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Effects of PEI-PEG Nanoparticles Loaded With CD44 siRNA on Inhibition of Growth, Invasion, and Migration of Glioblastoma Cells

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Abstract

Objectives: In this study, the inhibitory effects of polyethylene imine glycol (PEI-PEG)/ CD44 siRNA nanostructures on the proliferation, invasion, and apoptosis of U87MG GBM cancer cell line, as well as the expression levels of *ALDH1*, *RANKL*, and *NOTCH1* were evaluated.

Methods: In this experimental study, PEI-PEG/ CD44 siRNA nanoparticles were synthesized and characterized by atomic force microscopy (AFM), evaluation of size and zeta potential, and Fourier transform infrared (FTIR) spectroscopy. The MTT assay was adopted to evaluate the cytotoxicity of the nanoparticles. The expression levels of target genes were assessed by qRT-PCR. Flow cytometry was used for apoptosis evaluation and Trans well matrigel assay and scratch-migration were employed for investigating the invasion and migration of glioma cells.

Results: The size and zeta potential of PEI-PEG were influenced after CD44 siRNA loading. PEI-PEG loaded with CD44 siRNAs resulted in significant inhibition of glioblastoma cell line in the concentration of 60 pmol ($P<0.05$). In addition, transfection of glioma cells with CD44 siRNA led to significant downregulation of *ALDH1*, *NOTCH1*, and *RANKL1* ($P<0.05$). Transfection of this siRNA also resulted in significant suppression of invasion and migration ($P<0.05$).

Conclusions: PEI-PEG could effectively form the polyplex in combination with siRNA, be transfected into the U87MG glioma cancer cell line, and inhibit the proliferation, invasion and migration of glioma cells via suppression of *ALDH1* and *NOTCH1*, as well as *RANKL1* expression levels.

Keywords: Glioblastoma, CD44, siRNA, PEI-PEG

Introduction

Glioblastoma is an aggressive and malignant tumor starting from brain in adults (1). The five-year survival rate in glioblastoma is estimated to be below 5% (2). In spite of the rapid progression in developing innovative and more effective strategies for battling against this type of cancer, the complete cure of glioblastoma has remained an extremely challenging issue (3). Therefore, a clear and comprehensive explanation about underlying molecular mechanisms contributing to the glioblastoma initiation/ progression is believed to play a pivotal role in developing new therapeutic strategies (4, 5).

As a cell membrane glycoprotein, CD44 is believed to contribute to the multiple biological events including proliferation, apoptosis, cell motility, and angiogenesis (6). CD44 appears to have critical functions in the central nervous system, particularly in neuron development (7). More importantly, increased expression levels of this transmembrane protein is reported to exist in the glioblastoma (8).

RNA interference (RNAi) technology by non-coding small interfering RNAs (siRNAs) is a promising methodology for targeting and degrading specific mRNA molecules (9, 10). However, because of low transfection efficacy of naked siRNAs, susceptibility to nucleases, insufficient distribution and – more importantly, presence of biological burdens including blood-brain barrier, the efficacy of siRNA delivery into neurons remains extremely limited (11).

Polyethylenimine is considered as a commonly used cationic polymer which bears high cationic charge density, and has an efficient siRNA delivery capacity due to its native endosome buffering capacity (12-14). The complexation of PEI nanoparticles with siRNA into PEI/ siRNA polyplex is introduced as one of the highly efficient non-viral nucleic acid carriers in cancers (15). Therefore, the present study aimed to assess the effects of CD44 knockdown through transfecting of glioblastoma cells with PEI/siRNA nanoparticles on the aggressive behavior of these cells.

Materials and Methods

Preparation of PEI Nanoparticles

In this experimental study and in order for preparing PEI

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Key Messages

- CD44 siRNA loaded PEI-PEG exerted cytotoxic effects on glioma cells
- CD44 siRNA loaded PEI-PEG modulated the expression of *ALDH1, RANKL,* and *NOTCH1* genes in glioma cells
- CD44 siRNA loaded PEI-PEG induced apoptosis in glioma cells
- CD44 siRNA loaded PEI-PEG inhibited migration invasion of glioma cells

