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Transduction of an optimized recombinant lentivirus expressing E-cadherin shRNA resulted in stable downregulation of CDH1 gene and obvious cell morphological change in the human colorectal cancer cell line HT29

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ABSTRACT

BACKGROUND: E-cadherin (encoded by CDH1 gene) is a protein associated with invasiveness, metastasis, and poor prognosis of tumors and a critical protein in maintaining the structural integrity of epithelial sheets. The inhibition of E-cadherin expression has been reported in several types of cancers, including colorectal cancer, esophageal adenocarcinoma, gastric cancer, and pancreatic cancer. The aim of this study was to prepare an optimized recombinant lentivirus expressing short hairpin RNAs to stable downregulation of E-cadherin and evaluation of its transduction effects on cell morphology. METHODS: Human pGIPZ lentiviral shRNA vector was used to downregulation of the E-cadherin in the HT29 human colorectal cancer cell line. The expression level of mRNA was assessed using qRT-PCR. The changes in protein expression were confirmed by western blotting and ICC. Non-transduced HT29 and transduced HT29 with pGIPZ non-silencing lentiviral shRNA vector were used as controls. Furthermore, morphology of the transduced cells was monitored for 40 days using light and fluorescent microscopy. RESULTS: While the morphology of HT29 cells was epithelial-like, they changed gradually into fibroblast-like appearance after transduction. Our results showed that these molecular and morphological changes were stable in our monitoring time. CONCLUSION: It can be concluded that the suppression of CDH1 gene by shRNA method leads to depletion of E-cadherin protein expression and morphological changes in the human colorectal cancer cell line HT29.

Keywords: pGIPZ lentiviral vector, CDH1 Gene, E-cadherin, shRNAs

INTRODUCTION

"Cadherins are a calcium ion-dependent cell surface glycoprotein family. It has been shown that they are fundamental in cell-cell adhesion" [1, 2]. E-cadherin (encoded by CDH1 gene) which is a single-span trans-membrane glycoprotein [3, 4] and the primary cell adhesion molecule within the epithelium plays an important role in maintaining the structural integrity of the epithelial sheets. In many cancers, loss of E-cadherin is related to a more aggressive and poor prognosis of tumors [1, 2]. Loss of E-cadherin is a feature of epithelial-mesenchymal transition (EMT), a phenomenon related to metastasis [1, 2]. The role of E-cadherin as a metastasis inhibitor has been well established [1, 5]. Inhibition of E-cadherin expression has been reported in several types of cancers, including esophageal adenocarcinoma, gastric cancer, vulvar squamous cell carcinoma, pancreatic cancer and

colorectal cancer [6–8]. Regarding the role of E-cadherin in metastasis and EMT, several studies have used knockdown of E-cadherin for cancer related studies [9-11]. Moreover, there is substantial evidence that downregulation of E-cadherin is able to enrichment of cancer stem cells in cancer cell lines through induction of EMT [9].

Since, interference RNA (RNAi) is as a powerful tool to reduce gene expression, in most studies it uses for downregulation of different genes [12]. RNAi can silence selective gene by two mechanisms based on nucleic acids. The first one is cytoplasmic delivery of short double-stranded (ds) interfering RNA oligonucleotides (siRNA), which leads to unstable gene silencing and the second one is nuclear delivery using plasmids that express short hairpin RNA (shRNA), which are similar to endogenous interfering RNA and leads to stable gene silencing [13]. shRNAs with stem-loop structures can be used in gene silencing via RNA interference after DICER processing (14). Some studies showed that shRNAs are more efficient than siRNAs in silencing of gene expression [15–17]. These days, lentiviruses are used to transfer shRNA-expression cassettes due to high efficiency delivery of shRNAs into cells [13]. The Lentiviruses (LV) are a subclass of retroviruses, they carry two copies of a single-stranded RNA genome in an enveloped capsid, can integrate into host genomes and cause long-term gene silencing in dividing and non-dividing cells [13, 18].

