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Cationic microRNA-delivering nanovectors with bifunctional peptides for efficient treatment of PANC-1 xenograft model

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ABSTRACT

Therapeutic strategies based on modulation of microRNA activity possess much promise in cancer therapy, but the in vivo delivery of microRNA to target sites and its penetration into tumor tissues remain great challenge. In this work, miR-34a-delivering therapeutic nanocomplexes with a tumor-targeting and -penetrating bifunctional CC9 peptide were proposed for efficient treatment of pancreatic cancers. In vitro study indicated that the nanoparticle-based miR-34a delivery systems could effectively facilitate cellular uptake and greatly up-regulate the mRNA level of miR-34a in PANC-1 cell lines. The up-regulation of miR-34a remarkably induced cell cycle arrest and apoptosis, suppressed the tumor cell migration and inhibited the target gene expressions such as E2F3, Bcl-2, c-myc and cyclin D1. More importantly, the in vivo systemic administration of the developed targeting miR-34a delivery systems in a pancreatic cancer model significantly inhibited tumor growth and induced cancer cell apoptosis. Such bifunctional peptide-conjugated miRNA-delivering nanocomplexes should have great potential applications in cancer therapy.

1. Introduction

MicroRNAs (miRNAs) are a diverse class of highly conserved small noncoding RNA molecules that determine cell fate by regulating multiple gene products and cellular pathways [1–3]. Misregulation of miRNAs has been implicated in various human cancers, and miRNAs as novel types of tumor suppressors or oncogenes play an important role in human carcinogenesis [4–6]. Tumor suppressor miRNAs provide a new opportunity to treat cancer. The reintroduction of miRNAs in down-regulation cancer cells reactivates cellular pathways and induces a therapeutic response [7–9]. MicroRNA-34a (miR-34a) is a potential down-regulated tumor suppressor in some cancer cell lines. It also can target multiple genes associated with cell proliferation, apoptosis and differentiation [10–14]. For example, at the molecular level, miR-34a can target the E2F3-encoding mRNA and suppress the expression of E2F3 protein, a key regulator of cell cycle progression [15]. Furthermore, miR-34a can inhibit the cancer cell proliferation and migration, and trigger apoptosis via the activation of p53 and down-regulation of c-Met [16,17]. Due to the important functions provided by miR-34a, the therapeutic approaches based on miR-34a restoration are promising. However, the systemic administration of miRNA to the target tissue without potential side effects is challenging.

Adenovirus-associated vectors (AAVs) are widely applied for the systemic delivery of tumor-suppressor miRNAs and have shown the promising efficacy in gene silencing and the treatment of cancer [18]. Due to their safety and amenability to surface functionalization, the nanoparticle-based delivery systems are extended for miRNA-based therapeutics delivery. Until now, few literature had been reported on the in vivo applications of nanoparticle-based miRNA delivery for cancer therapy. One example is the recently reported by Huang's group, which developed the antibody-modified liposome/polycation based nanovectors for specific and efficient delivery of miRNA in vivo [19]. Another example is that the antibody-coated porous silica nanoparticles can systemically deliver miR-34a to inhibit neuroblastoma tumor growth [20]. Our

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previous studies showed that the β-cyclodextrin-polyethylenimine (PEI-CD, PC) conjugates, where β-CD was crosslinked by low molecular weight PEI (600Da), are capable of efficiently mediating gene transfection in various tumor cell lines and different mouse models [21]. PC is amenable to surface modification by installing various functional molecules to achieve multifunctional vectors [22–25].

In this work, CC9 (CRGDKGPDC), a specific tumor-homing and -penetrating bifunctional peptide, was chemically conjugated or physically combined with the cationic PC vectors for effectively carrying tumor-suppressor miRNAs (Fig. 1). CC9 can home to tumors though specific RGD motif and is then proteolytically cleaved in the tumor to produce CRGDK, which gain affinity for neuropilin-1 (NRP-1) and NRP-1 binding triggers tissue penetration [26,27]. The CC9-containing PC nanovectors can complex with miR-34a. Such resultant nanocomplexes were employed for effectively treating pancreatic cancers, and their cancer therapeutic effects were systematically investigated through a series of experiments in vitro and in vivo.

