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Inhibiting mir-29 and targeting ADAM12: lidocaine inhibits breast cancer development

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Research article

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Abstract

Breast cancer is the most common cancers among women in the world. For hundreds of years, researchers are devoted for developing new strategy against cancer. As a rapid and effective local anesthetic, lidocaine is reported having multiple-physiological functions in clinic treatment, such as anti-cancer and anti-inflammatory activity. Besides, the microRNAs (miRNAs) have been demonstrated to be involved in the cancer development, and the miRNA-29 family is abnormally expressed in a variety of cancers, which could not only regulate the cancer cell proliferation, migration and invasion, but also promote cancer cell apoptosis by binding to target proteins. However, the protective effect of lidocaine on breast cancer cells and the mechanism was still unclear. In the present study, the relative expression level of the miRNA-29 in cancer cells and tissues was measured with quantitative RT-PCR. Bioinformatic analysis was performed to predict the potential target of the miRNA-29 in breast cancer cells, and the luciferase reporter assay was employed to validate the direct binding of the target protein and the miRNA-29 in breast cancer cells. Cell Counting Kit-8 (CCK-8) and the Cell Apoptosis Assay Kit were utilized to analyze the cancer cell proliferation and apoptosis after lidocaine treatment.

Instruction

At present, breast cancer has become one of the most common cancers among women in the world, especially in developed countries. It is worthy of recognition that the treatment and care about breast cancer has improved markedly in recent decades, combined with the mechanism exploration of the breast cancer [1].

Lidocaine is an amide anesthetic commonly widely used as a rapid and effective local anesthetic in local skin, mucosal anesthesia and regional nerve block [2]. In recent years, there have been many literatures reported the multiple-physiological functions about lidocaine in clinic. For example, as early as 1999, Martinsson T has reported that the Ropivacaine, a new long-acting local anesthetic, showed excellent anti-proliferative activity on human colon adenocarcinoma cells [3]. In another study, Sakaguchi M suggested that lidocaine may suppress the proliferation of human tongue cancer cells by oral and exert anti-cancer activity [4]. Besides, M. Hirata also confirmed that Lidocaine would inhibit tyrosine kinase activity of the epidermal growth factor receptor (EGFR) and then suppress proliferation of colorectal cancer cells in 2004 [5]. In recent years, with the increasing of researches on the pathogenesis of tumors, researchers have gradually realized that the application of different anesthetics in perioperative period has certain effects on tumor metastasis, recurrence and postoperative survival of tumor patients. Moreover, it has been found that lidocaine, as a cytotoxic and inexpensive drug, has a good application prospect in cancer treatment and prevention [6]. However, the mechanism by which lidocaine negatively regulate the tumor development was still unclear. This article further confirmed the protective effect of the lidocaine against breast cancer development and explored the mechanism of lidocaine in vitro anti-tumor effect.

Encoded by endogenous genes, microRNAs (miRNAs) are discovered belong to a class of endogenous non-coding small RNAs with about 22 nucleotides in recent years. MiRNAs participate in vital important life processes such as embryonic development and cell proliferation *in vivo* [7]. in addition to this, the miRNAs could also regulate cell differentiation, growth and apoptosis, and indirectly promote and inhibit cancer development. The miRNA-29 family is abnormally expressed in a variety of cancers [8], and the relationship of the miRNA-29 expression level with the breast cancer development was evaluated in this research, and whether the anti-cancer activity of the lidocaine on breast cancer was also explored in this present research.

In the present research, the results indicated that lidocaine could significantly inhibit the breast cancer cell proliferation and induce the cell apoptosis at the treatment concentration of 100, 200 and 500 μM. Lidocaine (200 μM) obviously increased the expression of miRNA-29 and rescued the reduction of miRNA-29 caused by miRNA-29 inhibitor. And prediction suggested that the miRNA-29 might target the A Disintegrin and Metalloprotease 12 (ADAM12) and then regulate the cancer cell procession [9]. Compared with the control group, the expression of ADAM12 was suppressed by lidocaine (200 μM) in breast cancer cells. All the results in this research revealed that the lidocaine could up-regulating the miRNA-29 expression and then suppress the expression of ADAM12, finally induce apoptosis and inhibit proliferation of breast cancer cells. Lidocaine has the potential to be a therapeutic regimen for breast cancer.

Materials And Methods

Reagents

Lidocaine used in this experiment was purchased from Sigma-Aldrich (USA), The lidocaine was dissolved in standard growth medium at the concentration of 1 mg/mL for stock at -20℃. And final treatment concentrations were achieved by diluting with standard growth medium.

