Suppression of Breast Cancer Cells *In Vitro* by Polyamidoamine-Dendrimer-Mediated 5-Fluorouracil Chemotherapy Combined with Antisense Micro-RNA 21 Gene Therapy

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ABSTRACT: A specific micro-RNA (miRNA), micro-RNA 21 (miR-21), is strongly overexpressed in breast cancer cells. Antisense inhibition of miRNA function, an important tool for uncovering miRNA biology, which is often used to knockdown miRNA, can cause a notable inhibition of cell growth. In this study, 5-fluorouracil (5-FU) was conjugated to polyamidoamine dendrimers via direct encapsulation; this method was then combined with antisense micro-RNA 21 (as-miR-21) strategies to evaluate the effects of the growth suppression of breast cancer cells. Our results show that as-miR-21 strategies significantly improved the chemosensitivity of free 5-FU on breast cancer cells (MCF-7). In addition, not only could as-miR-21 effectively increase the apoptotic cell numbers but it could also bring down the migration ability of MCF-7 cells. Our results provide invaluable information for the future design of drug–polymer complexes for multimodal cancer treatments. © 2009 Wiley Periodicals, Inc. J Appl Polym Sci 114: 3760–3766, 2009

Key words: dendrimers; drug delivery systems; nanotechnology

INTRODUCTION

Breast cancer is the most frequent cancer in women and is a leading cause of death. 5-Fluorouracil (5-FU),[1] as one of the primary antineoplastic chemotherapy drugs, has been used in clinical practice for decades. However, current traditional chemotherapy comes with systemic toxicity and severe side effects; as a result, this limits tumor-specific treatments.

With the aim of increasing the therapeutic efficacy and reducing the toxicity of anticancer drugs, various drug carriers,[2] including polymer micelles and coated nanoparticles, have been synthesized and extensively studied. Recently, a great deal of attention has been given to polyamidoamine (PAMAM) dendrimers; these are one of the most appropriate candidates for suitable carrier systems. PAMAM dendrimers[3] represent an exciting new class of macromolecular architecture called *dense star polymers.* Unlike classical polymers, dendrimers[4] have a high degree of molecular uniformity, a narrow molecular weight distribution, specific size and shape characteristics, and a highly functionalized terminal surface. To take advantage of hydrogen-bond interaction,[5] 5-FU was encapsulated in PAMAM nanoparticles simply by the membrane dialysis method.

Dendrimer formulation of anticancer drugs offers a number of advantages over conventional dosage forms. In particular, it provides a sustained release of drug targeting the desired site. Hence, not only the therapeutic efficiency of the drug increases, but

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this also allows a reduction in the amount of drug administered and, therefore, minimizes undesirable side effects. Patri et al.[6] used generation five (G5) PAMAM dendrimers in the targeted drug delivery of methotrexate and significantly reduced the toxicity and increased the aqueous solubility. Kono et al.[7] combined the anticancer drug Adriamycin with the side chains of the Glu residues of a generation four PAMAM dendrimer using an amide bond; a remarkable extent of Adriamycin release was induced at pH 5.5. In addition, there were positively charged primary amino groups on the surface, which made them feasibly interact with biomolecules to form complexes through their charge-based interactions and protected them from rapid degradation by cellular endonucleases and exonucleases.[8]

Chemotherapy is the preferred therapeutic approach for breast cancer, but successful long-term treatment is prevented by the development of drug sensitivity. Recent advances in our understanding of the molecular mechanism implicated in the rise of sensitivity in cellular models of breast cancers have emphasized its biological complexity, including increased antiapoptotic regulator activity,[9] growth factor receptor deregulation,[10] and posttranslational modification or alteration. In the past few years, we discovered a new class of genomic regulators, named *micro-RNAs* (miRNAs), that are now universally recognized as central players in virtually all neoplasm development and progression. Notably, the discovery of mammalian miRNAs has uncovered a new set of genetic elements that act directly as repressors of gene expression and have been causally linked to several types of cancer.

miRNAs are a new class of small noncoding RNAs, naturally 19–25 nucleotides in length, and control the expression of a wide variety of genes by binding to the complementary sequences in the 3′-untranslated regions of the target messenger RNAs, inducing translational repression messenger RNA cleavage, or destabilization.[11,12] miRNAs have diverse functions in biological processes, including the regulation of cellular proliferation, differentiation, and cell death.[13–15] As dysregulation of these biological processes frequently occur in human cancer, miRNAs may, therefore, play a critical role in the process of tumorigenesis.