Table 1. siRNA Sequences

siRNA	Strands	Sequence (5'to 3')
CD44 (1)	Sense	UUUUGGAAAUCACUAAUAGtt
	Antisense	CUAUUAGUGAUUUCCAAAAtt
CD44 (2)	Sense	AAUGCAAACUGCAAGAAUCtt
	Antisense	GAUUCUUGCAGUUUGCAUUtt
CD44 (3)	Sense	AAGAGAAAGGAAGUUUUUCtt
	Antisense	GAAAAACUUCCUUUCUCUUtt

nanoparticles, first polyethylene glycol (PEG2000) groups were functionalized using bromoacetyl chloride and, then, attached to the PEI surface. PEG2000 (5 g, 2.5 mmol), TEA (1.4 mL, 10 mmol), and bromoacetyl chloride (875 µL, 10 mmol) were dissolved in 30 mL CH_2Cl_2. After stirring the reaction mixture under N_2 for 12 h, the product was deposited in diethyl ether and dissolved in brine. The PEG-bromoacetyl was extracted with 50 mL CH_2Cl_2, the solvent was evaporated and the product was used for conjugation to PEI.

For Modification of PEI with PEG2000, 5% and 10% of primary amines of PEI were substituted by PEG-bromoacetyl. Hence, a solution of PEG-bromoacetyl in 10 mL dichloromethane was added dropwise to a solution of PEI in 5 mL of dichloromethane. Then the reaction mixture was stirred under N_2 for 12 hours. Afterwards, the solvent was evaporated and the residue was purified by dialysis bag (8–12 kDa cut off) against distilled water for 24 hours, and then it was lyophilized. The conjugation was confirmed by the FT-IR.

After purification, the number of amine groups on the PEI molecule was calculated by determining the number of first-order amines through using TNBS (5% w/v) [Trinitrobenzenesulfonic acid solution]. TNBS reacted with amines of the first type and produced dye derivatives. Applying this method, the amount of PEG created on PEI was determined.

Complexation of siRNA in PEIs-PEG

The siRNA against CD44 and non-targeting siRNA as a negative control (Table 1) were designed and synthesized by Microsynth company (Switzerland). Complexation of siRNAs then was performed. Briefly, siRNA and PEI were solved in PBS buffer separately. After 5 minutes incubation, the PEI-PEG solution was added to the siRNA-containing vial, mixed and incubated for 30 minutes.

Evaluation of the Morphology of NPs

Atomic force microscopy (AFM) analysis was used to investigate the morphology of the PEI-PEG loaded with CD44 siRNA.

Evaluation of Size and Zeta Potential of PEI-PEG Loaded With CD44 siRNA

Some important features of loaded PEI-PEG nanoparticles including zeta potential and size were assessed by dynamic light scattering and zeta sizer (Malvern). All assessments were carried out in triplicate at wavelength of 633 nm.

Fourier Transform Infrared Spectroscopy (FTIR)

The molecular structure and formation of PEI-PEG nanoparticles were evaluated by FTIR, such that the sample was accumulated by the KBr pellet method and compressed to a plate. FTIR was done using a FTIR spectrometer (Spectrum 2000; Perkin-Elmer, Waltham, MA, USA) in the spectral region 400 to 4000 cm^{-1}.

Cell Line and Cell Culture

Glioma U87MG cell line was taken from Pasteur Institute (Tehran, Iran). The glioma cells were cultured in the RPMI-1640 medium with 10% fetal bovine serum (Gibco, USA) and 1% penicillin-streptomycin. The U87MG cells were cultured (5% CO2) at a temperature of 37°C, and then were used in the logarithmic phase of the experiments.

MTT Assay

First, 15×10^3 cells per well were seeded into 96 well cell culture plate. The cells were treated in 60-80% confluent cells. Briefly, in one of the tubes 1.6 µL of PEI-PEG was diluted in 10 µL PBS (N/P ratio: 70), and in the other tube different concentration of siRNA (40-80 pmol) was diluted in 10 µL PBS. Then the contents of both tubes were mixed. After 30 minutes incubation in room temperature, prepared polyplexes were added to each well in serum free medium and incubated for 6 hours. Afterwards, the media was replaced with complete media.

