Anti-microRNA-21/221 and microRNA-199a transfected by ultrasound microbubbles induces the apoptosis of human hepatoma HepG2 cells

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Abstract. Gene therapy, particularly microRNA (miRNA), is a promising candidate in the treatment of cancer; however, it is challenging to develop gene delivery systems. Ultrasound microbubbles have been used for gene delivery with excellent results. The present study aimed to investigate the transfection efficiency of HepG2 cells using ultrasound microbubbles. The effects of three miRNAs (miR-21, miR-221 and miR-199a) on HepG2 cells were also determined by performing ultrasound microbubble-mediated gene transfection. Three recombinant plasmids containing anti-miR-21, anti-miR-221 and miR-199a were fused with enhanced green fluorescent protein. For the transfection of genes, the type of contrast agent, the concentration of microbubble contrast agent and the exposure intensity of ultrasound were optimized. The expression of miRNAs was detected using reverse transcription-polymerase chain reaction. To determine the effect of anti-miR-21, anti-miR-221 and miR-199a on HepG2 cells, MTT, cell cycle analysis and Annexin V-PE/7-ADD apoptosis assays were performed. The optimal condition was 10% sulfur hexafluoride microbubbles at an ultrasound frequency of 2.0 MHz and mechanical index of 0.28. When cells were transfected with three recombinant plasmids using ultrasound microbubbles, there was significant downregulation of miR-21 and miR-221 and upregulation of miR-199a ($P<0.05$). All three treatments inhibited cell proliferation and promoted the apoptosis of cells. The present data indicated that the delivery of anti-miR-21, anti-miR-221 and miR-199a may be mediated by ultrasound microbubble contrast agents. With this approach, cell proliferation may be effectively inhibited and cell apoptosis may be induced. These are novel cancer therapy targets.

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Introduction

Liver cancer is the sixth most common type of cancer and the second-leading cause of cancer-associated mortality worldwide (1). As estimated by the World Health Organization, 782,000 people are diagnosed with liver cancer and 521,000 liver cancer-associated mortalities were reported globally in 2012 (1). Hepatocellular carcinoma (HCC) is the most common form of primary liver cancer, accounting for 70-85% of cases (2-4). In general, the majority of patients are diagnosed when HCC progresses to the middle to late stages. Despite providing several effective therapies, including transarterial chemoembolization, radioembolization, percutaneous ethanol injection, ablation and chemotherapy, the 5-year survival rate is only 17-34% in these patients (5-9). In addition, HCC is associated with chronic hepatitis infection, chronic alcohol consumption and non-alcoholic fatty liver disease (3,4). However, the underlying molecular pathogenesis has not yet been completely elucidated.

MicroRNAs (miRNAs) are a group of small, evolutionarily conserved, non-coding RNA molecules, which negatively regulate the expression of genes by interacting with 3' untranslated regions of targeted mRNA. miRNAs are involved in numerous biological processes, including cell proliferation, differentiation, apoptosis and metabolism (10,11). The misregulation of miRNAs is often associated with various human diseases, ranging from inflammatory disorders to cancers (12-14). Presently, it is established that >80 miRNAs are involved in the regulation of tumorigenesis and metastasis signaling networks that cause HCC (3). In patients diagnosed with HCC, miR-199a is downregulated (15-17), while miR-21 and miR-221 are upregulated (16-19).

Presently, traditional gene transfection is generally mediated by viral vectors or non-viral vectors. However, due to the security of viral vectors and the low transfection efficiency of non-viral vectors, additional application of these vectors is limited (20). Ultrasound microbubbles are nanobubbles with good biological compatibility and stability (20). The ultrasound images are enhanced using ultrasound contrast

agents. Furthermore, microbubbles are used in non-invasive gene/drug delivery systems (20). Compared with traditional transfection vectors, ultrasound microbubbles have the advantages of high safety, stability and transfection efficiency (20). Ultrasound microbubbles have been widely used to investigate the functions of genes and miRNA (21-25).

The present study aimed to optimize the parameters of ultrasound microbubbles, which mediate the transfection of miRNA into the human hepatoma HepG2 cell. In addition, the effects of anti-miR-21, anti-miR-221 and miR-199a on HepG2 were also investigated. In the present study, anti-miR-21, anti-miR-221 and miR-199a were transfected with ultrasound microbubbles.

