

Suppressing the Migration of Human Breast Cancer Cell Line by Targeting VAMP3 with miR-199a-3p

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Abstract

Deregulation of microRNAs contributes to multiple processes in cancer growth and progression. miR-199a-3p is decreased in highly metastatic breast cancer cells, MDA-MB-231, and its ectopic expression has a potent antimetastatic effect on these cells. However, the mechanism by which miR-199a-3p mediates its antimetastatic function has yet to be elucidated. Because miR-199a-3p reduces the expression levels of its target genes, it is likely to observe an inverse association between miR-199a-3p and its prometastatic target genes at the expression level. The current work determines that the Vesicle-associated membrane protein 3 (VAMP3) expression is increased in highly metastatic breast cancer cells compared to less metastatic cells, Michigan Cancer Foundation-7. The ectopic expression of miR-199a-3p strongly inhibits VAMP3 Messenger RNA and protein in vitro. Herein, it is confirmed that two sites within the 3'-untranslated sequence of VAMP3 Messenger RNA are actively targeted by miR-199a-3p, discovering a new regulatory mechanism for VAMP3 expression. Functional studies reveal that the suppression of VAMP3 contributes to miR-199a-3p antimetastatic effect, particularly cellular migration in vitro. In conclusion, these results indicate that miR-199a-3p targeting of VAMP3 possesses a significant potential impact in preventing or curing metastatic breast cancers.

Keywords

microRNA; Breast cancer; Metastasis; miR-199a-3p; Vesicle-associated membrane protein

Introduction

Breast cancer is the most frequently occurring cancer among females worldwide, representing one in four of all cancers. The estimated number of diagnosed cases has increased from 1.28 million in 2008 to 1.67 million by 2012^[1]. Despite recent therapeutic advances, around one-third of patients died of metastatic breast

cancer. In order to improve the therapeutic effect and survival rate, it is crucial to further our understanding in the molecular mechanisms of breast cancer. Several recent studies have focused on the role of microRNAs (miRNA) in tumorigenesis and metastasis of breast cancer cells. MicroRNAs, endogenous short noncoding Ribonucleic acids (RNAs) with 18 to 25 nucleotides, act as regulators of gene expression. MicroRNAs are capable

of directing RNA-induced silencing complex (RISC) to their target messenger RNAs (mRNAs) and imperfectly interact with the 3'-untranslated regions (3'UTRs). These interactions can mediate post-transcriptional repression and/or translational inhibition^[2,3]. Some miRNAs are known to function as oncogenes or tumor suppressor genes, and aberration in their expression is a common feature of various cancers and diseases^[4]. Several reports have demonstrated that miR-199a-3p is suppressed in various cancers, including hepatocellular carcinoma^[5,6], endometrioid adenocarcinoma^[7], ovarian cancer^[8], prostate cancer^[9], testicular cancer^[10], renal cell carcinoma^[11], bladder cancer^[12], osteosarcoma^[13], and papillary thyroid carcinoma^[14]. Previous studies have also demonstrated the strong inhibition of miR-199a-3p in patients with stage 3 metastatic breast cancer samples^[15] as well as in breast cancer cells with high potential of metastasis compared to these cells with low metastatic potential^[16]. The suppression of miR-199a-3p are found to be able to enhance the proliferation, survival, migration and invasion of breast cancer cells, partially through its direct targets, caveolin-2 and p21 Serine/threonine-protein kinase (PAK4)^[16,17]. As it is believed that individual miRNA targets a large number of genes, understanding the importance of miR-199a-3p in breast cancer requires the identification of more of its functional targets^[18,19]. This current work is designed to investigate the impact of miR-199a-3p on its potential target vesicle-associated membrane protein 3 (VAMP3) in human highly metastatic breast cancer cells. Vesicle associated membrane protein 3 (VAMP3) participates in the formation of SNARE protein complex that directs the movement and fusion of the trans-Golgi compartments with endosomal compartments or plasma membrane^[20,21]. There is a growing evidence that VAMP3 protein is involved in the intracellular trafficking and secretion of matrix metalloproteinase (MMPs)^[22,23]. The MMPs are essential in the regulation of extracellular matrix remodeling and subsequent results of cancer invasion and metastasis^[24]. The focus of the current work is the impact of miR-199a-3p on the expression and biological function of VAMP3 in human highly metastatic breast cancer cells.