Cell culture

Human breast cancer cell lines MDA-MB-453 (ER^-, $HER2^{high}$), MCF-7/HER2 (ER^+, $HER2^{high}$) and MCF-7 (ER+, $HER2^{low}$) were purchased from Shanghai Institutes for Biological Sciences, CAS. MDA-MB-453 cells were cultured in Leibovitz's L-15 Medium (Gibco, Carlsbad, CA, USA). MCF-7/HER2 and MCF-7 cells were cultured in Dulbecco's Minimum Essential Medium (Gibco, Carlsbad, CA, USA). All the culture medium was supplemented with 10% heated- Fetal Bovine Serum (FBS) and 1% penicillin/G-streptomycin sulfate solution. All the cells were cultured in an incubator at the condition of 37℃ and 5% CO_2.

RNA isolation and RT-PCR

After treated with 100, 200 and 500 μM Lidocaine, the total RNA in breast cancer cell lines MDA-MB-453 (ER^-, $HER2^{high}$), MCF-7/HER2 (ER^+, $HER2^{high}$) and MCF-7 (ER+, $HER2^{low}$) was extracted using TRIzol Reagent (Sigma, St. Louis, MO, USA) according to the manufacturer's instructions [10]. Following that, the

NanoDrop 2000C instrument was used to determine the quantity and quality of extracted RNA, and then the total RNA was reverse transcripted into cDNA according to the instructions of Reverse Transcription Kit (TaKaRa, Dalian, China). The SYBR Green Master Mix (Roche) was conducted for the RT-PCR detection of miRNA-29 and ADAM12, with the *gapdh* gene used as the internal control. Primer sequences used in this study were showed in Table 1. All data were normalized by using $2^{-\Delta\Delta Ct}$ method as relative quantification.

Table 1
Sequences of the primers used in this research.

Genes	Sequences
miRNA-29	CGGGTACCGGTCCTTTCTAG GTT
	CGGAATT CAAAAATGTGGGC
Adam12	CGCTCGAAATTACACGGGTC
	CAGCGAGGTTTGGTGTGTTG
gapdh	AATGGGCAGCCGTTAGGAAA
	GCGCCCAATACGACCAAATC

Cell proliferation assay

To detect the inhibitory effect of the Lidocaine on breast cancer cell lines MDA-MB-453 (ER^-, $HER2^{high}$), MCF-7/HER2 (ER^+, $HER2^{high}$) and MCF-7 (ER+, $HER2^{low}$), the CCK-8 assay (96992-500TESTS-F, Sigma-Aldric, USA) was carried out for the cancer cell proliferation detection after indicated treatment. This experiment was performed under the guidance of the manufactures' instruction with a little modification [11]. In brief, these three cell lines were seeded in 96-well culture plates (1×10^4 cells/well, 100 μl each well) and cultured inappropriate medium containing 10% FBS in a 37°C, 5% CO_2 humidified environment for 24 h. Subsequently, CCK-8 reagents (10 μl/well) were added to each well and cells were incubated according to the manufacturer's protocol, and the absorbance (optical density, OD) values of each well were detected at a wavelength of 450 nm on a microplate spectrophotometer system (Molecular Devices, Sunnyvale, CA, USA). This experiment was performed in triplicate.

Cell apoptosis assay

The percentages of the apoptotic breast cancer cells were measured with Cell Apoptosis Assay Kit (Life Technologies) in flow cytometry according to the manufacturer's instruction with some modifications [12]. In short, the breast cancer cells in each group were collected, planted into 6-well plates and cultured in an incubator at the 37°C, 5% CO_2 environment. After indicated treatment, the cells in each group were dissociated into single cells with trypsin, and further washed with PBS, then and resuspended in 1 × binding buffer at a concentration of 1×10^6 cells/mL. 10 Mm Annexin V-FITC and propidium iodide (PI) solution was subjected into suspension for 15 minutes incubation in the dark. Cell lines incubated with

nothing were used as the negative group. Finally, all of cell samples were analyzed using a FACSCalibur cytometer (BD). All experiments with triplicate were performed.

MiRNA transfection experiments

To overexpress or knock down miR-29 expression, the miR-29 mimic, miR-29 mimic control, miR-29 mimic inhibitor, as well as the miR-29 mimic inhibitor control were designed and synthesized by Ruibobio (Guangzhou, China). The miRNAs were transfected into breast cancer cells using Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA) under the guidance of the manufacturer's protocols [13]. Transfection efficiency of these miRNAs was examined by quantitative Real Time-Polymerase Chain Reaction (RT-PCR).

