

This article is licensed under a Creative Commons Attribution-NonCommercial NoDerivatives 4.0 International License.

MicroRNA-548m Suppresses Cell Migration and Invasion by Targeting Aryl Hydrocarbon Receptor in Breast Cancer Cells

WM Farhan Syafiq B. WM Nor,*† Ivy Chung,‡§ and Nur Akmarina B. M. Said†

*Faculty of Medicine, University of Malaya, Kuala Lumpur, Malaysia

†Department of Pharmaceutical Life Sciences, Faculty of Pharmacy, University of Malaya, Kuala Lumpur, Malaysia

‡Department of Pharmacology, Faculty of Medicine, University of Malaya, Kuala Lumpur, Malaysia

§University of Malaya Cancer Research Institute, Faculty of Medicine, University of Malaya, Kuala Lumpur, Malaysia

Breast cancer is the most commonly diagnosed cancer among women and one of the leading causes of cancer mortality worldwide, in which the most severe form happens when it metastasizes to other regions of the body. Metastasis is responsible for most treatment failures in advanced breast cancer. Epithelial–mesenchymal transition (EMT) plays a significant role in promoting metastatic processes in breast cancer. MicroRNAs (miRNAs) are highly conserved endogenous short noncoding RNAs that play a role in regulating a broad range of biological processes, including cancer initiation and development, by functioning as tumor promoters or tumor suppressors. Expression of miR-548m has been found in various types of cancers, but the biological function and molecular mechanisms of miR-548m in cancers have not been fully studied. Here we demonstrated the role of miR-548m in modulating EMT in the breast cancer cell lines MDA-MB-231 and MCF-7. Expression data for primary breast cancer obtained from NCBI GEO data sets showed that miR-548m expression was downregulated in breast cancer patients compared with healthy group. We hypothesize that miR-548m acts as a tumor suppressor in breast cancer. Overexpression of miR-548m in both cell lines increased E-cadherin expression and decreased the EMT-associated transcription factors SNAI1, SNAI2, ZEB1, and ZEB2, as well as MMP9 expression. Consequently, migration and invasion capabilities of both MDA-MB-231 and MCF-7 cells were significantly inhibited in miR-548m-overexpressing cells. Analysis of 1,059 putative target genes of miR-548m revealed common pathways involving both tight junction and the mTOR signaling pathway, which has potential impacts on cell migration and invasion. Furthermore, this study identified aryl hydrocarbon receptor (AHR) as a direct target of miR-548m in breast cancer cells. Taken together, our findings suggest a novel function of miR-548m in reversing the EMT of breast cancer by reducing their migratory and invasive potentials, at least in part via targeting AHR expression.

Key words: Breast cancer; MicroRNA-548m; Aryl hydrocarbon receptor; Epithelial–mesenchymal transition (EMT); Invasion; Migration

INTRODUCTION

Breast cancer is the most commonly diagnosed cancer among women and is one of the leading causes of cancer mortality worldwide, especially in low- and middle-income countries¹. Breast cancer alone is expected to account for 30% of all new cancer cases and 15% of all cancer-related deaths in women². It is characterized by heterogeneity of genetic modification and comprises variable phenotypes that are driven by distinctive molecular alteration and variabilities³. Breast cancers could be fatal when it metastasizes to other regions of the body, which contributes to most treatment failures⁴. Epithelial–mesenchymal

transition (EMT) has been suggested as a key mechanism in promoting the metastatic processes in cancer cells. EMT is a biological process that allows polarized epithelial cells to undergo various biochemical modifications, including losing its junction and apical–basal polarity⁵. This allows the cells to adopt a mesenchymal phenotype that defines cell shape, reprogram gene expression, and, consequently, enhance the motility and acquisition of invasive properties⁶. Therefore, it is crucial to find a new biomarker or regulator to suppress motility and invasion processes as a potential strategy in the treatment of metastatic breast cancer.

MicroRNAs (miRNAs) are a class of highly conserved endogenous short noncoding RNAs that play a pivotal role

Address correspondence to Nur Akmarina B. M. Said, Department of Pharmaceutical Life Sciences, Faculty of Pharmacy, Jalan Universiti, University of Malaya, 50603 Kuala Lumpur, Malaysia. E-mail: nur_akmarina@um.edu.my

in various developmental and psychological processes. These processes include cancer initiation and development⁷, apoptosis⁸, cell cycle progression⁹, cellular proliferation¹⁰, differentiation, and metastasis¹¹. A miRNA may be found to be upregulated in some cancer types, and thus indicated as oncomiR, or downregulated in other type of cancers, thus displaying a tumor suppressor function¹². miRNAs are important genetic regulators wherein each miRNA can possibly regulate and target the expression of hundreds of genes. On the other hand, a single transcript can be targeted by multiple miRNAs to concurrently downregulate multiple proteins in the same pathway¹³. Several miRNAs have been recognized in regulating EMT processes in breast cancer development by modulation of EMT-related genes. For instance, miR-138¹⁴, miR-221/222¹⁵, miR-9¹⁶, and miR-21¹⁷ act as either promoters or inhibitors of EMT-related genes such as E-cadherin and vimentin, which later affect breast cancer invasion and metastasis. Understanding the mechanism and functional role of miRNAs may provide better understanding and insight into EMT regulation. In this study, we are interested to investigate the role of miR-548m in breast cancer.

miR-548 is a newly discovered miRNA family, which are poorly conserved in their sequences¹⁸. Recent evidence has shown that members of the miR-548 family have a close correlation with metastatic behaviors^{19–22} and are more commonly downregulated in breast cancer^{22–25}. From our previous study, miR-548m was identified as an inducer of mesenchymal phenotype in bladder cancer using multiparametric screening for EMT modulators²⁶. However, the biological function and molecular mechanisms of miR-548m in cancers have not been fully elucidated. Moreover, a particular miRNA may also be found to be upregulated in some cancer types, and thus supposedly oncogenic, but downregulated in other cancers, which is indicative of tumor suppressor function¹². In breast cancer, treatment with 5-fluorouracil²⁷ and polyphenon-60²⁸ in MCF-7 cell lines showed upregulation of miR-548m expression upon treatment, suggesting possible tumor suppressor function of miR-548m. In this present study, we assessed the expression levels of miR-548m in human breast cancer and examined its effects on cell proliferation, migration, invasion, and EMT-related gene expression. Furthermore, we explored the target of miR-548m in breast cancer cells and the possible mechanism of its function.