Materials and Methods

Cell Culture: Two human breast carcinoma cell lines, named as NCI-PBCF-HTB26 (MDA-MB-231) and Michigan Cancer Foundation-7 (MCF-7) were grown in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% FCS, penicillin (100 U/ml), and

streptomycin (100 µg/ml), 2 mM L-glutamine (Lonza Braine SA, Braine-l'Alleud, Belgium). MDA-MB-231 and MCF-7 cells were cultivated at 37°C in a 5% CO₂ incubator.

MicroRNA Transfection: MDA-MB-231 cells were reverse-transfected with miR-199a-3p and Negative Control mimics at final concentration of 25 nM. MicroRNAs were mixed with 0.3% DharmaFECT4 (GE Healthcare Dharmacon Inc., Lafayette, CO USA) in OPTI-MEM (GIBCO®, Thermo Fisher Scientific Inc, Invitrogen, UK) for 20 min 1.5×10^4 or 3×10^5 MDA-MB-231 cells per 96 or 6-well plate, respectively. Cells were seeded directly onto the lipid- mixture in antibiotics-free media.

Quantitative real-time polymerase chain or Quantitative reverse transcription-polymerase chain Analysis: Total RNA was harvested using Invitrogen TRIzol™ Reagent (Thermo Fisher Scientific Inc, Waltham, MA USA) and isolated by phenol-chloroform extraction and ethanol precipitation. 200 ng of total RNA was then reverse transcribed using miScript II RT Kit (Qiagen N. V., Hilden, Germany). For analysis of mRNAs, the RT reactions were used as templates for real time amplification using Green I Master Mix (F. Hoffmann-La Roche Ltd, Basel, Switzerland). For miRNAs detection, miScript SYBR Green PCR Kit and miScript Primer Assay from QIAGEN were used. Primers for qRT-PCR were designed online using the ProbeFinder software (Roche Molecular Systems, Inc, Mannheim, Germany) at (<http://qpcr.probefinder.com/roche3.html>) and manufactured by Invitrogen (Thermo Fisher Scientific Inc, Waltham, MA USA). The following primers were used; GAPDH (fwd, AGCCACATCGCTCAGACAC; rev, GCCCAATACGACCAATCC), VAMP3 (fwd, GACTTCAGCAGACACAAAATCAA; rev, TGCACGGTCGTCTAACTCAG). The expression of target genes or miRNA were calculated relative to the Ct-values of the control sample and normalized to the reference gene.

Western Blot Analysis: MDA-MB-231 cells were lysed by NP40 buffer and total protein level was measured using bicinchoninic acid assay (BCA assay) reagents (Thermo Fisher Scientific Inc, Waltham, MA USA). 20 µg of total protein was separated in 12% SDS-polyacrylamide gel, and transferred onto a PDVF membrane (Merck Millipore, Billerica, MA USA). After an hour of preincubation with 5% non-fat milk powder (Cell Signaling Technology, Inc., Danvers, MA USA), blots were incubated overnight with primary antibodies

VAMP3 (Thermo Fisher Scientific Inc, Waltham, MA USA) and Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Cell Signaling Technology, Inc., Danvers, MA USA) at 4°C. Blots were then incubated for two hour with secondary HRP-conjugated antibodies (Cell Signaling Technology, Inc., Danvers, MA USA) at room temperature. Antibody binding was exposed using an ECL reagent (GE Healthcare Life Sciences, Buckinghamshire, UK).

Luciferase Assay: Wild type (WT) 3'UTR of VAMP3 was cloned into the NotI and XhoI sites of digested psiCHECK-2 vector (Promega Corp., Fitchburg, WI USA) using T4 DNA ligase (New England Biolabs (UK) Ltd. Hitchin, UK). Mutations in the 3'UTR of Phosphatidylinositol-4,5-Bisphosphate 3-Kinase Catalytic Subunit Beta (PIK3CB) were carried out using QuikChange lightning site-directed mutagenesis kit (Stratagene Inc., La Jolla, CA USA). MDA-MB-231 cells were reverse-transfected in five replicates in 96 well plates (1.5x10⁴ cells/well). 48 hrs post-transfection, the cells were lysed and luciferase reagents (Promega Corp., Fitchburg, WI USA) were added to each well and the *FireFly* and *Renilla* luminescent signals were quantified using POLARstar Omega multifunctional microplate reader (BMG LABTECH, Allmendgrün, Germany). The luciferase activity of *FireFly* serves as an internal control to normalize transfection efficiency.