Bioinformatics methods and luciferase assay

To predicate the potential target genes of miR-29, four different available databases TargetScan, miRWalk, MiRanda and RNA22 were performed in this experiment. Screening for the common targets predicated by the above databases, the protein with the highest score was selected for the following binding assay.

Based on the bioinformatics results, the 3'-untranslated region (UTR) of ADAM12 gene was predicated having the binding site with the miR-29. In this experiment the luciferase assay was finished to confirm the interaction between the miR-29 and ADAM12 [14]. Firstly, the wild-type ADAM12 3'-UTR sequence and mutant 3'-UTR sequence of ADAM12 gene were synthesized and cloned into pMIR-REPORT luciferase reporter plasmids (Promega Corporation, Madison, WI, USA). Plasmids with WT or mutant 3'-UTR DNA sequences were co-transfected with miR-520a-3p mimic, inhibitor or negative control. After 24 cultivation at 37°C, 5% CO_2 environment, cells were lysed using a dual luciferase reporter assay kit (Promega Corporation) according to the manufacturer's manual, and fluorescence intensity was measured using GloMax 20/20 illuminometer (Promega Corporation). All experiments were performed in triplicate.

Western blot

After indicated treatment, the relative expression of the ADAM12 in breast cancer cells was evaluated by western blot as previous described [15]. Briefly, the MDA-MB-453 (ER^-, $HER2^{high}$), MCF-7/HER2 (ER^+, $HER2^{high}$) and MCF-7 (ER+, $HER2^{low}$) in this experiment were lysed with lysis buffer containing protease inhibitor cocktail (78437, Thermo Fisher Scientific, Inc.), and total protein was detected using BCA Protein Assay kit (23225, Pierce, USA). Then the BCA Protein Assay kit (23225, Pierce, USA) was used for the detection of the concentrations of protein samples. Next, equal amounts of protein samples were loaded on 10% sodium dodecyl sulfate-polyacrylamide denaturing gels and transferred onto a polyvinylidene difluoride membrane (PVDF, EMD Millipore, Billerica, MA). After blocked in 5% nonfat milk for 2 hours at room temperature, the membranes were incubated with the appropriate primary antibody against ADAM12 or GAPDH overnight at 4 °C. after incubated with horseradish peroxidase-conjugated secondary antibody for 1 hour, the proteins were visualized using an enhanced chemiluminescence (ECL) detection

kit (Thermo Fisher Scientific) and quantified using ImageJ software (BIO RAD). The experiment was repeated three times independently.

Statistical analysis

In this present research, the SPSS 19.0 Software (IBM, Armonk, NY, USA) was used for statistical analyses. All the data were presented as means ± SD from triplicate repeats. The student's t-test was used for un-paired comparisons between two groups, and pearson correlation was performed for correlation analysis between miR-29 and ADAM12 gene expression. $p < 0.05$ was considered statistically significant.

Results And Discussion

Lidocaine inhibits proliferation of breast cancer cells

To detect the anti-cancer activity of the lidocaine *in vitro*, the inhibitory effect of the lidocaine on the proliferation of the breast cancer cells was evaluated with CCK-8 assay. As there are different subtypes in breast cancer, three representative breast cancer cell lines MDA-MB-453 (ER-, HER2high), MCF-7/HER2 (ER+, HER2high) and MCF-7 (ER+, HER2low) was used for the anti-cancer activity evaluation of lidocaine. All these cell lines were treated with 100, 200 and 500 μM lidocaine for 12, 24 and 48 h, and the CCK-8 results showed that lidocaine could significantly reduce the proliferation abilities of MDA-MB-453 (ER-, HER2high) (Fig. 1A) and MCF-7/HER2 (ER+, HER2high) (Fig. 1B) cells and showed an obviously concentration-dependently relationship after 24 h. While the lidocaine has slight effect on the MCF-7 (ER+, HER2low) breast cancer cells (Fig. 1C).

