Ultrasound-targeted microbubble destruction-mediated miR-205 enhances cisplatin cytotoxicity in prostate cancer cells

DINGWEN QIN¹, HAIGE LI¹ and HONGLIN XIE²

¹Department of Imaging, The Second Affiliated Hospital of Nanjing Medical University, Nanjing, Jiangsu 210011; ²Department of Urology, The Affiliated Hospital of Hangzhou Normal University, Hangzhou, Zhejiang 310003, P.R. China

Received July 26, 2017; Accepted December 18, 2017

Abstract. MicroRNAs (miRNAs) are non-coding ~20 nucleotides long sequences that function in the initiation and development of a number of cancers. Ultrasound-targeted microbubble destruction (UTMD) is an effective method for miRNA delivery. The aim of the present study was to investigate the potential roles of UTMD-mediated miRNA (miR)-205 delivery in the development of prostate cancer (PCa). In the present study, miR-205 expression was examined by reverse transcription-quantitative polymerase chain reaction assay. miR-205 mimics were transfected into PC-3 cells using the UTMD method, and the PC-3 cells were also treated with cisplatin. Cell proliferation, apoptosis, migration and invasion abilities were detected using Cell Counting kit-8, flow cytometry, wound healing and Transwell assays, respectively. In addition, the protein expression levels of caspase-9, cleaved-caspase 9, cytochrome c (cytoc), epithelial (E)-cadherin, matrix metalloproteinase-9 (MMP-9), phosphorylated (p)-extracellular signal-regulated kinase (ERK) and ERK were measured by western blot analysis. The results of the present study demonstrated that miR-205 expression was low in human PCa cell lines compared with healthy cells and that UTMD-mediated miR-205 delivery inhibited PCa cell proliferation, migration and invasion, and promoted apoptosis modulated by cisplatin compared with UTMD-mediated miR-negative control group and miR-205-treated group. Furthermore, it was demonstrated that UTMD-mediated miR-205 transfection increased the expression of caspase-9, cleaved-caspase 9, cytochrome c and E-cadherin, and decreased the expression of MMP-9 and p-ERK. Therefore, UTMD-mediated miR-205 delivery may be a promising method for the treatment of PCa.

Introduction

Prostate cancer (PCa) is the most common malignant tumor in the male genitourinary system, and the incidence of cancer is fifth in the world (1). It is well known that the incidence of PCa in China ranks one hundred seventieth in the country, which has been rising from 2008 to 2012 (2,3), speculating that the number of people with PCa will continue to increase in the future (2).

MicroRNAs (miRNAs) are a class of non-coding small RNAs that regulate gene expression in a post-transcriptional manner (4,5). Recent studies have demonstrated that miRNA regulation serves a role in health and disease (6,7). Abnormal expression of miRNAs may lead to initiation and development of tumors (8-10). Previous studies have also indicated that a number of miRNAs may be delivered to human cells by food intake, therefore, miRNAs are relevant to human health (11,12). It has also been demonstrated that incidence, development, treatment, prognosis and recurrence of PCa are associated with abnormal expression of certain miRNAs (13-15). The authors of the present study hypothesized that miRNAs may aid in our understanding of pathogenesis and the basis for molecular diagnosis of PCa, and have attempted to evaluate the prognosis and to suggest novel treatment methods for PCa.

Microbubble ultrasound contrast agent is a safe, novel, stable and efficient gene transfer vector (13-15). In this technique, the gene of interest is contained in a microbubble, and when the microbubble breaks its released. Microbubble destruction induced by vibration increases the permeability of local cells and produces an irreversible sound hole, which may promote entry of a gene into the nucleus and increase its expression and transfection efficiency (16). Furthermore, microbubbles transport genes or drugs efficiently to avoid degradation by blood endonucleases and other lytic enzymes (17,18). By using this method of ultrasound-targeted microbubble destruction (UTMD), genes and drugs may reach target tissues or organs through the blood circulatory system. The goal of UTMD is to reduce the extent of adverse systemic responses (19). Research and clinical studies of UTMD primarily focus on cancer, anti-tumor therapies, thrombosis, thrombolytic therapy, inflammation, drug delivery and gene therapies (20,21). It has been widely demonstrated that microbubble-based techniques may improve gene transfection efficiency (reviewed in 20) and are considered as a novel approach to cancer treatment (18).
Based on the potential role of miR-205 in the molecular mechanism underlying PCA development (22), the aim of the present study was to investigate the transfection efficiency and safety of UTMD-mediated transfection of miR-205 to PC-3 cells. Furthermore, the present study attempted to investigate the role served by miRNAs in the development of PCA and the feasibility of UTMD-mediated gene therapy.

