Regulation of the P2X7R by microRNA-216b in human breast cancer

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ABSTRACT
Breast cancer is the most common cancer in women around the world. However, the molecular mechanisms underlying breast cancer pathogenesis are only partially understood. Here, in this study, we found that P2X7R was up-regulated and miR-216b was down-regulated in breast cancer cell lines and tissues. Using bioinformatic analysis and 3’UTR luciferase reporter assay, we determined P2X7R can be directly targeted by miR-216b, which can down-regulate endogenous P2X7R mRNA and protein levels. Ectopic expression of miR-216b mimics leads to inhibited cell growth and apoptosis, while blocking expression of the miR-216b results in increased cell proliferation. Furthermore, our findings demonstrate that knock-down of P2X7R promotes apoptosis in breast cancer cells through down-regulating Bcl-2 and increasing the cleavage caspase-3 protein level. Finally, we confirmed that down-regulation of miR-216b in breast cancer is inversely associated with P2X7R expression level. Together, these findings establish miR-216b as a novel regulator of P2X7R and a potential therapeutic target for breast cancer.

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1. Introduction
Breast cancer is the most common cancer in women around the world, which affects 1.38 million women worldwide per year [1]. Although the implementation of screening programs and the development of new therapeutics in the last 20 years have significantly reduced mortality rates, the molecular mechanisms underlying breast cancer pathogenesis are only partially understood [2].

Recently, P2X7 receptor (P2X7R) has been found to be associated with breast cancer pathogenesis [3,4]. P2X7R belongs to the P2X receptor families which are ATP-regulated (ligand-gated) ion channels. When P2X7R is activated by ATP binding, it opens a pore through which molecules up to 900 Da can pass. Binding of P2X7 receptor by ATP can stimulate various signaling pathways, which can induce proliferation, differentiation and apoptosis [5,6]. Studies have reported on the effect of P2X7R in inflammation. P2X7R can enhance the release of endogenous cytokines involved in the immune-response, such as interleukin-1beta (IL-1β) [7]. Recent studies have focused the function of P2X7R in tumors. It has been described that P2X7R was significantly higher in papillary thyroid carcinoma patients with tumor multifocality, lymphovascular invasion, and extrathyroid extension [8]. A multicentric study showed that patients that have glomas with high P2X7R expression present longer survival, when compared to patients with glomas expressing lower P2X7R levels [9]. Evidence that P2X7 triggers NFATc1, PI3K/Akt, ROCK, and VEGF pathways in osteoblasts promoting either primary tumor development or osteoblastic lesions is also reported [10]. Using breast and cervical cancer cell lines (MDA-MB-231, HeLa and MCF-7), researchers demonstrated that in the presence of hypoxia stimulus, the increase in P2X7R expression plays a role in inducing cell invasion through phosphorylation of Akt and nuclear accumulation of NF-κB [11].

MicroRNAs (miRNAs) are naturally existed small non-coding RNAs that can modulate gene expression post-transcriptionally through either protein translation repression or mRNA degradation of target genes [12]. Over the past decade, miRNAs are emerging as critical regulators in carcinogenesis and tumor progression by acting either as oncogenes or tumor suppressor genes [13]. Recently, researchers have demonstrated that P2X7R can be regulated by miRNAs. miR-186 and miR-150 has been reported to target P2X7R and inhibit its expression [14]. Furthermore, miR-150 can inhibit endogenous P2X7R protein expression in lung cancer cells [15].
In the present study, we have investigated the role of miR-216b in the regulation of P2X7R expression in breast cancer cells. Our findings demonstrated that the 3' UTR of P2X7R contains a putative binding site for miR-216b. Furthermore, we experimentally showed that miR-216b directly targets the 3' UTR of P2X7R to suppress its expression. We determined that P2X7R was up-regulated and miR-216b was down-regulated in breast cancer tissues and cells. Our findings also demonstrated that miR-216b over-expression leads to inhibited growth, clonogenicity and increased apoptosis in breast cancer cells. Furthermore, our data revealed a discordant expression of P2X7R at the transcript, which is inversely associated with miR-216b expression in breast cancer tissues. Finally, our findings demonstrate that knockdown of P2X7R promotes apoptosis in breast cancer cells through down-regulating Bcl-2 and increasing the cleavage caspase-3 protein level. Therefore, miR-216b may mediate its tumor suppressor function, at least in part, by suppressing downstream pathways of P2X7R, such as the Bcl-2/caspase-3 pathways. Altogether, our study characterized a novel microRNA-mediated mechanism of P2X7R regulation and suggests tumor inhibiting actions of miR-216b in breast cancer cells.