Lidocaine reduced the relative expression of miR-29 in breast cancer cells

In the previous experiment, we have observed that the lidocaine showed excellent inhibitory effect against breast cancer cells proliferation, especially the MDA-MB-453 (ER-, $HER2^{high}$) and MCF-7/HER2 (ER+, $HER2^{high}$) cells. Besides, according to the importance role of the miR-29 in the cancer cell development, we analyzed the relative expression of the miR-29 in these three cell lines after lidocaine treatment for 24 h. As the results showed in Fig. 2, we can see the relative expression level of miR-29 was significantly reduced in the MDA-MB-453 (ER-, $HER2^{high}$) (A) and MCF-7/HER2 (ER+, $HER2^{high}$) (B) cells after treatment, compared with the normal group. However, the lidocaine has slight inhibitory activity of the miR-29 expression in MCF-7 (ER+, $HER2^{low}$) (C). This result is consistence with the data in Fig. 1, suggesting that the anti-cancer activity of the lidocaine is mediated by miR-29 in breast cancer cells.

Lidocaine suppresses the expression of ADAM12 via miR-29 in human breast cancer cells

Based on the results above, we confirmed the vital important role of miR-29 in breast cancer cells proliferation for the first time. However, the detail mechanism of the anti-cancer activity of the miR-29 was still unclear. After exposure to different concentration of lidocaine, the expression of ADAM12 in MDA-MB-453 (ER-, $HER2^{high}$) (Fig. 3A) and MCF-7/HER2 (ER+, $HER2^{high}$) (Fig. 3B) was measured with western blot, the statistical analysis was showed in Fig. 3C & 3D. Besides, in this present research, four different publicly available databases were used to predicate the potential target of the miR-29 in breast cancer cells. Among hundreds of proteins, ADAM12 was predicted as the downstream target of miR-29 with the highest score (Fig. 3E). To confirm this prediction, the dual-Luciferase reporter assay was performed to examine the interaction between miR-29 and ADAM12. Results in Fig. 3F suggested that after co-transfected with the miR-29 mimic and ADAM12 reporter plasmid, a high luciferase activity was observed in cells, and the luciferase activity was significantly reduced in the miR-29 inhibitor transfection group. Finally, the correlation of miR-29 and ADAM12 was explored, and the results in Fig. 3G indicated that the miR-29 regulates ADAM12 expression negatively in breast cancer tissues ($p < 0.001$).

Lidocaine inhibits proliferation and induces apoptosis of breast cancer cells by down-regulating miR-29

In the previous, we have revealed the lidocaine could regulate the relative expression of miR-29 as well as the ADAM12 expression in breast cancer cells. However, whether the lidocaine inhibits the proliferation activity and induces cancer apoptosis was also mediated by the miR-29 was unclear. So, in this experiment, the MDA-MB-453 (ER-, $HER2^{high}$) and MCF-7/HER2 (ER+, $HER2^{high}$) breast cancer cells were treated with lidocaine (200 μM) after transfected with miR-29 mimics and negative control of mimics for 24 h. As the results showed in Fig. 4A & 4B, after transfected with miR-29 mimics, the expression level of the miR-29 was significantly increased compared with the control group, and this increased level could be reversed by the lidocaine (200 μM) addition. the lidocaine significantly reduced the proliferation ability of the breast cancer cells compared with the control groups (Fig. 4C & 4D). Besides, the flow cytometry also suggested that the lidocaine (200 μM) could induce a higher level of apoptosis cells over 48 h. The percentage of the apoptotic cancer cells was up-regulated to 30.96% and31.66%, which could be reversed by miR-520a-3p mimics (Fig. 4E & 4F).

Conclusion

Lidocaine is widely known as a rapid and effective local anesthetic commonly used in clinic, and it is usually used for pain relief via epidural, intravenous or intraperitoneal injection. As reported, the plasma concentration of lidocaine is ranging from 1 to 5 μ g/mL (about 3.5–17.3 μM). To block the afferent nociceptive fibers in the tissue, approximately 600 μM of lidocaine is needed. While, in the present research, we also confirmed the anti-cancer activity of lidocaine *in vitro*. In breast cancer cell lines MDA-MB-453 (ER-, $HER2^{high}$) and MCF-7/HER2 (ER+, $HER2^{high}$), 200 μM of lidocaine was adequate for the inhibition of the cancer cell proliferation without cytotoxic effects. In addition to this the 200 μM of lidocaine could significantly induce the apoptosis of breast cancer cell, but also effective against MDA-

MB-453 (ER-, HER2^{high}) and MCF-7/HER2 (ER+, HER2^{high}) cell lines. This difference may be explained by the differential expression of certain genes.

