted into HT-29 cells. MTT assay was employed to evaluate the cytotoxic effects of transfection on the cells. Flow cytometry was used to evaluate the apoptosis rate. The mRNA expression of apoptosis and metastasis related genes were assessed by quantitative Real-Time PCR (qRT-PCR). Wound healing assay was used to assess the migration potency of the infected cells. **Results:** Expression of CD133 was significantly downregulated after transfection of CD133-specific siRNA. Moreover, the rate of apoptosis was significantly increased after transfection. The migration potential of cells was diminished after transfection. siRNA delivery resulted in the modulation of expression of apoptosis and metastasis-related genes. **Conclusion:** siRNA mediated targeting of CD133 could be considered as a promising approach to treat CRC through suppressing the cancerous behavior of tumor cells.

Received: 2019-02-18, Revised: 2019-06-26, Accepted: 2019-06-30.
Citation: Akbari M, Shanehbandi D, Asadi M, Shomali N, Faraji A, Khaze V, Pakdel A, Mokhtarzadeh A, Ebrahimi AA, Shabani A, Bardaran B. Effects of CD133 Silencing on Survival and Migration of HT-29 Colorectal Cancer Cells. *Iran J Immunol.* 2019; 16(3):246-257. doi: 10.22034/iji.2019.80275.

Keywords: Apoptosis, Colorectal Cancer, CD133, Metastasis, siRNA

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INTRODUCTION

Several decades ago, the incidence and prevalence of colorectal cancer (CRC) was not remarkably high. Nonetheless, it has become a prevalent cancer and today it is responsible for about 10% of cancer-related mortality worldwide (1). The underlying reasons for the increasing prevalence of CRC in developed countries are aging, dietary habits, as well as lifestyle-related risk factors such as low physical activity, obesity, and smoking. Genetics, along with environmental contributing factors, have been considered as major culprits in CRC onset (2-5). Impairment in the Genomic and/or epigenomic stability has been identified in most cases of early neoplastic lesions in colon tissue and is probably a central molecular and pathophysiological factor in the development of CRC (6-8). With the advent of novel treatment options for primary as well as metastatic CRC, new hopes have emerged. Such options include laparoscopic surgery in the case of primary CRC, and more-aggressive resection of metastatic CRC, radiotherapy, and chemotherapy (9,10). Nonetheless, these new treatment options have been accompanied by limited effectiveness of the therapy as well as long-term survival (11). As a result, investigating new treatment strategies has been on the agenda for researchers and clinics (12). CD133 (also called prominin-1) is a transmembrane glycoprotein that is expressed in several cells, such as endothelial cells, neuroepithelial cells, and hematopoietic cells (13). CRC cells expressing CD133 present stem cell characteristics, like self-renewal and multi-directional differentiation capability (14). CD133 is regarded as a specific marker of primary colorectal cancer stem cells (CSCs) and its expression has been linked to differentiation and tumor size in CRC (15). CD133 expressing CRC cells have been shown to be resistant to radiotherapy and chemotherapy (16,17). Nevertheless, findings about the aggressiveness of CRC cells expressing CD133 have been controversial (18,19). Despite some therapeutic successes in CRC treatment, the combination of drugs and new methods is still not effective in improving the condition and increasing the lifespan of patients. Therefore, the development of new therapeutic strategies, like gene therapy, is necessary. Gene therapy is a strategy that involves the transfer of specific genetic sequences to the cell in order to relieve the disease. In such approaches, transfection of cancer cells to silent genes involved in growth and proliferation through interference RNA (iRNA), like small interfering RNA has been investigated (20,21). In the current investigation, a HT-29 CRC cell line was transfected with CD133-targeted siRNA. Then, the apoptosis and invasive behavior of the cells were investigated.