MATERIALS AND METHODS

Gene Expression Analysis Using the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO) Database

Microarray profiles of selected breast cancers were obtained from the publicly available GEO database (<http://www.ncbi.nlm.nih.gov/geo/>). In the GEO data set search of miR-548m, the following search terms or keywords were

applied (microRNA OR miR OR miRNA) AND (Breast) AND (tumor OR cancer OR carcinoma OR neoplas* OR malignan*). The microarray data sets were considered eligible if they met the criteria as follows: (1) the samples in each data set must contain breast cancer and nontumor control groups; (2) data sets consist of miR-548m expression between breast cancer and normal breast tissues; (3) miRNA–microarray data with other intervening factors were removed, such as experiments with gene knock-down and other treatments; and (4) in terms of sample size, each data set should contain at least three samples ($n > 3$). GEO2R (<http://www.ncbi.nlm.nih.gov/geo/geo2r/>) was applied to screen differentially expressed miRNAs between breast cancer and normal tissue samples. The adjusted p values (adj. p) using the Benjamini and Hochberg (BH) false discovery rate (FDR) method by default were applied to correct for the occurrence of false-positive results.

Cell Lines and Cell Culture

Human breast cancer cell lines MDA-MB-231 and MCF-7 were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA) and cultured in high-glucose Dulbecco's modified Eagle's medium (DMEM; GIBCO, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; GIBCO) in the presence of 1% penicillin–streptomycin (GIBCO). The cells were maintained at 37°C humidified atmosphere in an incubator with 5% CO₂ saturation. Transfections of miRNA and starvation media were deprived of penicillin–streptomycin and FBS, respectively.

Cell Transfection

The miRIDIAN miR-548m mimic (C-301392-00, mature miRNA sequence), miRIDIAN mimic negative control #1 (CN-001000-01, mature miRNA sequence), and miRIDIAN miRNA mimic transfection control with Dy547 (fluorescently labeled mimic control) were purchased from Dharmacon (Lafayette, CO, USA). The breast cancer cell lines MDA-MB-231 and MCF-7 were reverse transfected at a final concentration of 25 nM using Lipofectamine® RNAiMAX (Life Technologies, Carlsbad, CA, USA) transfection reagent per the manufacturer's protocols. The efficiency of miR-548m overexpression was verified by visualization with a fluorescently labeled Dy547 mimic control. These controls were used under the same experimental conditions as miR-548m mimics, which allowed to assess cell transfection efficiency during each assay. All subsequent functional assays were performed 72-h posttransfection.

Quantitative Real-Time Polymerase Chain Reaction (RT-qPCR)

Large RNA and small RNA from breast cancer cell lines were isolated using a NucleoSpin® miRNA Kit

(Macherey-Nagel, Düren, Germany). The concentration and purity of the mRNA and miRNA samples were measured using a NanoDrop Spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE, USA). cDNA from mRNA was generated using a RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, Waltham, MA, USA) according to the manufacturer's instructions. The expression levels of mRNA were assessed with RT-qPCR using 5x HOT FIREPol® EvaGreen® qPCR Mix Plus (Solis BioDyne, Tartu, Estonia). Oligonucleotide primers as shown in Table 1 were synthesized by Integrated DNA Technologies (IDT). The expression level of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as reference gene. The small RNA sample was reverse transcribed with TaqMan® MicroRNA Reverse Transcription kit (Applied Biosystems, Weiterstadt, Germany) according to the manufacturer's instructions. cDNA was quantitated using a TaqMan microRNA assay specific (Applied Biosystem) for miR-548m and RNU48 according to the manufacturer's protocol. The expression level of RNU48 was used as reference gene.

Western Blot Analysis

Total protein was isolated in parallel with large RNA and small RNA with the NucleoSpin® miRNA kit (Macherey-Nagel) according to the manufacturer's protocol. Protein pellets were resuspended in protein solving buffer set PSB/TCEP (Macherey-Nagel). Proteins were loaded and separated in a 10% acrylamide gel (100v) and transferred onto a 0.2- μ m nitrocellulose membrane (Amersham Protran, Buckinghamshire, UK) using the Thermo-Pierce Fast Semi-Dry Blotter apparatus. The membrane was blocked with 5% nonfat milk for 1 h at room temperature with agitation, followed by overnight incubation at 4°C with primary antibodies against aryl hydrocarbon receptor (AHR) (1:500; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and GAPDH (1:1,000; Santa Cruz Biotechnology) in BSA with tris-buffered saline-Tween 2 (TBS-T; Santa Cruz Biotechnology). Subsequently, the membrane was washed and incubated with secondary

antibody (1:2,000; Sigma-Aldrich, St. Louis, MO, USA) for 1 h at room temperature. Protein signal was detected with enhanced chemiluminescence horseradish peroxidase (HRP) substrate (Radiance Plus; Azure Biosystems, Dublin, CA, USA) and recorded using the Azure Biosystems c600 quantitative Western blot imaging system (Azure Biosystems).