The groups included in the MTT assay were treated with various concentrations of siRNA (40-80 pmol) and PEI-PEG to determine the toxicity of PEI-PEG as negative control. Twenty-four hours after the incubation, the previous culture medium was incubated with MTT medium for 4 hours. Then DMSO and Sorenson's Phosphate buffer were added to the wells containing the cells, and the absorbance was measured at 570 nm by ELISA plate reader (Sunrise, Tecan, Australia).

RNA Isolation and qRT- PCR

The total RNA isolation from U87MG cells was carried out using RiboEx reagent (GeneAll, Korea) based on the manufacturer's guidelines. Then cDNA was synthesized

from total RNA using BioFact kit (BioFact, Korea) in line with the established guidelines. Quantitative real-time PCR (qRT-PCR) was conducted using SYBR Green master mix (BioFact, Korea) and Roche light cycle 96 system. The primer sequences of CD44, Noch-1, RANKL, and VEGF are presented in Table 2. Finally, the relative expression levels in various groups were calculated using $2^{-(\Delta\Delta Ct)}$ method by normalizing the mRNA expression level in β-actin as the reference gene. All PCR reactions were performed in triplicate.

Evaluation of Apoptosis by Flow Cytometry

U87MG cells were cultured in 6-well plates containing RPMI-1640 medium with 10% FBS. After treatment of cells with siRNA/PEI PEG for 24 h, cells were washed twice with PBS. Then cells were incubated with 5 µL of Annexin V-FITC and 5 µL of propidium Iodide (PI) (Annexin V-FITC/PI staining kit, EXBIO, Vestec, Czech Republic) for 15 min in the dark at room temperature. Finally, the flow cytometry analysis was conducted using MACSQuant Analyzer 10 flow cytometer instrument (Miltenyi Biotec, Germany), and the data were analyzed by FlowJo software.

Trans Well Matrigel Assay

To run this assay, 24-well Transwell chambers (Corning-Costar, Cambridge, MA) were employed for investigating cell invasion. Before running the experiments, 100 µL matrigel basement membrane matrix was used for coating the inserts. After the trypsinization was completed, 1×10^5 cells were transferred to the upper matrigel chamber. In addition, the lower chamber containing FBS supplemented culture medium was used as chemoattractant. Having gone through processes of filtering the cells, fixation with methanol, and staining with Giemsa stain, the invaded cells were counted under the inverted microscope.

Scratch-Migration Assays

Migration assay was used to determine the U87MG cell migration. After seeding of 2×10^5 U87MG cells/well in 24-well plates and reaching to a confluency of >90%, a scratch was produced via a sterile pipette tip. Then PBS was used to wash cell debris. The gap area at 0 and 48 hours left as the result of scratching was photographed under the light microscope. Quantification of migration rate was carried out using the NIH ImageJ software.

Table 2. Primer Sequence

Primer	Sequence (5′ to 3′)	
CD44	F	CAAGCCACTCCAGGACAAGG
	R	ATCCAAGTGAGGGACTACAACAG
Notch-1	F	GGCCACCCCTCCTAGTTTG
	R	CTCACTGGCATGACACACAACA
RANKL	F	AGCGTCGCCCTGTTCTTCTA
	R	CTGCTCTGATGTGCTGTGATCCAA
ALDH1	F	TCGTCAGGCACACAACCGTC
	R	GGCGTGGGGTGGTATCTGT
β-Actin	F	CACTCTTCCAGCCTTCCTTCCT
	R	GTGATCTCCTTCTGCATCCTGTCG

Statistical Analysis

To compare the groups, student *t* test and one/two way ANOVA were applied by GraphPad Prism. The findings are shown as mean ± SD, and *P* value less than 0.05 is statistically considered significant.

Results

Characteristics of Nanoparticle

The size and zeta potential of siRNA/PEI-PEG nanostructure were measured. The size of PEI, PEI-PEG, and PEI-PEG-siRNA were 115.1, 137.5, 174.3 nm, respectively. Zeta potential of PEI, PEI-PEG, and PEI-PEG-siRNA were 39.6, 30.5, 27.7 mV, respectively. Our results showed that zeta potential and size of PEI-PEG were partly affected after loading of CD44 siRNA (Table 3).