In the recent years, miRNA-29 has become a hotspot in life science research, and its mechanism and function has been gradually clarified. As a potential biomarker, miRNA-29 has attracted more and more attention of researchers in the occurrence and development of breast cancer. Within further research, miRNA-29 will play a greater role in the diagnosis, treatment and prognosis of breast cancer, and provide a research direction as a novel cancer therapeutic target. Combined with the cell proliferation and the miRNA-29 results, we could see that the lidocaine is only effective on the breast cancer cell lines with high miRNA-29 expression level, such as MDA-MB-453 (ER-, HER2^{high}) and MCF-7/HER2 (ER+, HER2^{high}).

In conclusion, the present research demonstrated that lidocaine may exert protective effect on the breast cancer treatment via reducing the relative expression of the miRNA-29, which may cause the increased expression of reduction of ADAM12, finally exhibit an inhibitory effect on cell proliferation and induction activity on breast cancer cell apoptosis. The miRNA-29- ADAM12 axis is a novel molecular mechanism that is vital important for the breast cancer development, and lidocaine may serve as potential therapeutic regimen for breast cancer.

Declarations

Compliance with ethical standards

Funding

The research did not receive any specific funding.

Conflict of interest

The authors declare that they have no conflict of interest.

Ethical approval

Our study did not require an ethical board approval because it did not contain human or animal trials.

Informed consent

Informed consent was obtained from all individual participants included in the study.

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References

1. Odle TG. Precision Medicine in Breast Cancer. Radiol Technol. 2017;88:401M–421M.
2. Martinsson T. Ropivacaine inhibits serum-induced proliferation of colon adenocarcinoma cells in vitro. J Pharmacol Exp Ther. 1999;288:660–4.
3. Araújo LD, Singi G, Gazola R. Verification of protector effect of the norepinephrine and felypressin upon the cardiovascular system under action of the lidocaine hydrochloride and prilocaine hydrochloride in anesthetized rats. Pharmacol Res. 2002;46:107–11.
4. Sakaguchi M, Kuroda Y, Hirose M. The antiproliferative effect of lidocaine on human tongue cancer cells with inhibition of the activity of epidermal growth factor receptor. Anesth Analg. 2006;102:1103–7.
5. Hirata M, Sakaguchi M, Mochida C, Sotozono C, Kageyama K, Kuroda Y, Hirose M. Lidocaine inhibits tyrosine kinase activity of the epidermal growth factor receptor and suppresses proliferation of corneal epithelial cells. Anesthesiology. 2004;100:1206–10.
6. Lirk P, Fiegl H, Weber NC, Hollmann MW. Epigenetics in the perioperative period. Br J Pharmacol. 2015;172:2748–55.
7. Zendjabil M, Favard S, Tse C, Abbou O, Hainque B. The microRNAs as biomarkers: What prospects? C R Biol. 2017, 340, 114–131.
8. Wang Y, Zhang X, Li H, Yu J, Ren X. The role of miRNA-29 family in cancer. Eur J Cell Biol. 2013;92:123–8.
9. Schmitt MJ, Margue C, Behrmann I, Kreis S. MiRNA-29: a microRNA family with tumor-suppressing and immune-modulating properties. Curr Mol Med. 2013;13:572–85.
10. Bachman J. Reverse-transcription PCR (RT-PCR). Methods Enzymol. 2013;530:67–74.
11. Zhu G, Cheng Z, Lin C, Hoffman RM, Huang Y, Singh SR, Zheng W, Yang S, Ye J. MyD88 Regulates LPS-induced NF-κB/MAPK Cytokines and Promotes Inflammation and Malignancy in Colorectal Cancer Cells. Cancer Genomics Proteomics. 2019;16:409–19.
12. Shen W, Liang B, Yin J, Li X, Cheng J. Noscapine Increases the Sensitivity of Drug-Resistant Ovarian Cancer Cell Line SKOV3/DDP to Cisplatin by Regulating Cell Cycle and Activating Apoptotic Pathways. Cell Biochem Biophys. 2015;72:203–13.
13. Lima CR, Geraldo MV, Fuziwara CS, Kimura ET, Santos MF. MiRNA-146b-5p upregulates migration and invasion of different Papillary Thyroid Carcinoma cells. BMC Cancer. 2016;16:108.
14. Lei Z, Shi H, Li W, Yu D, Shen F, Yu X, Lu D, Sun C, Liao K. miR185 inhibits nonsmall cell lung cancer cell proliferation and invasion through targeting of SOX9 and regulation of Wnt signaling. Mol Med Rep. 2018;17:1742–52.
15. Zhang C, Wang M, Shi C, Shi F, Pei C. Long non-coding RNA Linc00312 modulates the sensitivity of ovarian cancer to cisplatin via the Bcl-2/Caspase-3 signaling pathway. Biosci Trends. 2018;12:309–16.

