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Molecular imaging of a cancer-targeting theragnostics probe using a nucleolin aptamer- and microRNA-221 molecular beacon-conjugated nanoparticle

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ABSTRACT

MicroRNAs (miRNA, miR) have been reported as cancer biomarkers that regulate tumor suppressor genes. Hence, simultaneous detecting and inhibiting of miRNA function will be useful as a cancer theragnostics probe to minimize side effects and invasiveness. In this study, we developed a cancer-targeting therangostics probe in a single system using an AS1411 aptamer - and miRNA-221 molecular beacon (miR-221 MB)-conjugated magnetic fluorescence (MF) nanoparticle (MFAS miR-221 MB) to simultaneously target to cancer tissue, image intracellularly expressed miRNA-221 and treat miRNA-221-involved carcinogenesis. AS1411 aptamer-conjugated MF (MFAS) nanoparticles displayed a great selectivity and delivery into various cancer cell lines. The miR-221 MB detached from the MFAS miR-221 MB in the cytoplasm of C6 cells clearly imaged miRNA-221 biogenesis and simultaneously resulted in antitumor therapeutic effects by inhibiting miRNA function, indicating a successful astrocytoma-targeting theragnostics. MFAS miRNA MB can be easily applied to other cancers by simply changing a targeted miRNA highly expressed in cancers.

1. Introduction

The development of targeted theragnostics, which is a treatment approach that combines therapeutics with diagnostics, has increased with advances in molecular biology, nanotechnolgy and biomedical imaging and understanding of diseases. Targeted cancer theragnostics can be utilized as imaging probes and drugs discovered against a specifically important function of cancer biology. Nanotechnology and biomedicine are being integrated ever more frequently in the various phases of drug discovery and development. Modifying the surface of various inorganic nanoparticles has recently become important to the development of novel biomedical technologies including therapeutics, diagnosis and clinical imaging [1–9]. The functional moiety on nanoparticles allows for the conjugation of targeting probes such as antibody [10,11], peptide [12], aptamer [8,13], and protein [14,15] that can target receptors highly expressed in cancers. Among these molecular entities, aptamers, which are small oligonucleotides, have successfully been used to target cancers with favorable pharmacokinetic properties, such as small in size, lacking immunogenicity, ease of synthesis and high binding affinity [16–19]. The AS1411 aptamer targeting nucleolin protein, which is highly expressed in the membrane of cancer cells, has been successfully conjugated to various nanoparticles and when conjugated is internalizes into cancer cells by an unknown molecular mechanism [9,20–22]. In these previous studies, the AS1411 aptamer-conjugated nanoparticles were shown to have the capability to serve as a cancer-targeted gene delivery vehicle.

It is known that microRNAs (miRNA, miR), 20–22 nucleotides of non-coding small RNA, regulate various cellular functions including proliferation, differentiation and apoptosis as well as diseases [23–27]. miRNAs function by base-pairing with their target mRNAs, which are perfectly or imperfectly matched with the relevant miRNA sequences, resulting in mRNA degradation or translational inhibition of their targets [28,29]. Using the theory of miRNA hybridization with its target mRNA, a few non-invasive miRNA imaging methods using a luciferase reporter gene, stem loop- or linear-structured DNA molecular beacon (MB) containing perfectly complimentary oligonucleotides against a target miRNA have been used to visualize the biogenesis of various miRNAs during neurogenesis, carcinogenesis or myogenesis *in vitro* and *in vivo* [30–37]. Additionally, antagomiR (or antimiR), which is a synthetic oligonucleotide that fully complements a miRNA of interest, leads to a loss of function of the miRNA by binding to the miRNA-associated gene silencing complexes, resulting in

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a significant reduction in cell viability of cancers [38–40]. Thus, synthetic complimentary oligonucleotides against miRNAs can be potentially used as a molecular mechanism-based theragnotics biomarker in cancers by simultaneously imaging miRNA biogenesis and disrupting miRNA-dependent regulatory circuits in neoplasia.

In this study, we developed a new approach for creating aptamer- and miRNA-based cancer-targeting theragnostic nanoparticles by using the AS1411 aptamer and miR-221 MB, which is complementary to miRNA-221. The AS1411 aptamer was used for targeted delivery into cancer cells which have a high expression of nucleolin protein and miR-221 MB was used to detect and inhibit miRNA-221, which is highly expressed in papillary thyroid carcinoma [32,33] and is involved cancer development.