MATERIALS AND METHODS

Cell Culture. The colorectal cancer cell line (HT-29) was purchased from cell bank, the RIKEN BioResource Center through the National Bio-Resource Project of the MEXT (RIKEN; Tsukuba, Ibaraki, Japan). The CRC cells were cultured in RPMI 1640 culture medium (Sigma-Aldrich, St. Louis, MO, USA), including 10% fetal calf serum (FCS; Sigma-Aldrich, St. Louis, MO, USA), 1% antibiotics, 100 Unit/mL penicillin and 100 µg/ml streptomycin (Gibco Inc., Paisley, UK). The cells were cultured in 37°C, 95% humidity, and 5% CO_2. Culture medium was changed each day, and the cells were passaged after reaching a confluence of 70–90%.

MTT Assay. The toxicity of CD133 siRNA on HT-29 cells was evaluated by MTT assay after 24, 48 and 72 hours of treatment. Cells were seeded at a density of 15×10^3 cells/well in 96-well culture plates, and then transfected with CD133 specific siRNA using JetPEI reagent (Polyplus, France) according to the manufacturer's instructions. For this purpose, 6 µl of transfection reagent and 80 pmol of siRNA was utilized. Moreover, scrambled siRNA was employed as a negative control. After the incubation time, 100 µl of MTT reagent (0.5 mg/ml in PBS) was added to each well and the plates were incubated for 4 hours. The created formazan crystals were solubilized by adding 100 µl of the solubilization buffer (DMSO + Sorensen buffer) to each well. After 30 minutes of incubation, the optical density (OD) of each well was measured at 570 nm using an ELISA reader (Awareness Technology, Palm City, FL, USA).

Flow cytometry. Flow cytometry assay (Annexin V and PI staining) was employed to determine the effect of CD133 specific siRNA on HT-29 cell apoptosis. First, 100 µl of cells were washed in calcium buffer and then poured off in flow cytometry tubes for assessment of apoptosis using the Phosphatidyl Serine detection kit (IQ Products, The Netherlands). Then, 5 µl of FITC conjugated Annexin V (Annexin V-FITC) was added to the cell suspension of each tube, and gently mixed and incubated for 20 minutes at 4 °C in a dark chamber. Then, cells were washed with calcium buffer and 5 µl of Propidium Iodide (PI) and added to each tube and kept for 10 minutes at 4°C in a dark chamber. After staining, cells were washed twice and re-suspended in PBS. Then, 1×10^6 cells per sample were gated and evaluated by green fluorescence (FL1-H) filter using untreated cells as a control in a FACSCalibur system (Becton Dickinson, USA). The Cellquest software (Becton Dickinson, USA) was used to analyze the results.

Real-time PCR Gene Expression Quantification. To quantify the mRNA expression of target genes, including CD133, BCL2, Bax, Cas3, Cas8, Cas9, Vimentin, MMP-1, c-Myc, and MDR, first the total RNA was extracted from HT-29 cells using RNX-PLUS reagent based on the manufacturer's manuals. Afterwards, reverse-transcription of mRNA into complementary DNA (cDNA) was performed using a universal cDNA synthesis Kit (TAKARA, Japan). To perform the gene expression analysis, real-time PCR was employed utilizing SYBR Green-1 dye universal Master Mix in a LightCycler® 96 system (Roche, Germany). The transcript level of β-actin, as the housekeeping gene, was also measured. The relative amounts of mRNA expression were evaluated based on the comparative C_T approach through $2^{-\Delta\Delta CT}$ formula (22). The primers used in real-time PCR quantification are indicated in Table 1.

Wound Healing Assay (scratch test). To evaluate the effect of CD133 specific siRNA on the HT-29 cells migration, the wound healing assay (scratch test) was used. HT-29 cells were seeded in 24-well plates for 24, 48 and 72 h, and a scratching was performed using a sterile 100 µl pipette tip across the cell monolayer to make a gap region, at the time of >90% confluence. Cell debris was cleared by washing with PBS. Then HT-29 cells were transfected with 80 pmol CD133 targeting siRNA. The gap area at 0, 24, 48 and 72 h after scratching was photographed under the light microscope. Measurement of the migration rate was carried out using the NIH Image J software.