Transwell Invasion Assay

The invasive potential of MDA-MB-231 and MCF-7 was measured using Boyden Transwell 0.8- μ m pore polycarbonate membrane insert (Transwell-Costar, Corning, NY, USA) and Corning® Matrigel® Growth Factor Reduced (GFR) Basement Membrane Matrix (Corning) according to the manufacturer's protocol. Briefly, the filter of the top chamber was coated with diluted Matrigel (0.3 mg/ml) following standard procedure and incubated at 37°C for 5 h to allow the Matrigel to solidify. First, miR-548m mimics and its control were reverse transfected into the breast cancer cells in a six-well plate for 72 h. They were then reseeded onto the membrane of the upper chamber coated with Matrigel at 5×10^4 cells/well for MDA-MB-231 and at 1×10^5 cells/well for MCF-7 in 300 μ l serum-free media. DMEM (500 μ l) supplemented with 10% FBS were used as a chemoattractant in the lower chamber. After 24 h, the lower chamber was washed with Dulbecco's phosphate-buffered saline (DPBS; GIBCO). Invaded cells on the lower membrane surface were fixed in 3.7% formaldehyde (Sigma-Aldrich) for 2 min. Noninvasive cells in the top chamber were removed with cotton swabs. The membrane in the lower chamber was stained with 0.2% crystal violet (Sigma-Aldrich). The number of invaded cells was viewed with a microscope and counted in at least five fields.

Wound Healing Assay

For the in vitro wound healing assay (scratch migration analysis), MDA-MB-231 and MCF-7 cells transfected with miR-548m mimics and negative control were allowed to grow in a 24-well plate until cells became

Table 1. Sequence of Primers for RT-qPCR

Gene	Forward Sequence (5'-3')	Reverse Sequence (5'-3')
GAPDH	CCCATCACCATCTTCCAGGAG	GTTGTCATGGATGACCTTGGC
AHR	ACATCACCTACGCCAGTGG	CTCTATGCCGCTTGAAGGAT
SNAI1	GGTTCTTCTGCGCTACTGCT	TAGGGCTGCTGGAAGGTAAA
SNAI2	GGGGAGGAGGGAAAGATTAG	GCACTTGGAAGGGGTATTGT
ZEB1	GCACAACCAAGTGCAAGAAGA	CATTTGCAGATTGAGGCTGA
ZEB2	ATCTGCTCAGAGTCCAATGC	TTGTTCCCTCAGGTTGAGAGC
VIM	TGGCACGTCTTGACCTTGAA	GGTCATCGTGATGCTGAGAA
ZO1	CGGTCCTCTGAGCCTGTAAG	GGATCTACATGCGACGACAA
CDH1	CGACCCAACCCAAGAATCTA	AGGCTGTGCCTTCCTACAGA
MMP9	TGTACCGCTATGGTTACTACTCG	GGCAGGGACAGTTGCTTC

confluent after 72 h posttransfection. Briefly, a scratch was made by scraping the bottom of each well in straight lines using a sterile 10- μ l pipette tip and washed with DPBS to remove cell debris. Cells were then maintained in the incubator at 37°C with 5% CO₂. Cell images were captured every 2 h using an Olympus microscope (Olympus America Inc., Waltham, MA, USA) by live imaging for 24 h. Cells were maintained in a plate incubator at 37°C with 5% CO₂. The area of uncovered wound was measured by creating an area at the wound margin at given time points, and a migration was reported as percentage of wound closure at each time point.

Cell Proliferation Assay

Cell proliferation of MDA-MB-231 and MCF-7 was monitored by a colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay according to the manufacturer's instructions. Briefly, cells were seeded onto 96-well plates, and the cell proliferation after transfection was documented every 24 h for 3 days. After every 24 h posttransfection, the cells were incubated with 20 μ l MTT (Sigma-Aldrich, Germany) into each well at a final concentration of 0.5 mg/ml. The incubation was continued for 4 h at 37°C and 5% CO₂, allowing viable cells to bio-reduce the MTT reagent into a colored formazan product. SDS (100 μ l) was added to each well to dissolve the formazan crystals overnight. The staining intensity in the medium was measured using a SpectraMax M3® spectrophotometer (Molecular Devices, Sunnyvale, CA, USA) by determining the absorbance at 570 nm. The absorbance at 650 nm was used as a reference.

Computational miRNA Target Prediction

For identification of miR-548m targets, bioinformatics analysis was performed to predict the possible target genes of miR-548m. Three different software algorithms were used to find conserved target regions, including TargetScan release 7.1 (<http://www.targetscan.org>), microRNA.org (miRanda) database, and DIANA-microT database (<http://diana.imis.athena-innovation.gr>) as reviewed by Riffo-Campos et al.²⁹. The predicted target genes from each software algorithms were merged, and specific cutoff point from each prediction software was applied. Finally, target genes were further narrowed down to breast cancer-associated genes (hsa-miR-548m-associated broad phenotype) using the miR-Ontology database (miRò2; www.ferrolab.dmi.unict.it/miro). Selected target genes were submitted to online tools for functional annotation analysis, Database for Annotation, Visualization and Integrated Discovery (DAVID; <http://david.abcc.ncifcrf.gov/>). The significant enrichment analysis of target genes was assessed based on the Kyoto Encyclopedia of Genes and Genomes (KEGG; <http://www.genome.jp/kegg/kegg2>

html) with a value of $p < 0.05$ considered as a statistically significant difference.

Dual-Luciferase Reporter Assay

For construction of the AHR 3'-UTR-Luc reporter plasmid, a fragment of the 3'-untranslated region (3'-UTR) of the AHR gene was synthesized and cloned into pUC57 (Bio-Basic Inc., Amherst, NY, USA) within the *SacI* and *SalI* restriction sites. The pmirGLO Dual-Luciferase miRNA target expression vector (pmirGLO vector) containing both the firefly luciferase (*Fluc*) gene and the *Renilla* luciferase (*Rluc*) gene was purchased from Promega (Madison, WI, USA). The AHR 3'-UTR was then subcloned by 1st BASE Services (First BASE Laboratories, Selangor, Malaysia), directly downstream from a *Fluc* gene under the control of the PGK promoter, and *Rluc* gene under the control of an SV40 promoter (as a transfection control) within the *SacI* and *SalI* restriction sites. MDA-MB-231 and MCF-7 cells were cotransfected with 50 ng reporter plasmids and 25 nM miR-548m mimics and its control for 48 h. The luciferase activity was determined with Dual-Glo Luciferase Assay System (Promega) according to the manufacturer's instruction. Luciferase activity was measured with SpectraMax L luminometer (Molecular Devices). Normalized data were calculated as the ratio of *Rluc/Fluc* activities.