Transwell Migration Assay: MDA-MB-231 cells were serum-starved and plated at the lower chambers of the 6-well transwells (BD Biosciences, San Jose, CA USA) as chemoattractant. 48 hr after transfection with VAMP3 small interfering RNA (siRNA), RISC-free siRNA, miR-199a-3p, and negative-control mimics, cells were seeded on the upper chambers. 24 hr later, the transwells were fixed and stained using methanol and Giemsa solution (Sigma-Aldrich, Billerica, MA USA), respectively. To calculate the migrated cells, seven random images were captured on a light microscope per transwell. The migrated cells were counted per

image using ImageJ software (National Institutes of Health, Bethesda, MA USA).

Results

VAMP3 is a Potential Target of miR-199a-3p

Based on previous expression profiles of downregulated genes in miR-199a-3p mimic transfected cells versus miR-199a-3p inhibitor in fibroblast cells, 20 genes were predicted to be a direct target of miR-199a-3p^[25]. The algorithms of TargetScan (<http://www.targetscan.org/>) were used as it is a more sensitive prediction tool compared to others^[26]. Four of these potential targets have more than one binding site for miR-199a-3p in their 3'UTRs, as shown in Table 1. VAMP3 is the highest fold-change and most statistically significant target of miR-199a-3p. Therefore, VAMP3 was selected for further analysis and validation. Computational screening identified that there are two potential target sites of miR-199a-3p in human VAMP3 3'UTR, located at 400-406 nt and 496-503 nt (Fig. 1).

miR-199a-3p Expression is Inversely Correlated with VAMP3 in High and Low Metastatic Breast Cancer Cell Lines.

In order to investigate the expression correlation between miR-199a-3p and its potential target VAMP3, their expression levels were measured in low and high metastatic breast cancer cells, MCF-7 and MDA-MB-231, respectively. Consistent with previous reports, miR-199a-3p level is reduced by more than 10-fold in MDA-MB-231 cells with high metastatic potential compared to MCF-7 (Fig. 2a). However, VAMP3 mRNA is increased by three-fold in MDA-MB-231 cells (Fig. 2b). Moreover, the expression of VAMP3 mRNAs are indeed reflected in its protein levels in breast cancer cells (Fig. 2c). The data indicates that the expression levels of miR-199-3p and its target gene, VAMP3, correlate in an inverse manner in breast cancer cells, MCF-7 and MDA-MB-231.

Table 1. The top list of potential miR-199a-3p targets in human cells harboring more than one site of interaction between the miRNA and its target genes. The genes were predicted using TargetScan (<http://www.targetscan.org/>). The table also includes the official transcript Refseq, the number of target sites in humans, the fold-change and statistical significance, adapted from Santhakumar *et al.*^[25].

Gene Symbol	Ref Seq	Target Sites	Fold Change	p Value
VAMP3	NM_009498	2 sites	-2.12268	2.96E-09
ABL2	NM_001136104	3 sites	-1.34062	1.10E-06
PAK4	NM_027470	2 sites	-1.22894	9.28E-06
MCFD2	NM_176808	3 sites	-1.22626	2.54E-06

Abbr: miRNA: microRNA; VAMP3: Vesicle-Associated Membrane Protein 3; ABL2: ABL Proto-Oncogene 2; PAK4: Serine/threonine-protein kinase; MCFD2: Multiple Coagulation Factor Deficiency 2