Materials and methods

Cell culture. RWPE-1 normal prostate cells and PCA cell lines VCaP, LNCaP, PC-3, and DU145 were purchased from American Type Culture Collection (Manassas, VA, USA). All cells were cultured in RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.) and 2 mM L-glutamine in an atmosphere of 5% CO2 at 37°C.

Cell treatment. For cisplatin treatment, DU145 and PC-3 cells were seeded (1x10⁶ cells/well) in six-well plates, and treated with 0, 1, 2, 4, 6, 10 and 15 µg/ml cisplatin (Beijing Solarbio Science and Technology Co., Ltd., Beijing, China) at room temperature for 48 h. For miRNA transfection, the miR-205 mimics and miRNA negative controls (miR-NC) were purchased from Shanghai Gene Chem Co., Ltd. (Shanghai, China). PC-3 and DU145 cells were seeded (1x10⁶ cells/well) in six-well plates and transfected with miR-205 mimics (target sequence: 5'-GAT TTCAGGTTGAAGTTCAGGGCAT-3', Cai et al. 2016 µg/ml) and miR-NC (target sequence: 5'-CCGATTAATCTGTGCTGCTGA-3', C=1.3 µg/µl) using Lipofectamine® 3000 reagent (Invitrogen; Thermo Fisher Scientific, Inc.) for 48 h according to the manufacturer's protocol. Subsequently, cells were harvested for further experiments.

miRNA-microbubble preparation and transfection. Microbubbles were obtained by sonication of an aqueous dispersion comprising 1,2-distearoyl-3-trimethylammonium-propane (0.4 mg/ml; Avanti Polar Lipids Inc., Alabaster, AL, USA) with perfluoropropane gas, polyethyleneglycol-2000 stearate (1 mg/ml; Avanti; DSPCa; Avanti Polar Lipids Inc.) (23). Microbubbles were examined by an inverted microscope (Shanghai, China; www.shgmyq.com/). miR-205 and miR-NC were separately added into the microbubbles, and incubated at 37°C for 30 min. PC-3 cells were transfected with a mixture of miR-205/miR-NC and microbubbles using Lipofectamine® 3000 reagent (Invitrogen; Thermo Fisher Scientific, Inc.) for 48 h according to the manufacturer's protocol.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was isolated from cells using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. First-strand cDNA was reverse transcribed from the total RNA using the RevertAid First Strand cDNA synthesis kit (Thermo Fisher Scientific, Inc.). The temperature and time of the reaction were 85°C for 5 min, 4°C for 5 min. The qPCR assay was performed using SYBR-Green PCR Master Mix kit (Takara Biotechnology Co., Ltd., Dalian, China) and an ABI 7500 real-time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.). The following thermocycling conditions were used for the PCR: Initial denaturation at 95°C for 30 sec; 40 cycles of 95°C for 5 sec, 60°C for 34 sec. Primers for target genes and U6 (the internal loading control) were designed using the Primer Premier software version 5.0 (Premier Biosoft International, Palo Alto, CA, USA) and synthesized by Shanghai GenePharma Co., Ltd. (Shanghai, China). The following primer sequences were used for PCR: miR-205 forward, 5'-TGGGCTGATGCCTTCTT-3' and reverse, 5'-GAGGAGCGGTATGGCAGATTGG-3'; U6 forward, 5'-CTCGCTTCGCGACGACA-3' and reverse, 5'-AACGCTTACGAAATTTGCGT-3' (reverse). Expression levels were normalized to U6, and relative expression values were calculated using the 2-ΔΔCq method (24).

Western blot analysis. Total protein was extracted from cells using ProteoPrep Total Extraction Sample kit (Sigma-Aldrich; Merck KGaA). Cell lysates were collected following centrifugation at 12,000 g at 4°C for 20 min. Bradford assay (Bio-Rad Laboratories, Inc., Hercules, CA, USA) was used to detect protein concentrations. Each protein sample (30 µg) was separated by 10% SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes (Bio-Rad Laboratories, Inc.). The PVDF membranes were treated with the following primary antibodies: Caspase 9 (1:1,000; cat. no. ab25758; Abcam, Cambridge, UK), cleaved-caspase 9 (1:1,000; cat. no. ab23234; Abcam), cytochrome c (cyto c; 1:1,000; cat. no. ab28146; Abcam), epithelial (E)-cadherin (1:1,000; cat. no. ab133597; Abcam), matrix metalloproteinase 9 (MMP-9; 1:1,000; cat. no. ab73734; Abcam); phosphorylated (p)-extracellular signal-regulated kinase (ERK)1/2 (1:1,000; cat. no. 9101; New England BioLabs, Inc., Ipswich, MA, USA), ERK1/2 (1:1,000; cat. no. ab17942; Abcam), β-actin (1:1,000; cat. no. ab8226; Abcam) overnight at 4°C. The following day, the membranes were incubated with a horseradish conjugated-secondary antibody (Donkey anti-rabbit IgG H&L, 1:7,000, cat. no. ab98488; Goat anti-mouse IgG H&L, 1:8,000, cat no. ab150117; Rabbit anti-mouse IgG H&L, 1:8,000, cat. no. ab175743; Abcam) for 1 h at room temperature. Expression was visualized using the Enhanced Chemiluminescence Detection kit (EMD Millipore, Billerica, MA, USA).