As shown in the FT-IR spectrum (Figure 1), the main absorption peaks for PEG were intense bands of hydroxyl groups (3421 cm^{-1}), and ether linkage (C–O–C, 1110 cm^{-1}) existed. Explicit absorption peaks relating to the primary and tertiary amine of PEI (3000-3500 cm^{-1}) were found on raw PEI. In addition, the peaks between 1460 and 1690 cm^{-1} were the characteristic absorption bands of PEI. After conjugation of PEI with PEG, the existence of the peaks between 1243 cm^{-1} and 1645 cm^{-1} confirmed the presence of the C-N and N-H. Meanwhile, the amine groups of PEI and ether groups of PEG were detected in the PEI-PEG spectrum.

Gel Retardation Assay

PEI and its conjugated PEG at different N/P ratios (77, 154) were prepared. Then 5 µL siRNA whose concentration was fixed at 1 mM was added to each one of them from 1 to 20 (siRNA concentration was fixed at 1 mM) and incubated for 20 minutes at room temperature. It was transferred to the wells of 2% agarose gel containing 1 µg/mL of EtBr previously prepared, and then electrophoresed on a 1% agarose gel in 0.5 tris-borate-EDTA buffer (TBE buffer; 89 mM Tris, 89 mM boric acid, 2 mM EDTA, pH 8.3) at 80 mV for 30 min. Afterwards, bands were visualized by UV illuminator (Tanon GIS System, Shanghai, China) with ethidium bromide staining. It should be noted that the

Table 3. Characteristics of Pharmaceutical Groups

Groups	Size	Zeta Potential	PDI
PEI	115.1 nm	39.6 mV	0.697
PEI-PEG	137.5 nm	30.5 mV	0.203
PEI-PEG- siRNA	174.3 nm	27.7 mV	0.217

PDI; Polydispersity index, nm; Nanometer, mV; Millivolt.
Size by dynamic light scattering (DLS).

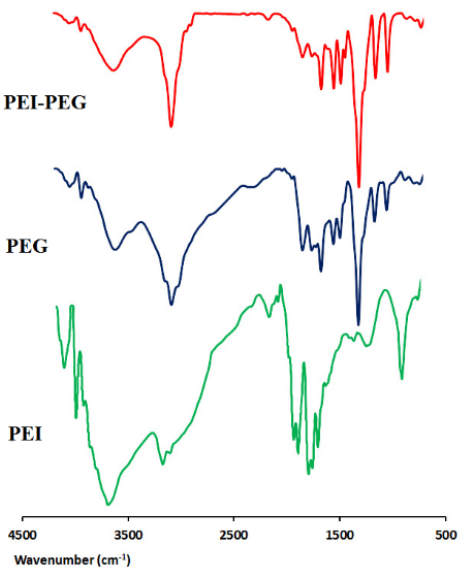

Figure 1. The FTIR Spectrum of PEG, PEG-acetyl, and PEG-PEI.

naked siRNA was used as a control (Figure 2A).

The results from cellular uptake assay showed that loading capacity of siRNA on PEI was 84% (Figure 2B). Moreover, AFM images indicated a multifaceted surface with spherical shapes and smooth surface of PEI-PEG loaded with CD44 siRNA (Figure 3). FTIR analysis confirmed the proper formation of PEI-PEG nanoparticles. In addition, the proper wavelength of the carboxyl group in PEI-PEG was also determined by this method. Overlapping of the wavelengths indicated that PEI-PEG compound was properly composed.

CD44 siRNA Loaded PEI-PEG Exerted Cytotoxic Effects on Glioma Cells

Prior to the evaluation of anticancer effects of siRNA loaded PEI, the MTT method was adopted to determine the effects of cytotoxicity regarding blank nanoparticles in different doses. PEI-PEG 5% at N/P was found to have a significantly lower cytotoxicity compared to higher N/Ps ($P<0.05$), and to have great transfection compared to lower N/Ps, which resulted in selecting the N/P direction for transfection of siRNA (Figure 4A). As shown in Figure 4B, moreover, PEI-PEG loaded with CD44 siRNA resulted in the significant inhibition of glioblastoma cell line in the concentration of 60 pmol ($P<0.05$).