Figures

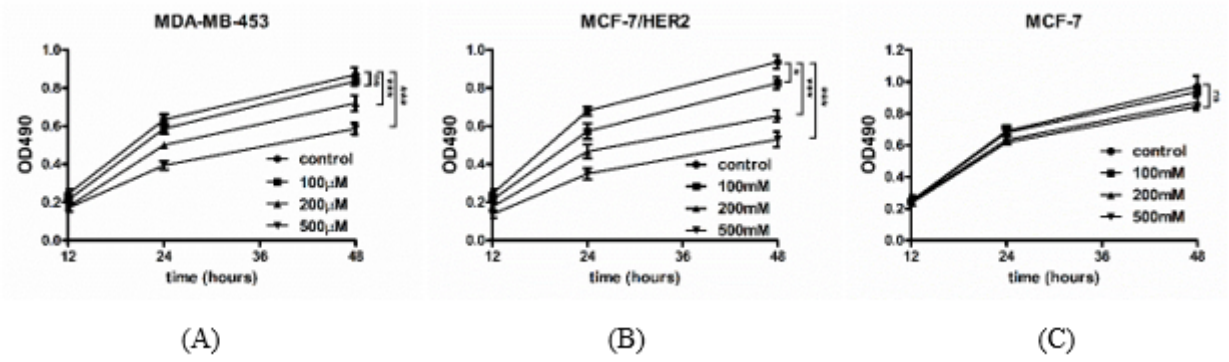

Figure 1

Lidocaine inhibits the proliferation ability of the breast cancer cells. MDA-MB-453 (ER-, HER2high), MCF-7/HER2 (ER+, HER2high) and MCF-7 (ER+, HER2low) breast cancer cells were treated with 100, 200 and 500 μM lidocaine for 12, 24 and 48 h by CCK-8 assay. This experiment was repeated at least three times and the data were presented as mean ± SD. *p < 0.05 vs. negative control.

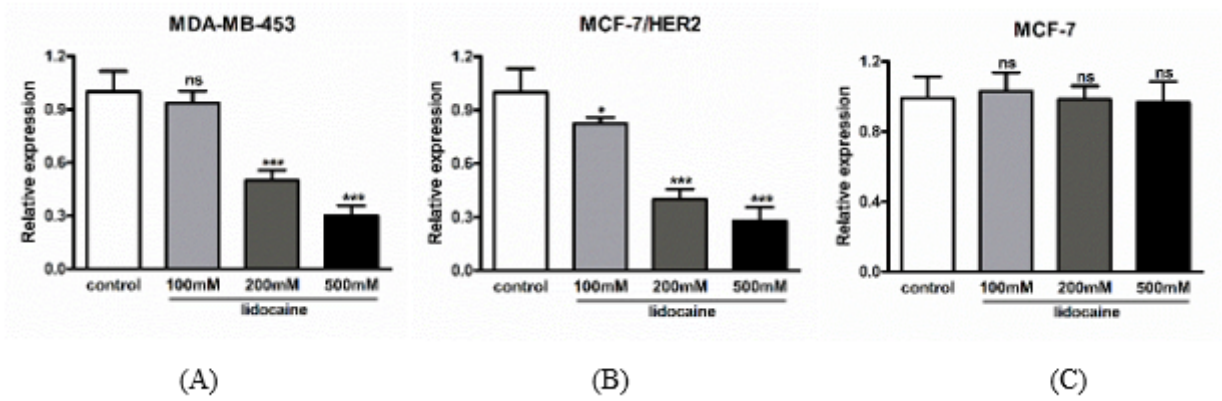

Figure 2

Reduced miR-29 expression level in breast cancer cells after compound treatment. MDA-MB-453 (ER-, HER2high), MCF-7/HER2 (ER+, HER2high) and MCF-7 (ER+, HER2low) breast cancer cells were treated with 100, 200 and 500 μM lidocaine for 24 h. The relative expression of the miR-29 was measured with RT-PCR.