The present study was conducted to production of an optimized recombinant lentivirus expressing E-cadherin shRNA to stable downregulation of the CDH1 gene in the human colorectal cancer cell line HT29. To produce of our recombinant lentivirus, the pGIPZ lentiviral shRNA vector was used as transfer vector. In this vector, shRNA constructs are expressed as human microRNA-30 (miR-30) primary transcripts. This feature adds a Drosha processing site to the hairpin stem loop and has been shown to increase the efficiency of gene silencing [19]. After recombinant lentivirus production, its transduction effects on mRNA and protein expression were evaluated in HT29 cell line. Moreover, cell morphology changes were monitored until 40 days. The final goal of this study was to downregulation of E-cadherin in the human colorectal cancer cell line HT29 for application in future studies on EMT, metastasis, and cancer.

MATERIALS AND METHODS

Cell culture: The HT29 human colorectal cancer cell line (IBRC C10097) was purchased from the Iranian Biological Resource Center (IBRC, Iran). Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco, USA) with 10% fetal bovine serum (Gibco, USA), 2 mM L-glutamine (Gibco, USA), 100 units/mL penicillin, and 100 µg/mL streptomycin (Invitrogen, USA) in 25 cm2 cell culture flasks and cultured overnight at 37°C in 5% CO_2 incubator.

Vectors: Human pGIPZ lentiviral shRNA (Cat# RHS4531, USA) and pGIPZ non-silencing lentiviral shRNA vectors were purchased from Dharmacon (Cat# RHS 4346, USA). pGIPZ shRNAs were available in glycerol stock as transformed in bacteria. The map of the vector has been shown in Figure 1A .

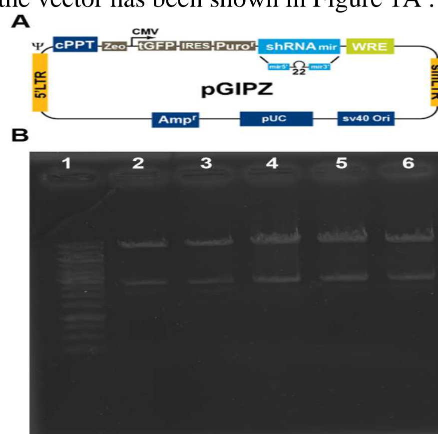

Figure 1: pGIPZ lentiviral vector. (A) Map of pGIPZ lentiviral vector. (B) Plasmid confirmation using EcoRI digestion: After digestion of pGIPZ vector by EcoRI, two bands (2716 and 8972 bp) were observed. Lane 1: ladder 100 kb; lane 2-6: pGIPZ lentiviral vector (repeated 5 times)

Plasmid preparation: For plasmid preparation, pGIPZ shRNA clones were grown at 37°C in 2X-LB (cat# BP1427500, Thermo Fisher Scientific, USA) broth (low salt) media in the presence of 100 µg/ml ampicillin (Invitrogen, USA). Plasmids were extracted according to the Plasmid Miniprep Kit (Qiagen, Germany) and confirmed by EcoRI (Thermo Fisher Scientific, USA) digestion which results in two bands (2716 and 8972 bp). The

DNA concentration was determined by measuring its absorption at 260 nm. The DNA purity was checked using the 260/280 nm ratio .

Lentivirus production: Each of the shRNA-pGIPZ (shE) and pGIPZ non-silencing (shC) vectors with 2 packaging plasmids (psPAX2, and pMD2.G) were transfected in HEK-293T (human embryonic kidney, ATCC CRL-3216) cells using the standard calcium phosphate precipitation method according to Tronolab protocol with some modifications [20]. At first, 5×106 HEK-293T cells were seeded in a 10 cm plate in DMEM (Gibco, USA) with 10% FBS (Gibco, USA). On the next day, 21 μg of transfer vector pGIPZ, 21 μg of psPAX2 vector, and 15 μg of pMD2.G vector were mixed with transfection buffer and added dropwise to the cells. 14 hours after the transfection, the medium was replaced with the fresh medium. Turbo green fluorescent protein (tGFP) expression was observed by fluorescent microscopy after 24 hours. The packaged recombinant lentiviruses were harvested from the supernatant of cell cultures 48 hours after transfection, centrifuged at 2000 rounds per minute (rpm) in 4 °C for 5 minutes, and filtered through a 0.22 μm filter. The recombinant viruses were stored at -70 °C for subsequent experiments. Viral titers were determined with counting the number of GFP-positive cells by flowcytometry.