CD44 siRNA Loaded PEI-PEG Inhibited the Expression of CD44 Protein in Glioma Cells

The expression levels of CD44 that had been treated with different concentrations of CD44 siRNA/PEI were measured in the glioma cell line (40, 60, 80 pmol) as well as in different times (24, 48, 72 hours), and were compared with non-treated control. Cells treated with CD44 siRNA/PEI in all concentrations significantly decreased the mRNA expression levels of CD44 compared to controls (Figure 5A). CD44 siRNA/PEI nanoparticles resulted in reduced expression levels of CD44, such that 60 pmol exerted more potent inhibitory effect ($P<0.05$). In addition, cells treated with nanoparticles for 48 hours reduced – more potently – the expression levels of CD44 in comparison to 24 and 72 hours ($P<0.05$).

CD44 siRNA Loaded PEI-PEG Modulated the Expression of *ALDH1, RANKL,* and *NOTCH1* Genes in Glioma Cells

The expression profiles of *ALDH1*, *RANKL*, and *NOTCH1* in PEI-PEG-siRNA treated group and control group were compared 24 hours after their being treated by quantitative RT-PCR technique. PEI-PEG-CD44-siRNA complexes resulted in a considerable drop in the levels of expression regarding *ALDH1* and *NOTCH1* in glioma cells, compared to non-treated control ($P<0.05$; Figure 6). In addition, nanoparticles loaded with siRNA downregulated RANKL significantly compared to control ($P<0.05$). No significant impact of blank nanoparticles on the expression levels of studied genes was detected.

CD44 siRNA Loaded PEI-PEG Induced Apoptosis in Glioma Cells

The effects of CD44 siRNA loaded PEI nanoparticles on the induction of apoptosis in glioma cells were analyzed

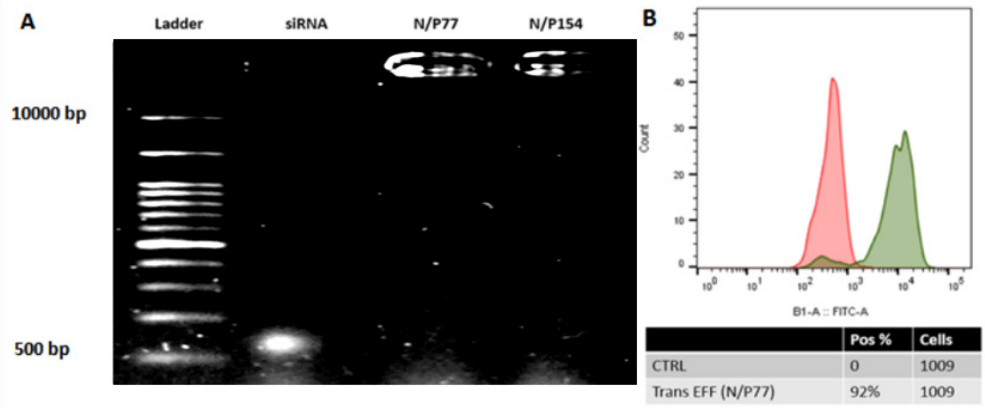

Figure 2. (A) Electrophoresis on a 2% Agarose Gel Showing Loading Capacity of CD44 siRNA on PEI-PEG, (B) Cellular Uptake Assay.

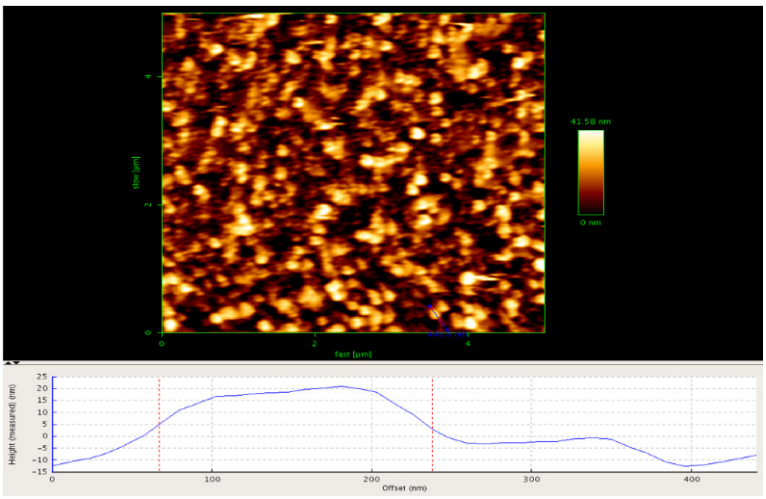

Figure 3. AFM of PEI PEG 5%. The Size of PEI PEG/siRNA Was 170 nm. Morphology of This NP Also Is Indicated. The White Points Are siRNA.