2. Materials and methods

2.1. Patient samples, cell culture and transfection

Breast cancer specimens and adjacent normal tissues were collected in Jinan Military General Hospital (Jinan, China). All the patients recruited into the present study did not receive radiotherapy or chemotherapy or any other treatment before and after operation. Written informed consent was obtained from all study participants. The use of tissue samples were approved by the ethical committees of the Jinan Military General Hospital. The characteristics of the patients involved in this study were shown in Table 1. The breast cancer cell lines (MDA-MB-468, MCF-7, MDA-MB-435s) and non-malignant breast epithelial cell (MCF-10A) were obtained from the ATCC and maintained in RPMI 1640 or Dulbecco’s Modified Eagle Medium (DMEM) with 10% fetal bovine serum (FBS) and 1% antibiotics (Invitrogen, USA). Transfection of the cells with miRNA mimics or miRNA inhibitors (GenePharma, China) was performed using Lipofectamine 2000 (Invitrogen, USA) according to the manufacturer’s instructions.

2.2. Detection of cell phenotypes

The effect of miR-216b on proliferation of breast cancer cells was evaluated by the MTT assay. MDA-MB-435s or MDA-MB-468 cells were plated in 96-well culture plates (3 x 10^3 per well). After 24 h incubation, the cells were transfected with miR-216 mimics or anti-miR-216b for 24 h. Then the MTT (0.5 mg/ml; Sigma–Aldrich, USA) was added to each well (20 μl/well). After 4 h of additional incubation, MTT solution was discarded and 200 ml of DMSO (Sigma, USA) was added and the plates shaken gently. The absorbance was measured on an ELISA reader at a wavelength of 570 nm. For colony formation assay, cells were counted and seeded in 12-well plates (in triplicate) at 100 cells per well. Fresh culture medium was replaced every 3 days. The number of viable cell colonies were determined after 14 days and colonies were fixed with methanol, stained with crystal violet, photographed and counted. Each experiment was performed in triplicate.

2.3. Western blotting and RT-PCR

Western blotting was performed to determine protein expression of P2X7R, Bcl-2 and caspase-3. Total protein extracted by Trizol reagent (Invitrogen, USA) and protein concentration in the supernatants was determined using Bradford protein dye reagent (Bio-Rad, Hercules, CA). The entire human P2X7R 3' UTR-mutated version (miR-216b-3' UTR-mut) were synthesized by GenPharm (Shanghai, China). The P2X7R 3' UTR reporter was generated by inserting the entire 3'UTR or 3'UTR-mut of human P2X7R mRNA into Xhol/NotI sites of psiCHECK-2 vector (Promega).