Statistical Analysis

Each experiment was performed three times at least. The results were expressed as the mean \pm standard error of the mean (SEM). Differences between values were statistically analyzed using Student's *t*-test or one-way analysis of variance (ANOVA). All statistical analyses were performed using GraphPad Prism 8 (GraphPad Software, San Diego, CA, USA). A value of $p < 0.05$ was considered statistically significant.

RESULTS

miR-548m Expression in Human Breast Cancer Tissues and Cell Lines

To examine the relevance of miR-548m in human breast cancer tissues and their expression, data analysis was performed using GEO2R on publicly available data sets obtained from the NCBI GEO. Nine data sets published in GEO reference series that met the entry criteria were queried for the expression of miR-548m in breast cancer³⁰⁻³⁶. The features of the included GEO data sets are described in Table 2. The expression data of miR-548m are shown on the scatter plots and receiver operating characteristic (ROC) curves in Figures 1 and 2. miR-548m expression was significantly downregulated in breast cancer tissues compared with normal breast tissues in two GEO microarrays (GSE45666 and GSE44899, all $p < 0.05$) (Fig. 1). The ROC curve of these two

Table 2. Information on Selected Gene Express Omnibus (GEO) Breast Cancer Microarrays Data Sets

GEO Accession	Platform	Year	Country	Number of Samples				Sources	Clinicopathological Characteristics in Patients (n)							
				Normal	Tumor	Metastasis	PMID		ER ⁺	ER ⁻	PR ⁺	PR ⁻	Her2 ⁺	Her2 ⁻	TNBC	
GSE80038	GPL18044	2016	China	4	4	4	28629464	Tissue	-	-	-	-	-	-	-	12
GSE44899	GPL7723	2014	Serbia	4	76	-	24104964	Tissue	27	33	23	38	11	50	26	26
GSE45666	GPL14767	2013	China	15	100	-	23722663	Tissue	52	63	39	76	41	74	36	36
GSE57897	GPL18722	2018	China	31	422	-	31209222	Tissue	246	176	234	188	120	302	46	46
GSE38167	GPL14943	2014	Australia	23	31	13	24479446	Tissue	-	-	-	-	-	-	-	31
GSE48088	GPL14613	2014	Spain	3	33	-	25047087	Tissue	26	8	22	12	13	21	4	4
GSE44124	GPL14767	2014	Spain	3	50	-	24098452	Tissue	-	-	-	-	-	-	-	7
GSE32922	GPL7723	2013	Spain	16	22	-	-	Tissue	-	-	-	-	-	-	-	-
GSE37407	GPL13703	2012	Sweden	6	19	31	22294488	Tissue	24	6	4	19	1	9	-	-

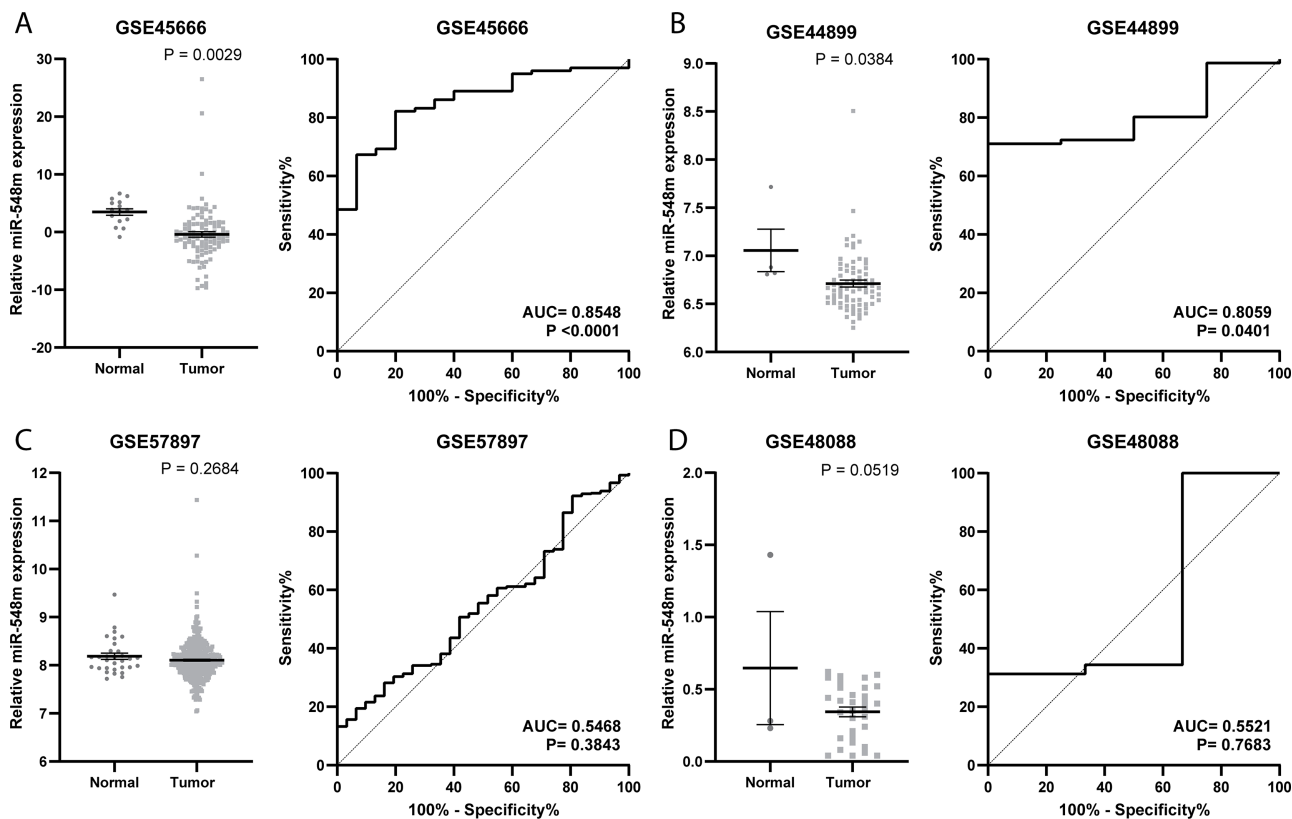
Abbreviations: GEO, Gene Express Omnibus; PMID, PubMed unique identifier; -, not available; n, number of patients; ER⁺, estrogen receptor positive; ER⁻, estrogen receptor negative; PR⁺, progesterone receptor positive; PR⁻, progesterone receptor negative; HER2⁺, human epidermal growth factor receptor 2 positive; HER2⁻, human epidermal growth factor receptor 2 negative; TNBC, triple negative breast cancer.