Statistical Analysis. The statistical analysis of data was conducted by SPSS software version 21 (SPSS, Chicago, IL, USA). Data were represented as mean ± standard deviation (SD). Differences between groups were analyzed with the Kruskal-Wallis test. A p-value less than 0.05 was considered as statistically significant. The graphing was carried out with GraphPad Prism v.6 software (GraphPad Software Inc., San Diego, CA; www.graphpad.com).

Table 1. Primer pairs and their related nucleotide sequence.

Gene	Sequence	Amplicon Size (bp)	Tm (°C)
CD133	Forward: 5′-CAGAGTACAACGCCAAACCA-3′ Reverse: 5′-AAATCACGATGAGGGTCAGC-3′	245	59
Bax	Forward: 5′-TTTGCTTCAGGGTTTCATCCA-3′ Reverse: 5′- CTCCATGTTACTGTCCAGTTCGT -3′	151	59
Bcl2	Forward: 5′- CCTGTGGATGACTGAGTACC -3′ Reverse: 5′- GAGACAGCCAGGAGAAATCA -3′	128	59
MMP1	Forward: 5′- GCGCACAAATCCCTTCTACC -3′ Reverse: 5′- ATCCGTGTAGCACATTCTGTCC -3′	170	59
Vimentin	Forward: 5′- CAGGCAAAGCAGGAGTCCA -3′ Reverse: 5′- AAGTTCTCTTCCATTTCACGCA -3′	122	59
Caspase3	Forward: 5′- GGCGCTCTGGTTTTCGTTAAT -3′ Reverse: 5′- CCAGAGTCCATTGATTCGCT -3′	120	59
Caspase8	Forward: 5′- TGAAAAGCAAACCTCGGGGA -3′ Reverse: 5′- TGAAGCTCTTCAAAGGTCGTG -3′	151	59
Caspase9	Forward: 5′- GCAGGCTCTGGATCTCGGC -3′ Reverse: 5′- GCTGCTTGCCTGTTAGTTCGC -3′	152	59
c-Myc	Forward: 5′-AGGCTCTCCTTGCAGCTGCT -3′ Reverse: 5′-AAGTTCTCCTCCTCGTCGCAGT - 3′	163	59
MDR	Forward: 5-′ATGGTCAGTGTTGATGGACAG -3′ Reverse: 5′-AGCTATCGTGGTGGCAAAC - 3′	108	59
GAPDH	Forward: 5′- CAAGATCATCACCAATGCCT -3′ Reverse: 5′- CCCATCACGCCACAGTTTCC -3′	166	59

RESULTS

Cytotoxic effect of CD133 specific siRNA on HT-29 cells.

Treatment with CD133 specific siRNA caused increased cytotoxicity in HT-29 cells compared to the control group. In comparison with the group, CD133 siRNA group demonstrated statistically significant decrease in the cell survival rate in a time-dependent manner (Figure 1).

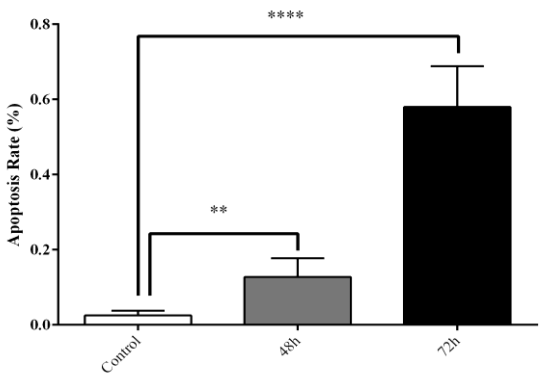

Figure 1. Evaluation of the effect of CD133 on cell viability. Cytotoxic effect of CD133-specific siRNA transfection of HT-29 cells was measured by MTT assay, indicating a statistically significant increase in apoptosis after 48 as well as 72 hours.