![Table 1](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3978578/bin/10155614table1.pdf)

2.4. Plasmid and luciferase assay

The entire human P2X7R 3’UTR harboring miR-216b, miR-125a, miR-1275, miR-588 target sequence as well as the seed-sequence mutated version (miR-216b-3’UTR-mut) were synthesized by GenPharm (Shanghai, China). The P2X7R 3’UTR reporter was generated by inserting the entire 3’UTR or 3’UTR-mut of human P2X7R mRNA into Xhol/NotI sites of psiCHECK-2 vector (Promega).
downstream of the Renilla luciferase gene. PCR primer sequences used for P2X7R 3′UTR were as follows: Forward 5′-AATCTCGAGCGGCCCTATCTGTCTGAT-3′ and Reverse 5′-TCGGGCCGCCACCAAGAATTCAACACTGGATC-3′. For knockdown of P2X7R, the siRNA targeting P2X7R was purchased from SANTA CRUZ (Arg siRNA (h): sc-38945). For the luciferase assay, 1 × 10^5 cells were transfected along with the P2X7R 3′UTR reporter and the miR-216b mimics, (or miR-125a mimics, miR-1275 mimics, miR-588 mimics) in a 24-well plate using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocol. After 24 h, firefly and Renilla luciferase activities were measured consecutively using Dual Luciferase Assay (Promega).

2.5. Apoptosis analysis

Terminal deoxynucleotidyl transferase-mediated dUTP labeling (TUNEL) assay was performed using an in situ apoptosis detection kit (R&D Systems, USA). Briefly, MDA-MB-435s were transfected with miR-216b or miR-control and MDA-MB-468 cells were transfected with anti-miR-216b or anti-NC, followed by Protease K digestion, then TdT reaction mix was applied to the cells for incubation at 37 °C for 60 min, followed by incubation with streptavidin horseradish peroxidase for 10 min. The final reaction of the product was visualized by 3,3′-diaminobenzidine. Approximately, 1000 tumor cells were counted in each section, and apoptotic index was expressed as the percentage of TUNEL-positive tumor cells.

For Annexin V assay, siRNA-P2X7R or siRNA-NC was transfected into MDA-MB-435s cells. After 48 h, DNA content was determined by propidium iodide staining as described by Hwang et al. [16], and Annexin V staining was performed with the Vybrant Apoptosis Assay Kit (Invitrogen).

2.6. Statistical analysis

A Student’s test was performed to analyze the significance of differences between the samples means obtained from three independent experiments. Differences were considered statistically significant at p < 0.05.

3. Results

3.1. The expression of P2X7R in breast cancer cell lines and tissues

Here, we used quantitative real-time PCR (qRT-PCR) to measure P2X7R mRNA expression levels in three breast cancer cell lines, MDA-MB-468, MCF-7, MDA-MB-435s and a non-malignant breast epithelial cell MCF-10A. Compared to MCF-10A cells, the expression of P2X7R were obviously reduced in MDA-MB-468 (0.2-fold) and MCF-7 (0.4-fold) cells, and the expression of P2X7R in MDA-MB-435s was 1.8-fold to that of MCF-10A cells (Fig. 1A). Highly metastatic cells MDA-MB-435s expressed the highest levels of P2X7R compared with their non-metastatic counterpart (MDA-MB-468) and low metastatic counterpart (MCF-7) in vitro. Furthermore, the protein expression of P2X7R also showed a similar pattern in the four cell lines (Fig. 1B).

To further confirm the role of P2X7R during breast cancer progression, we determined the expression of P2X7R in fresh tumor tissue samples and matched adjacent non-tumor tissue samples (Fig. 1C). The expression of P2X7R in ductal breast carcinoma was significantly higher than in adjacent normal tissue (*p < 0.05).