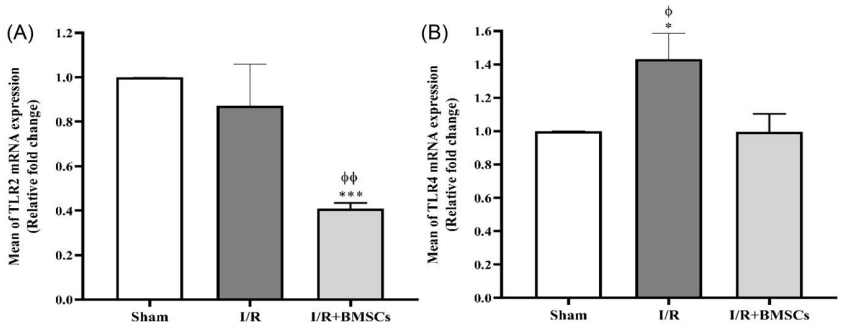

Figure 4. (A) The Effects of PEI, PEI-PEG_5% and PEI-PEG 10% at the Indicated N/P Ratios on the Cell Viability of Glioma Cells, Comparing the Cytotoxicity Effect of Various PEI PEG 5% and 10% With Various N/P. As it is Shown, the Cytotoxicity Rate of N/P 77 PEG 5% Is Better Than Others. (B) The Effects of Various Concentration of PEI-PEG CD44 siRNA on the Cell Viability of Glioma Cells.

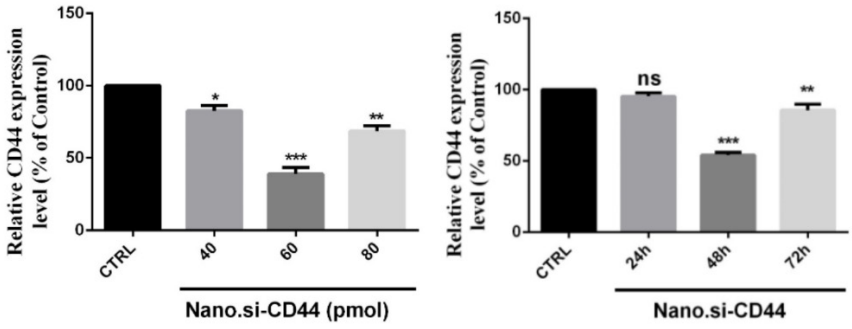

Figure 5. (A) The Effects of PEI-PEG/CD44 siRNA on the Expression Levels of CD44 With Various Concentrations of siRNA in Glioma Cell Line (***P=0.0001, ** P<0.001, *P<0.05). (B) The Effects of PEI-PEG/CD44 siRNA on the Expression Levels of CD44 in Various Times in Glioma Cell Line (***P=0.0001, ** P<0.001).

through flow cytometry analysis. Treatment of glioma cells with siRNA/PEI caused considerable increment in the apoptosis of the glioblastoma cells in comparison to control cells (P<0.05; Figure 7).

CD44 siRNA Loaded PEI-PEG Suppressed Invasion and Migration of Glioma Cells

For investigating the effects of CD44 siRNA loaded PEI PEG nanoparticles on the migration and invasion of the glioma cells, trans well matrigel assay and wound healing assay were employed. Through comparing parts of the cells in the denuded district using Image J software (Figure 8A-C), it was found that the migration of glioma cells treated with siRNA/PEI nanoparticles decreased

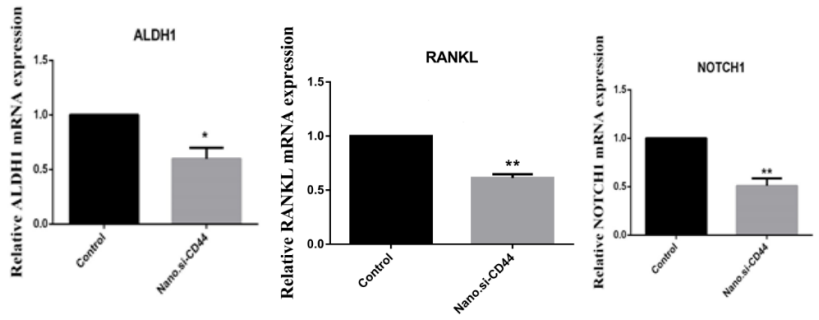

Figure 6. The Effects of PEI-PEG/CD44 siRNA on the Expression Levels of ALDH1, RANKL, NOTCH1 in Glioma Cell Line (*P=0.01, ** P<0.001).