Effect of CD133 targeted siRNA on apoptosis of HT-29 cells.
After treating HT-29 cells with CD133 targeted siRNA, a decreased number of vital cells after 24 (1.00 vs. 0.656; p=0.011), 48 (1.00 vs. 0.467; p=0.001), and 72 (1.00 vs. 0.504; p=0.005) hours was evident in comparison to the control group.

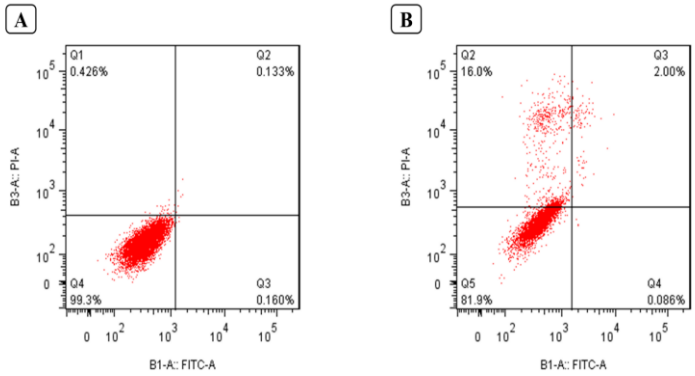

Figure 2. **Evaluation of effect of CD133 on apoptosis**. The apoptosis rate of cells was measured by flow cytometry after CD133-specific siRNA transfection of HT-29 cells. A; control, and B; apoptosis rate after 48 hours.

In addition, no significant difference in apoptosis rate was detected between the negative control/siRNA group and treatment groups (Figure 2 and 3).

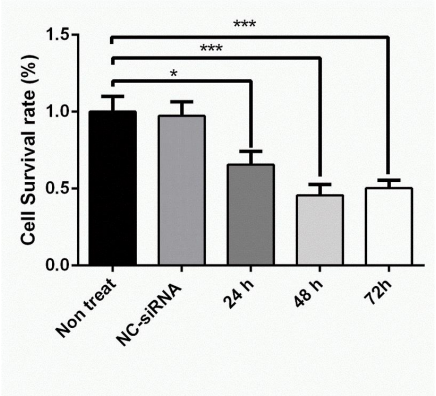

Figure 3. Evaluation of effect of CD133 on cell survival rate. Bar graphs illustrated the cell survival rate in HT-29 cells after delivering CD133-specific siRNA. After treating HT-29 cells with CD133 targeted siRNA, a decreased number of vital cells after 24 (p=0.011), 48 (p=0.001), and 72 (p=0.005) hours was observed in comparison to the control group. There was no significant difference in apoptosis rate between the negative control/siRNA group and treatment groups.

Effect of siRNA transfection on CD133 mRNA levels.
In comparison to the non-treated group, it was observed that after 24 hours since transfection, the expression of CD133 was significantly downregulated (0.656 vs. 1.00; p=0.016). As such, CD133 was lowly expressed in HT-29 cells after 48 (0.467 vs. 1.00; p=0.004) and 72 (0.503 vs. 1.00; p=0.001) hours from transfection in comparison to

control group. There was no significant difference between the negative control/siRNA group and treatment groups (Figure 4).