microarrays also implied that miR-548m exerted discriminatory capabilities that could be used to differentiate between breast cancer tissue and normal breast tissue (Fig. 1). By contrast, no significant difference in miR-548m expression was detected between breast cancer tissue and normal breast tissue in other microarrays (GSE57897, GSE48088, GSE44124, and GSE32922) (Fig. 1). In addition, the expression data of different types of breast cancer and normal breast tissue group were also collected on the GEO database. The expression of miR-548m was significantly downregulated in tissues of breast cancer with lung metastasis and primary breast tumor, compared to normal adjacent breast tissue (GSE80038; $p < 0.05$) (Fig. 2). By contrast, no significant difference in miR-548m expression was detected between metastatic breast cancer tissue, primary breast cancer tissue, and normal breast tissue group in other microarrays (GSE37407 and GSE38167) (Fig. 2).

Next, we analyzed miR-548m expression in a GEO microarray data set (GSE146477) generated from primary mammary epithelial cells and a panel of breast cancer cell lines that range in tumorigenic and invasive potential³⁷. miR-548m expression was higher in the less invasive MCF-7 cells and nontumorigenic human mammary epithelial cells (HMEC) and 184A1 cells, compared with the more aggressive breast cancer cell lines (MDA-MB-231, SKBR3, and T-47D) (Fig. 2D). However, when the endogenous expression of miR-548m in different types of breast cancer cell lines (MCF-7, HS578T, and MDA-MB-231) and nontumorigenic mammary cell line (MCF-10A) was assessed, our lab could not detect the expression of miR-548m in either breast cancer cell lines or normal human mammary cell (data not shown).

miR-548m Modulates Expression of EMT Markers in Breast Cancer Cells

To investigate the involvement of miR-548m in regulating EMT, we analyzed the expression of several EMT-related genes in MDA-MB-231 and MCF-7 cells following transfection of miR-548m mimics. The transfection resulted in significant upregulation of miR-548m in mimic-transfected cell lines compared to the negative controls (data not shown). As shown in Figure 3A and B, in both MDA-MB-231 and MCF-7 cells transfected with miR-548m mimics, E-cadherin (CDH1) expression was upregulated 1.23-fold and 1.35-fold, respectively, while no significant changes were seen on vimentin expression. In MDA-MB-231 cells, the expressions of SNAI1, ZEB2, and matrix metalloproteinase 9 (MMP9) were downregulated 1.32-, 1.30-, and 1.50-fold, respectively, following miR-548m mimics treatment (Fig. 3A). In MCF-7 cells, the upregulation of E-cadherin (CDH1) was accompanied by downregulation of SNAI2, ZEB2, and MMP9 1.45-, 2.12-, and 1.58-fold, respectively (Fig. 3B).



miR-548m Reduces the Migration and Invasion of Breast Cancer Cells

We hypothesized that overexpression of miR-548m would inhibit cancer cell migration and invasion. To test this postulation, wound healing assays and Transwell invasion assay were performed on MDA-MB-231 and MCF-7 cell lines transfected with miR-548m mimics. Overexpression of miR-548m significantly inhibited the potential of both breast cancer cell migration. Transfection with miR-548m mimics caused only a 47% reduction in wound closure in MDA-MB-231, compared with 62% reduction in negative control (Fig. 4A). In MCF-7 cells, miR-548m mimics caused a 37% reduction in wound closure compared with 75% reduction in negative control after 24 h. Transwell invasion assay with Matrigel demonstrated that miR-548m mimics reduced invasion of MDA-MB-231 and MCF-7 by 45% (Fig. 4B). However, the MTT assay showed that MDA-MB-231 and MCF-7 cells transfected with negative control or miR-548m mimics for 24, 48, and 72 h had no significant effects on proliferation (Fig. 4C). These results suggested that overexpression of miR-548m reduces migration and invasion capabilities of breast cancer cells.

miR-548m Is Involved in Key Signaling Pathways and Alters its Target Gene Expression

To identify the potential target genes of miR-548m whose modulations resulted in the decrease in migration

and invasion, we systematically screened through bioinformatic tools: TargetScan, microRNA.org (miRanda), and DIANA-microT-CDS. A total of 1,059 commonly predicted target genes were identified. The functional enrichment tool was employed to identify common pathways and the molecular network of the 1,059 potential target genes using KEGG analysis within the DAVID tools. The “tight junction” was found to be the most enriched pathway, followed by the “mTOR signaling pathway” (Fig. 5B). These predicted targets were eventually cross-checked with the miR-Ontology 2 (miR²O) database to identify the potential targets associated with breast cancer (Fig. 5A). A total of 31 genes were identified as potential targets of miR-548m in breast cancer, and we further categorized them into the pathobiological processes involved. As a result, six of them were more closely related to EMT, cell migration, and invasion in breast cancer based on published literature (Table 3). Subsequent gene profiling on those six selected potential targets revealed AHR to be consistently and significantly downregulated in both MDA-MB-231 and MCF-7 cells (data not shown).