Materials and methods

Plasmid construction. The experimental protocol was established according to the ethical guidelines of the Helsinki Declaration and was approved by the Human Ethics Committee of Guangzhou Red Cross Hospital (Guangdong, China). Written informed consent was obtained from individual patients.

Sequences of anti-hsa-miR-21-5p, anti-hsa-miR-221-3p and hsa-mir-199a-1 were synthesized (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and inserted into *Bam*HI and *Hind*III sites of the GV249 vector [a vector containing enhanced green fluorescent protein (EGFP); Shanghai Jikai Communication Technology Co., Ltd., Shanghai, China]. The recombinant plasmids were named EGFP-anti-miR21, EGFP-anti-miR221 and EGFP-miR199a. These plasmids were confirmed by commercial sequencing (Invitrogen; Thermo Fisher Scientific, Inc.) using an ABI3730XL capillary sequencer. The primers used for cloning were as follows: miR-21 sense, 5'-AGCTAAAAA TAGCTTATCAGACTGATGTTGAG-3' and antisense, 5'-GATCCTCAACATCAGTCTGATAAGCTATTTTT-3'; miR-221 sense, 5'-AGCTAAAAAAGCTACATTGTCTGC TGGGTTTCG-3' and antisense, 5'-GATCCGAAACCC AGCAGACAATGTAGCTTTTTT-3'; and miR-199a sense, 5'-TGGGATCCGGAAGAGTGGTGGTTTCCTTG-3' and antisense, 5'-ACCGAAGCTTAAAAAAAATCTTCTATG CGAGGCTCTG-3'.

Cells. The human hepatoma HepG2 cell line (Dongguang BioJet Biotechnology Co., Ltd., Guangdong, China) was maintained in Dulbecco's modified Eagle's medium (DMEM; HyClone; GE Healthcare Life Sciences, Logan, UT, USA) using 10% fetal bovine serum (FBS; HyClone; GE Healthcare Life Sciences) at 37˚C in an atmosphere containing 5% CO_2. The cells were routinely passaged every 1-2 days.

Preparation and optimization of the concentration of microbubbles. Two ultrasound microbubble contrast agents, sulfur hexafluoride (SF6; Bracco Suisse SA, Manno, Switzerland) and perfluoropropane (C3F8; Kanrun Technology Co., Ltd., Hunan, China), were used in the present study. Microbubbles were prepared according to the manufacturer's protocol. To prepare SF6, 5 ml of saline solution was injected into a vial containing freeze-dried powder. The vial was then agitated until the powder was completely dissolved in the saline solution. The suspension was used within 6 h. To prepare C3F8, perfluoropropane-albumin microsphere injection was performed.

A total of 10,000 cells were seeded onto a 96-well plate 24 h prior to subjecting the cells to treatment. Prior to conducting the treatment, cells were divided into 10 groups: Blank control (no cells); negative control (cells without treatment); SF6 treatment group (four subgroups treated with 1, 5, 10 and 20% SF6); and C3F8 treatment group (four subgroups treated with 1, 5, 10 and 20% C3F8). Contrast agents were suspended in DMEM containing 10% FBS. The culture was maintained for 48 h at 37˚C, and subsequently, a MTT assay was performed. Each treatment was performed in 6 wells, and the experiment was performed in triplicate.

MTT assay. MTT (20 μl; 5 mg/ml dissolved in PBS; Genview; Sigma-Aldrich; EMD Millipore, Billerica, MA, USA) was added to the well, and the cell culture was incubated for 4 h at 37˚C. The culture medium mixture was then discarded, and the cells were dissolved in 150 μl dimethyl sulfoxide for 10 min. The absorbance of the sample was determined at 490 nm on a Microplate Spectrophotometer (BioTek Elx800; BioTek Instruments, Inc., Winooski, VT, USA). Controls were a blank control (no cells) and a negative control (untreated cells).