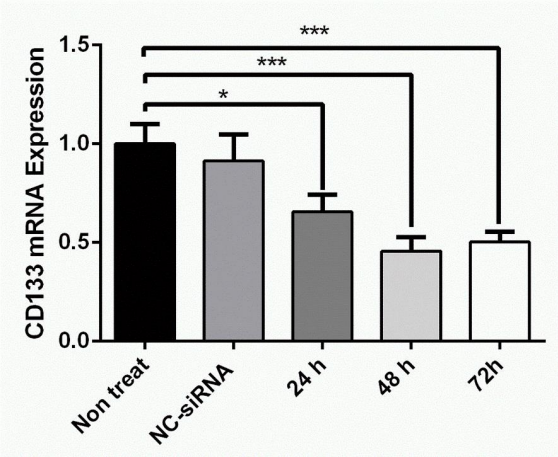

Figure 4. Evaluation of CD133 mRNA expression. The mRNA expression of CD133 in HT-29 cells in control groups as well as in siRNA transfected cells after 24, 48, and 72 hours. In comparison to the non-treated group, the expression of CD133 was significantly downregulated (p=0.016) after 24 hours since transfection. CD133 was downregulated in HT-29 cells after 48 (p=0.004) and 72 (p=0.001) hours from transfection in comparison to control group. No significant difference was observed between the negative control/siRNA group and treatment groups.

Effect of CD133 siRNA transfection on the expression of apoptosis and migration-related genes

Assessment of apoptosis-related genes revealed that CD133 targeting siRNA caused upregulation of Bax mRNA in HT-29 cells in comparison to the negative control group (1.3 vs. 1.00; p=0.031; Figure 5A, Table 2). However, Bcl-2 mRNA expression was downregulated in HT-29 cells compared with the negative control group, although the difference was not statistically significant (0.873 vs. 1.00; p=0.214; Figure 5B, Table 2). The Bax/Bcl-2 ratio Has been recently considered as a predictor of susceptibility to apoptosis (23). In the present study, the Bax/Bcl-2 ratio was significantly higher in the HT-29 cells of the treated group compared to the negative control group (1.487 vs. 1.0; p=0.003; Figure 5C, Table 2). According to the results, caspase 3 expression did not show a statistically significant difference between the treated group and the negative control group (1.19 vs. 1.0; p=0.172; Figure 5D, Table 2). As such, both caspase8 (1.22 vs. 1.0; p=0.081; Figure 5E, Table 2) and Caspase9 (1.057 vs. 1.0; p=0.297; Figure 5F, Table 2) expressions were upregulated insignificantly in siRNA treated HT-29 cells in comparison to the negative control group. Regarding metastasis-related genes, Vimentin mRNA expression was downregulated in CD133 siRNA treated HT-29 cells in comparison to the negative control group (0.4267 vs. 1.000; p=0.00079; Figure 5G, Table 2). Moreover, MMP-1 mRNA expression demonstrated significant downregulation in HT-29 treated cells compared with the negative control group (0.150 vs. 1.000; p=0.000043; Figure 5J, Table 2).

