

Original Research Article

Oxymatrine inhibits proliferation and migration of breast cancer cells by inhibiting miRNA-188 and upregulating its target gene, PTEN

Xiaoying Ma¹, Zijiang Sang¹, Qinghua Zhang², Wenbiao Ma^{1*}

¹Surgical Oncology Ward 2 (Breast and Thyroid Surgery), ²Department of Interventional Ultrasound, Qinghai Provincial People's Hospital, Xining, China

*For correspondence: **Email:** MWBLJ@163.com; **Tel:** +86-0971-8065169

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Abstract

Purpose: To explore the potential biological functions of oxymatrine on breast cancer (BCa) cells and the underlying molecular mechanism.

Methods: Relative levels of microRNA-188 (miRNA-188) and PTEN (gene of phosphate and tension homology deleted on chromosome ten) in BCa cells, MDA-MB-231 and TB549, were determined. The influence of oxymatrine treatment, miRNA-188 and PTEN on proliferative and migratory abilities in BCa cells were assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), cell counting kit-8 (CCK-8) and Transwell assay, respectively. The binding relationship between miRNA-188 and PTEN was evaluated by dual-luciferase reporter gene assay.

Results: Oxymatrine downregulated miRNA-188 and upregulated PTEN in BCa cells. Proliferative and migratory activities in BCa were inhibited by treatment of oxymatrine ($p < 0.05$). Dual-luciferase reporter gene assay results indicated that PTEN was the target gene of miRNA-188. Furthermore, rescue experiments demonstrated that the regulatory loop, oxymatrine/miRNA-188/PTEN, was involved in the regulation of the migration and proliferation of BCa.

Conclusion: Oxymatrine treatment inhibits BCa progression by downregulating miRNA-188, leading to the upregulation of PTEN. The results of the current study may provide new insight into the diagnosis and treatment of BCa.

Keywords: Breast cancer, Oxymatrine, MicroRNAs, Signaling pathway, Proliferation, Migration

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INTRODUCTION

Breast cancer (BCa) is the second leading malignancy globally, and about one-eighth of females suffer from BCa [1]. Annually, BCa affects 1.3 million women, and it ranks at 23 % of cancer cases and 14 % of cancer deaths [2]. A therapeutic strategy for metastatic breast cancer

(MBC) is limited. About 5% of BCa patients develop distant metastases at the time of diagnosis [2,3]. It is necessary to clarify mechanisms underlying the tumorigenesis and tumor progression of BCa, thus improving diagnostic accuracy and prognosis of BCa patients.

Accumulated evidence has identified critical functions of microRNA (miRNAs) in human diseases [4]. Abnormally expressed miRNAs are closely linked to the occurrence and progression of cancers [5,6]. In BCa, dysregulated miRNAs are able to affect tumor cell behavior, thereby mediating tumor progression [7]. A previous study demonstrated that miRNAs serve as biological markers and therapeutic tools for BCa [8]. Determination of miRNA levels helps to classify subtypes of BCa and evaluate therapeutic outcomes [9]. MicroRNAs can be utilized as non-invasive tool for effectively monitoring disease progression in BCa [9,10].

The aim of the present study was to determine the potential influence of oxymatrine on the biological activities of breast cancer (BCa) cells as well as the underlying mechanism of action.

EXPERIMENTAL

Cell culture and transfection

The MDA-MB-231 and TB549 cells were purchased from American Type Culture Collection (ATCC) (Manassas, VA, USA). The cells were cultured at 37 °C in Roswell Park Memorial Institute 1640 (RPMI 1640) (HyClone, South Logan, UT, USA) containing 10 % fetal bovine serum (FBS) (HyClone, South Logan, UT, USA), 100 U/mL penicillin and 100 mg/mL streptomycin in an incubator with 5 % CO₂. The cells were inoculated into 6-well plates and cultured overnight. Transfection was conducted using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA).

MTT (3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay

The cells were prepared to suspension with 2×10⁴/mL and applied into 96-well plates. At the appointed time points, cells were reacted with 20 μL of MTT solution (5 mg/mL) (Sigma-Aldrich, St. Louis, MO, USA) per well for 4 hours.

Afterwards, medium was replaced and 150 μL of dimethyl sulfoxide (DMSO) (Sigma-Aldrich, St. Louis, MO, USA) was added. The mixture was shaken at a low speed for 10 min to dissolve the Formazan crystals. Absorbance was determined at 450 nm with an enzyme-linked immunosorbent detector. Each experiment was repeated in triplicate.

