PUMA is a novel target of miR-221/222 in human epithelial cancers

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Abstract. miR-221 and miR-222 (miR-221/222) are frequently up-regulated in human epithelial cancers. However, the mechanism of miR-221/222 action involved in carcinogenesis has not been extensively studied. Here, we found that reduction of miR-221/222 inhibited cell proliferation and induced mitochondrial-mediated apoptosis in human epithelial cancer cells (A549 lung cancer and MCF-7 breast cancer cells). Bioinformatics and luciferase reporter assays showed that miR-221/222 co-modulated the p53 up-regulated modulator of apoptosis (PUMA) expression by directly targeting the binding site within the 3'UTR. Together, these findings suggest that PUMA is a direct target of miR-221/222 that functions as an endogenous apoptosis regulator in these epithelial cancers.

Introduction

Lung cancer and breast cancer, the common forms of human epithelial cancers, are the most frequent causes of cancer-related death worldwide, and their incidences are increasing (1,2). In most instances, multiple genetic changes including activation of proto-oncogenes and inactivation of tumor suppressor gene are involved in the development and progression of human epithelial cancers (3). microRNAs (miRNAs), recently identified small non-coding RNAs, can regulate gene expression by sequence specific pairing with target mRNA 3' untranslated region (3'UTR) and subsequent inhibition of mRNA translation. Previous studies have indicated that miRNAs can function as oncogenic miRNAs (oncomiRs) or tumor suppressor miRNAs, playing crucial roles in transformation and carcinogenesis (4,5).

Among oncomiRs, miR-221 and miR-222 (miR-221/222) share the same 'seed' sequence, evolutionarily conserved and short regions at their 5' ends through which they bind their target sites in mRNA 3'UTR. Both miRNAs are clustered within a 1-kb genomic interval and are transcribed from the same strand. In epithelial cancers (glioma, prostate carcinoma, hepatocellular cancer, lung cancer, breast cancer), several genes have been found to be common targets of these two miRNAs, such as p27, Bmf, PTEN and TIMP3 (6-14). Our recent data showed that co-suppression of miR-221/222 inhibits human glioma cell growth by targeting the 3'UTR of p27 mRNA *in vitro* and *in vivo* (11). Moreover, miR-221/222, target PTEN and TIMP3 tumor suppressors, then induce TRAIL resistance and enhance cellular migration (14). However, the mechanism of the biological function of miR-221/222 in epithelial cancers is still unknown and under further investigation.

In this study, we identified the p53 up-regulated modulator of apoptosis (PUMA) as a novel target of miR-221/222-induced apoptosis. Inhibiting miR-221/222 can directly regulate PUMA protein expression by targeting binding sites in the 3'UTR, subsequently resulting in an increase in mitochondrial membrane potential and caspase-3/7 activity, thereby leading to apoptosis in the A549 and MCF-7 cell lines.

Materials and methods

Cell culture and transfection. Human breast cancer cell line MCF-7, human lung cancer cell line A549 and human embryonic kidney cell line HEK293 were maintained in Dulbecco's modified Eagle's medium (DMEM) (Gibco, USA) supplemented with 10% fetal bovine serum (Gibco), 2 mM glutamine (Sigma), 100 units of penicillin/ml (Sigma), and 100 μg of streptomycin/ml (Sigma), and incubated at 37˚C with 5% CO_2. 2'-O-methyl (OMe)-oligonucleotides were chemically synthesized and purified by high-performance liquid chromatography by GenePharma Co. Ltd. (Shanghai, China). All the

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bases were 2'-OMe modified entirely and had the following sequences: 2'-OMe-anti-miR-221(As-miR-221), 5'-AGCUA CAUUGUCUGCUGGGUUUC-3'; 2'-OMe-anti-miR-222 (AS-miR-222), 5'-AGCUACAUCUGGCUACUGGGU-3'. 2'-OMe-oligonucleotides (200 pmol) were transfected using Lipofectamine 2000 (Invitrogen). Scrambled oligonucleotides (Scr) were also transfected as a control and had the following sequence: 5'-UCUACUCUUUCUAGGAGGUUGUGA-3'. Three days after transfection, cells were used for further analysis and divided into five groups, including control, scramble, As-miR-221, As-miR-222 and As-miR-221/222.