Fig. 1. Expression P2X7R in breast cancer cell lines and tissues. (A and B) Representative qRT-PCR and Western blot experiments analyzing the expression of mRNA for the P2X7R in non-malignant breast epithelial cell MCF-10A, non-metastatic counterpart (MDA-MB-468), low metastatic counterpart (MCF-7) and highly invasive (MDA-MB-435s) human breast cancer cells; (C) relative expression of P2X7R (normalized to GAPDH) was detected by using a qRT-PCR in ductal breast carcinoma tissue samples and matched adjacent non-tumor tissue samples; (D) relative expression of P2X7R (normalized to GAPDH) was detected by using a qRT-PCR in ductal breast carcinoma tissue samples, mammary gland hyperplasia tissue samples and matched adjacent non-tumor tissue samples. *p < 0.05.
specimen and adjacent normal breast tissues from 23 patients by using qRT-PCR. We observed that P2X7R expression was significantly increased in breast cancer tissue compared with adjacent normal breast tissues (Fig. 1C). Tumors with high malignancy for ductal breast carcinoma (23 case) expressed highest levels of P2X7R compared with mammary gland hyperplasia (11 case) and normal tissues, suggesting that P2X7R up-regulation was associated with tumor progression (Fig. 1D).

3.2. miR-216b directly targets and inhibits P2X7R

In order to explore the regulation of P2X7R by miRNAs, three computational algorithms, TargetScan, miRD and miRanda, were used to search for potential miRNAs that may target P2X7R and a large number of different miRNAs were predicted. Among these candidate miRNAs, four miRNAs (hsa-miR-125a, hsa-miR-1275, hsa-miR-588, hsa-miR-216b), which were predicted by all three algorithms, attracted our attention immediately (Fig. 2A).

To verify whether the four predicted miRNAs (miR-125a, miR-1275, miR-588, miR-216b) can directly target P2X7R, we subcloned the full-length P2X7R 3'-UTR into a luciferase reporter vector. Fig. 2B shows that addition of miR-216b mimics, but not the mimics of miR-125a, miR-1275 and miR-588, dramatically suppressed the luciferase activity of the P2X7R 3'-UTR upon co-transfection with the luciferase vector. The profound inhibition was abolished when the seed sequences of the miR-216b target sequences were mutated in the Luc-mut vector (Fig. 2C). Moreover, the mutated miR-216b had no further inhibition effect on the wild-type P2X7R 3'-UTR luciferase reporter vector (Fig. 2D). We then assessed the effect of miR-216b on P2X7R expression. As shown in Fig. 2E and F, miR-216b mimics reduced P2X7R protein and mRNA levels, while anti-miR-216b transfection increased P2X7R protein and mRNA levels. These results provide evidence that miR-216b directly targets the 3'-UTR of P2X7R mRNA, resulting P2X7R degradation and inhibits its expression.

3.3. miR-216b induces cell apoptosis, inhibits cell proliferation and invasion

To corroborate the function of miR-216b during tumorigenesis, breast cancer cells lines, MDA-MB-435s and MDA-MB-468, were transfected with miR-216b mimics or anti-miR-216b. As shown in Fig. 3A, miR-216b mimics increased 5-fold of the expression of miR-216b, while anti-miR-216b decreased 70% of the miR-216b expression. MTT and colony formation assays were performed to examine the effects of miR-216b on in vitro cell growth. Our data demonstrated that relative cell growth was significantly facilitated in miR-216b mimics transfected cells. As expected, cells receiving anti-miR-216b displayed the opposite effect (Fig. 3B, C, D and E). As shown in Fig. 3F, our data showed that cell apoptosis was induced obviously in miR-216b mimics transfected MDA-MB-435s (18.1% in miR-control group vs. 38.4% in miR-216b mimics group) and inhibited in anti-miR-216b transfected MDA-MB-435s (23.1% in miR-control group vs. 19.6% in Anti-miR-216b group). Then, transwell invasion assay was performed to claim the effect of miR-216b on breast cancer cells’ invasion abilities. The results shown in Fig. 3G and H indicated miR-216b inhibits breast cancer cell invasion abilities, and play a tumor suppressor role in breast cancer.