Cell viability. Cell viability was measured using the Cell Counting Kit (CCK)-8 assay (Beyotime Institute of Biotechnology Co., Ltd., Shanghai, China). Cells (1x10⁵ cells/well) were seeded into a 96-well plate and cultured at 37°C in a 5% CO2 incubator for 48 h. CCK-8 solution (10 µl) was added into each well and incubated at 37°C for 4 h. The absorbance was measured at a wavelength of 450 nm with a microplate reader (Molecular Devices, LLC, Sunnyvale, CA, USA).

Flow cytometry. After PC-3 cells (1x10⁶ cells/ml) were treated with: i) 1X PBS (blank); ii) cisplatin (2 µg/ml); iii) cisplatin (2 µg/ml) + scrambled-miRNA (miR-NC; 100 µg) + UTMD; iv) miR-205 (100 µg) + UTMD; v) miR-205 (100 µg) + U6 + UTMD; vi) miR-205 (100 µg) + U6 + cisplatin (2 µg/ml); vii) miR-205 (100 µg) + U6 + cisplatin (2 µg/ml) + UTMD. Cells were digested with
0.25% EDTA-trypsin (Weike; Shanghai, China; www.weike21.com/), and dispersed. Cell suspension was centrifuged in 500 x g at 37°C for 5 min, and collected. Subsequently, cells were washed with 1X PBS, and re-suspended using 1X binding buffer and double stained with the Annexin V-FITC/PI staining kit (BD Biosciences, Franklin Lakes, NJ, USA). Finally, apoptotic cells were detected by flow cytometry (Taomsun, TMS-2050 [FlowJo 10; Version: 10.2.64 (Bit), Suzhou, Jiangsu, China). Apoptotic rates of treated PC-3 cells were quantified by graphPad prism 7 software.

Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) and 4',6-diamidino-2-phenylindole (DAPI) staining. Cell apoptosis was detected using the In-Situ Cell Death Detection kit (R&D Systems, Inc., Minneapolis, MN, USA), according to the manufacturer’s protocol. Cells (1x10³ cells/well) were seeded in 24-well plates, and treated with: i) 1X PBS (blank); ii) cisplatin (2 µg/ml); iii) cisplatin (2 µg/ml) + scrambled-miRNA (miR-NC, 100 µg) + UTMD, also referred as UTMD-mediated miR-NC group; iv) cisplatin (2 µg/ml) + miR-205 (100 µg) + UTMD; v) cisplatin (2 µg/ml) + miR-205 (100 µg), also referred as miR-205 group; and vi) cisplatin (2 µg/ml) + UTMD. Similarly, cells were fixed in 4% paraformaldehyde at 4°C for 30 min, permeabilized in 0.1% Triton X-100, and treated with 50 µl fluorescein-12-dUTP or 60 µl DAPI at room temperature for 30 min. The fluorescence of cells in the middle of each well was detected by fluorescence microscope (Zeiss Axiosvert 100 M; Zeiss GmbH, Jena, Germany).

Wound-healing assay. The wound-healing assay was used to determine the effects of UTMD-mediated miR-205 delivery on cell migration. Treated cells were seeded in the six-well plates (1x10³ cells/well) and cultured in RPMI-1640 medium for 12 h at 37°C in a 5% CO₂ incubator. A 100 µl pipette tip was used to create a straight scratch and the images captured were used as the baseline. Subsequently, cells were treated as mentioned earlier. Finally, cells were washed three times with 1X PBS to remove the suspended cells, and new images were captured.

Invasion assay. Cell invasion assay was performed using Transwell chambers. Matrigel inserts were placed in the upper compartment and incubated for 30 min in an incubator at 5% CO₂ and 37°C. A 200 µl solution with serum-free medium containing treated cells (5x10⁵ cells/well) was seeded in the upper compartment. The lower compartment was filled with 600 µl medium supplemented with 10% FBS. Following 24 h of incubation, the migratory cells were fixed with methanol and stained with crystal violet at room temperature for 25 min. Cell numbers were counted in five representative fields and images were captured under a fluorescence microscope.