Optimization of ultrasound microbubble-mediated transfection. Cells were seeded on a 6-well plate and subjected to transfection until 70-80% confluency was attained. Prior to subjecting the cells to treatments, cells were divided into 6 groups: Empty control (no treatment); negative control (plasmid); positive control [plasmid + liposome (Lipofectamine® 2000, Invitrogen; Thermo Fisher Scientific, Inc.)]; ultrasound control (plasmid + ultrasound exposure); ultrasound SF6 microbubbles (plasmid + ultrasound exposure + SF6); and ultrasound C3F8 microbubbles (plasmid + ultrasound exposure + C3F8). The latter three groups, which were treated with ultrasound, were divided into 4 subgroups according to their different ultrasound parameters: 2.0 MHz and MI, 0.12; 2.0 MHz and MI, 0.20; 2.0 MHz and MI, 0.28; and 2.0 MHz and MI, 0.35. Prior to treatment, cells were rinsed with DMEM. Ultrasound treatment was then provided with different parameters to the latter 3 groups for 30 sec. Following transfection, cells were maintained in DMEM. To this medium, 10% FBS was added 6-8 h post-transfection. Lipofectamine 2000-mediated gene transfection was performed according to the manufacturer's protocol, as described previously (23). Following 48 h transfection, cells were subjected to fluorescence microscopy and flow cytometry. Each treatment was performed in 3 wells, and the experiment was performed in triplicate. While performing fluorescence microscopy, EGFP-positive cells and total cells of each group were recorded in 9 fields under a magnification of x200 (Zeiss GmbH, Jena, Germany). Thereafter, transfection efficiency was calculated using the following formula: Transfection efficiency (%) = (positive cells / total cells) x 100.

Flow cytometry analysis was performed using FACSCalibur (BD Biosciences, Franklin Lakes, NJ, USA). This procedure was performed at an excitation wavelength of 488 nm and an emission wavelength of 530±15 nm.

RNA extraction and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Cells were transfected with three recombinant plasmids (EGFP-anti-miR21, EGFP-anti-miR221 and EGFP-miR199a) and vector plasmid

(GV249) through ultrasound microbubble SF6. Cells were then collected 48 h post-transfection. Cells that were not transfected were set as a blank control. Total RNA was extracted using TRIzol (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol.

RT-qPCR was performed using the reverse Tra Ace RT-qPCR kit (Toyobo Co., Ltd., Osaka, Japan) according to the manufacturer's protocol. The primers for miR-21, miR-221, miR-199 (as aforementioned for plasmid construction) and the internal U6 control (sense, 5'-TCGCTTCGGCAGCACA-3' and antisense, 5'-AACGCTTCACGAATTTGCGT-3') were obtained from Ruibo Bio-Technology Co., Ltd. (Shanghai, China).

Cell cycle and apoptosis assay. Cells were transfected with three recombinant plasmids (EGFP-anti-miR21, EGFP-anti-miR221 and EGFP-miR199a) and vector plasmid (GV249) through ultrasound microbubble SF6. Cells were then collected 48 h post-transfection. Cells that were not subjected to transfection were set as the blank control. Cells were stained with propidium iodide and subjected to cell cycle analysis on FACSCalibur. The apoptosis of cells was determined using an Annexin V-PE/7-AAD apoptosis detection kit (Beijing Bioco Laibo Technology Co., Ltd., Beijing, China) according to the manufacturer's protocol. Flow cytometry was performed using FACSCalibur.

Statistical analysis. The data were analyzed using SPSS 16.0 (SPSS, Inc., Chicago, IL, USA). Continuous data was expressed as the mean ± standard deviation. For group comparisons, the homogeneity of variance was first tested using the Levene test. To compare equal variances, one-way analysis of variance (ANOVA) was performed, followed by Fisher's least significant difference post-hoc test. To compare unequal variances, one-way ANOVA modified with Welch or Brown-Forsythe tests was performed, followed by Dunnett's T3 test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Optimization of the concentration of contrast agent microbubbles and ultrasound parameters. HepG2 cells were treated with different concentrations of ultrasound contrast agent microbubbles, and the cytotoxicity of microbubbles was detected. The results are shown in Fig. 1. The two contrast agents exhibited similar cytotoxicity. When the concentration of the two contrast agents was increased, cell activity decreased significantly ($P<0.05$). When the concentration of the contrast agents was <10%, no evident cytotoxicity was observed in the two agents as the cell viability was >80%. However, 20% of microbubble agents exhibited evident cytotoxicity when the cell viability was <65%. A previous study established that transfection efficiency was positively associated with the concentration of ultrasound microbubble agents when the microbubbles did not affect the proliferation of cells (9). Therefore, it was ensured that the concentration of contrast agents was 10% in the subsequent experiments.