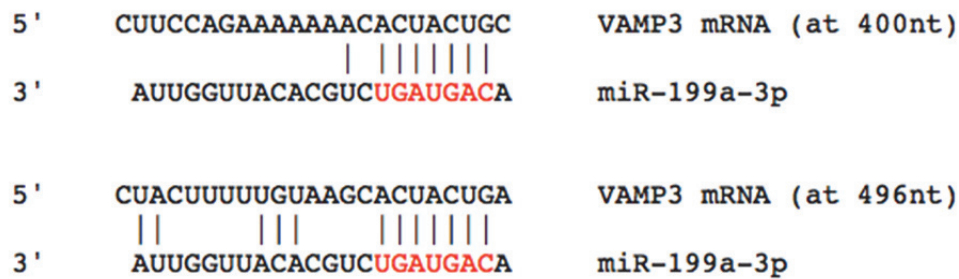


Figure 1. A schematic diagram illustrating the potential miR-199a-3p binding sites in VAMP3 3'UTR at nucleotides 400–406 and nucleotides 496–503. The seed sites are written in red.

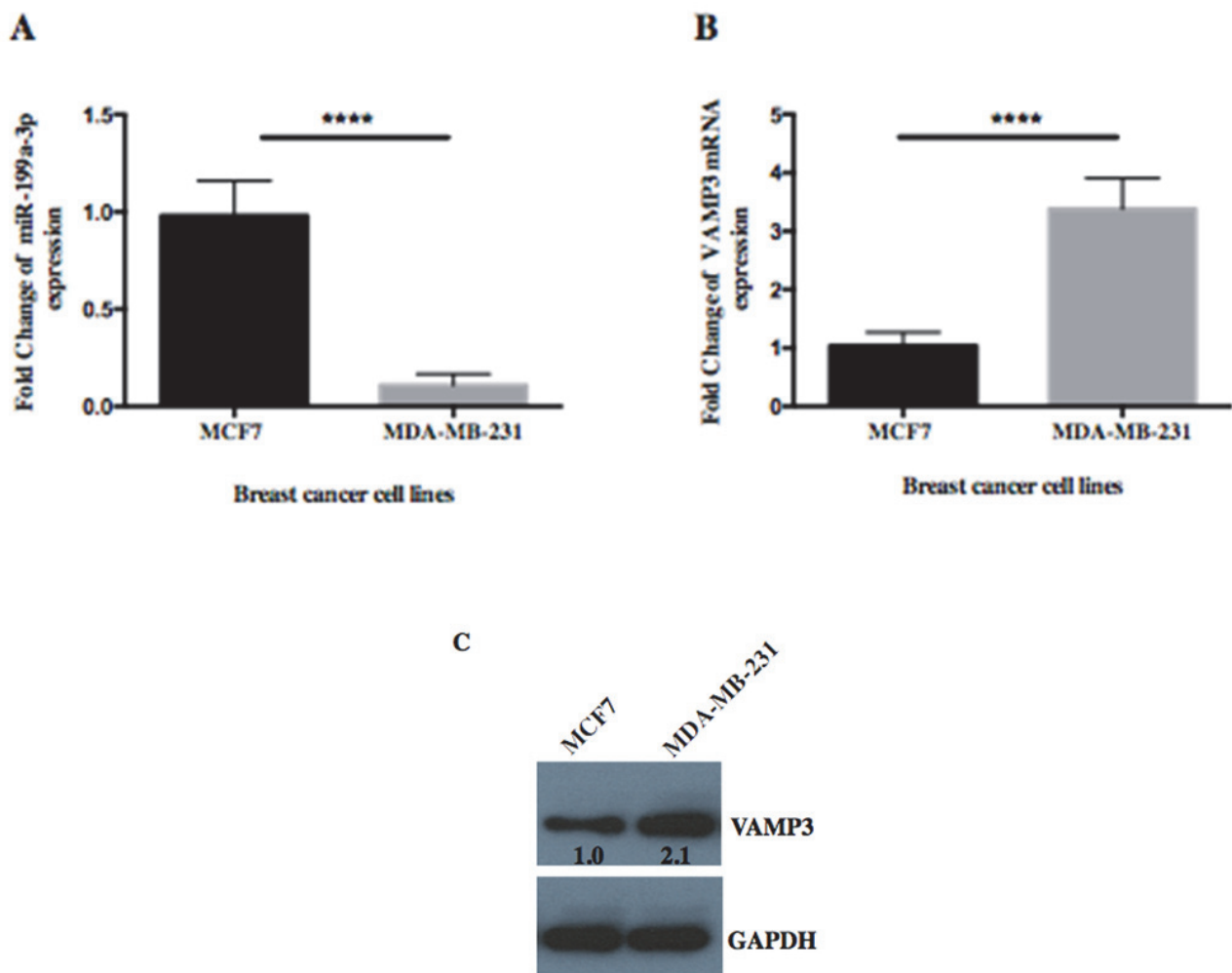


Figure 2. Inverse relationship between miR-199a-3p and VAMP3 mRNA and protein levels in MCF-7 and MDA-MB-231 cell lines. A) The level of miR-199a-3p expression in MCF-7 and MDA-MB-231. B) Expression of VAMP3 mRNA in MCF-7 and MDA-MB-231. miR-199a-3p was quantified using qRT-PCR, normalized to miR-16, and fold changes calculated versus MCF-7 cells. mRNAs were measured by qRT-PCR, normalized to control GAPDH mRNA, and fold changes calculated versus MCF-7 cells. Data represent mean \pm SD. **** P < 0.0001; Experiments were reproduced three times in laboratory. C) WB analysis of VAMP3 protein expression in MCF-7 and MDA-MB-231 cells. Numbers indicate the ratio between the protein expression of VAMP3 and GAPDH, loading control.