Northern blot analysis. Total RNA was extracted with TRIzol reagent (Invitrogen). Total RNAs (20 μg) were separated on a 12% denaturing polyacrylamide gel, and then transferred to Hybond N+nylon membrane (Ambion, USA). The membrane was dried, UV cross-linked, hybridized with Digoxigenin labeled probes overnight at 37˚C in a buffer containing 5X SSC, 20 mmol/l Na_2HPO_4 (pH 7.2), 7% SDS, 1X Denhardt's, and 0.2 mg/ml salmon sperm DNA. The specific probes, end-labeled with DIG, were miRNA-221, 5'-gaaacccagcagacaat gtagct-3'; miRNA-222, 5'-gagacccagtagccagatgtagct-3'; U6, 5'-atttgcgtgtcatccttgcg-3'. The probes were purchased from Proligo Primer & Probes (Sigma, USA). The membrane was washed with 1x SSC/1% SDS buffer at 50˚C. After equilibration in detection buffer, blots were detected with Dig Luminescent Detection kit (Roche, USA) and analyzed by GeneGenius.

Cell viability assay. Cells were seeded into 96-well plates at 4,000 cells/well. After transfection as described previously, 20 μl of MTT (5 g/l) was added into each well at each day of consecutive 6 days after treatment and incubated for 4 h, and the supernatant was then discarded. Finally, 200 μl of DMSO was added to each well to dissolve the precipitate. Optical density (OD) was measured at the wavelength of 570 nm. The data are presented as the mean ± SD, derived from triplicate samples of at least three independent experiments.

Apoptosis assays. Cell apoptosis in cultured cells was evaluated with annexin V labeling, caspase 3/7 activity and mitochondrial membrane potential. For the annexin V assay, an annexin V-FITC labeled Apoptosis Detection kit (Abcam, USA) was used according to the manufacturer's protocol. Caspase 3/7 activity was measured using Caspase-Glo 3/7 reagent (Promega, Madison, WI). Mitochondrial membrane potential was determined with cationic dye JC-1 (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine-chloride, C25H27 Cl3N4) staining. Briefly cells were incubated with 10 mg/ml JC-1 for 20 min at room temperature and then analyzed with FACSCalibur to detect green fluorescence at excitation/emission wavelengths of 485/530 nm and red fluorescence at excitation/emission wavelengths of 485/590 nm.

Western blots. Equal amounts of protein per lane were separated by 8% SDS-polyacrylamide gel and transferred to PVDF membrane. The membrane was blocked in 5% skim milk for 1 h and then incubated with a specific antibody for 2 h. The antibodies used in this study were: antibodies to PUMA, Bcl-2 and Bax (Santa Cruz, USA). The antibody against ß-actin (Santa Cruz, USA) was used as control. The specific protein was detected by using a SuperSignal protein detection kit (Pierce, USA). The band density of specific proteins was quantified after normalization with the density of ß-actin.

Bioinformatic analysis of miR-221/222 and target gene validation. The miRNA targets of miR-221/222 predicted by computer-aided algorithms were obtained from miRNA Viewer (http://cbio.mskcc.org/cgi-bin/mirnaviewer/mirnaviewer.pl) and PicTar (http://pictar.bio.nyu.edu). The human 3'UTR of the PUMA gene was amplified by PCR using the following primers: PUMA-3'UTR-Forward: 5'-CGATTCTAGAAATC ATGTTCTGGTGG-3' and PUMA-3'UTR-Reverse: 5'-GCA TTCTAGAATTCTGCACAGTAAGCATA-3', and then cloned into the *Xba*I/*Xba*I site of the pGL3-control vector (Promega, USA), downstream of the luciferase gene, to generate the vector pGL3- PUMA. For reporter assays, cells were cultured in 96-well plates and transfected with 0.2 μg each of three plasmids and 5 pmol of the interest oligonucleotides (As-miR-221 and/or As-miR-222) by Lipofectamine 2000. Luciferase activity was measured 72 h after transfection using a Dual-luciferase reporter assay system (Promega).