AHR as a Direct Downstream Target of miR-548m in Breast Cancer Cells

Among the genes explored, AHR (SVR score: -0.8462) showed consistent downregulation in both cell lines at

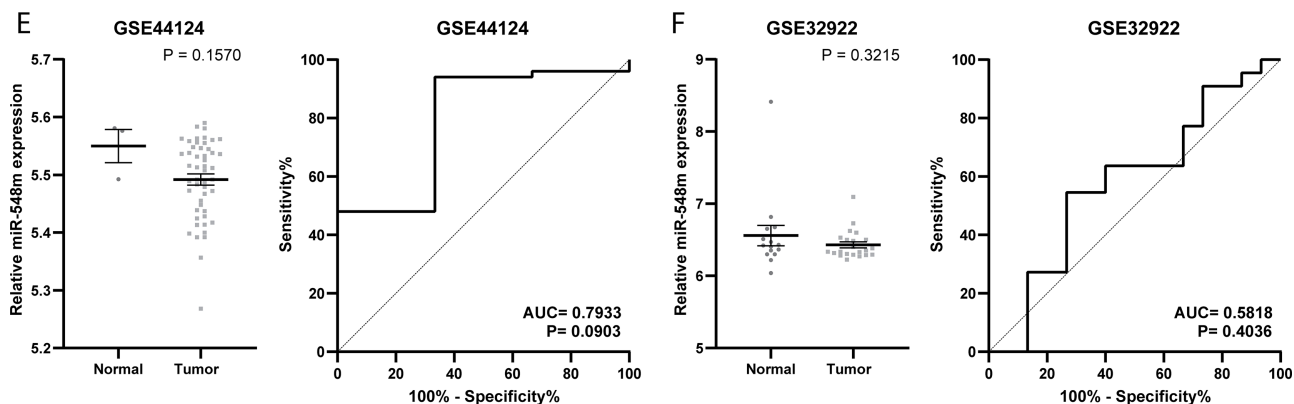


Figure 1. Above and facing page. Expression of miR-548m in normal breast and breast cancer tissues based on the Gene Expression Omnibus (GEO) database. The miR-548m expression and the ROC curve from (A) GSE45666, (B) GSE44899, (C) GSE57897, (D) GSE48088, (E) GSE44124, and (F) GSE32922. All data are presented as the means \pm standard error of the mean (SEM).

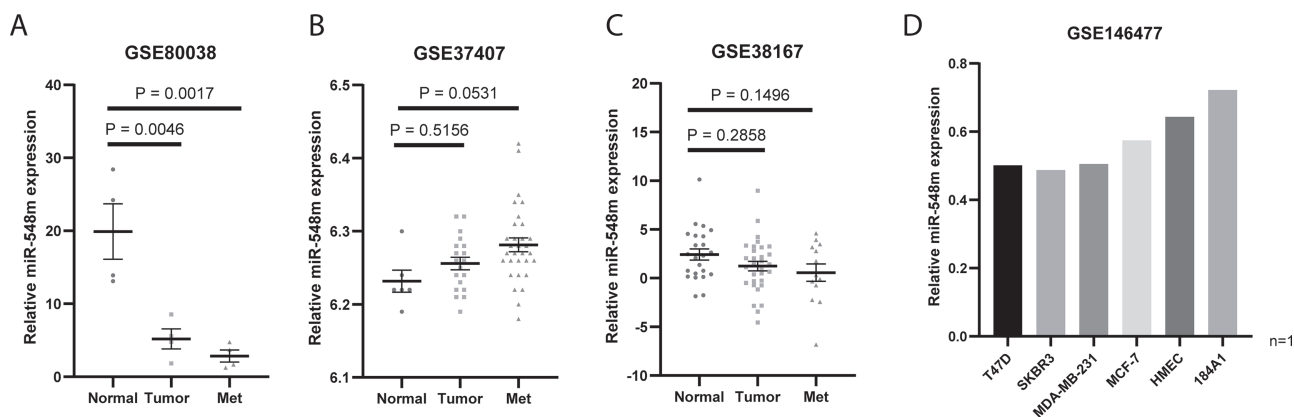


Figure 2. Expression of miR-548m in breast cancer tissues and cell lines based on the GEO database. The miR-548m expression and the ROC curve from (A) GSE80038, (B) GSE37407, (C) GSE38167, and (D) GSE146477. All data are presented as the means \pm SEM, except for (D) where $n = 1$.

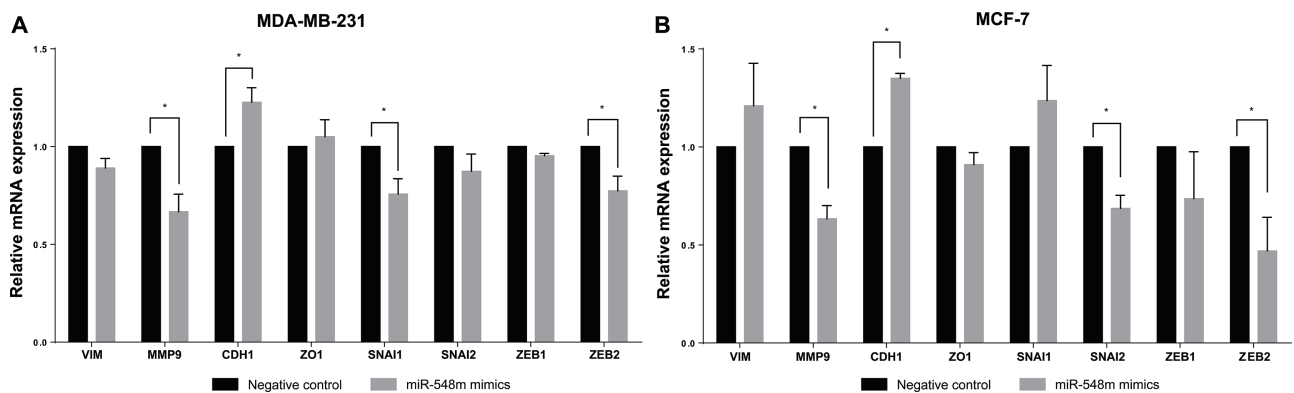


Figure 3. miR-548m modulates the expression of epithelial-mesenchymal transition (EMT) markers. Quantitative real-time polymerase chain reaction (RT-qPCR) analyses of relative selected EMT marker expression in (A) MDA-MB-231 and (B) MCF-7 cells transfected with miR-548m mimics compared with negative control. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal control to normalize the results to 1. Graphs are displayed as mean \pm SEM, $n = 3$. * $p < 0.05$.