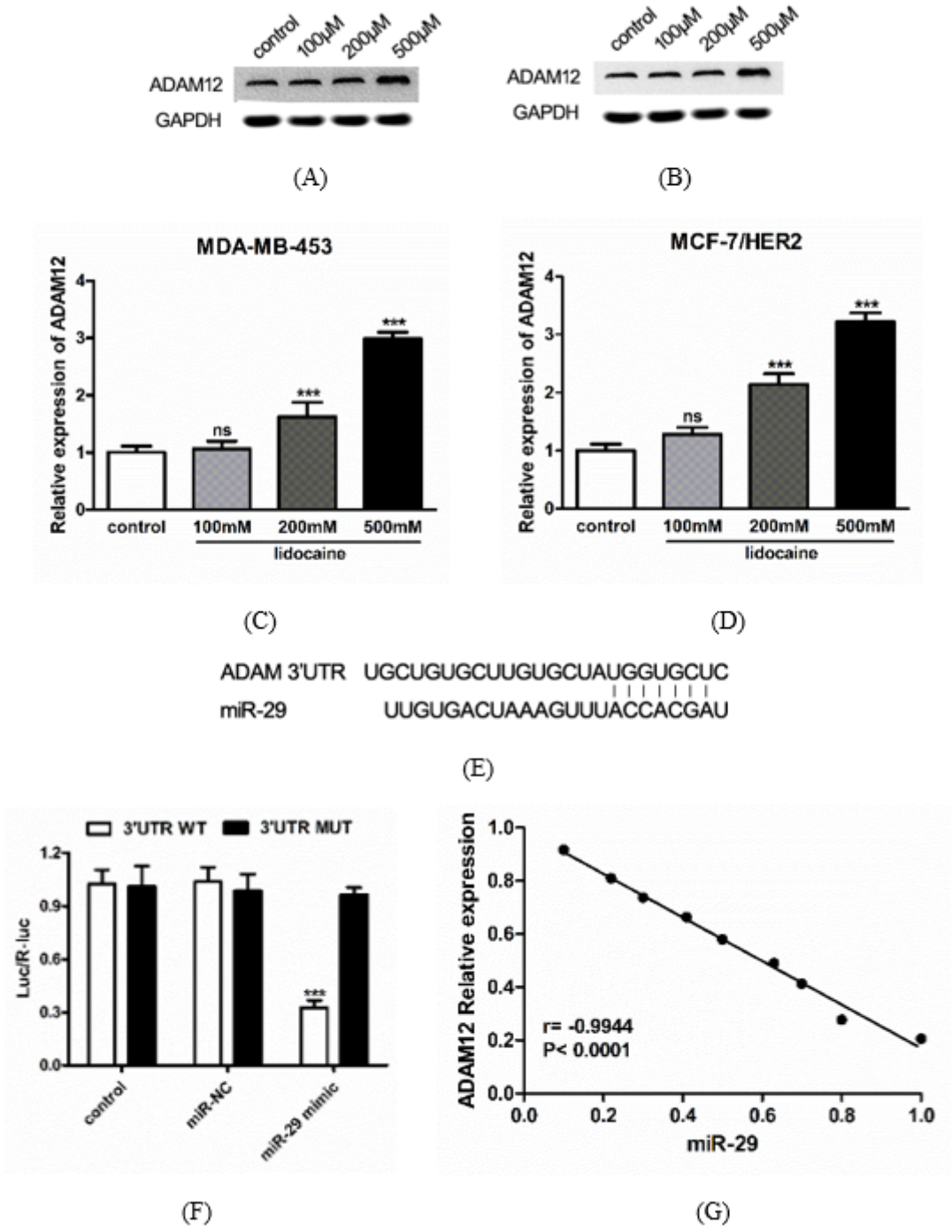

Figure 3

miR-29 targets and negatively regulates ADAM12 expression in breast cancer cells. After exposure to different concentration of lidocaine, the expression of ADAM12 in MDA-MB-453 (ER-, HER2high) (A) and MCF-7/HER2 (ER+, HER2high) (B) was determined with western blot. The relative expression of ADAM12 expression was analyzed (C, D). The ADAM12 was predicted as the downstream target of miR-29 (E). The binding of the miR-29 on 3'-UTR of ADAM12 was confirmed by luciferase activity assay (F). The correlation of miR-29 and ADAM12 was explored by pearson correlation analysis (G).