Statistical analysis. Statistics was determined by ANOVA, or t-test using SPSS11.0. Statistical significance was set at $P<0.05$.

Results

As-miR221/222 inhibites expression of miR-221/222-upregulated MCF-7 and A549 cells. In contrast to HEK293 cells (normal epithelial control cells), miR-221/222 were overexpressed in MCF-7 and A549 cells. In particular, the expression of miR-221/222 was significantly up-regulated in A549 cells. To knock down endogenous miR-221/222, chemically engineered oligonucleotides were synthesized and transfected into MCF-7 and A549 cells. Northern blot analysis showed that As-miR-221 and As-miR-222 efficiently and specifically silenced endogenous miR-221 and miR-222, respectively. U6 was present as loading control in five groups (Fig. 1).

As-miR-221/222 induces cancer cell apoptosis. As shown in Fig. 2A, cells transfected with As-miR-221/222 proliferated at a significantly lower rate than the other four groups as evaluated by MTT assay. Our previous study showed that co-suppression of miR-221/222 by AS-miR-221/222 resulted in up-regulation of p27 in glioma cells, thereby affecting their proliferation potential via reducing a G1 to S shift in the cell cycle (11). In order to test whether As-miR-221/222 inhibits cell proliferation through induction of cell apoptosis, apoptosis assays were employed. By Annexin V analysis, the apoptotic cells were significantly increased in cells transfected with AS-miR-221/222 as compared to that in parental cells and cells transfected with scramble (Fig. 2B), suggesting that apoptosis was significantly induced in cells transfected with AS-miR-221/222. Collapse of mitochondrial membrane potential is one of the earliest events in apoptosis. AS-miR-221/222 triggered a significant induction of collapse in mito-

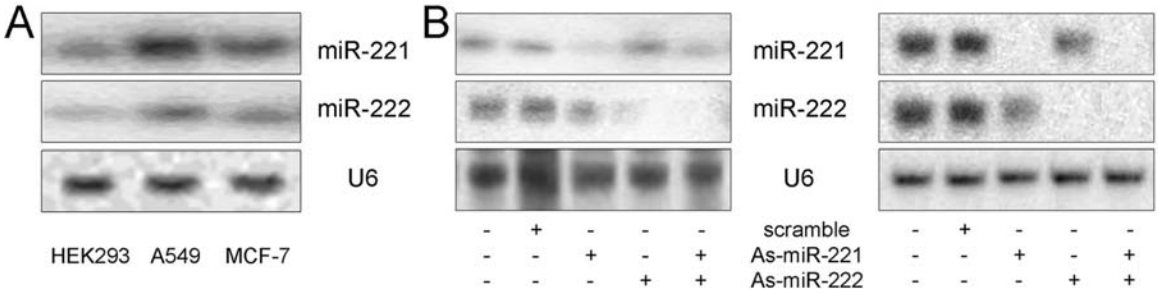

Figure 1. As-miR-221/222 inhibits the expression of miR-221/222. (A) Northern blot analysis showed that miR-221/222 was overexpressed in A549 and MCF-7 cell lines versus HEK293 cell. (B) miR-221/222 was significantly suppressed by transfection with miR-221/222 antisense oligonucleotides, whereas their expression was unchanged in cells transfected with scrambled oligonucleotides and control.