2. Material and methods

2.1. Preparation of multimodal nanoparticle-based cancer-targeting theragnostic probe

MNP@SiO_2(RITC)-PEG/COOH/pro-N/NH_2 nanoparticles (MF, 2 mg/ml) were purchased from Biterials (Seoul, Republic of Korea). 5′- NH_2-modified oligos, AS1411 (5′-TTGGTGGTGGTGGTTGTGGTGGTGGTGG-3′) and 5′- NH_2-modified AS1411 mutant (mt) oligos, (5′-TTCCTCCTCCTCCTTCTCCTCCTCCTCC-3′) were kindly provided by Postech Biotech Center. Both miR-221 MB and miR-126 MB were designed to form a partially double stranded oligonucleotide. The long oligonucleotide containing the miRNA-221 or miRNA-126 binding sequence, which is a perfectly reverse complimentary sequence against mature miRNA-221 (5′ AGCUACAUUGUCUGCUGGGUUUC 3′) or miRNA-126 (5′ UCGUACCGUGAGUAAUA-AUGCG 3′), was 5′- NH_2-S-S-gaaacc-cagcagacaatgtagctTGTCGCTT-Cy 5.0—3′ or 5′- NH_2-S-S-cgcattattactcacggtacgaTGTCG CTT-Cy 5.0—3′ and the short oligonucleotide was 3′-atcgaACAGCG-BHQ 2-5′ or 3′-atgctACAGCG-BHQ 2-5′. Both oligonucleotides were manufactured by Bionics (Bionics, Inc, Korea). Conjugation of AS1411 or miRNA MB with MF nanoparticles was previously described in details with slight modifications [9,22,36]. The AS1411 aptamer or/and miR-221 MB were covalently conjugated using the coupling reagent, *N*-(3-dimethylaminopropyl)-*N*′-ethyl-carbodiimide hydrochloride (40 nmol) at an MF/AS1411, MF/miR-221 MB and MF/AS1411/miR-221 MB molar ratio of 1:6, 1:6 and 1:3:3 for 1 h at room temperature. After each conjugation, the particles were analyzed by TEM using a JEM 1010 system (JEOL, Japan) and DLS was conducted using a Zetasizer Nono ZS system (Malvern Instruments, Worcestershire, UK) to characterize the distribution and size of the nanoparticles.

The conjugation efficiency between MF and AS1411, miR-221 MB or both was determined by collecting unconjugated oligonucleotides in the supernatant after centrifugal filtration of the MF reaction mixutre and measuring the concentration using NanoDrop ND-1000 Spectrometer (NanoDrop products, Wilmington, DE, USA). These data were presented as mean ± SD calculated from quadruple wells.

2.2. Cell culture

Cancer cell lines, U-87MG, PC-12, F11, C6, HepG-2, SK-Hep-1, Caco-2, CT-26, TPC-1, NPA, U-2OS, HeLa, A549, PC-3 and F9 cells, and normal cell lines, MSC, F3, CHO, 293, L132 and TM-4 cells were cultured according to ATCC protocols. The C6 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM, Grand Island, NY), supplemented with 10% heat-inactivated fetal bovine serum (FBS, Invitrogen, Grand Island, NY) and 1% Antibiotics (Invitrogen, Grand Island, NY), in a standard incubator (5% CO_2 atmosphere at 37 °C). The cells were split at regular intervals.

2.3. Confocal laser microscopy assay

Cancer and normal cells (1×10^5 cells/one 24 well) were seeded on 25-mm-diameter glass cover slips and the cells were grown for 24 h at 37 °C. Before treatment with MFAS, MFASmt or MFAS miR-221 MB, the cells were incubated for 30 min at 4 °C and then washed with phosphate buffered saline (PBS). Each nanoparticle was added to the cells in tris-buffer (pH 7). After incubation for 30 min at 4 °C, the cells were fixed by gently shaking for 20 min with 4% formaldehyde solution (Sigma, Saint Louis, MO). The cells were then washed three times with PBS for 10 min, and cover-slipped with mounting medium containing a 4′, 6-diamidino-2-phenylindole dihydrochloride (DAPI) solution (Vector Laboratories, Inc, CA). The cells were imaged by confocal laser scanning microscopy (Carl Zeiss LSM 510, Weimer, Germany) using filters specific for Rhodamine B isothiocyanate (RITC, Excitation/Emission = 555/578 nm) and Cyanine 5.0 (Cy 5.0, Excitation/Emission = 649/670 nm). For live cell imaging, C6 cells were plated on glass coverslips and incubated with MFAS nanoparticles. Live-cell imaging was performed using a Carl Zeiss LSM 510 inverted confocal fluorescence microscope equipped with a 100X/1.40NA oil-immersion objective. Image streams were captured for 10 s.

2.4. Fluorescence intensity

Measurement of the fluorescence intensity of MFAS, MFASmt or miRNA MB nanoparticles was previously described in details with slight modifications [9,22,36]. Briefly, 1×10^5 C6 cells per well were seeded into 24 well plates and then incubated in a 5% CO_2-humidified chamber for 24 h. MFAS, MFASmt or miR-221 MB nanoparticle was added to the prepared cells at 4 °C for 30 min under serum-free conditions. After washing two times for 10 min at room temperature (RT) under mild shaking and lysing with RIPA buffer (Thermo Fisher Scientific Inc., Waltham, MA), the fluorescence intensity was measured (100 μL) using an Infinite M200 system (Tecan, GmbH, SZ, Austria). All data are presented as the means ± SD calculated from quadruple wells and significant differences between samples were assessed using a Student's *t*-test ($P < 0.05$).