Cell counting kit-8 (CCK-8) assay

The cells were inoculated into 96-well plates using 3.0×10³ cells per well. At the appointed time points, 10 μL of CCK-8 solution (Dojindo, Kumamoto, Japan) was added into each well. The absorbance of each sample was read at 450 nm in a microplate reader.

Transwell assay

Cells were prepared in suspension of 1×10⁵ cells/mL. An aliquot of 100 μL of suspension was applied in the upper side of Transwell chamber (Corning, Corning, NY, USA). In the bottom side, 500 μL of medium containing 20 % FBS was applied. After 48 h of incubation, cells penetrated to the bottom side were fixed in methanol for 15 min, stained with crystal violet for 20 min and counted using a microscope. Migratory cell number was counted in 5 randomly selected fields per sample (magnification 200×).

Quantitative real-time polymerase chain reaction (qRT-PCR)

Extraction of total RNA in cells was performed using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). The RNA was subjected to reverse transcription, and the complementary deoxyribose nucleic acid (cDNA) was applied for PCR using SYBR Green method (TaKaRa, Tokyo, Japan). The U6 was considered as the internal reference. Primer sequences were synthesized by Invitrogen Co., Ltd. (Carlsbad, CA, USA). The primer sequences used are listed in Table 1.

Table 1: Primer sequences used in PCR

Gene		Primer sequence
miRNA-188	Forward (5'>3')	ACACTCCAGCTGGGTGGGAGGTGGTACGTT
	Reverse (5'>3')	CTCAACTGGTGTCTCGTGGAGTCGGCAAATTCA
GAPDH	Forward (5'>3')	GAAGAGAGAGACCCTCACGCTG
	Reverse (5'>3')	ACTGTGAGGAGGGGAGATTTCAGT
PTEN	Forward (5'>3')	TGGATTCTGACTTAGACTTGACCT
	Reverse (5'>3')	GGTGGGTTATGGTCTTCAAAAGG
U6	Forward (5'>3')	CTCGCTTCGGCAGCAGCACATATA
	Reverse (5'>3')	AAATATGGAACGCTTCACGA

Dual-luciferase reporter gene assay

PTEN-3'UTR-MUT and PTEN-3'UTR-WT luciferase vectors were constructed based on the binding sites in promoter regions of miRNA-188 and PTEN. The cells were co-transfected with miRNA-188 mimics/NC and PTEN WT/PTEN MUT. After 48 h transfection, the cells were lysed for the determination of relative luciferase activity (Promega, Madison, WI, USA).

Statistical analysis

IBM SPSS 18.0 software (IBM Inc., New York, USA) was used for data analysis. All data are expressed as mean \pm SD (standard deviation). Paired two-tailed *t*-test or chi-square test was used for comparing differences between two groups. $P < 0.05$ was considered statistically significant.

RESULTS

Oxymatrine treatment blocked the proliferative and migratory activities of BCa

MDA-MB-231 and TB549 cells were induced with 10, 50 or 100 μ M oxymatrine for 24 h, respectively. Results from MTT assay revealed that the proliferative rate was dose-dependently reduced in BCa cells (Figure 1 A). Subsequently, BCa cells were treated with 50 μ M Oxymatrine for 24, 48 or 72 h, respectively. Results from CCK-8 assay revealed the declined viability in BCa cells treated with Oxymatrine compared to those of control (Figure 1 B and C). Similarly, migratory ability of BCa cells was suppressed by oxymatrine treatment as well (Figure 1 D and E).

Oxymatrine treatment downregulated miRNA-188 in BCa

Relative level of miRNA-188 was dose-dependently downregulated in MDA-MB-231 and TB549 cells induced with 0, 10, 50 or 100 μ M Oxymatrine for 24 h, respectively (Figure 2 A). To uncover potential functions of miRNA-188 in BCa, we constructed miRNA-188 inhibitor and tested its transfection efficacy (Figure 2 B). Knockdown of miRNA-188 markedly elevated viability and migratory cell number in BCa (Figure 2 C - E). It is suggested that miRNA-188 exerted an anti-tumor role in BCa.