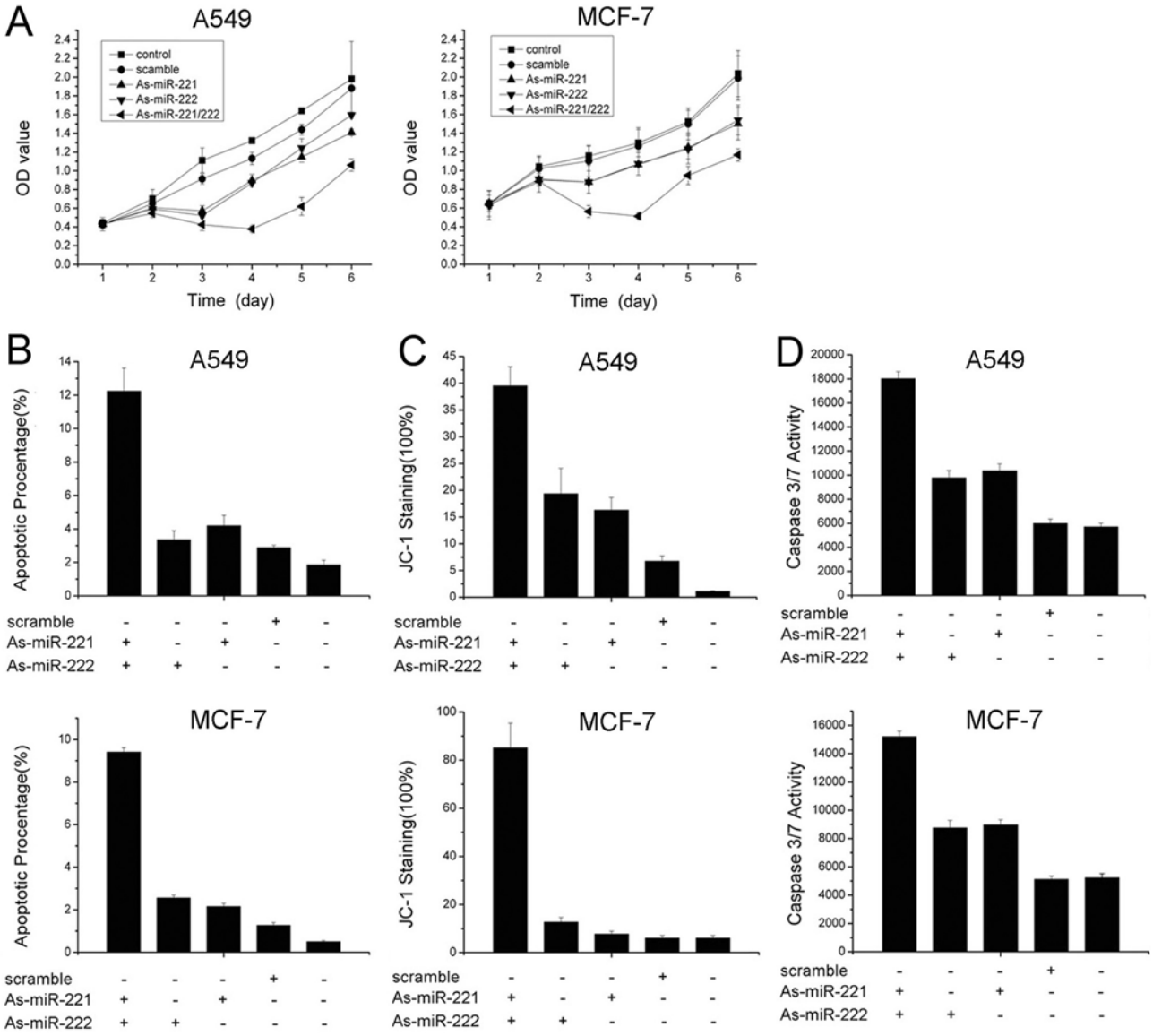

Figure 2. As-miR-221/222 increases cell apoptosis to inhibit cancer cell growth. (A) MTT assay showed that the cells transfected with As-miR-221/222 proliferated at a significantly lower rate than the other four groups. (B) Annexin V analysis showed that the cells transfected with As-miR-221/222 were apoptosis at a significantly higher rate than the other four groups. (C) Cells transfected with AS-miR-221/222 show a significantly greater collapse in mitochondrial membrane potential compared with the other four groups. (D) A strong increase of caspase3/7 activity was detected in cells transfected with AS-miR-221/222.