2. Materials and methods

2.1. Materials

Branched polyethyleneimine (PEI, MW 600D), β-cyclodextrin (β-CD, MW 1135), 1,1′-carbonyldiimidazole (CDI, MW 162.15) [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (MTT, MW 218.1), bis-Benzimide H 33342 trihydrochloride (Hoechst 33342), fluorecein isothiocyanate isomer I (FITC), triethylamine (Et_3N, ≥99%), 1,1′-dioctadecyl-3,3,3′,3′- tetramethylindocarbocyanine perchlorate (DiI, 60010), and succinimidyl-3-(2-pyridyl-dithio) propionate (SPDP, MW 312.37) were obtained from Sigma (St. Louis, MO, USA). SDS-PAGE (P0015), peptide CC9 (Sequence: NH2-Cys-Arg-Gly-Asp-Lys-Gly-Pro-Asp-Cys, MW 1342), miR-34a and miR-control were obtained from National Laboratory for Oncogenes and Related Genes, Cancer Institute of Shanghai Jiaotong University. The control RNA sequence is the widely used negative control siRNA (Sense sequence: UUCUCCGAACGUGUCACGUdTdT; Antisense sequence: ACGUGACACGUUCGGAGAAdTdT). Other chemicals and solvents were of analytical reagent grade.

2.2. Polymer synthesis

The details of synthesis and characterization of PEI_{600}-CD (PC) were described in our previous publication [21]. Briefly, β-CD (0.42 g, 0.37 mmoL) and CDI (0.51 g, 3 mmoL) were dissolved in anhydrous DMF (6 mL). The mixture was stirred at room temperature for 1 h and was then precipitated with cold diethyl ether. The resulting CDI-activated CD (CD-CDI) was filtered, dissolved in 5 mL DMSO, and stored at 4°C. Next, PEI 600 (1.5 g) was dissolved in 3 ml DMSO. The described CD-CDI in 5 ml DMSO and 0.3 ml triethylamine (Et3N) was added dropwise to the PEI solution over 1.5 h with stirring, followed by reaction for an additional 5 h. After the reaction, the mixture was dialyzed in water for 2 days and freeze-dried for other 2 days to produce PEI_{600}-CD (PC). The CC9 peptide was conjugated quantitatively onto PC by succinimidyl-3-(2-pyridyl-dithio) propionate (SPDP) as previously reported [22,25]. Briefly, the PC (150 mg) was dissolved in 10 mL PBS and SPDP (3.92 mg, 0.0125 mmol) was dissolved in a mixture of 0.5 mL PBS and 0.5 mL DMSO, respectively. The two solutions were mixed, followed by addition of 0.1 mL triethylamine. The mixture was stirred in the dark for 2 h. The CC9 peptide (7.92 mg, 0.0083 mmol) in 2 mL PBS buffer was added slowly under stirring in the dark for another 4 h. The final mixture was dialyzed with a dialysis tube (MW 8000–14000) for 2 days and then freeze dried to produce PC-CC9 (Fig. 1) for further use. The conjugation of CC9 onto the PC backbone was confirmed by ^1H NMR. The ^1H spectrum of each sample was recorded on a Bruker DRX-400 spectrometer (Bruker, Ettlingen, Germany) at room temperature. Gel electrophoresis was performed at room temperature in TAE buffer in 1% (w/w) agarose gel at 80 V for 40 min. DNA was visualized by UV (254 nm) illuminator.

2.3. Preparation of nanocomplexes and characterization

The schematic preparation processes of different conjugation nanoparticles (PC/miRNsA + CC9 and PC-CC9/miRNA) were shown in Fig. 1b and c. The polymer solutions were added to the solutions of the miRNA in the same volume, mixed and incubated for 30 min at room temperature to form the nanoparticles with different N/P ratios, where "N"" is the molar amount of nitrogen from the cationic polymer and "P"" is the molar amount of phosphate from miRNA. The PC-CC9/miRNA complexes were prepared by adding mixing solution of PC-CC9 to miRNA solution. For PC/miRNA + CC9, the complexes were prepared by adding CC9 solution to the PC/miNRA solutions for another 30 min.

For the characterization of complexes, the particle sizes and zeta potentials of the complexes were measured at 25°C by dynamic light scattering (DLS) using Zetasizer 3000 (Malvern Instruments, Worcestershire, UK). Complex solutions (200 μL) containing 2 μg (0.75 μM) of miNRA were prepared at various N/P ratios. Average mean (nm) and zeta potential (mV) were used for data analysis. The complexes were also characterized by transmission electron microscopy (TEM). Briefly, complexe solution was dropped to carbon-coated copper grids. After 5-min drying at room temperature, images were recorded by electron microscope (JEM-2010 TEM).