Micro-RNA 21 (miR-21) is one of the founding members of the miRNA family. Yan et al.[16] showed that miR-21 is overexpressed in primary breast cancer cells. Iorio et al.[17] also demonstrated that compared with normal breast tissue, miR-21 is also aberrantly expressed in human breast cancer tissue.

Our previous studies suggested that miR-21 is a tumor oncogene, as the involvement of the down-regulation of miR-21 expression with antisense oligonucleotide technology can not only effectively increase the apoptotic cell numbers but also bring down the migration ability of breast cancer cells. The possible involvement of antisense micro-RNA 21 (as-miR-21) for cancer cell growth suppression prompt us to speculate that it may have something to do with the cellular response to therapeutic drugs.

Despite the progress in gene therapy and chemotherapy, however, more and more attention is being paid to the combination of gene therapy and chemotherapy[18] because many studies have demonstrated that the use of a single treatment strategy against cancer is generally ineffective because of the multifactorial nature of this disease. Furthermore, the combination of more than one drug to maximize the anticancer response is being increasingly used.[19] It has been demonstrated that there is a synergistic relationship between antisense oligonucleotide gene therapy and 5-FU chemotherapy.[20]

In these studies, we combined chemotherapy and gene treatment to enhance the sensitivity of 5-FU to obtain a better therapeutic effect. A G5–PAMAM dendrimer was chosen as the carrier to synchronously delivery 5-FU and then conjugate with as-miR-21, which was proven to selectively knock down the expression of miR-21.The physical properties and drug-release behavior of 5-FU–PAMAM complexes were investigated with transmission electronic microscopy and ultraviolet–visible absorbance measurements. Through their charge-based interactions, 5-FU–PAMAM could conjugate with as-miR-21. The transfect efficiency was evaluated with the use of a flow cytometry system. The suppression effect of tumor cells by chemotherapy combined with as-miR-21was evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, apoptosis, and transwell assessment. Our studies are presented here to illustrate the possibility of a new perspective for the treatment of malignant tumors and to indicate the potential utility of these conjugates for medical applications.

EXPERIMENTAL

Cell lines and reagents

Human breast adenocarcinoma cells (MCF-7) were a kind gift from J. Hao (William T. Gossett Neurology Laboratories, Department of Neurology, Henry Ford Health System, Detroit, MI). The cell cultures were incubated at 37°C in a 10% CO_2 atmosphere and maintained routinely in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and 2 m*M* L-glutamine (Invitrogen, Carlsbad, CA). A methanolic solution of PAMAM dendrimer, G5 containing 128 surface amino groups (Generation 5–PAMAM, G5D), and fluorescein

isothiocyanate (FITC) were purchased from Sigma-Aldrich (St. Louis, MO). Trypsin–ethylene diamine tetraacetic acid, phosphate-buffered solution (PBS), and DMEM were obtained from Invitrogen (Gaithersburg, MD). 5-FU for injection was provided by Tianjin Jinyao Pharmaceutical Group Corp. (Tianjin, China).

Drug loading in the formulations

5-FU/PAMAM nanoparticles were prepared by a diafiltration method. Before the synthesis of the drug–dendrimer complex, the G5 PAMAM dendrimers were first filtered and dialyzed against PBS for 1 day and then deionized water for another day to remove methanol. Briefly, we dissolved the PAMAM dendrimer and 5-FU (1:100 molar ratio) and dialyzed the solution (with a molecular weight cutoff of 7000 g/mol; Spectrum Medical Industries, Inc., Houston, TX) in double-distilled water for 24 h. The solution inside the dialysis bags was collected and filtered with a 0.22-μm filter. The samples were then freeze-dried for subsequent use. A 640 UV spectrophotometer (Bechman, Fullerton, CA) was used to identify the 5-FU/PAMAM nanoparticles by scanning from 200 to 400 nm.

Morphologies of the 5-FU/PAMAM nanoparticles

The transmission electron microscope was used to examine particle morphology. One drop of the 5-FU–PAMAM sample was added to a copper-supported mesh membrane, and the excess solution was removed with filter paper. Then, 1% phosphotungstic acid was added to the mesh membrane. Excess solution was removed after 1 min, and the sample was dried at room temperature. The concentration of the prepared sample was 1 mg/mL.