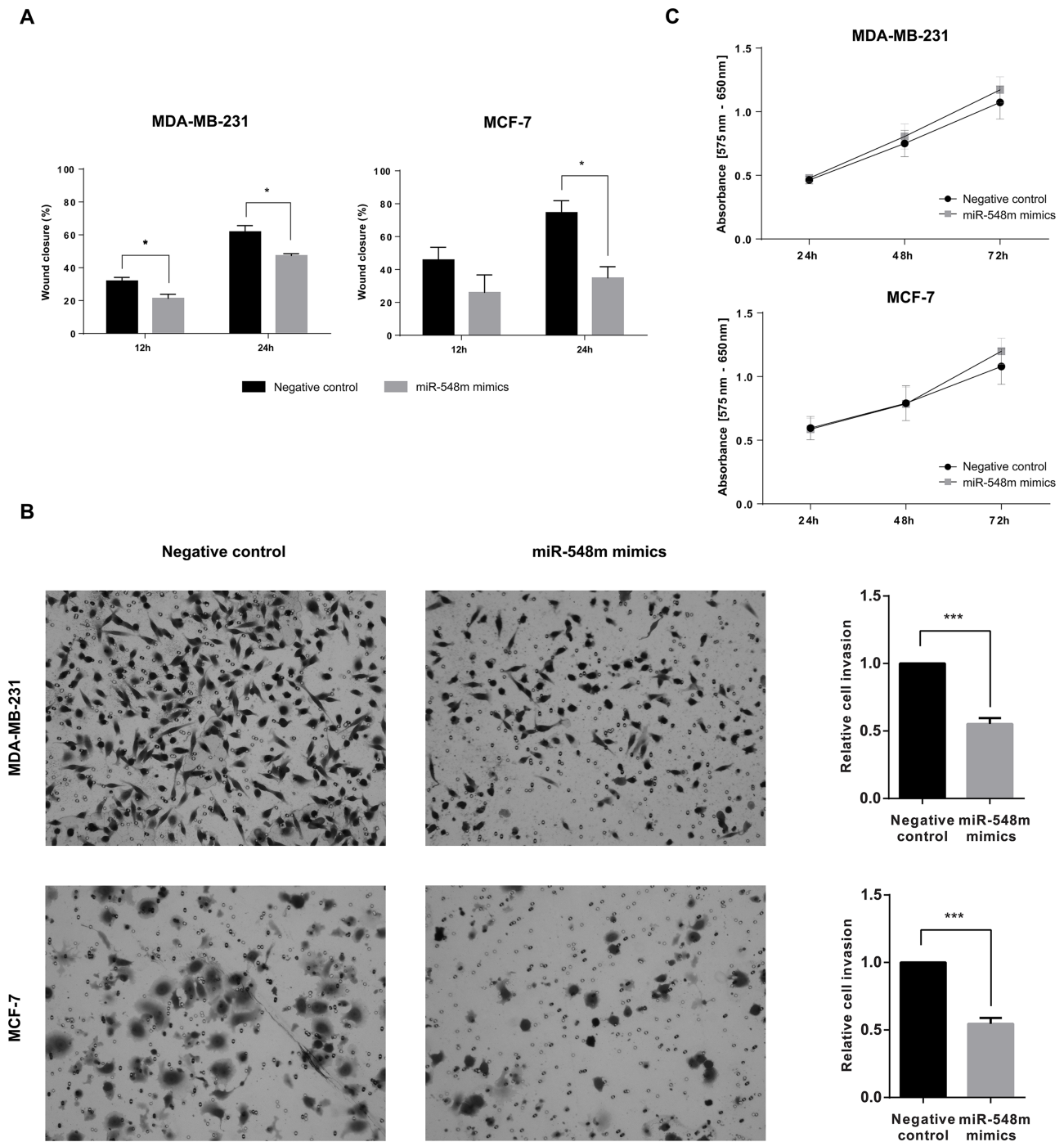


Figure 4. miR-548m reduces the migration and invasion of breast cancer cells. (A) The migration abilities of MDA-MB-231 and MCF-7 cells were measured by wound healing analysis between miR-548m mimics compared with negative control. For migration area quantification, the cell-free areas of the images were defined manually by NIS-Elements packages. (B) The invasion abilities of MDA-MB-231 and MCF-7 cells were measured using Transwell coated with Matrigel and stained with crystal violet (magnification: 10 \times). The graph was normalized to negative control. Representative images (left) and quantification of invasive cells (right) are shown. (C) The effects of miR-548m on proliferation of MDA-MB-231 and MCF-7 were evaluated as the value of absorbance at 575 nm with reference at 650-nm wavelength. Graphs are displayed as mean \pm SEM, $n = 3$. * $p < 0.05$; *** $p < 0.001$.