Transduction: To determine the optimal transduction efficiency for the HT29 cell line a range of MOI (multiplicity of infection) values of 0.3, 1, 3, 5, and 10 were used. At first, HT29 cell line were cultured and washed with PBS. Then the recombinant viruses were added to each well with optimal MOI. For efficient transduction, the "Spin-fection" method (2000 rpm for 60 minutes at a temperature of 25°C) was used. After "Spin-fection," plates were incubated at 37°C and the medium was changed 14 and 20 hours after "Spin-fection." After 24, 48, and 72 hours, transductions were assayed with GFP expression [20, 21]. pGIPZ lentiviral shRNA transduced HT29 (HT29-shE) and pGIPZ lentiviral non-silencing shRNA transduced HT29 cells (HT29-shC) were purified using puromycin (Sigma, Germany). To determine the appropriate concentration of puromycin for the HT29 cell line, a serial titration with the following concentrations of 0, 1, 2.5, 5, 10 and 15 μg/m was formed. Each experiment was repeated three times.

Transfection and transduction efficiency evaluation: To evaluation of transfection and transduction efficiency, observed cells were counted at least in 5 random fields. Percentage of efficiency was calculated using the following formula:

%Efficiency = (number of GFP positive cells /total number of cells) × 100

SYBR-Green Real-Time-PCR: Total RNA was extracted from 2 × 106 cells (for HT29-shE at 10 and 40 days after transduction) using the RNeasy mini kit (Qiagen, Cat#Q74134, Germany). The cDNA was synthesized using the TaKaRa kit (PrimeScriptTMRT reagent Kit, Cat# RR037A, USA). Real Time PCR assay was replicated three times for each sample and the difference of the Ct values between the sample's replications was no more than 0.5. The average Ct was used for analysis. All reactions were performed using Power SYBR Green PCR Master Mix (ABI Bio systems, Cat# 4367659, USA) at the default setting on an ABI Bio systems Step One Plus Real-Time PCR Machine with the following temperature profiles: denature at 95°C for 5 minutes, 40 cycles of 95°C for 35 seconds, and then hold at 60°C for 1 minute. Relative expression levels were determined from collected data as threshold cycle numbers. Table 1 shows the sequence of primers. The GAPDH gene served as an internal control.

Western blotting: Cells were lysed using M-PER (mammalian protein extraction reagent) (Thermo Fisher Scientific, USA) supplemented with protease inhibitor cocktail (Sigma, Germany). After blocking with 5% non-fat milk in TBST for 1 hour, the membranes were incubated with primary antibodies dissolved in 5% bovine serum albumin (BSA) (Sigma, Germany) in TBST overnight at 4°C. The following primary antibody was used: anti-human-E-cadherin (Cat# sc7870, USA), at a dilution of 1:2,000. The membranes were washed with TBST 3 times for 5 minutes each and then incubated for 1 hour at room temperature with secondary antibody (1:3,000, goat anti-rabbit IgG/HRP; Cat# 65-6120, Invitrogen, USA) dissolved in 5% non-fat milk in TBST. Anti-human-beta Actin (Abcam, USA) was used as an internal reference at a 1:5,000 dilution.

Immunocytochemistry of cells: Cells were washed 3 times with PBS and fixed with 4% paraformaldehyde for 30 minutes at room temperature. The fixed cells were incubated for 25 minutes with 1:9 dilution of normal goat serum in PBS to block nonspecific binding of the primary antibody. The cells were incubated in 3% H2O2 in PBS for 15 minutes to block endogenous peroxidase and then incubated with E-cadherin antibody (Cat# ab15148, USA) overnight at 4°C. The slides were washed 3 times with PBS and incubated with goat anti-rabbit IgG-PE (Cat# ab72465, USA) for 1 hour at room temperature. Cells were washed and incubated with DAPI (Sigma Aldrich, St. Louis, USA) for 5 minutes to detect the nuclei. Cells were evaluated under the microscope.