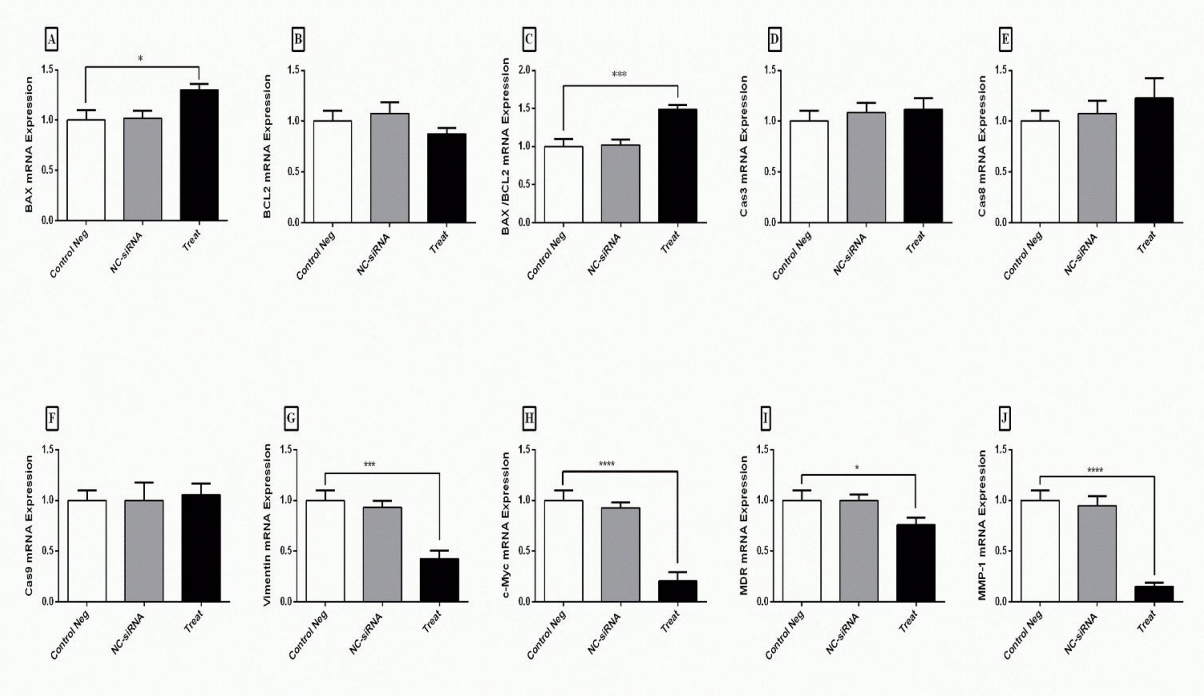

Figure 5. Evaluation of effect of CD133 on apoptosis and migration-related genes. Bar graphs illustrates the mRNA expression of apoptosis-, metastasis-, and drug resistance related genes in HT-29 cells after transfection with CD133-specific siRNA. CD133 targeting siRNA caused upregulation of Bax mRNA (A) in HT-29 cells in comparison to the negative control group (p=0.031). The Bax/Bcl-2 ratio (C) was significantly higher in the treated group in HT-29 cells in comparison to the negative control group (p=0.003). Vimentin mRNA (G) expression was downregulated in CD133 siRNA treated HT-29 cells in comparison to negative control group (p=0.00079). MMP-1 mRNA (J) expression demonstrated significant downregulation in HT-29 treated cells compared with negative control group (p=0.000043). Both c-Myc (p=0.000092) (H) and MDR (p=0.026) (I) expressions were significantly downregulated in the siRNA transfected cells compared with the negative control group.

Finally, both c-Myc (0.2067 vs. 1.000; p=0.000092; Figure 5H, Table 2) and MDR (0.7600 vs. 1.000; p=0.026; Figure 5I, Table 2) expressions were significantly downregulated in the siRNA transfected cells compared with the negative control group.

Table 2. Fold change of the gene expression differences in different groups and time points.

Gene	Fold change (Treat vs. Negative control)	p-value
Bax	1.30	0.031
Bcl2	0.87	0.214
Bax/Bcl2	1.48	0.003
Caspase3	1.19	0.172
Caspase8	1.22	0.081
Caspase9	1.05	0.29
Vimentin	0.42	0.00079
MMP-1	0.15	0.000043
c-Myc	0.20	0.000092
MDR	0.76	0.026

Effect of CD133 siRNA on the migration of HT-29 cells.

The metastatic potential of HT-29 cells was evaluated using the migration assay. The potential of transfected cells in filling the gap area over time was assessed (24). The migration assay indicated a reduction in the total area of coverage after the knocking down of CD133 in comparison to the control cells after 24, 48, and 72 hours (Figure 6A). According to the Figure 6B, the number of migrated cells was decreased at three time points of 24 (800 ± 40 vs. 1000 ± 50; p=0.024), 48 (850 ± 45 vs. 1200 ± 55; p=0.0043) and 72 (900 ± 55 vs. 1600 ± 60; p=0.00071) hours in the CD133-targeted siRNA group in comparison to the control group.