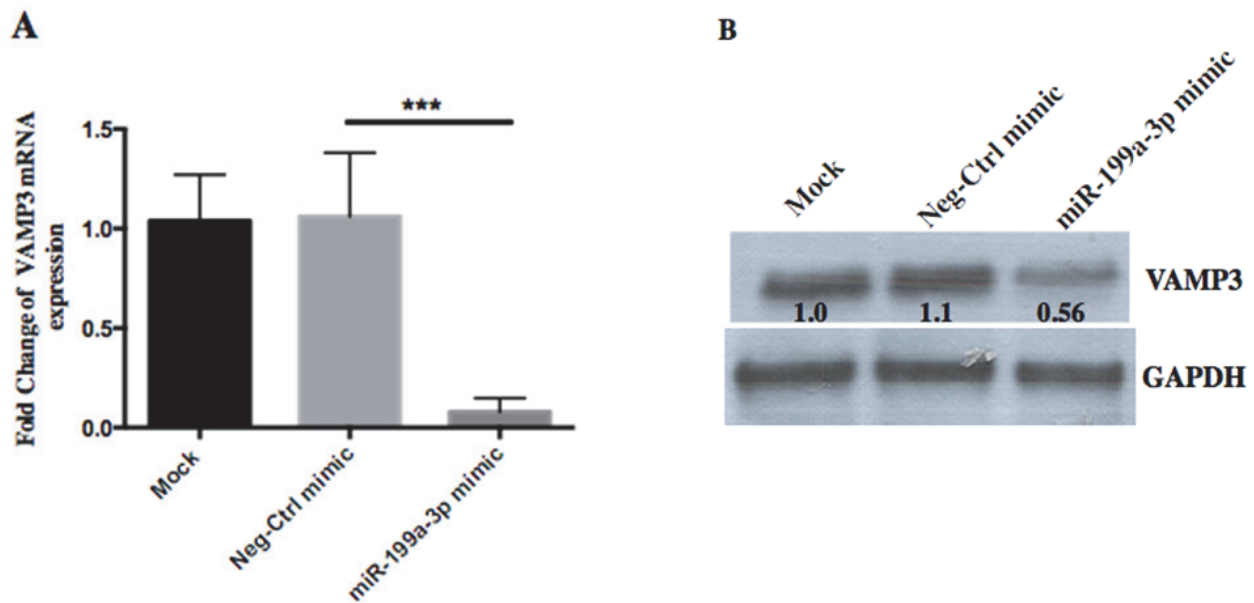


Figure 3. Inhibitory effects of miR-199a-3p mimic on the expression levels of VAMP3 mRNA and protein. MDA-MB-231 cells were mock and transfected with miR-199a-3p or Neg-Ctrl (Cel- miR-67) mimics at a final concentration of 25nM. A) The expression of VAMP3 mRNA was detected by qRT-PCR after two days of transfection. Data were normalized to miR-16 and fold changes calculated versus Neg-Ctrl mimic. Data represent mean \pm SD. *** $P < 0.001$; Experiments were reproduced three times in laboratory. B) Cell lysate was collected from mock and transfected MDA-MB-231 cells, followed by western blot analysis probed with anti-VAMP3 and GAPDH antibodies. Numbers indicate the ratio between VAMP3 and control GAPDH..