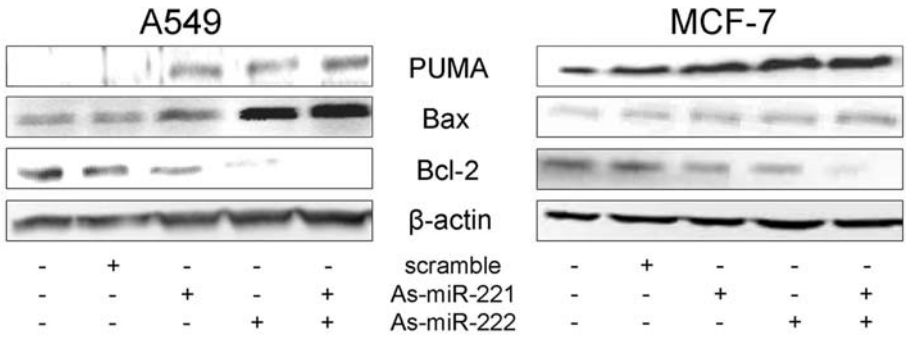

Figure 3. The apoptotic protein expression after As-miR-221/222 transfection. By Western blot assay, the expression of PUMA, Bax and caspase 3 in As-miR-221/222 group was significantly more up-regulated than the other four groups. The expression of Bcl-2 in As-miR-221/222 group was significantly more down-regulated than the other four groups.