Furthermore, a knockdown plasmid, siRNA-P2X7R was generated, which decreased the expression of P2X7R by 70%. To investigate the effect of P2X7R on proteins involved in apoptosis, we examined the anti-apoptotic protein, Bcl-2, and a apoptotic protein, caspase 3. Through Western blot analysis. The results demonstrated that siRNA-P2X7R significantly decreased the content of Bcl-2 protein and induced a cleavage caspase-3 level. Moreover, annexin V-FITC/PI double staining in cells was performed by flow cytometry. In the nonapoptotic, viable control cells, the annexin V-FITC staining and PI negative staining were located in the bottom left quadrant of the dots (Fig. 3I, left panel). After transfection the cells with siRNA-P2X7R, a significant number of cells showed annexin V-FITC positive and PI negative staining, which increased the dot numbers in the bottom right quadrant from 11.0% of siRNA-NC transfected cells to 17.1% (Fig. 3I). Furthermore, cells in advanced apoptosis stained positive with annexin V-FITC and PI (upper right quadrant) and were significantly augmented from 1.7% of siRNA-NC transfected cells to 20.0% (Fig. 3I). These results demonstrated a similar effect of P2X7R knockdown with miR-216b mimics, which further confirmed that P2X7R was a direct target of miR-216b.

3.4. The expression of miR-216b in tissues and cell lines

Since we have demonstrated the expression levels of P2X7R in breast cancer cell lines and tissues, we then used quantitative real-time PCR (qRT-PCR) to measure miR-216b mRNA expression levels in the cell lines, MDA-MB-468, MCF-7, MCF-10A and MDA-MB-435s. Compared to MCF-10A cells, the expression of miR-216b were obviously increased in MDA-MB-468 (3.2-fold) and MCF-7 (2.4-fold) cells, and the expression of miR-216b in MDA-MB-435s was 0.3-fold to that of MCF-10A cells (Fig. 4A). High metastatic cells MDA-MB-435 expressed the lowest levels of miR-216b compared with their non-metastatic counterpart (MDA-MB-468) and low metastatic counterpart (MCF-7) in vitro. Furthermore, the expression of miR-216b also down-regulated in ductal breast carcinoma compared with adjacent normal tissues (Fig. 4B) and there was an inverse correlation between the level of P2X7R mRNA and the level of miR-216b expression assessed by qRT-PCR in the breast cancer tissues (Fig. 4C).

It has been demonstrated that ATP can bind P2X7R to stimulate various signaling pathways [5], therefore in this study we showed that targeting P2X7R by miR-216b can attenuate ATP/P2X7R signaling pathways and induced Bcl-2/caspase-3 pathway, leading to the inhibited cell proliferation and induction of cell apoptosis (Fig. 4D).

4. Discussion

Recent studies have shown that the P2X7R expressed at much higher or increased levels in several tumors compared with normal tissues, including thyroid carcinoma and lymphoid neoplasm. Furthermore, some of these studies have demonstrated that P2X7R can exhibit antiapoptotic effects [17] or sustaining cell growth [18] on several cancer types. Jelassi et al. has provided evidence to support that activation of the P2X7Rs promotes cancer cell invasiveness [19]. In this study, our findings demonstrated that P2X7R expressed at higher levels in ductal breast carcinoma compared with normal tissues. Furthermore, ductal breast carcinoma with higher malignancy expressed highest levels of P2X7R compared with mammary gland hyperplasia and normal tissue. And in highly metastatic cells MDA-MB-435, P2X7R expressed the highest levels compared with their non-metastatic counterpart (MDA-MB-468) and low metastatic counterpart (MCF-7) in vitro, suggesting that P2X7R up-regulation was associated with tumor progression.