Loading capacity, drug encapsulation, and *in vitro* release

5-FU/PAMAM nanoparticles were placed into dialysis bags, and the bags were introduced into double-distilled water. After it was stirred at 37°C for 0.5 h, the drug concentration of the dialyzed sample was determined by measurement of the absorbance at 266 nm (the characteristic wavelength of FU in water). The drug-loading capacity and drug encapsulation were calculated by the following formulas:

$$\text{Drug-loading capacity} = M_{\text{5-FU}}/M_{\text{5-FU/PAMAM}}$$

$$\text{Drug encapsulation} = M_{\text{5-FU}}/M_{\text{drugdevoted}}$$

where $M_{\text{5-FU}}$ is the drug content detected in the solution, $M_{\text{5-FU/PAMAM}}$ is the quantity of 5-FU-loaded PAMAM nanoparticles detected in the solution, and $M_{\text{drugdevoted}}$ is the initial quantity of 5-FU. For the *in vitro* release studies, 5-FU/PAMAM nanoparticles were placed into dialysis bags, and the bags were introduced into PBS at pH 7.4. Pure 5-FU was dissolved in water and used as a control. At a scheduled interval of time, a 0.5-mL sample was taken out of the dialysis bag to measure the concentration of 5-FU, and the outer phase was again replenished with 500 mL of distilled water at variable periods of time up to 24 h. We determined the concentration of the 5-FU that was released into the PBS by measuring the absorbance at 266 nm.

Oligonucleotide transfection

The as-miR-21 with a sequences of 5′-GTC CAC TCT TGT CCT CAA TG-3′ was synthesized and purified by the use of a high-pressure liquid chromatography system, dissolved with diethylpyrocarbonate water, and frozen at −20°C.

After seeding the cells onto six-well plates for 24 h in DMEM medium containing 10% fetal bovine serum, we starved the cells for 4 h at 37°C before transfection. After seeding at a 3×10^5 cells/well density (70% confluence), we transferred the MCF-7 cells grown on poly(D-lysine)-coated plates. The as-miR-21 solution (20 μmol/1) was incubated with 5-FU/PAMAM solution at 16 N/P ratios (ratio of the number of terminal amino groups in the dendrimer to the number of phosphate groups in the as-miR-21) for 20 min and these were added directly to the cells to a final oligonucleotide concentration of 100 nmol/L. Transfection complexes were prepared. The transfection medium was replaced 4 h after transfection.

Annexin-V binding assay

All cells were seeded and treated with the free drugs or as-miR-21 as described before. Thereafter, the cells were trypsinized, resuspended in binding buffer [10 m*M* 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid, 140 m*M* NaCl, and 2.5 m*M* $CaCl_2$] (Santa Cruz Biotechnology, CA), and stained with an Annexin V-FITC/PI Apoptosis Detection Kit for 15 min in the dark at room temperature according to the protocol provided by the manufacturer. The cell populations were analyzed with a FACS flow cytometer (Becton Dickinson, San Jose, CA). Five thousand cells were counted for each sample.

Cytotoxicity assessments

The sensitivity of human breast cancer cells, MCF-7 cells, to the drug was determined by MTT [3-(4,5-dimethylthiazole)-2,5-diphenyltetrazoliumbromide] assay. Briefly, 10^4 cells/well were seeded in 96-well plates and allowed to attach overnight. Cells were cultured for 6 days in a medium supplemented with free 5-FU, 5-FU–PAMAM, or as-miR-21/5-FU–