The gene transfection efficiency mediated by PC/pDNA + CC9 and PC-CC9/pDNA nanoparticles in PANC-1, BxPC-3 and 293T cell lines were evaluated as before [21]. The indicated cells were cultured in medium supplemented with 10% fetal bovine serum (FBS). Briefly, the cells were seeded with a density of 3×10^4/well in 48-well plates and grown for 18 h at 37°C in a humidified 5% CO_2 incubator. The medium was replaced with 0.3 ml of fresh serum-free medium, and 100 ml of freshly prepared vectors/pDNA complex containing 1 μg of DNA was added to each well. After 4 h of transfection, the medium was replaced by a fresh growth medium and then the cells were incubated for an additional 24 h. Luciferase activity was measured according to standard protocols of luciferase assay system (Promega). The total protein was measured according to a BCA protein assay kit (Pierce) and luciferase activity was reported in terms of RLU/mg cellular protein.

The cytotoxicity of the complexes was evaluated using the MTT assay in different cell lines including the PANC-1, BxPC-3 or 293T cells. The cells were seeded at 8×10^3 cells/well in 200 μL serum-medium in 96-well plates. After 24 h, the medium was replaced with 200 μL serum-free culture media containing serial dilutions of complex solutions at a series of N/P ratios for 4 h, then the culture medium was replaced by 100 μL medium with 10% FBS and 0.5 mg/mL filtered MTT solution for another 4 h. Finally, each well was replaced with 100 μL DMSO and measured spectrophotometrically in an ELISA plate reader (Model 550, Bio-Rad) at a wavelength 570 nm. The relative cell growth (%) related to control cells cultured in media without polymer was calculated by [A]test/[A]control × 100.

2.4. Cellular uptake

For microscopic observation, 5×10^4/well PANC-1 and 293T cells were seeded onto 24-well plates and grown for 18 h. 0.5 μg (0.375 μM) of FAM-siRNA was complexed with PC-CC9, PC + CC9 and PC at N/P of 25 100 μL solution at room temperature for 20 min before use. After transfection for 2 h, the cells were washed with PBS and fixed with fresh 4% paraformaldehyde. Cell membrane (DiI, red) and nucleus (Hoechst, blue) were stained according to the corresponding protocols of related kits provided by Biotium 60010 and Sigma Hoechst 33342. The cells were visualized with a confocal laser scanning microscopy (*CLSM*, Radiance *2100*, *Bio-Rad*) with a 100 × oil immersion lens.

For flow cytometric analysis, the PANC-1 and 293T cells were cultured and transfected as described above. The dose of FAM-siRNA is 2 μg (0.15 μM) per well. To study the target specificity of PC-CC9, a competition assay with free CC9 was also performed. PANC-1 cells were incubated with free CC9 (100 mg/mL) for 30 min before the addition of the complexes. After transfection for 2 h, the cells were washed with PBS and trypsinized, and re-suspended in PBS. The samples were analyzed by flow cytometry (Caliber, CA, USA). Transfected with free FAM-siRNA PANC-1 cells were used to set the background.

2.5. Quantitative real-time PCR

Quantitative Real-Time RT-PCR (qRT-PCR) was applied to assess the expression level of miR-34a. The target genes include E2F3, Bcl-2, c-myc and cyclin D1. The desired cells were seeded into 6-well plates with a density of 2×10^5/well and grown for 24 h to achieve full confluence. The concentration of FAM-siRNA is 0.15 μM per well. The Total RNA was extracted with Trizol according to the manufacturer's instructions. Quantization of PCR product was performed in 1 × TE buffer using absorption values at 260 nm and 280 nm: c(RNA) = OD_{260} × dilution factor × 0.04 μg/μL. For microRNA expression analysis, 100 ng of total RNA was used along with miR-34a–specific primers supplied with miR-34a Taqman MicroRNA Assay. cDNA was synthesized using Taqman MicroRNA Reverse Transcription kit. Each RT-PCR reaction was consisted of 35 cycles of 1 min at 94 °C, 1 min at 60 °C and 1 min at 72 °C. Quantization of PCR product was performed after electrophoresis on 1.5% agarose gels and ethidium bromide staining. The miR-34a expression level in tumor tissue was also assayed using the same procedures.

To assess the up-regulation capability of the miR-34a delivery system, PANC-1 cells were seeded into 6-well plates with a density of 2×10^5/well. After 16 h of culture, the medium was removed, and the cells were incubated in the free-serum media containing PBS control, free miR-34a, PC/miR-34a, PC-CC9/miR-34a, PC/miR-34a + CC9 or PC/miR-con for 4 h. Then, the transfection medium was replaced with fresh growth medium and the cells were incubated for an additional 48 h. The miR-34a expression level was assayed as described above.

2.6. Western blot assay

In the western blotting analysis, the cells were transfected as described above for 24 h and 48 h. The cell proteins were then extracted after transfection. The total