The cells were transfected with EGFP-miR-199a using two ultrasound contrast agent microbubbles with different mechanical indexes (MIs; 0.12, 0.2, 0.28 and 0.35). Thereafter,

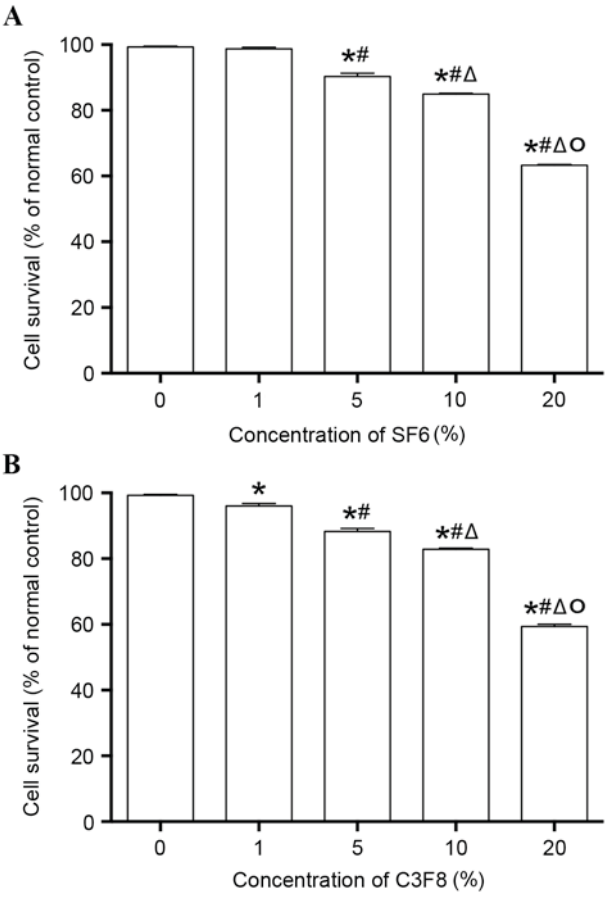

Figure 1. The cytotoxicity of contrast agent microbubbles at different concentrations. HepG2 cells were treated for 48 h with different concentrations (1, 5, 10 and 20%) of the contrast agents (A) SF6 and (B) C3F8. A MTT assay was then performed. $^{*}P<0.05$ compared with the blank control; $^{\#}P<0.05$ compared with the 1% group; $^{\Delta}P<0.05$ compared with the 5% group; $^{\circ}P<0.05$ compared with the 10% group. SF6, sulfur hexafluoride; C3F8, perfluoropropane.

transfection efficiency was determined using fluorescence microscopy and flow cytometry. Similar trends were observed in the data obtained from the two assays. Compared with ultrasound-treated cells, the two ultrasound contrast agent microbubbles significantly improved transfection efficiency ($P<0.05$; Fig. 2). When MI was increased, transfection efficiency increased initially, and then decreased.

Transfection efficiency was the highest when MI was 0.28. The transfection efficiency in the ultrasound SF6 microbubble group was a little higher than in the ultrasound C3F8 microbubble group but this did not reach statistical significance. However, no statistically significant difference was observed between the transfection efficiency of the ultrasound SF6 microbubble group and the C3F8 microbubble group ($P>0.05$). In the ultrasound SF6 microbubble group, transfection efficiency was highest when MI was 0.28, which was significantly increased compared with that of the positive control group (plasmid + liposome; 26.31±0.72% vs. 24.70±0.67%; $P<0.05$). Therefore, in the remaining experiments, ultrasound SF6 microbubbles with an MI of 0.28 were used.

miRNA expression in cells transfected with recombinant plasmids through ultrasound SF6 microbubbles. The three recombinant plasmids were transfected into HepG2 cells