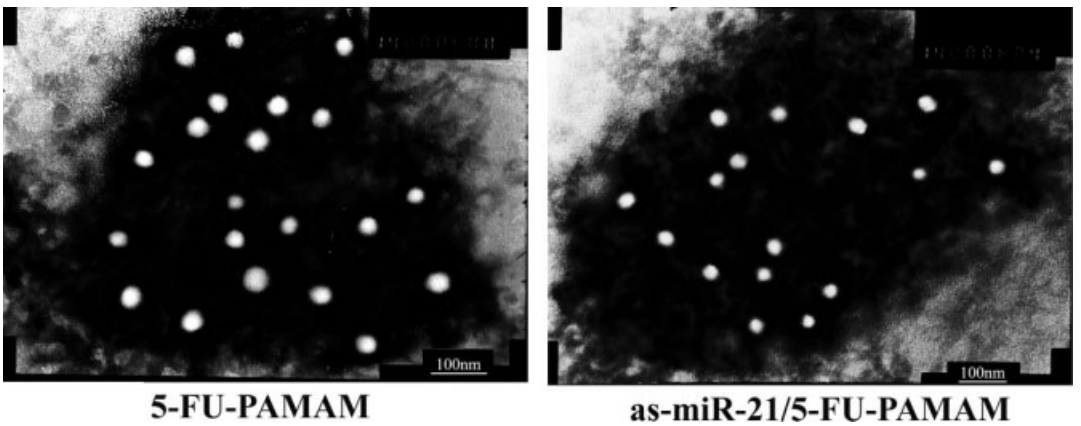

Figure 1 Morphologies of the drug-loading PAMAM complex and the drug–PAMAM/miRNA complex.

PAMAM. The concentrations of 5-FU and as-miR-21 were 5 μg/mL and 20 μmol/L, respectively. Each group contained 8 wells. On each day of the consecutive 6 days, 20 μL of MTT (0.5 mg/mL) was added to each well, and the cells were incubated at 37°C for an additional 4 h. We then stopped the reaction by lysing the cell with 200 μL of dimethyl sulfoxide at room temperature for 30 min. The level of absorbance was read with a Fluostar Optima (BMG Labtech, Offenburg, Germany) multiplate reader at a wavelength of 570 nm. The results are presented as a percentage of the control.

Cell invasion assessment

In vitro invasion assays were carried out with modified Boyden chambers consisting of Transwell membrane filter inserts (pore size = 8 μm, Corning Costar Corp., Cambridge, MA) in 24-well tissue culture plates. The cell invasion chambers were prepared by the placement of 100 μL of a 1:5 dilution of Matrigel onto the filter and incubation at 37°C for 30 min to allow Matrigel polymerization. Cells (1×10^5) suspended in serum-free DMEM were added to each Transwell chamber and allowed to invade toward the underside of the chamber for 48 h at 37°C in a humid atmosphere of 5% CO_2/95% air. Cells that passed through the membranes were fixed in 95% ethanol and stained with hematoxylin and counted under a light microscope.

RESULTS AND DISCUSSION

The elevated expression of miR-21 has been commonly reported to be associated with different cancers and has been shown to be involved in oncomir-modulating tumor growth and apoptosis.[21] The possible involvement of as-miR-21 for cancer cell growth inhibition prompts us to speculate that it may play an important role in the cellular response to therapeutic drugs.

In these studies, a combination of as-miRNA gene therapy and chemotherapy for the enhancement of drug chemosensitivity for better suppression of human breast tumors was evaluated. The dendrimers had unique characteristics, including monodispersity and modifiable surface functionality, along with a highly defined size and structure. This makes these polymers attractive candidates as carriers in drug-delivery applications. Hydrophobic drugs could be complexed within the dendrimer interior to make them water soluble as its interior shells and core structure. Furthermore, by virtue of the multivalent surface containing a larger number of positive charges, which made it feasible for them to interact with as-miR-21 and form complexes through their charge-based interactions, both the drug and as-miR-21 were effectively delivered to the same cells for combined actions and synergistic effects.

Nanoparticles can enhance the solubilization of a hydrophobic drug, protect drug activity, increase

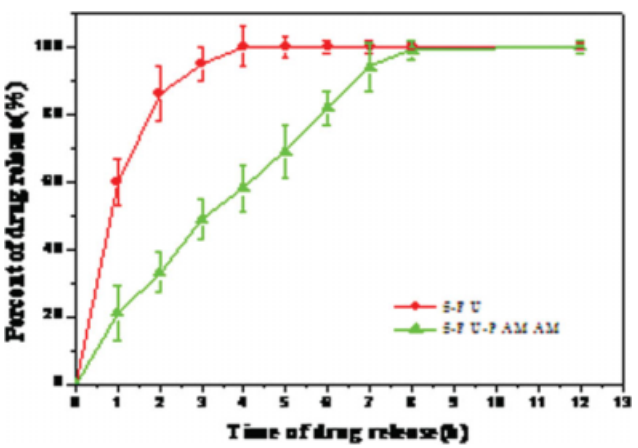

Figure 2 Release profiles of 5-FU from the 5-FU–PAMAM conjugate complexes in comparison with the free drug. The 5-FU–PAMAM conjugates were dialyzed with pH 7.4 PBS, and the released 5-FU were quantified as described in the Experimental section. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