Cell morphology monitoring: The morphology of the transduced cells was monitored for 40 days with light and florescent inverted microscope (Olympus, Japan) and compared to control cells.

Statistical analysis: All experiments were carried out in triplicate and statistical analyses were performed using GraphPad Prism software 6.01 by one-way ANOVA and a paired t-test. Results are expressed as means ± the standard error of the mean (SEM). For relative quantification of gene expression, differences were calculated as $2^{\Delta\Delta CT}$. P <0.05 was considered statistically significant.

RESULTS

Lentiviral vector, production and titration evaluation

Plasmid digestion with EcoRI restriction endonuclease confirmed vector and two bands (2716 and 8972 bp) were appeared on the agarose gel (Figure 1B). The production of the recombinant lentivirus was verified using the expression of tGFP using the fluo¬rescence microscope 24, 48 and 72 h after transfection. As shown in figure (2A, B), more than 90% of HEK293T cells were transfected by vectors. The optimum titer of the recombinant virus was 1.5–2×106. IU/mL.

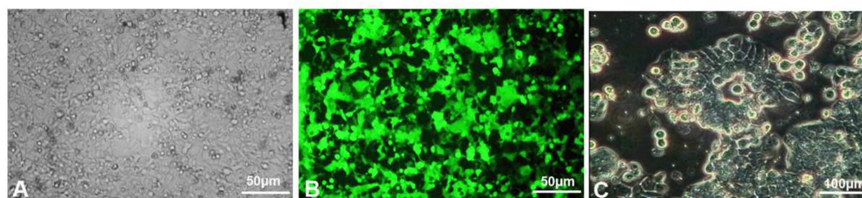

Figure 2: (A, B) Transfected HEK 293T cells after 48 hours GFP expression; (A) Light microscopy image, (B) microscopy fluorescent image (the same field). (C) HT29 morphology; Prior to transduction, the morphology of non-transduced HT29 cell line was epitelial-like.

Transduction assessment

The results of transduction using the fluo¬rescence microscope showed that, at MOI of 5, more than 80% of the transduced cells were found to be tGFP-positive. Moreover, the optimal concentration of puromycin for HT29 cell line obtained at 1μg/mL.

pGIPZ lentiviral vector carrying shRNA reduced expression level of E-Cadherin mRNA and protein

Real time-PCR data analysis showed that the mRNA expression of E-cadherin was reduced in HT29-shE cells compared to non-transduced HT29 and HT29-shC cells. There were no significant differences in the mRNA expression of the E-cadherin after 10 and 40 days (Figure 3A). Western blot analysis showed that the protein expression of E-cadherin was reduced in HT29-shE cells (Figure 3B, C). By contrast, the protein expression of E-cadherin in non-transduced HT29 and the HT29-shC cells did not change. Western blot results were compatible with real time-PCR results. ICC analysis of protein expression showed that different levels of E-cadherin expression were observed between HT29-shE cells with non-transduced HT29 and HT29-shC cells, and the abundance of E-cadherin was lower in HT29-shE cells than in the control cells (Figure 4). These results were compatible with real time-PCR and western blot results.