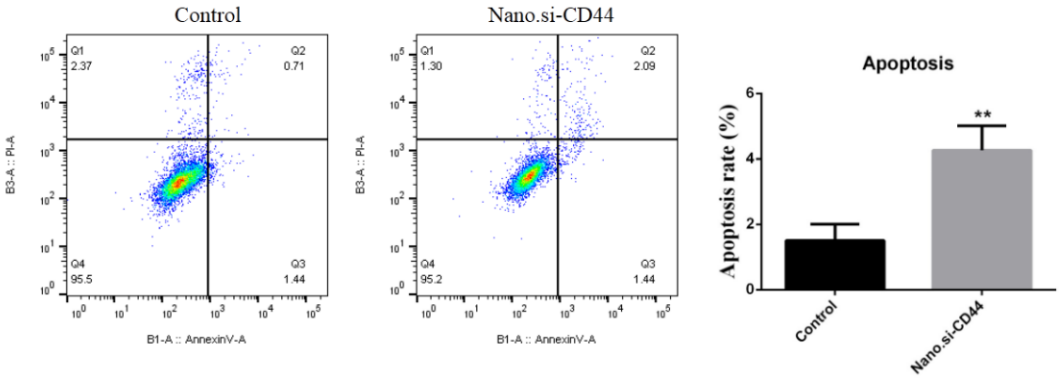

Figure 7. The Effects of PEI-PEG/CD44 siRNA on Apoptosis Induction in Glioma Cell Line.

significantly compared to control cells after 72 hours of incubation (P<0.05).

Discussion

In the present study, PEI-PEG nanoparticles were synthesized and applied as a carrier for delivery of CD44 siRNA. Characterizing techniques revealed that CD44 siRNA loading on PEI-PEG nanoparticles altered the NPs sizes. The results from cellular uptake assay showed higher encapsulation capacity of siRNA on PEI-PEG. Because of rapid degradation, low penetration of cellular membranes, and instability, naked siRNAs are not appropriate approaches to knockdown the expression of a gene (16). Therefore, loading of siRNAs into PEI-PEG and other polymers provides an appropriate delivery platform, which protects nucleic acids from the degradation, and increases their efficacy and cellular uptake. In addition, they are useful tools for delivering which helps overcome brain barriers (16). In our study, it was shown that the transfection of glioma cells with PEI-PEG/CD44 siRNA – comparing to naked CD44 siRNA, suppressed the cell proliferation significantly, which is a proof of better cellular uptake of siRNA and increased cytotoxicity effects.

CD44 is strongly considered as a prognostic factor for glioblastoma since increased expression levels of this biomarker are frequently seen in patients with glioblastoma. Wei et al found that brain tumors showed a high expression levels of CD44. Obviously, CD44 has critical function in malignant neoplasia and metastasis, particularly in glioblastoma (17). In vitro studies have already reported an increased tumor cells invasion and metastasis in the presence of CD44 (8, 18). Merzak et al (19) demonstrated that suppression of CD44 by a monoclonal antibody decreased the metastatic and invasive behavior of glioblastoma cell line significantly. On the other hand, an increase in the expression levels of CD44 was discovered to enhance the invasion of astrocytoma lines (20). In this study, it was indicated that the efficient delivery of CD44 siRNA into glioblastoma cell lines mediated by PEI-PEG nanoparticles led to significant suppression of cells invasion and increased apoptotic rates of glioma cells.