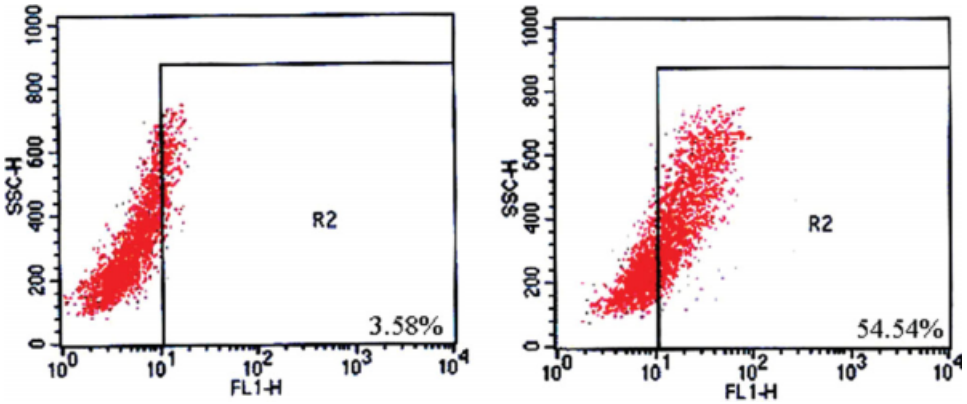

Figure 3 Transfection efficiency (detected by flow cytometry) of the MCF-7 cells after transfection with a 5-FU–PAMAM complex in comparison with a negative control. SSC-H represents side scatter height. FL1-H represents FITC fluorescence height. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

drug stability, improve the drug's therapeutic index, and decrease its adverse side effects.[22,23] Taking advantage of hydrogen-bond interaction, we encapsulated 5-FU in the PAMAM nanoparticles simply by a membrane dialysis method.

Analysis by UV spectrophotometry (data not shown) made it clear that a solution of 5-FU had a high absorbance at 266 nm. However, the absorbance of the 5-FU/PAMAM nanoparticles at 269 nm greatly decreased. This indicated that 5-FU was loaded into the PAMAM dendrimers by diafiltration. On the basis of the decrease in absorbance at 266 nm, the encapsulation efficiency and loading efficiency of the drug were determined by UV spectroscopy to be 66.21 and 31.77%, respectively.

Like many other polymeric nanoparticles, the morphology of our 5-FU/PEG–PBLG nanoparticles was spherical or elliptical (Fig. 1). A representative transmission electron microscopy scan of the 5-FU/PAMAM nanoparticle is shown in Figure 1. Previous research has shown that nanoparticles are not easily phagocytized, which indicated that the reticuloendothelial system (RES) should not have taken up the 5-FU/PAMAM nanoparticles examined in this study. The size of the PAMAM slightly increased after drug loading and as-miR-21 binding, but the size was always smaller than 100 nm. The small size of the complexes facilitated their uptake by the tumor cells.

Sustained release is the slow release of a drug that is entrapped within nanoparticles. This allows the drug to stay at an effective concentration in the circulation over time. Figure 2 shows the *in vitro* 5-FU release profiles from the PAMAM dendrimer in pH 7.4 PBS. This brackets the normal pH of human blood (pH $\approx$ 7.4). The 5-FU conjugated in G5 PAMAM exhibited a much slower release compared with the free drug. As shown in Figure 2, in the case of the routine formulation, almost 60% of the drug was released within 1 h; on the contrary, less than 20% was released from the 5-FU–dendrimer complexes. The release of 5-FU from the host lasted for about 12 h. This result is consistent with previous reports. The binding of as-miR-21 seemed to have a