miR-199a-3p Inhibits the Expression of VAMP3 in Metastatic Breast Cancer Cells

To determine the effect of miR-199a-3p on the expression of VAMP3, we used miR-199a-3p mimic for transfection in MDA-MB-231 cell line. The over-expression of miR-199a-3p mimic significantly reduces VAMP3 mRNA in the MDA-MB-231 cells compared with that of Neg-Ctrl mimic (Fig. 3a). Consistent with this, the western blot demonstrates that miR-199-3p is able to reduce the protein level of VAMP3 in human MDA-MB-231 cells (Fig. 3b).

VAMP3 is a Direct Target of miR-199a-3p

To directly assess whether miR-199a-3p was able to inhibit VAMP3 expression through a sequence-specific interaction with the 3'UTR of VAMP3 mRNA, a luciferase plasmid carrying a 3'UTR of the VAMP3 mRNA was generated. In addition, directed mutagenesis was introduced at both or each potential binding site of miR-199a-3p within the 3'UTR of VAMP3 mRNA (Fig. 4a). The miR-199a-3p mimic effectively decreased luciferase activity of the Luc-VAMP3 3'UTR-transfected MDA-MB-231 cells when co-transfected with miR-

199a-3p mimic compared with that of the control construct (Fig. 4b). By contrast, mutations in either potential site of miR-199a-3p partially rescued the decreased luciferase activity by miR-199a-3p mimic. A double mutant completely rescued the decreased luciferase activity by miR-199a-3p (Fig. 4b). Based on these results, it is confirmed that miR-199a-3p has two functional binding sites with 3'UTR of VAMP3 mRNA.

Silencing of VAMP3 Attenuates the Migration Capacity of MDA-MB-231 Cells

Based on previous observations that VAMP3 regulates the metastatic and invasive capacity of pancreatic cancer cells and fibrosarcoma cells^[21,22], it was hypothesized that VAMP3 can induce migration in highly metastatic breast cancer cells. To examine the effect of VAMP3 expression on cell migration, VAMP3 was knocked down in MDA-MB-231 cells and compared with control RISC-free siRNA transfected cells. MDA-MB-231 cells transfected with VAMP3 siRNA decreased their migration capacities compared with those transfected with control siRNA 48 hr after transfection (Fig. 5). There was a 60% reduction in the cell count