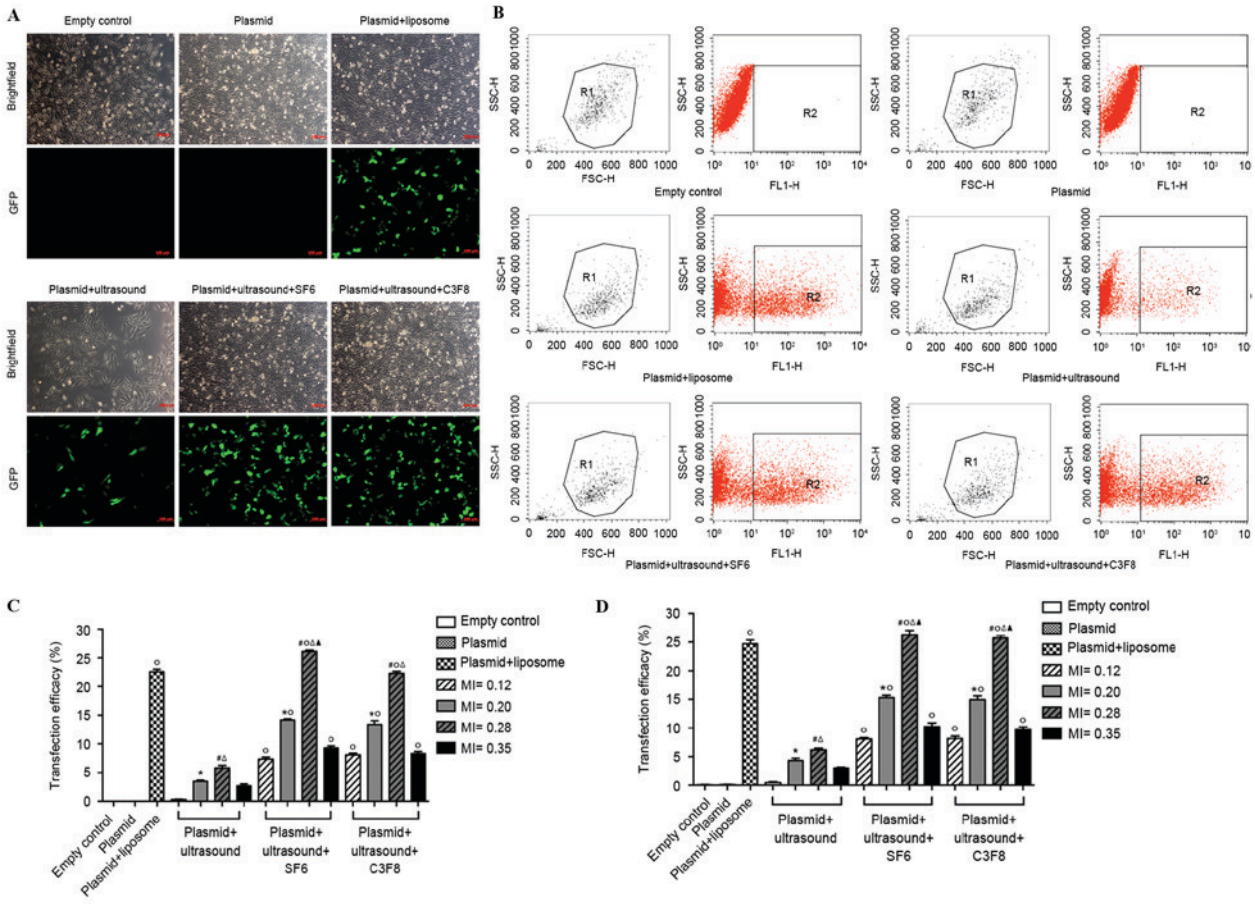

Figure 2. Transfection efficiency under different MIs. Cells were divided into 6 groups and treated with ultrasound at different MIs. Following treatment for 48 h, cells were subjected to (A) fluorescence microscopy and (B) flow cytometry. The quantitative analysis of (C) fluorescence microscopy and (D) flow cytometry, respectively. Each treatment was performed in three wells, and the experiment was performed in triplicate. $^{\circ}$P<0.05 compared with the ultrasound control group; *P<0.05 compared with the MI=0.12 group; $^{\#}$P<0.05 compared with the MI=0.20 group; $^{\Delta}$P<0.05 compared with the MI=0.35 group; $^{\blacktriangle}$P<0.05 compared with the lipofection control group. MI, mechanical index; SF6, sulfur hexafluoride; C3F8, perfluoropropane; GFP, green fluorescent protein.

using ultrasound SF6 microbubbles. The expression of miR-21, miR-221 and miR-199a was determined using RT-qPCR. Following transfection, miR-21 and miR-221 were significantly downregulated, while miR-199a was significantly upregulated compared with the negative control (P<0.05; Fig. 3).