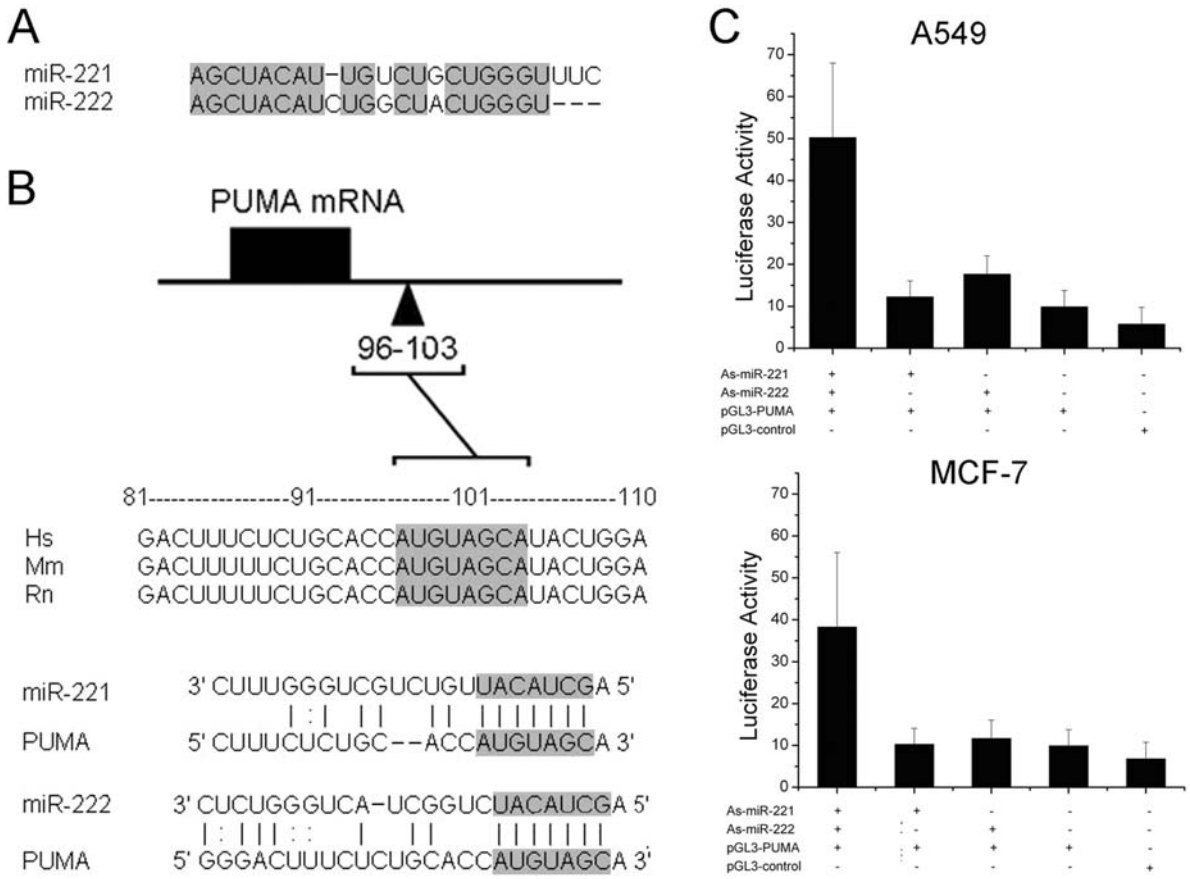

Figure 4. PUMA was identified as a target gene of miR-221 and miR-222. (A) A schematic representation showing the mature miR-221 and miR-222 sequences (identical seed sequences AGCUACAU as shown). (B) PUMA mRNA 3'UTR depicting the putative target site for miR-221 and miR-222. The 3'UTR of PUMA contains one site expected to be recognized by both miR-221 and miR-222, respectively. Numbers represent the position of the seed match within the UTR sequences. Hs, human; Mm, mouse; Rn, rat. (C) p3'UTR-PUMA luciferase constructs containing a PUMA 3'UTR were transfected into A549 and MCF-7 cells stably transduced with As-miR-221 and/or As-miR-222. Luciferase activity was determined 48 h after transfection. The ratio of normalized sensor to control luciferase activity is shown.

chondrial membrane potential compared with the other four groups (Fig. 2C). Cytochrome c release and activation of caspase 3/7 are a series of subsequent events after collapse of mitochondrial membrane potential. Accordingly, we validated caspase 3/7 activity. A strong increase of caspase 3/7 activity was detected in cells transfected with AS-miR-221/222 (Fig. 2D). These results supported that As-miR-221/222 triggered MCF-7 and A549 cell apoptosis.

As-miR-221/222 alters the apoptotic protein expression. In order to further explore the molecular mechanism of miR-221/222 involvement in MCF-7 and A549 cell apoptosis, apoptosis-

related protein expression was measured by Western blot analysis. A marked increase of pro-apoptosis proteins including PUMA and Bax was observed in MCF-7 cells in the As-miR-221/222, As-miR-221 and As-miR-222 groups. In contrast, Bcl-2 expression in As-miR-221/222 group, As-miR-221 group and As-miR-222 group was significantly down-regulated compared to the scramble and control groups. However, As-miR-221 or As-miR-222 exerted lower effects on these proteins than As-miR-221/222. A similar trend in these protein expression changes was detected in A549 cells (Fig. 3). The data indicated that As-miR-221/222 induced cancer cell apoptosis through activation of PUMA.

miR-221/222 acts directly at the PUMA mRNA 3'UTR. Next we hypothesized that PUMA maybe a direct target of miR-221/222. First, using bioinformatics, we obtained mature sequences and seed sequences of miR-221 and miR-222 (Fig. 4A). By querying target prediction programs PicTar and miRNA Review, PUMA, was identified in both programs as a putative miR-221/222 target gene. PUMA contains seed sequences and base-pairing potential in the 3'UTR for miR-221 and miR-222 (Fig. 4B). The free energy of miR-221 and miR-222 binding to the PUMA targeting site was calculated using RNA hybrid (mfe: miR-221,-14.5 kcal/mol; miR-222, -17.8 kcal/mol).