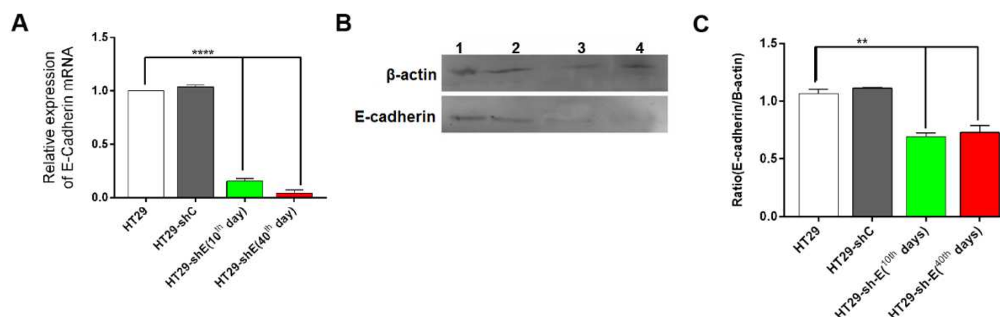

Figure 3: Reduced expression level of E-Cadherin mRNA and protein. (A) E-Cadherin mRNA expression in HT29-shE cells was reduced compared to non-transduced HT29. There was no significant difference between mRNA expression of E-cadherin after 10 and 40 days. Values normalized to the housekeeping GAPDH (P <0.05). (B, C) E-Cadherin protein expression in HT29-shE cells was reduced compared to non-transduced HT29. There was no significant difference between protein expression of E-cadherin after 10 and 40 days; (B) Lane 1: HT29; lane 2: HT29-shC; lane 3: HT29-shE (10th day); lane 4: HT29-shE (40th day). (C) Densitometry analysis of protein content was normalized against its corresponding β-act data point (P <0.05)

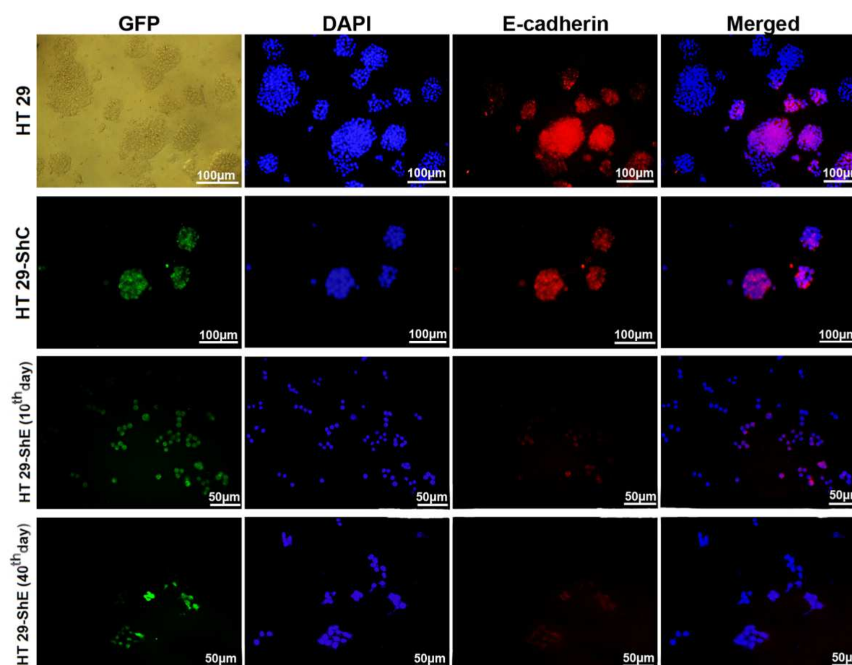

Figure 4: Immunofluorescence staining of E-cadherin showed reduced expression of E-cadherin protein. The red signal represents expression of GFP, The blue signal represents the nuclear DNA staining by 4´, 6-diamidino-2-phenylindole. The red signal represents expression of E-cadherin.

Downregulation of E-cadherin changed the morphology of HT29 cells

Prior to transduction, morphology of non-transduced HT29 cell lines was epithelial-like (Figure 2C). Following transduction, cell morphology changes were monitored daily using light and fluorescent microscopy and photographed (Figure 5). After transduction, cell morphology monitoring showed that at first, HT29-shE cells were dispersed and presented with single and double-cell morphology (Figure 5A, B). After about 10 days, HT29-shE cells gradually became elongated and lost their epithelial morphology with disrupted intracellular adhesion, and acquired fibroblast-like appearance (Figure 5C, D). Finally, most cells presented fibroblast-like shape and this morphology was stable during our monitoring time (Figure 5E, F). In this period, no morphological changes in the HT29-shC cells were observed (Figure 5G, H). It is notable that only GFP-positive cells were subjected to morphological changes and GFP- negative cells have no sign of morphology changes (Figure 5I, J).