2.5. Magnetic resonance analysis

5×10^5 C6 cells treated with MFAS or MFASmt were washed with PBS three times. C6 cells that had been detached by trypsin/ethylenediaminetetraacetic acid (EDTA) were fixed with 4% paraformaldehyde and imaged using a 1.5 T micro-MRI (GE Medical Systems, Milwaukee, WI). To determine if the transplanted cells were visible by MRI, T_2^*-weighted images were obtained using a fast low angle shot (FLASH) sequence.

2.6. Real time PCR

Real time PCR for mature miRNA-221 was previously described in details [33]. Small RNA was isolated from CHO, TPC-1, NPA or C6 cells using *mir*Vana™ miRNA isolation kits (Ambion, USA). qRT-PCR of mature miRNA-221 and -126 was then conducted using the *mir*Vana™ qRT-PCR primer Set and the *mir*Vana™ qRT-PCR miRNA kit (both from Ambion, USA). Real time-PCR primer set for HOXB5 was as follows: 5′ GGCGCATGAAGTGGAAGAAGGACA 3′ and 5′ GAGAGGGAGCCACAGGAA-GACC 3′. The qRT-PCR program was as follows: 95 °C for 3 min and 40 cycles of 95 °C for 15 s and 60 °C for 30 s. The relative amounts of each mature miRNA were normalized versus the U6 snRNA primer set (Ambion, USA).

2.7. Bioluminescence assay

C6 cells were seeded into 24-well plates at a density of 1×10^5 cells per well. After 24 h incubation, transfection of the CMV/Gluc (vector containing Gaussia luciferase (Gluc) reporter gene controlled by the cytomegalovirus (CMV) promoter), CMV/Gluc/3xPT_miR-221 (CMV/Gluc vector containing 3 copies of the reverse complimentary sequences against miRNA-221 which was cloned into the 3′ UTR of the Gluc reporter gene) or NF-kB/Fluc (vector containing Firefly luciferase (Fluc) reporter gene that was regulated by a synthetic promoter containing a response element sequence for nuclear factor kappa B (NF-kB)) vector was performed using LipofectAMINE Plus reagent (Invitrogen, USA). Fourty-eight hours after transfection, cells were washed with phosphate buffered saline (PBS) and treated with 100 μl of lysis solution. Supernatants were plated in microplates and Gluc and Fluc activities were measured using a Synergy MX (Bio-Tek, Ltd.) with a luciferase assay kit (TargetingSystems, USA). All results are displayed as means ± standard deviation (SD; $n = 4$).

2.8. Cell viability assays

1×10^5 C6 cells were plated into 96-well plates with 200 μl of culture medium. Seven different doses (0, 0.05, 0.1, 0.2, 0.3, 0.6, 1.2 and 2.4 nmol) of miR-221 MB or, MF, MFAS or MFAS miR-221 MB was added to the cells for 48 h. These cells were exposed to 20 μl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT, 1 mg/ml) solution, and incubated for 4 h at 37 °C. The 200 μl of dimethyl sulfoxide (DMSO) was added to solubilize the formazan crystals. The optical density (OD) was read using a microplate reader (Microplate Reader 680, BIORAD, Hercules, CA, USA).

2.9. In vivo theragnostics effects of MFAS miR-221 MB

All animals used in the *in vivo* experiments were housed under specific pathogen-free conditions and the experiments were approved by institutional animal care and use committee in CHA University. For *in vivo* imaging of cancer-targeting theragnostics, MFAS miR-221 MB and NF-kB/Fluc were co-transfected into C6 cells. 1×10^6 C6 cells were injected subcutaneously into the right thigh of a nude mice (male BALB/c, 7 weeks old). Meanwhile, only NF-kB/Fluc was transfected into the left thigh of a nude mouse as a control. The mice was scanned for 6 days using a IVIS spectrum (Xenogen, CA) ($n = 4$, mice). For *in vivo* bioluminescence and fluorescence imaging, mice were anesthetized in an IVIS imaging chamber equipped with a small holder connected to an isoflurane and oxygen tank. Anesthetic gas was administered with 2% isoflurane in O_2 gas at a flow rate of 1 L min^{-1} through a nose cone. For bioluminescence imaging, 4 mg of D-luciferin for Fluc imaging was intraperitoneally injected. The *in vivo* fluorescence imaging was conducted using a red filter (605/30 nm, excitation filter; 660/20 nm, emission filter). A camera was used to acquire images at a constant exposure time (1 s). For antitumor