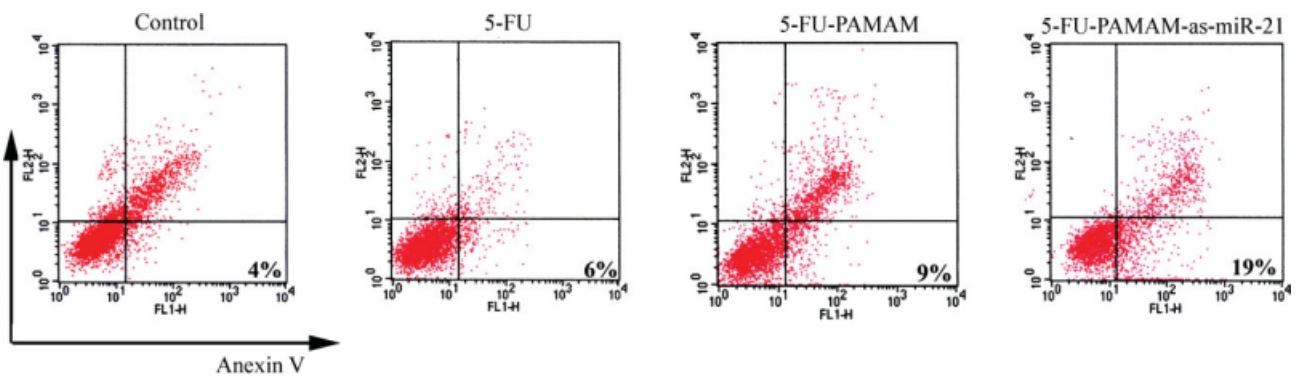

Figure 4 As-miR-21 increased drug-induced apoptotic cell death in the MCF-7 cells. The cells were transfected with 5-FU, 5-FU–PAMAM, and as-miR-21 for 48 h, and this was followed by an Annexin V binding assay. The experiments were repeated at least three times, and the results are similar to those shown. The number represents the percentage of cells in the fourth quadrant. FL1-H represents FITC fluorescence height. FL2-H represents propidium iodide (PI) fluorescence height. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

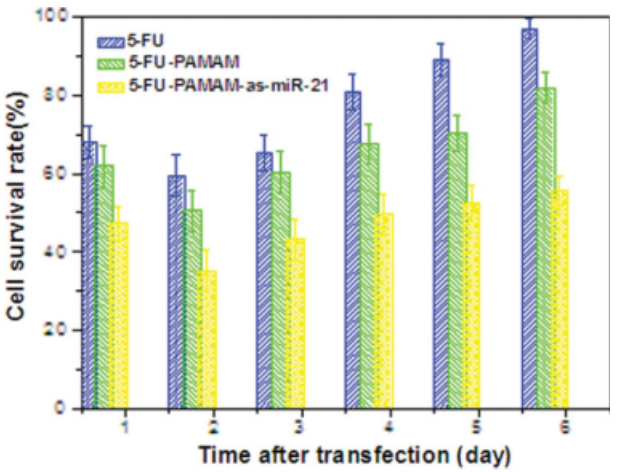

Figure 5 As-miR-21 modified drug sensitivity of the MCF-7 cells. The cells were transfected with 5-FU, 5-FU–PAMAM, and as-miR-21 for 6 days. Cell survival was then assessed by MTT assay. The data represented the mean values of three independent experiments (mean ± standard error of the mean). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

tiny effect on the release of 5-FU, as similar release profiles were presented by the two formulations.

As evidenced by fluorescence microscopy observation and flow cytometer measurement (Fig. 3), after binding to 5-FU–PAMAM, the cell uptake of as-miR-21 dramatically increased from 3.58 to 54.54%.

The method of flow cytometric analysis of cells staining with Annexin V was used to investigate the role of as-miR-21 in drug-induced apoptosis in MCF-7 cells (Fig. 4). The drugs used in the study were 5-FU. The knockdown of miR-21 expression increased the apoptotic cell population of MCF-7 cells (19%) compared with that of the control (4%) or that induced by 5-FU–PAMAM as a single agent (9%). That is to say, as-miR-21 combined with 5-FU significantly enhanced the percentage of apoptosis. It was reported that as-miR-21 may up-regulate caspase-3 expression,[24] which is the key factor influencing apoptosis. Our results confirm the close link between miR-21 and apoptosis-related genes.

Because as-miR-21 was found to regulate the process of drug-induced apoptosis, it may, therefore, also likely modulate the sensitivity of cells to anticancer drugs. By MTT assay, transfection with as-miR-21 by 5-FU–PAMAM significantly improved the sensitivity of MCF-7 cells to 5-FU (Fig. 5). Although the 5-FU–PAMAM group was superior to the free-drug group, it only exhibited a moderately suppression. In contrast, codelivery of 5-FU and as-miR-21 to MCF-7 cells, however, at any time, showed the best tumor-growth-suppression effect, which suggested a enhanced chemosensitivity effect associated with as-miR-21. Therefore, miR-21 may also likely play a role in the modification of drug response in human breast cancer cells.