Statistical analysis. All results are presented as the mean ± standard deviation of three independent experiments. Statistical analysis was performed using SPSS software (version 13.0; SPSS, Inc., Chicago, IL, USA). The differences between groups were assessed by one-way analysis of variance followed by Dunnett’s test. P<0.05 was considered to indicate a statistically significant difference.

Results

miR-205 expression is decreased in human PCa cells. RT-qPCR assays were performed to detect the expression level of miR-205 in the normal prostate cell line RWPE-1 and in the PCa cell lines VCaP, LnCaP, PC-3 and DU145. The results indicated that the expression levels of miR-205 were significantly lower in PCa cells compared with RWPE-1 cells (P<0.001; Fig. 1).

miR-205 inhibits prostate cancer cell proliferation. The effects of miR-205 overexpression on PCa cells were detected. PC-3 and DU145 cells were treated with PBS (Blank), miRNA-NC (Control) and miR-205 mimics (miR-205). RT-qPCR results demonstrated that miR-205 expression was significantly increased in the miR-205 mimics treated groups compared with the respective control groups (P<0.001; Fig. 2A and B). In addition, miR-205 mimics-treated PC-3 and DU145 cells exhibited a significant reduction in proliferation compared with the respective control groups at 48 h (Fig. 2C and D).

UTMD-mediated miR-205 transfection inhibits cisplatin-modulated cell proliferation. Cationic microbubble technology is an effective method for miRNA delivery (25). As presented in Fig. 3A, the microbubbles were imaged using an inverted microscope. It is proven that cisplatin has an anti-PCa effect (26). In this experiment, to screen for the optimum cisplatin concentration to treat DU145 and PC-3 cells, CCK-8 assay was performed to detect viability of DU145 and PC-3 cells treated with 0, 1, 2, 4, 6, 10 and 15 µg/ml cisplatin. The results demonstrated that cisplatin markedly decreased the viability of DU145 and PC-3 cells in a dose dependent manner, compared with the untreated control group (Fig. 3B). Additionally, cisplatin inhibited the viability of PC-3 cells more than that of DU145 cells. The IC50 of cisplatin was 2 µg/ml in PC-3 cells. Therefore, subsequent experiments were performed using 2 µg/ml cisplatin in PC-3 cells.

To investigate whether UTMD-mediated miR-205 serves a role in PCa, PC-3 cells were treated with: i) 1X PBS (blank); ii) cisplatin(2µg/ml); iii) cisplatin(2µg/ml)+scrambled-miRNA...
Figure 3. UTMD-mediated miR-205 transfection inhibits cell proliferation modulated by cisplatin. (A) Microbubbles were examined by an inverted microscope. Scale bar, 5 µm; magnification, x100. (B) Cell viability was detected by Cell Counting kit-8 assay in DU145 and PC-3 cells treated with 0, 1, 2, 4, 6, 10, 15 µg/ml cisplatin for 48 h. *P<0.05, **P<0.01, ***P<0.001 vs. the respective untreated control group at 48 h. (C) Relative expression level of miR-205 determined by reverse transcription-quantitative polymerase chain reaction assay in PC-3 cells. ***P<0.001. (D) PC-3 cell proliferation. Data are presented as the mean ± standard deviation; *P<0.05, **P<0.01 and ***P<0.001 vs. the cisplatin + miR-NC + UTMD group; #P<0.05 and ###P<0.001 vs. the cisplatin+miR-205 group. miR, microRNA; NC, negative control; OD, optical density; UTMD, ultrasound-targeted microbubble destruction.
UTMD-mediated miR-205 transfection promotes apoptosis modulated by cisplatin. (A) Apoptosis in treated PC-3 cells was detected by flow cytometry. The abscissa of FL1-H was FITC. The longitudinal coordinate was PI and (B) quantification of it. ***P<0.001. (C) Apoptosis was also measured by TUNEL in treated PC-3 cells (magnification, x400). miR, microRNA; NC, negative control; OD, optical density; UTMD, ultrasound-targeted microbubble destruction.

Results demonstrated that UTMD-mediated miR-205 transfection significantly increased apoptotic rates in PC-3 cells compared with the UTMD-mediated miR-NC transfected group and the miR-205 group (Fig. 4A and B). In addition, the results of the TUNEL assay indicated that about 30 apoptotic cells were stained by fluorescence in UTMD-mediated miR-205 transfection group, which was higher compared with the UTMD-mediated miR-NC transfection group and with the miR-205 group (Fig. 4C).