Various hypotheses have been proposed about CD44 through evaluating putative mechanisms underlying CD44 mediated increase in the invasion and metastatic behavior of glioblastoma. For example, it has been reported that CD44 acts as a hyaluronic acid (HA) receptor in the glioma cell line, which is involved in invasiveness properties. It has been also suggested that CD44 may interact with other signaling pathways, and regulate the invasive and metastatic phenotype of tumor cells. NOTCH1, RANKL, and ALDH1 are among the most important pathways that might be controlled by

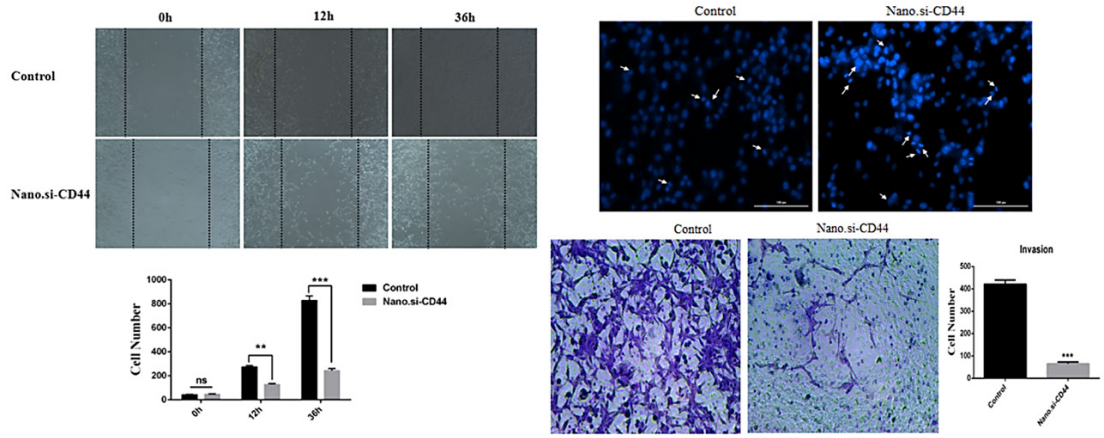

Figure 8. (A) The Effect of PEI-PEG/CD44 siRNA on Migration of Glioma Cell Line. (B) The Effect of PEI-PEG/CD44 siRNA on the Nucleus Fragmentation in Various Times in Glioma Cell Line. (C) The Effect of PEI-PEG/CD44 on Invasion of Glioma Cells

CD44. Expression level of NOTCH1 has increased in various human cancers including breast, colon, pancreas, and central nervous system cancers (21,22). NOTCH1 signaling is a critical pathway for cellular proliferation and tumor progression in many brain tumors (23, 24). In the U87MG glioblastoma cell line, this pathway results in the activation of AKT stimulating the WNT/β-catenin and NF-κB signal transductions and, therefore, increases cellular invasion and migration (25,26). RANKL is a protein that plays the role of ligand for RANK and is also known as an apoptotic regulator in many cancers. Overexpression of RANK L in U87MG cells results in tumor invasion (27). Its upregulation causes an increase in the number of astrocytes which release the causes of invasion of cancer cells (27,28). ALDH1 is a group of enzymes, which their overexpression are related to the progression of stem cell-based tumors. In glioblastoma, overexpression of ALDH1A1 has been seen in astrocytes (29-32). Therefore, targeting these signaling has important implications for preventing glioblastoma invasion. In this study, it was demonstrated that glioblastoma cells treated with nanoparticles loaded with CD44 siRNA suppressed the expression levels of CD44 significantly, and further resulted in the inhibition of cell invasion. In addition, CD44 knockdown significantly suppressed the expression levels of NOTCH1, RANKL, and ALDH1 in glioblastoma cells.

Conclusions

Given the importance of key genes including Notch-1, RANKL, and ALDH1 in the invasion and metastasis of glioblastoma cell line, it was concluded that successful and efficient knockdown of CD44 in the glioblastoma cell line resulted in the inhibition of invasion, partly through modulation of NOTCH1, RANKL, and ALDH1 expression. Furthermore, PEI-PEG nanoparticles may be employed as an appropriate strategy for effectively and efficiently carrying and delivering of siRNAs to glioma cells.

Authors' Contribution

PM implemented *in vitro* techniques concerning nanoparticle synthesis and characterization, cell culture and flowcytometry, and etc.; EST wrote the article; and BB and AM guided the present scientific team, wrote and revised the article. All the authors studied and approved the final manuscript.

Conflict of Interests

Authors declared no conflict of interests.

Ethical Issues

This article performed by the authors contains no studies with human or animal participants.

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