PTEN was the direct target of miRNA-188

Through database prediction, potential binding sequences in the promoter regions of PTEN and miRNA-188 were depicted (Figure 3 A). Transfection efficacy of miRNA-188 mimics was

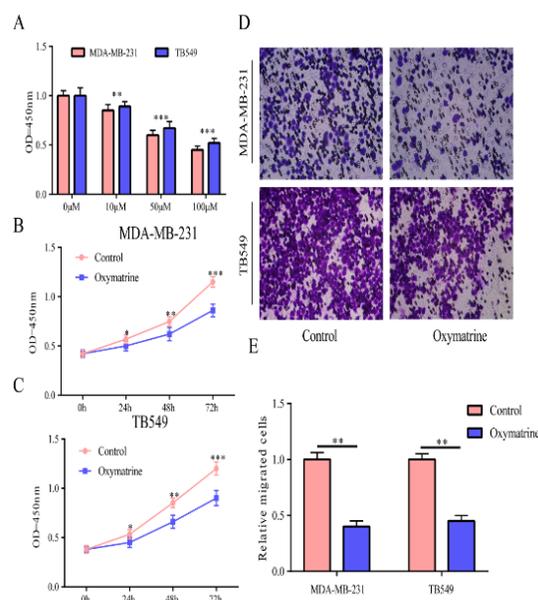


Figure 1: Oxymatrine treatment blocked proliferative and migratory abilities of BCa. (A) Proliferative rate detected using MTT assay in MDA-MB-231 and TB549 cells treated with 10, 50 or 100 μ M Oxymatrine for 24 h. (B, C) Viability detected by CCK-8 assay in MDA-MB-231 and TB549 cells treated with 50 μ M Oxymatrine for 24, 48 or 72 h. (D, E) Migration determined by Transwell assay in MDA-MB-231 and TB549 cells treated with 50 μ M oxymatrine for 24 h

excellent in MDA-MB-231 and TB549 cells (Figure 3 B). It is shown that luciferase activity markedly declined in BCa cells co-transfected with PTEN WT and miRNA-188 mimics, verifying their binding relationship (Figure 3C). Moreover, the relative level of PTEN was remarkably downregulated in BCa cells overexpressing miRNA-188, while it was upregulated in those transfected with miRNA-188 inhibitor (Figure 3 D and E). Thus, PTEN was the direct target of miRNA-188 and its level was negatively regulated by miRNA-188.

Oxymatrine treatment downregulated miRNA-188 and upregulated PTEN in BCa

Interestingly, PTEN level was dose-dependently upregulated in BCa cells treated with increasing doses of oxymatrine (Figure 4A & 4B). In MDA-MB-231 and TB549 cells, 50 μ M oxymatrine markedly upregulated PTEN which was downregulated by overexpression of miRNA-188 (Figure 4C & 4D). In addition, decreased viability in Oxymatrine-treated BCa cells was partially reversed by transfection of miRNA-188 mimics (Figure 4E, 4F). Therefore, it is considered that Oxymatrine treatment affected BCa progression by downregulating miRNA-188 to target PTEN.

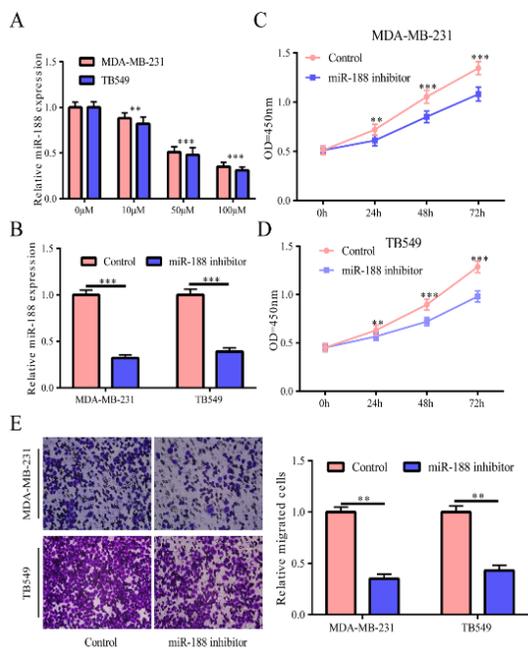


Figure 2: Oxymatrine treatment downregulated miRNA-188 in BCa. (A) MiRNA-188 level in MDA-MB-231 and TB549 cells treated with 0, 10, 50 or 100 μ M Oxymatrine for 24 h. (B) Transfection efficacy of miRNA-188 inhibitor in MDA-MB-231 and TB549 cells. (C, D) Viability in MDA-MB-231 and TB549 cells transfected with control or miRNA-188 inhibitor. (E) Migration of MDA-MB-231 and TB549 cells transfected with control or miRNA-188 inhibitor.