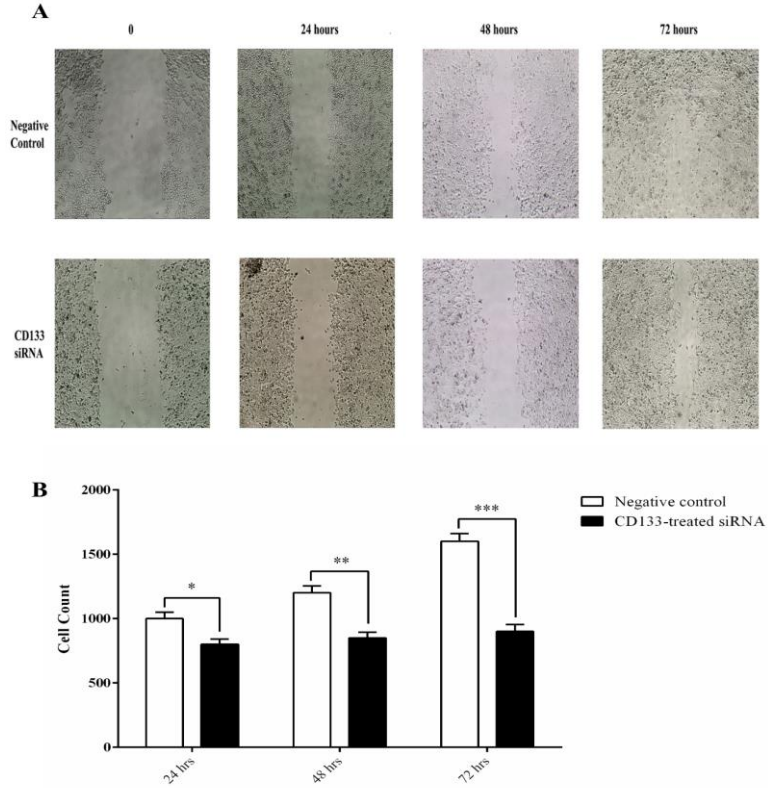

Figure 6. **Evaluation of effect of CD133 on cell migration.** A) Evaluation of migration ability of HT-29 cells after transfecting with CD133-specific siRNA. In order to assess the CD133 effect on cell migration, a confluent monolayer of HT-29 cells was scratched and then treated with CD133 siRNA. The scratched area was assessed after 0, 24, 48, and 72 hours by light microscopy and direct observation. A reduction was observed in the total area coverage after CD133 knockdown in comparison to the control cells after 24, 48, and 72 hours. B) Bar graphs show the number of migrated cells.

DISCUSSION

In the current study, we endeavoured to clarify whether or not targeting CD133 with siRNA could offer therapeutic prospects with respect to increased apoptosis and migration suppression of cancerous cells. In this study, we purchased CD133 specific siRNAs from "Santa Cruz Biotech" company. This company provides siRNA products as a pool of three to five target-specific oligonucleotides. Use of pooled siRNAs,