Anti-miRNA-21/221 and miRNA-199a induce apoptosis of HepG2 cells. The impact of anti-miRNA-21/221 and miRNA-199a on HepG2 cells was determined in the present study. First, cell proliferation was detected using MTT at different time points (24, 48, 72 and 96 h). Compared with the negative control, the growth of the transfected cells was significantly inhibited (P<0.05; Fig. 4A). Compared with anti-miR-21/miR-221, miR-199a exhibited the most significant inhibition (P<0.05).

The cell cycle of transfected cells was analyzed. While performing flow cytometry, the cell cycle was divided into four stages: M1, cells in G0/G1 phase with diploid DNA; M2, cells whose DNA lies between the diploid and tetraploid stages; M3, cells in the S phase with tetraplid DNA; and M4, cells that have undergone apoptosis. In the M4 stage, the cell percentage was 0.46% in the blank group and 2% in the negative control. In comparative terms, the cell percentage in the M4 stage was significantly increased in anti-miR-21,

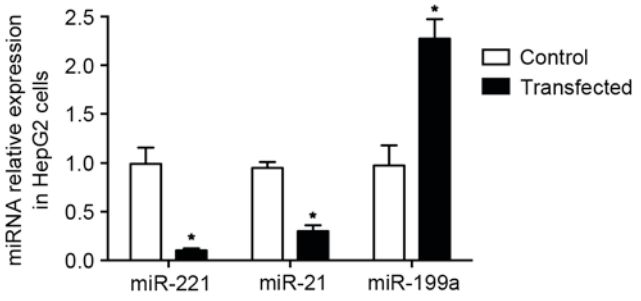

Figure 3. The expression of miRNAs following microbubble-mediated transfection of recombinant plasmids EGFP-anti-miR21, EGFP-anti-miR221 or EGFP-miR199a hepatoma HepG2 cells. *P<0.05 compared with the control group. EGFP, enhanced green fluorescent protein; miRNA and miR, microRNA.

anti-miR-221 and miR-199a-transfected groups (all P<0.05; Fig. 4B). The percentage in miR-199a-transfected cells was the highest. These data revealed that treatment with anti-miR-21, anti-miR-221 and miR-199a induces apoptosis of HepG2 cells.

Furthermore, the apoptosis of transfected cells was confirmed by performing an Annexin V-PE/7-AAD double staining assay. Compared with negative controls, the apoptotic rate was significantly increased in anti-miR-21, anti-miR-221