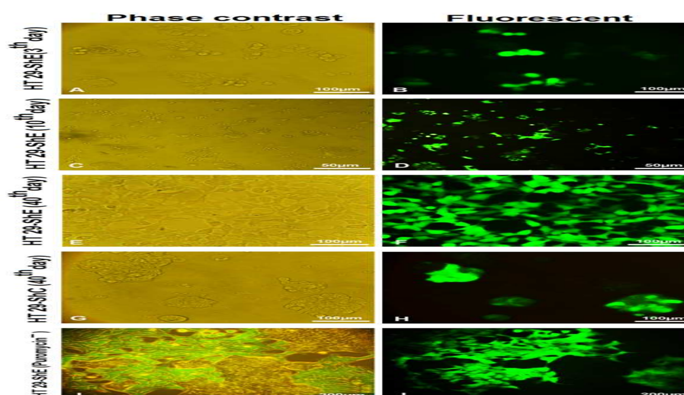

Figure 5: Monitoring morphological changes in transduced HT29 by lentiviral virus at different times. (A, B) 72h after transduction, HT29-shE cells were dispersed and presented with single and double-cell morphology. (C, D) 10th day, HT29-shE cells gradually became elongated, lost their epithelial morphology, and acquired fibroblast-like appearance with a long tubular shape. (E, F) 40th day, the most cells presented fibroblast-like appearances. (G, H) 40th day, HT29-shC without any morphological changes. (I, J) Only GFP-positive cells were subjected to morphological changes and GFP-negative cells have no sign of morphology changes

DISCUSSION

Colorectal cancer is the second leading cause of cancer-related deaths [6]. Since, many deaths of patients with cancer arise from metastasis, its related processes such as EMT, are of great significance. Extensive studies have

showed that EMT has an important role in the onset of metastasis, invasion, and relapse of cancer [9, 10, 22]. The E-cadherin is an important molecule in cancer progression and EMT process, its downregulation is a main feature of EMT [23]. Since, E-cadherin has a key role in cell-cell adhesion and maintaining the structural integrity of the epithelial sheets it is expected that its downregulation leads to cell morphological changes.

Some studies showed that downregulation of E-cadherin in the human mammary epithelial cells (HMLE) led to a fibroblast-like appearance for these cells. Their results showed that the expression of epithelial markers such as E-cadherin decreased and the expression of mesenchymal markers such as vimentin increased. Moreover, it has been determined that EMT occurred in these HMLE cells and they had a similar phenotype to the CSCs [9-11].

In the present study, we used pGIPZ lentiviral vector for transfer of shRNA to downregulate the E-cadherin in HT29 colorectal cancer cell line. Transfer vectors accompanied by two packaging vectors were co-transfected into HEK-293T cells. pGIPZ lentiviral shRNA consist of a Turbo GFP, which is an improved variant of the green fluorescent protein CopGFP cloned from copepod Pontellina plumata [24]. TurboGFP and shRNAmir are a part of a bicistronic transcript allowing the visual marking of shRNAmir expressing cells. As the pGIPZ vector have a puromycin drug resistance marker, it is possible to select the transduced cells. To reach a high efficiency transduction, we optimized MOIs and concentration of puromycin for HT29 cell line. Following lentiviral transduction, to confirm reduced E-cadherin expression, the expression level of E-cadherin mRNA and protein was evaluated using qRT-PCR, western blot, and ICC. The non-silencing lentiviral shRNA vector and non-transduced HT29 have been used as controls. To evaluation of the morphological changes, the cells were monitored with light and fluorescent microscopy until 40 days. Results showed that reduced expression of E-cadherin led to gradually morphological changes in HT29 cell line which is accompanied with the loose of epithelial-like shape and acquiring a fibroblast-like appearance. Furthermore, our analysis showed that molecular and morphological changes along with the GFP expression were stable in our monitoring time.

It is clear that to evaluate the mesenchymal cell markers such as vimentin and/or fibronectin and EMT phenotype such as invasion/migration assay in our transduced cells, more studies needs to be done. Moreover, these cells can be characterized in terms of colorectal cancer stem cells markers such as CD133 and CD44.

CONCLUSION

Generally, it can be concluded that downregulation of CDH1 gene using lentiviral vectors expressing shRNA can lead to a stable decrease in the E-cadherin expression and an obvious cell morphological change. Our results implies that this route of E-cadherin suppression, can be considered as a useful method for the development of an EMT model to further studies on metastasis, cancer stem cells, and their related phenomena .

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