To substantiate that miR-221 and miR-222 are direct regulators of PUMA, the 3'UTR region of PUMA encompassing the highly conserved putative miR-221/222 binding sites was fused downstream of the coding sequences for a luciferase reporter. Upon co-transfection in MCF-7 and A549 cells, AS-miR-221/222 increased the activity of the reporter construct relative to a vector-only control (pGL3-vector) (Fig. 4C).

Discussion

miR-221/222 have been reported to be involved in controlling proliferation in breast cancer and lung cancer (12,15). p27, PTEN and TIMP3, are successfully identified as a direct target of miR-221/222. Here, we report the modulating effect of miR-221/222 on pro-apoptotic PUMA by directly targeting the 3'UTR of PUMA mRNA in lung cancer (A549) and breast cancer (MCF-7) cell lines. The results of PUMA targeted by miR-221/222 may have implications in the pathogenesis of the two kinds of epithelial cancers.

PUMA, also called Bcl-2 binding component 3 (BBC3), shares homology with Bcl-2 family proteins within a short stretch of amino acids termed the BH3 domain, a region that allows interaction among the family members. Proteins with similar homology, called BH3-only proteins, constitute the third subgroup of the Bcl-2 family. Like all the BH3-only proteins, PUMA binds to and neutralizes prosurvival members of the Bcl-2 family to promote apoptosis (16). Although most BH3-only proteins interact with only a subset of prosurvival Bcl-2 proteins, PUMA targets all of them (17). In addition to neutralizing prosurvival proteins, BH3-only proteins also activate either Bax or Bak protein to induce cell death (18,19). These characteristics make PUMA a particularly potent effector of apoptosis. In our study, up-regulation of pro-apoptosis proteins (PUMA and Bax) and down-regulation of apoptotic protein Bcl-2 were observed after reduction of miR-221/222. Further, we found that the 3'UTR of PUMA mRNA exists in the highly conserved putative miR-221/miR-222 binding sites by bioinformatics method. Luciferase reporter assay validated that PUMA was a direct target of miR-221/222. These findings suggest that PUMA is direct regulated by As-miR-221/222 in breast and lung cancer cells.

PUMA initiates apoptosis by activating caspases through the intrinsic, mitochondrial-mediated pathway (20). Activation of this pathway is characterized by BAX and BAK insertion into mitochondrial membrane, and release of cytochrome c from the mitochondrial inter-membrane space into the cytosol, subsequent activation of caspase 9 and 3, and inevitably, cell death. Additionally, a mitochondria-dependent apoptosis involves mitochondrial outer membrane permeabilization and collapse of mitochondrial membrane potential. Cytochrome c release and activation of caspase 3/7 are a series of event after collapse of mitochondrial membrane potential (21). It has been reported that PUMA suppresses the growth of A549 cells through induction of apoptosis and sensitizes cells to chemotherapeutic agents and irradiation (22). In addition, up-regulation of PUMA expression mediated by adenovirus inhibits MCF-7 cell survival and enhances radiosensitivity (23). In this study, we found that cells transfected with AS-miR-221/222 show a significantly greater collapse in mitochondrial membrane potential and a strong increase of caspase 3/7 activity, whereas, this effect is mediated by PUMA.

In conclusion, our study demonstrates that knocking down miR-221/222 by oligonucleotides results in a significant increase in collapse of mitochondrial membrane potential and caspase 3/7 activity, ultimately leading to cell apoptosis in A549 and MCF-7 cells. This apoptotic effect was initiated by PUMA, which was directly regulated by miR-221/222 through targeting the binding site within the 3'UTR. The data suggest modulation of miR-221/222 activity may represent a novel approach for treating epithelial cancers.

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