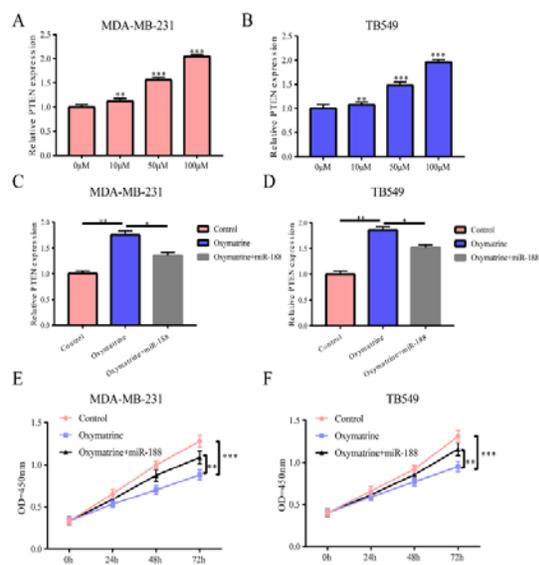


Figure 4: Oxymatrine treatment downregulated miRNA-188 and thereby upregulated PTEN in BCa. (A, B) PTEN levels in MDA-MB-231 and TB549 cells treated with 0, 10, 50 or 100 μ M Oxymatrine for 24 h, respectively. (C, D) PTEN levels in MDA-MB-231 and TB549 cells treated with blank control, 50 μ M Oxymatrine or 50 μ M Oxymatrine + miRNA-188 mimics. (E, F) Viability of MDA-MB-231 and TB549 cells treated with blank control, 50 μ M Oxymatrine or 50 μ M Oxymatrine + miRNA-188 mimics

DISCUSSION

Traditional Chinese Medicine is a tremendous treasure in clinical practice. Oxymatrine is extracted from the root of *Sophora flavescens*, which is widely distributed in Asia and Pacific Islands. Oxymatrine has been identified as the main active ingredient of *Sophora flavescens* and it exerts multiple biological functions. In many types of cancer, Oxymatrine presents anti-tumor properties [11-14]. For instance, oxymatrine suppresses EMT in colorectal cancer via the activation of the NF- κ B pathway. By regulating the levels of Bcl-2 and Bax, oxymatrine induces apoptosis and blocks proliferation in lung cancer cells A549. The findings revealed that oxymatrine treatment downregulated miRNA-188, thus inhibiting the capacity of BCa cells to proliferate and migrate.

Changes in miRNA activities and functions are closely linked to cancer phenotypes. Molecular regulation of miRNAs is of great significance in developing cancer treatment strategies. Based on literature review, miRNA-188 is generally considered as a tumor-suppressor gene [15-17]. In the present work, miRNA-188 was downregulated in BCa cells following Oxymatrine treatment. Knockdown of miRNA-188

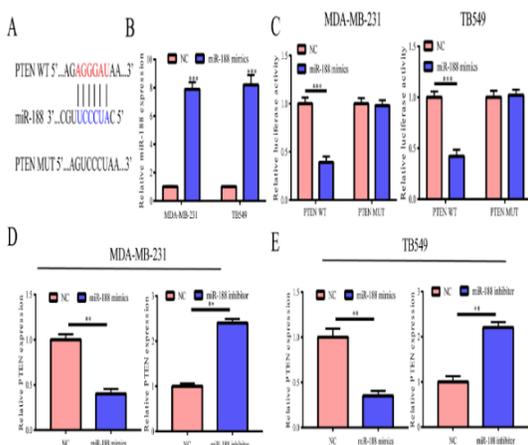


Figure 3: PTEN was the direct target of miRNA-188. (A) Binding sequences in the promoter regions of PTEN and miRNA-188. (B) Transfection efficacy of miRNA-188 mimics in MDA-MB-231 and TB549 cells. (C) Luciferase activity in MDA-MB-231 and TB549 cells co-transfected with NC/miRNA-188 mimics and PTEN WT/PTEN MUT. (D, E) PTEN level in MDA-MB-231 and TB549 cells transfected with miRNA-188 mimics or inhibitor

accelerated proliferative and migratory activities in BCa cells. PTEN was found to be the direct target of miRNA-188 through dual-luciferase reporter gene assay. PTEN, a tumor-suppressor gene with dual-specificity phosphatase activities, is involved in the inhibition of tumor cell growth and tumorigenesis [18,19]. PTEN mutation is responsible for the formation of primary and metastatic malignancies [20]. This study showed that PTEN is the target of miRNA-188, and more importantly, it was involved in BCa progression regulated by miRNA-188.

CONCLUSION

Oxymatrine treatment mitigates BCa progression by downregulating the ability of miRNA-188 and upregulating PTEN. These findings provide a new strategy for the diagnosis and treatment of BCa.

DECLARATIONS

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them.

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