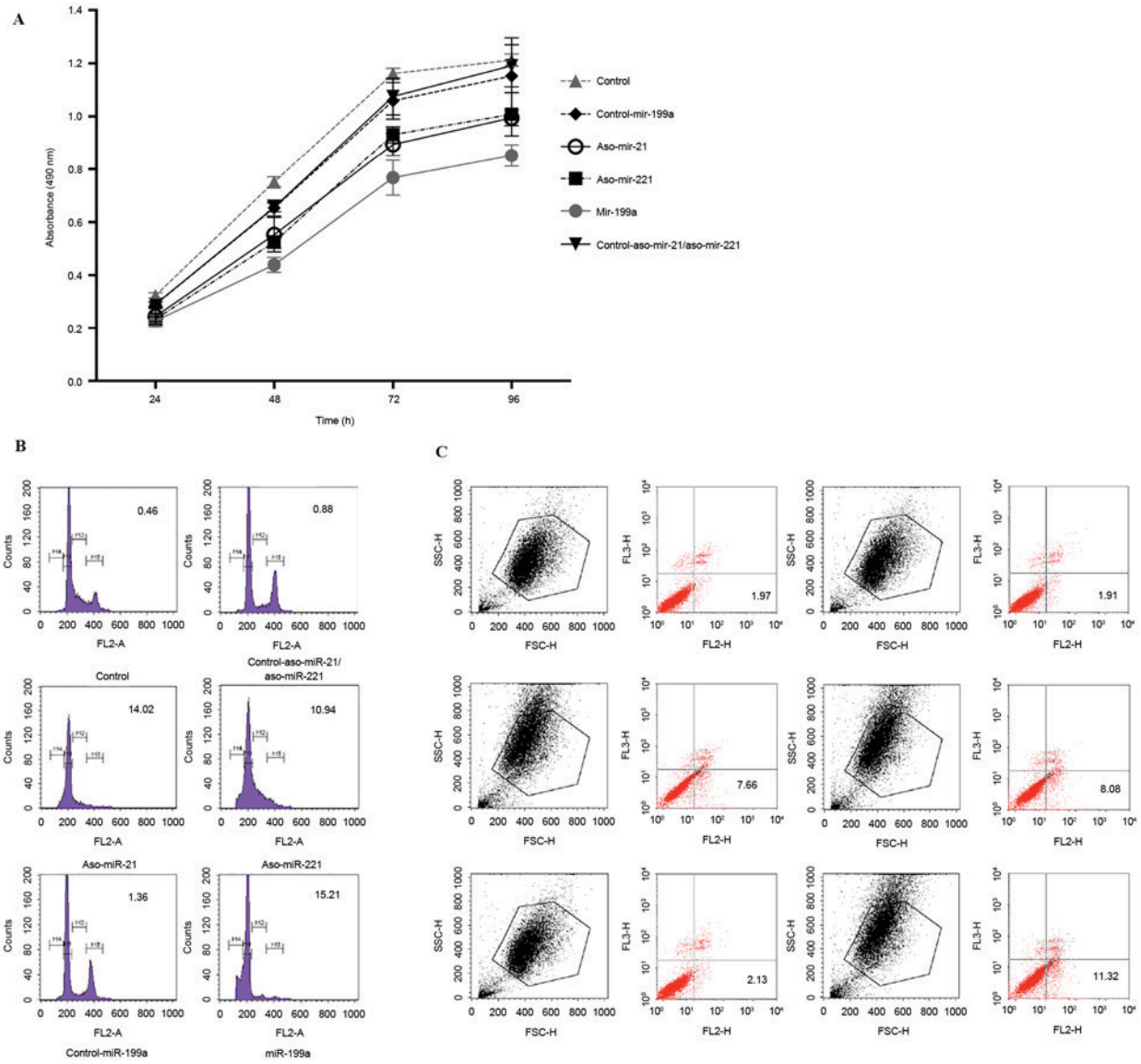

Figure 4. Anti-miR21/miR221 and miR-199a induced apoptosis of HepG2 cells. HepG2 cells were transfected with recombinant EGFP-anti-miR21, EGFP-anti-miR221 or EGFP-miR199a. (A) Cell proliferation was detected by MTT assay at the indicated time points (24, 48, 72 and 96 h). (B) After 48 h, cell cycle analysis and (C) apoptosis assays were performed on the cells. Aso-miR-, anti-microRNA.

and miR-199a-transfected groups (P<0.05; Fig. 4C; all >7% vs. <3%). In miR-199a-transfected cells, the apoptotic rate was highest at 11.10±0.46%. Thus, the apoptotic rate was statistically increased compared with that observed in two anti-miRNA-transfected groups (P<0.05).

Discussion

miRNAs are involved in the occurrence, development and prognosis of cancers, making them a promising target for cancer gene therapy (12,26). Previous studies demonstrated that numerous miRNAs are involved in the pathogenesis of HCC (3-4,15-17). Gene therapy, particularly miRNA-targeted therapy, is a promising candidate in the treatment of cancer, including HCC. However, this area of study is hampered by the shortage of available delivery vectors (27,28). The conventional viral vectors are marred by safety problems, while non-viral vectors have a major drawback of low transfection efficiency (27,28). Previous studies established that under ultrasound exposure, contrast agent microbubbles improve transfection efficiency and the expression of DNA in local tissue or cells (25,29,30). The present results also indicated that ultrasound contrast agent microbubbles significantly improved the transfection efficiency of DNA (P<0.05).