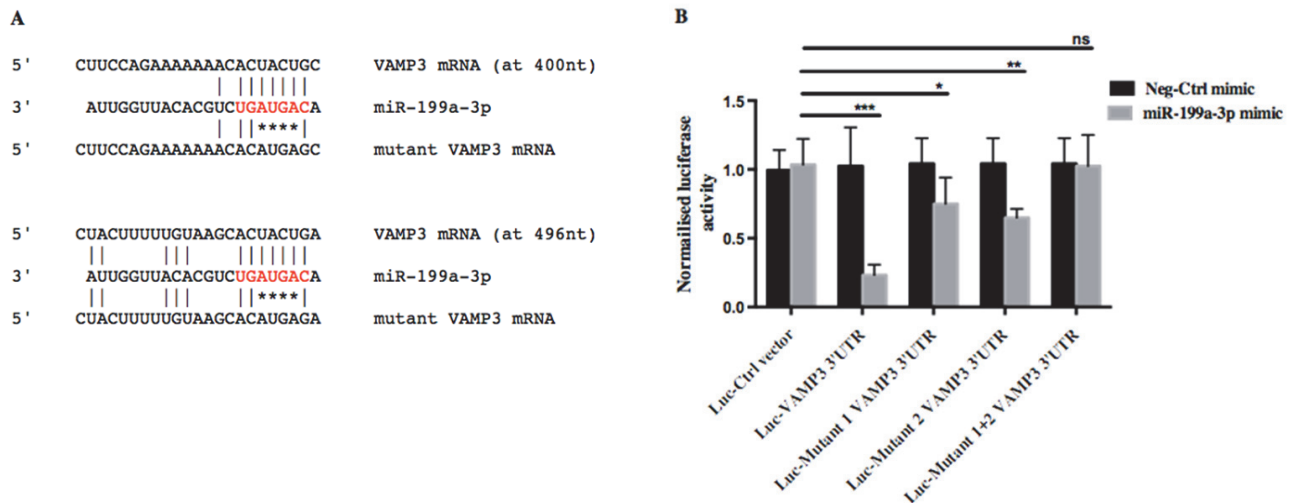


Figure 4. Binding site specific mutagenesis blocks the modulation of VAMP3 expression by miR-199a-3p. A) Schematic diagram showing the location and mutation of the potential miR-199-3p binding sites within the 3'UTR of VAMP3 mRNA. Red-colored nucleotides indicate the seed match in the VAMP3 3'UTR and stars (*) represent the sites of the point mutation. B) Dual-luciferase assays showing inhibition of wild-type 3'UTR or mutant 3'UTR after transfection of miR-199a-3p or Neg-Ctrl mimics in MDA-MB-231 cells. Data represent mean \pm SD. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; Experiments were reproduced three times in laboratory.

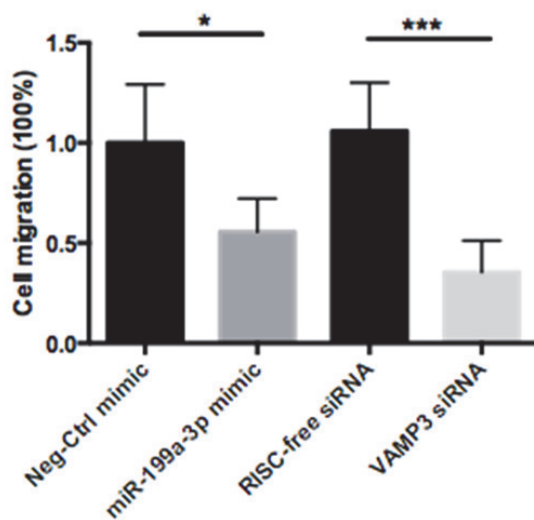


Figure 5. Transfection of VAMP3 siRNA or mimics of miR-199a-3p into MDA-MB-231 suppresses cell migration. Cells were transfected with either VAMP3 siRNA or RISC-free siRNA. Cell migration was determined using transwell chambers at 48 hr after transfection with VAMP3 siRNA, miR-199a-3p mimics, or controls. Migrated cells in the transwell were methanol-fixed and Giemsa-stained. Six different microscopic images were then counted for each transfection. The count of migrated cells for each transfection group. * $P < 0.05$. NC, negative control siRNA. The average number of migrated cells was calculated for each transfection group. Then the count of migrated cells transfected with the VAMP3 siRNAs or miR-199a-3p mimic were normalized to those transfected with the controls. Data represent mean \pm SD. * $P < 0.05$; *** $P < 0.001$; Experiments were reproduced three times in laboratory.

of migrated cells transfected with the VAMP3 siRNA, suggesting it is required to positively influence the migration capacity of breast cancer cells. Consistent with previous reports^[11,15], the over-expression of miR-199a-3p in MDA-MB-231 cells results in inhibition of cancer cell migration (Fig. 5).

Discussion

Cancer metastasis is the main cause of all cancer-related deaths, its prevention could be a promising approach in treating or at least controlling the migration of cancer cells. Previous work has detected the significant reduction of miR-199a-3p in highly metastatic breast cancer cell lines and grade 3 cancer tissue samples^[14,15]. The fact that miR-199a-3p expression is largely decreased in highly metastatic breast cancer cells indicates a potential role in the invasion and metastasis of cancer. Li *et al.*^[16] reported that the suppression of miR-199a-3p increased PAK4 expression and activated its signaling pathway. PAK4/MEK/ERK signaling pathway contributes to promotion of the metastatic phenotype of breast cancer cells^[15]. In addition, miR-199a-3p also targets caveolin-2 for suppression in breast cancer cells^[16]. In this work, VAMP3 level was inversely related to miR-199a-3p expression in MCF-7 and MDA-MB-231. As the expression of miR-199a-3p is decreased in high metastatic cancer cells, VAMP3 expression is increased. Moreover, the ectopic expression of miR-199a-3p significantly inhibited the expression of endogenous