UTMD-mediated miR-205 transfection inhibits PC-3 cell migration and invasion modulated by cisplatin. In order to further investigate the biological significance of UTMD-mediated miR-205 transfection in PCa cells, wound-healing and Matrigel invasion assays were performed.
The results demonstrated that UTMD-mediated miR-205 delivery decreased PC-3 cell migration and invasion in cells co-treated with cisplatin compared with the UTMD-mediated miR-NC transfection group and with the miR-205 group (Fig. 5A and B).

**UTMD-mediated miR-205 transfection increases expression of caspase-9, cleaved-caspase 9, cytroc and E-cadherin, and decreases expression of MMP-9 and p-ERK.** To investigate the potential mechanism of UTMD-mediated miR-205 delivery on the inhibition of apoptosis and invasion of PC-3 cells modulated by cisplatin, the protein expression levels of apoptosis-associated genes (including caspase-9, cleaved-caspase 9 and cytroc), E-cadherin, MMP-9, ERK and p-ERK were evaluated using western blot analysis. The results demonstrated that in comparison with the UTMD-mediated miR-NC transfection group and with the miR-205 group, UTMD-mediated miR-205 transfection notably upregulated the expression of caspase-9, cleaved-caspase 9 and cytroc, which suggested that miR-205 may promote cell apoptosis. UTMD-mediated miR-205 transfection also resulted in increased protein expression levels of the epithelial marker E-cadherin and in decreased expression of MMP-9, which suggested that miR-205 may inhibit epithelial-mesenchymal transition (EMT). Furthermore, results demonstrated that UTMD-mediated miR-205 delivery markedly decreased p-ERK expression, which suggested that miR-205 may down-regulate the ERK signaling pathway (Fig. 6).

**Discussion**

miRNAs serve roles in proliferation, differentiation, cell cycle, apoptosis, migration and invasion (27,28). A number of miRNAs have been previously identified that may serve roles as novel markers for diagnosis of multifarious tumors (29). miRNAs, acting as oncogenes or tumor suppressor genes, may become novel therapies against cancer (30). miR-205 is a highly conserved miRNA that was identified based on the conserved sequence of mouse and Takifugu rubripes (31), and was subsequently identified in human and zebrafish (32,33). Previous studies have indicated that miR-205 serves roles in the development of various tumors, including colorectal cancer, PCA, adenocarcinoma, endometrial cancer, non-small cell lung cancer and nasopharyngeal carcinoma (34). In the present study, miR-205 was down-regulated in PCA cells and inhibited PCA cell proliferation.
UTMD is a novel, safe, non-invasive technology (35). Compared with viral vector transfection technology, the combined use of UTMD and non-viral vectors is a safer and more effective method of increasing the gene and drug transfection efficiencies (36-38). UTMD involves the attachment of genes to microbubbles that may subsequently be injected and circulated through blood vessels and then destroyed by ultrasound ionization at the target site (39). Microbubble destruction leads to increased capillary permeability, generating holes in the cell membrane releasing the ‘payload’, which is subsequently incorporated intracellularly (40).

Previous studies have demonstrated that miRNAs can enhance cisplatin anti-PCa effects (41-43). The present study evaluated UTMD-mediated miR-205 delivery in PCa cells to determine whether this delivery system facilitated gene delivery in PCa cells, with the aim to investigate alterations in cell proliferation, apoptosis, migration and invasion, and to elucidate the regulatory functions of miR-205 in PCa. The results of the present study demonstrated that UTMD-mediated miR-205 transfection inhibited PCa cell proliferation, migration and invasion, and promoted apoptosis modulated by cisplatin. Therefore, UTMD-mediated miR-205 delivery may inhibit EMT in PC-3 cells. In conclusion, the present study used UTMD to successfully transfect PCa cells with miR-205 mimics plasmid; the results demonstrated that cell proliferation, migration and invasion were suppressed, and apoptosis was increased, which may aid in future efforts of miRNA inhibition in vivo. Furthermore, the present study demonstrated that UTMD-mediated miR-205 delivery increased the expression levels of E-cadherin and decreased the expression of MMP-9 and p-ERK, which suggested that the ERK signaling pathway may play a role in the development and progression of PCa. UTMD-mediated miR-205 delivery may be a novel molecular targeted therapy for the treatment of PCa.

Competing interests

The authors declare that they have no competing interests.
References


This work is licensed under a Creative Commons Attribution-NonCommercial-NoDerivatives 4.0 International (CC BY-NC-ND 4.0) License.