targeting the gene of interest, decreases the off-target effects. It has shown that the low concentration of each individual siRNA reduces the off-target effects below detection limits (1). This eliminates the need for a CD133 negative cell line as a negative control for normalization of the off-target effects. The decreased expression of CD133 in this study is a consequence of the accumulative effects of 3-5 target specific siRNA oligonucleotides. This means in the concentration used, each siRNA is responsible for 20-30% of CD133 downregulation. In the case of off-targeting, this trace amount of each individual sequence cannot significantly affect the expression of non-targeted genes (25). Apoptosis of HT-29 CRC cells was increased after the transfecting of CD133-specific siRNA, in spite of controversial results in the expression of apoptosis-related genes. Furthermore, the migration potency of the cells was diminished. In advanced cancer therapy, two major targets, including the epidermal growth factor receptor (EGFR) and the vascular endothelial growth factor (VEGF), have already been suggested (26). In addition, the Wnt signaling pathway has been considered as a potential target for CRC therapy. It has been showned that the activity of the Wnt pathway accounts for the chemoresistance of CD133-expressing CRC cells (27,28). 5-fluorouracil was seen to upregulate Wnt activity in CD133-expressing CSCs from CRC subjects (29). As an inhibitor of Wnt signaling, Dickkopf-1 (DKK-1) caused a decline in the expression of CD133. Moreover, DKK-1 suppressed the proliferation, migration, and invasion of CRC cells (30). As a consequence, blocking the Wnt pathway might be an approach which could resolve the issue of chemoresistance. It is possible to eliminate CSCs through targeting membrane proteins like CD133 and then delivering drugs that can particularly trigger apoptosis. The majorityof attempts have been made using CD133 in the therapy of cancers. The conjugation of a CD133 monoclonal antibody with a cytolethal distending toxin, which inhibits the proliferation of CD133-expressing cells in head and neck squamous cell carcinomas, has resulted in promising outcomes (31). Furthermore, using short hairpin RNAs, the repression of CD133 molecules was diminished in melanoma cells. This implementation culminated in declined cell motility and cell growth (32). Wang *et al*. (33) applied single-walled carbon nanotubes (SWNTs) conjugated with CD133 monoclonal antibodies in glioblastoma. They ascertained an increased apoptosis of CD133-expressing cells, and that the tumorigenic and self-renewal behaviors of CD133-expressing cells were inhibited. In this study, we transfected CD133-targeting siRNA into HT-29 cells. This experiment increased the apoptosis rate of cancer cells and suppressed their migration potency. Nonetheless, the exact mechanism of such suppression of cancer cells by targeting CD133 is still unknown and should be further clarified. A number of studies have investigated the underlying mechanisms of metastasis in cells expressing CD133 molecule. In a study, chemokine CCL21/CCR7 increased the metastasis and survival of CD133 expressing pancreatic CSCs. The regulation of metastasis in CD133 expressing pancreatic CSCs was suggested to be through modulating epithelial–mesenchymal transition (EMT) and Erk/NF-κB pathways (27). Although we did not investigate the exact molecular mechanism of metastasis in HT-29 CRC cells, the metastasis-related genes were observed to be decreased after delivering CD33-specific siRNA, resulting in a reduced metastasis potency of cancer cells. The resistance of cancer cells to chemical drugs and radiation therapy is a challenging issue in the treatment of cancer. The resistance can be due to mutations in the genome of individuals, which leads to an increase in the expression of drug targets, thereby disabling the drug or removing the drug from the cell. Sometimes, after the initial chemotherapy, patients develop tumors that are

resistant to several drugs. CSCs have also been shown to play a major role in the resistance of tumor cells that have an inherent resistance to chemotherapy drugs and play a major role in tumor growth and tumor reversal to new tumor cells (34). Additionally, it was observed that breast cancer cells exhibited more drug resistance potency when they overexpressed CD133. Therefore, targeting the CD133 marker, along with other surface markers on breast cancer cells, can be a useful strategy to eradicate breast cancer cells in the clinic (35). Researches have also suggested that CD133 is related to multi-drug resistant genes, such as ABCG1 and ABCG2 (36,37). In this study, CD133-targeting siRNA transfection in HT-29 cells resulted in a decreased expression of MDR, suggesting a beneficial effect of transfection in potentially decreasing the resistance of CRC cells to chemotherapeutic drugs.

Considering all the facts, in the current study, we observed the benefits of CD133-targeting siRNA transfection in CRC related HT-29 cells. These beneficial effects might stem from increased apoptosis of cancer cells along with decreased metastasis potency of a cell after the suppression of CD133. This investigation provides evidence for the therapeutic potential of CD133-specific siRNA in ameliorating CRC, which needs animal studies and clinical trials to further validate its efficiency.

ACKNOWLEDGEMENTS

This study was supported by the Immunology Research Center, Tabriz University of Medical Science, Tabriz, Iran and Semnan Biotechnology Research Center, Semnan University of Medical Sciences, Semnan, Iran.

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