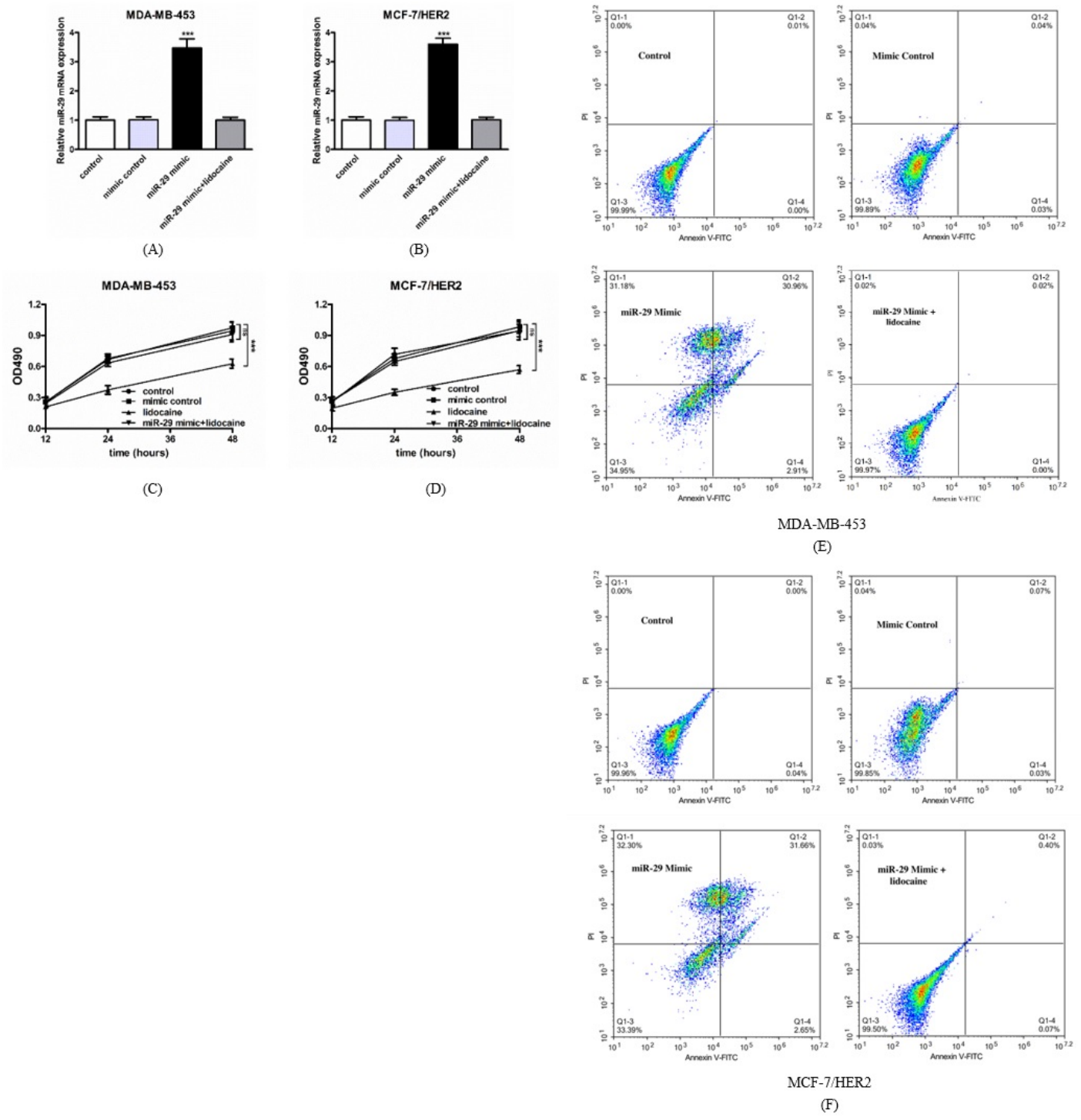

Figure 4

Inhibits proliferation and induced apoptosis of breast cancer cells mediated by miR-29 after lidocaine treatment. MDA-MB-453 (ER-, HER2high) and MCF-7/HER2 (ER+, HER2high) breast cancer cells were treated with lidocaine (200μM) after transfected with miR-29 and negative control. The relative expression of miR-29 was measured by RT-PCR after miR-29-mimic transfection or lidocaine (200μM) exposure in MDA-MB-453 (ER-, HER2high) and MCF-7/HER2 (ER+, HER2high) breast cancer cells (A, B). CCK-8 was performed to detect the cancer cell proliferation after lidocaine treatment (C, D). The Cell

Apoptosis Assay Kit was used to measure the percentage of apoptotic breast cancer cells after lidocaine (200μM) exposure (E, F).