It has been demonstrated that miRNAs can play specific roles in cancer cell proliferation, differentiation, migration and metastasis. Numerous miRNAs have been reported to be differentially expressed in breast cancer cells and tissues, suggesting their involvement in breast cancer pathogenesis [20]. We explored the possible miRNAs that may target P2X7R in breast cancer cells through different computational algorithms and screened four possible miRNAs (hsa-miR-125a, hsa-miR-1275, hsa-miR-588 and
hsa-miR-216b). Only miR-216b had obvious inhibiting effect on the luciferase intensity of P2X7R-3’UTR reporter, which indicates that P2X7R maybe a direct target for miR-216b. Then we confirmed that miR-216b had no further inhibiting effect on the P2X7R-3’UTR-mut luciferase reporter, in which the miR-216b seed sequence was mutated. Furthermore, miR-216b-mut mimics also had no effect on the wild-type P2X7R-3’UTR reporter. And we showed that miR-216b can significantly suppressed the protein and mRNA levels of P2X7R.

Recent studies have indicated the role of miR-216b as a tumor suppressor of tumor cell growth in several cancers. Researchers revealed that miR-216b attenuated nasopharyngeal cancer cell...
proliferation, invasion and tumor growth through inhibition of the KRAS-related AKT and ERK pathways. The inversely correlation of the expression of miR-216b and KRAS protein during nasopharyngeal tumorigenesis was also indicated [21]. Shadan Ali et al. demonstrated that re-expression of miR-216b in pancreatic tumor cells showed inhibition of cell proliferation and colony formation through targeting and inhibiting Ras expression [22]. Another study indicated that miR-216b promoted cellular senescence through the p53/p21 pathway by CKII downregulation-mediated ROS production [23]. Therefore, miR-216b seems as a tumor suppressor for various kinds of malignant tumors. In this study, we focused on the miR-216b potential effectiveness in breast cancer. We found highly metastatic cells MDA-MB-435 expressed the lowest levels of miR-216b compared with their non-metastatic counterpart (MDA-MB-468) and low metastatic counterpart (MCF-7) in vitro. Furthermore, the expression of miR-216b also down-regulated in ductal breast carcinoma compared with adjacent normal. In the in vitro study, miR-216b was showed to inhibit cell growth and induced apoptosis of breast cancer cell lines.

The induction of apoptotic cell death in many cell types is controlled by Bcl-2 family and caspases. Bcl-2 is a central player in the genetic program of eukaryotic cells, favoring survival by inhibiting cell death. Bcl-2 blocks a major apoptotic pathway by inhibiting the release of cytochrome C from the mitochondria, thereby preventing caspase-induced apoptosis [24]. Our investigation revealed that down regulation of P2X7R can induce cell apoptosis through down-regulating Bcl-2 protein and increasing cleavage caspase-3 protein levels, further indicating that miR-216b may directly target P2X7R to induce cell apoptosis through Bcl-2/caspase-3 pathway. Furthermore, we found a reverse-correlation in the expression of

Fig. 3. miR-216b inhibits the cell growth and induces the cell apoptosis of breast cancer cells. (A) QRT-PCR was used to detect the transfection efficiency of miR-216b mimics in MDA-MB-231 and anti-miR-216b in MDA-MB-468 cells; (B and C) cell survival was determined by MTT assay; (D and E) the long term cell growth as determined by colony formation assay; (F) TUNEL-TMR staining was used to detect DNA fragmentation during programmed cell death. in MDA-MB-435s cells transfected with miR-216b mimics and MDA-MB-468 cells transfected with anti-miR-216b; (G and H) cell invasion ability was determined by Transwell invasion assay; (I) Western blot was conducted to detect the protein expression of P2X7R, Bcl-2 and caspase-3; (J) Annexin V-FITC/PI double staining in MDA-MB-435s cells was performed by flow cytometry.
miR-216b and its target protein P2X7R in examined malignant tissues. Therefore, targeting P2X7R by miR-216b can attenuate ATP/P2X7R signaling pathways, leading to the inhibition of cell growth and induction of apoptosis through Bcl-2/caspase-3 pathway.

In conclusion, the study sheds new light on the specific function of miR-216b and its mechanism in breast cancer proliferation, and suggests that targeting miR-216b may provide a potential therapeutic strategy for blocking proliferation in breast cancer.

Conflict of interests

The authors declare no conflict of interests.

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