VAMP3 at both mRNA and protein levels *in vitro*. Thus, these results suggest that VAMP3 can be a potential target of miR-199a-3p in breast cancer cells. The data from luciferase assay confirmed that the regulation mechanism of VAMP3 by miR-199a-3p in breast cancer cells is mediated *via* two sites in 3'UTR region of VAMP3. This work provides the first evidence of the significant impact of VAMP3 in the migration of breast cancer cells. VAMP3 mediated the chemotactic migration of MDA-MB-231 cells *in vitro*. This agrees with studies demonstrating the positive influence of VAMP3 in the migration and malignant phenotypes of pancreatic cancer cells (PANC-1) and invasive human fibrosarcoma cells (HT-1080)^[21,22]. Although the mechanism by which VAMP3 regulates the migration in breast cancer cells remains to be studied, one possible scenario could be that VAMP3 mediates the intracellular trafficking of integrin to the cell membrane and initiates its downstream signaling cascades in cell adhesion and spreading^[26,27]. Consistent with this, VAMP3 is important for the trafficking of 1 matrix metalloproteinase (MT1-MMP), which contributes to cancer cell invasion^[24]. In conclusion, the results of VAMP3 as a novel target of miR-199a-3p refined our insights into how this miRNA contributes to cancer metastasis. Targeting VAMP3 by miR-199a-3p appears to have a significant role in breast cancer metastasis and that could be a promising strategy for cancer intervention.

Conflict of Interests

The authors declare that there is no conflict of interest regarding the publication of this paper.

Disclosure

None of the authors received any type of commercial support either in forms of compensation or financial for this study. They have no financial interest in any of the products or devices, or drugs mentioned in this article.

Ethical Approval

Obtained.

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miR-199a-3p ينظم هجرة خلايا سرطان الثدي من خلال استهداف جين VAMP3

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المستخلص. التغييرات في مستويات microRNAs الخلوية تتدخل في مراحل متعددة من نمو الاورام السرطانية أو تقدمها. مستوى miR-199a-3p ينخفض بدرجة كبيرة في خلايا سرطان الثدي الخبيثة مثل خلايا MDA-MB-231، ولذلك فإن رفع مستواه داخل الخلايا يؤدي الى تأثير مضاد لصفات الورم الخبيث (antimetastatic). وعموما فإن الآلية التي يعمل بها miR-199a-3p لإحداث تأثيراته المضاد للورم الخبيث (antimetastatic) تحتاج إلى دراسة. ولأن miR-199a-3p يعمل على التقليل من مستويات تعبير الجينات التي يستهدفها، فمن المتوقع أن نلاحظ وجود علاقة عكسية بين التعبير miR-199a-3p وجيناته المستهدفة والتي ستحفز انتشار الورم الخبيث (prometastatic). تم اثبات أن تعبير جين VAMP3 كان عالي جدا في خلايا سرطان الثدي الخبيثة وسريعة الانتشار MDA-MB-231 مقارنة بتلك الأقل خبثا وانتشارا MCF-7 وأن رفع مستوى miR-199a-3p داخل خلايا سرطان الثدي الخبيثة أدى الى تثبيط تعبير VAMP3. إضافة الى انه تم اكتشاف مكاني التفاعل بين VAMP3 و miR-199a-3p والتي تؤكد على وجود آلية جديدة لتعديل تعبير VAMP3 عن طريق miR-199a-3p. الدراسات الوظيفية أكدت ان التخلص من تعبير VAMP3 الموجود في خلايا سرطان الثدي الخبيثة وتؤدي الى التقليل من خصائصها الخبيثة، تحديدا الهجرة الخلوية من مكان لآخر. ختاماً تشير النتائج إلى أن miR-199a-3p والقادر على استهداف الجين الخبيث VAMP3 يمكن ان يحمل تأثير كبيراً في منع حدوث او علاج سرطان الثدي الخبيث.