In general, transfection efficiency that is mediated by ultrasound microbubbles is affected by the following parameters: Ultrasound exposure condition; type and concentration of microbubbles; and cell types (20). Therefore, it is important to optimize the conditions of ultrasound intensity and concentration of microbubbles in gene delivery systems that are mediated by ultrasound microbubbles. In HepG2 cells, to the best of our knowledge, previous studies have not identified DNA transfection that is mediated by ultrasound microbubbles. Therefore, the present study first optimized the concentration of contrast agent microbubbles and ultrasound intensity. In the present study, two contrast agents, SF6 and C3F8, were used.

The present data indicated that the two agents did not exhibit any marked cytotoxicity when the concentration was <10%. When the ultrasound intensity was 2.0 MHz, the MI was 0.28, and the transfection efficiency was significantly increased compared with that of lipofection-mediated transfection. In addition, SF6 and C3F8 exhibited high transfection efficiency. This indicated that two ultrasound microbubbles were efficacious gene delivery vectors. Since SF6 microbubbles exhibited the highest transfection efficiency, 10% of SF6 microbubbles were applied at an ultrasound frequency of 2.0 MHz (MI, 0.28) in order to determine the effect of miR-21, miR-221 and miR-199a on HepG2 cells.

Using ultrasound microbubble-mediated transfection, anti-miR-21/miR-221 and miR-199a were revealed to inhibit cell proliferation and induce cell apoptosis in HepG2 cells. Previous studies have established that miR-21 was overexpressed in numerous cancers, including HCC, breast cancer, cervical cancer, lung cancer, colon cancer, adenocarcinoma and glioma (31-33). Furthermore, miR-21 was involved in the proliferation of tumor cells, invasion of tumor vascular phase and tumor staging (31-33). These previous studies indicated that miR-21 acts as an oncogene, promoting the occurrence and progression of tumors. The present study demonstrated that anti-miR-21 inhibited the proliferation and induced apoptosis of hepatoma cells. However, miR-21 was reported to target the suppressor gene phosphatase and tensin homolog (PTEN), thereby negatively regulating programmed cell death factor 4 (PDCD4) (34). In the present study, with an increase in the expression of anti-miR-21, the expression of miR-21 decreased. As a result, there was upregulation in the expression of PTEN and PDCD4, and the apoptosis of HepG2 cells was induced. Previous studies revealed that miR-221 affects several tumorigenic pathways in the early stages (35-38). An overexpression of miR-221 was associated with the invasion phenotype in patients with HCC, while miR-21 inhibited apoptosis by targeting B-cell lymphoma-2 modifying factor (BMF) (35,36). A number of studies indicated that miR-221 was negatively associated with cyclin dependent kinase inhibitors (CDKN1B/p27 and DKN1C/p57) in patients diagnosed with HCC (37,38). miR-221 initiates tumorigenesis by targeting p27 and p57. Thus, cell proliferation is promoted to target BMF and inhibit apoptosis. The present data also revealed that anti-miR-221 inhibits cell proliferation and promotes cell apoptosis. With the expression of anti-miR-21, the expression of miR-21 decreased. Consequently, there was upregulation in the expression of CDKN1B/p27 and DKN1C/p57, while the expression of BMF was downregulated. Together, these events led to the apoptosis of HepG2 cells.

In the present study, miR-199a was downregulated in patients diagnosed with HCC, leading to poor prognosis of patients diagnosed with HCC (39). In the present study, miR-199a inhibited cell proliferation to the greatest extent. It was reported that miR-199a inhibits the proliferation of hepatoma cells by targeting hypoxia-inducible factor-1a and cluster of differentiation 44, as well as by regulating the cell cycle. In addition, miR-199a upregulates CDKNlB/p27 and CDKN1A/p21 to inhibit the progression of the cell cycle, thereby inducing apoptosis (40).

In conclusion, the parameters for ultrasound SF6 microbubble-mediated gene delivery were optimized. The conditions for the transfection of recombinant plasmids were then optimized, which contained anti-miR-21, anti-miR-221 and miR-199a. Finally, it was identified that anti-miR-21/miR-221 and miR-199a inhibited cell proliferation and induced cell apoptosis in HepG2 cells. This indicated that these three miRNAs may be novel gene therapy targets.

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