The effects of the combination of as-miR-21 and 5-FU on cell invasion *in vitro* are shown in Figure 6. An *in vitro* cell invasion assay was performed on the basis of the principle of the Transwell chamber assay. The Matrigel matrix served as a reconstituted basement membrane *in vitro*.

In these largely used devices, we put cells in an upper chamber to migrate, whereas the migration stimuli (molecules or cells) were placed in the bottom chambers or in an intermediate filter. Again, the total numbers of migrating cells that reached the bottom chamber could be counted.

In brief, the black dots in the figure stand for the migrating cells, whereas the transparent dots represent the unmigrated cells. In other words, the higher the total numbers were, the stronger the invasion ability of the cells was.

The number of cells migrating through the Matrigel matrix was counted, and the result is presented

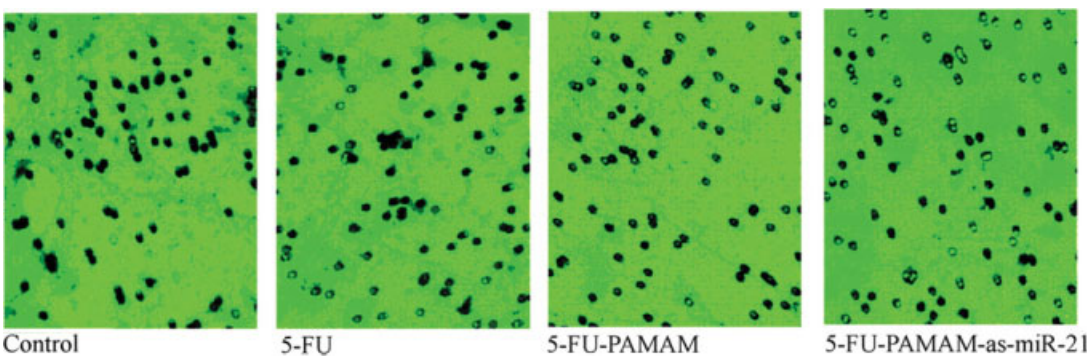

Figure 6 Invasion ability of the MCF-7 cells treated with different complexes, assayed with a modified Transwell chamber. (A) After 24 h, the number of cells that had migrated through the membrane was counted under a microscope in five random fields at a magnification of 100×. The pCMV-PRL3 miRNA-1249-A2 cells showed significantly reduced invasiveness in comparison with parental SGC7901 and pCMV-PRL3 miRNA-neg-A1 cells (*$p < 0.05$). (B) Image of MCF-7 cell invasion. The slides were stained with hematoxylin and eosin and magnified 10×. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

in Figure 6. As shown in Figure 6, a significantly alternation of cell invasive number among the 5-FU–PAMAM conjugate, free drug, and 5-FU–PAMAM/as-miR-21 complex was observed. The mean numbers plus or minus the standard deviation of three separate experiments with these cells attached to the lower surface of the filters were as follows: control, 46 ± 3/HP; 5-FU, 40 ± 1/HP; 5-FU–PAMAM, 30 ± 2/HP, and as-miR-21/5-FU–PAMAM, 19 ± 4/HP. The as-miR-21combined with 5-FU knockdown cells showed a significantly reduced invasiveness compared to the negative control cells ($p < 0.05$). The results also verify the conclusion we drew previously: the combined drug and gene therapies did give a better suppression effect for the tumor cells. This is also demonstrates the potential applications for the codelivery of the antisense miRNA and chemotherapy drugs in cancer therapy.

CONCLUSIONS

A PAMAM dendrimer, a good delivery agent for both chemotherapy and gene therapy, effectively conjugated with 5-FU and as-miR-21 simultaneously, forming complexes with sizes lower than 100 nm. The codelivery of as-miR-21 not only significantly improved the cytotoxicity and chemosensitivity of 5-FU and dramatically increased the apoptotic percentage of the MCF-7 cells but also brought down the migration ability of the tumor cells. Our results provide invaluable information for the future application of drug–polymer complexes combined with gene therapy for cancer treatments.

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