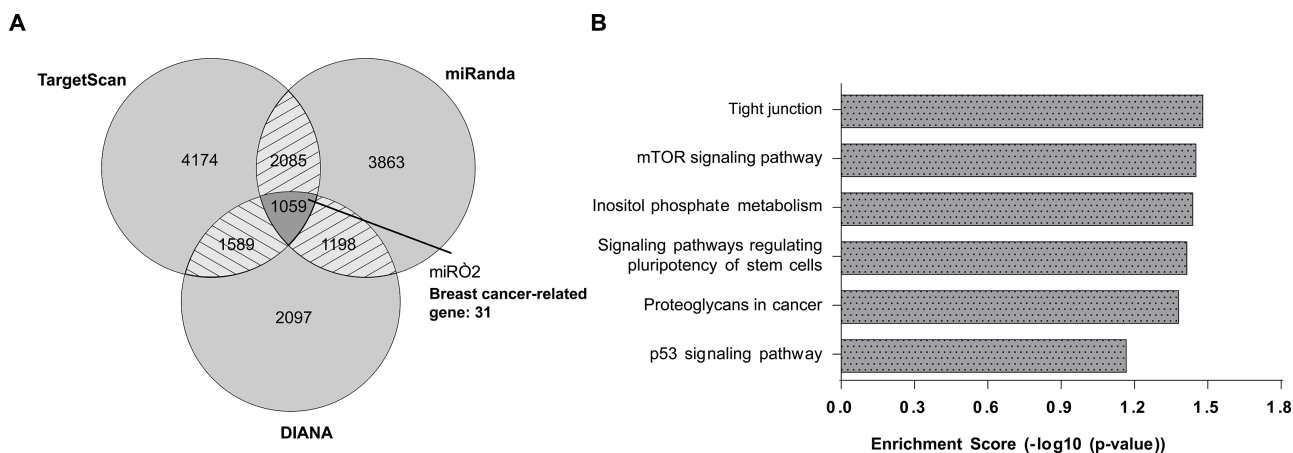


Figure 5. Possible target genes of miR-548m analysis. (A) miR-548m target genes were predicted using TargetScan release 7.1, DIANA-microT-CDS database and microRNA.org (miRanda) database, and the lists of predicted target genes were merged. Subsequently, those predicted target genes were adopted into the miR-Ontology 2 (miR02) database to identify the potential targets within breast cancer-associated classification. (B) Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis of predicted miR-548m target genes within the Database for Annotation, Visualization and Integrated Discovery (DAVID) tools.

the mRNA and protein levels following introduction of miR548m mimic (Fig. 6A). The predicted binding sites of miR-548m in AHR was identified using TargetScan. The miRNA:mRNA alignment analysis showed that the 3'-UTR of AHR contains one putative binding site for miR-548m located from nucleotide position (np) 4,089 to np 4,096, 5'-UACCUUUA-3' (Fig. 6B). To validate the 3'-UTR of AHR as a direct target of miR-548m, dual-luciferase assays were performed. Overexpression of miR-548m significantly reduced the luciferase activity of the reporter vector containing the 3'-UTR of AHR, by 27% and 23% in MDA-MB-231 and MCF-7, respectively, indicating the putative binding sites were functional in both cells and confirming AHR as a direct target of miR-548m (Fig. 6C).

DISCUSSION

Functional identification of miRNAs and their target genes is crucial in understanding their role in cancer progression. The role of miRNAs in breast cancer has drawn much interest due to their critical roles in cancer development including initiation and progression, cell proliferation, apoptosis, differentiation, invasion, and metastasis. In the present study, we identified that miR-548m regulates EMT through inhibiting migration and invasion in breast cancer cell lines. Data mining from the NCBI GEO database suggests the possible role of miR-548m as a tumor-suppressive miRNA, although the variation could be attributed to miRNA-specific function in different breast cancer histological group and subtype. There were also very low available numbers of normal tissues to be compared with tumor tissues in these data sets, affecting the differential expression and proper breast tumor

miR-548m-related analyses. Interestingly, in the GSE80038 data set, which consists of triple-negative breast cancer (TNBC) subtypes, expression of miR-548m was found to be significantly downregulated in this subtype of breast cancer with lung metastasis and primary breast tumor, compared to normal adjacent breast tissue ($p < 0.05$). In parallel to our in vitro work in the TNBC cell line MDA-MB-231, overexpression of miR-548m was able to reduce the expression of several EMT markers including MMP9, SNAI1, and ZEB2, as well as inhibit migration and invasion of the cells. This further supports the role of miR-548m as a tumor suppressor miRNA in breast cancer, especially TNBC subtypes. Although in this study we were unable to detect the in vitro expression of miR-548m, this miRNA has been shown to be expressed in the same cell lines in other studies^{27,28,37}. This discrepancy might be attributed by the phenotypic and genotypic variation in the cell line over a certain period of time as well as heterogeneity caused by genetic drifts in cultures at a single point in time³⁸.

Nevertheless, the introduction of miR-548m mimics in MDA-MB-231 and MCF-7 resulted in increased expression of E-cadherin and downregulation of several EMT markers. Having identified the role of miR-548m in reducing breast cancer cell migration and invasion while having no effect on cell proliferation, we searched possible target genes that participated in the following processes. KEGG pathway analysis demonstrated that the putative target genes of miR-548m are enriched in several pathways, including the tight junction and mTOR signaling pathways, which are both involved in regulating migration and invasion in cancer^{39,40}. AHR was then further identified as a direct target of miR-548m. Taken together, our study

Table 3. List of Selected Putative miR-548m Target Genes Related to Breast Cancer

Gene Symbol	Description	miTG score*	Cumulative Weighted Score†	mirSVR Score‡	Pathobiological Processes Involved
PTE	Phosphatase and tensin homolog	0.9986	-0.29	-0.9921	Breast tumorigenesis and tumor progression
CALCR	Calcitonin receptor	0.9487	-0.15	-1.0759	Migration, metastasis
RRP1B	Ribosomal RNA processing 1B	0.9452	-0.26	-1.0334	Polymorphism, gene for BRCA progression
DNASE1	Deoxyribonuclease 1	0.9222	-0.62	-1.1741	Cell death
FCGR2A	Fc fragment of IgG, low affinity IIa, receptor (CD32)	0.9219	-0.20	-1.1450	Polymorphism
TFRC	Transferrin receptor	0.9181	-0.63	-0.5357	Iron metabolisms
GADD45A	Growth arrest and DNA damage-inducible, alpha	0.9098	-0.24	-1.0404	Stress response gene
PPP1CB	Protein phosphatase 1, catalytic subunit, beta isozyme	0.8792	-0.12	-1.0589	Cell morphology
LATS2	Large tumor suppressor kinase 2	0.8650	-0.19	-0.4257	Cell death and differentiation
NUF2	NUF2, NDC80 kinetochore complex component	0.8472	-0.14	-1.0979	Tumor growth and apoptosis
ATF1	Activating transcription factor 1	0.8459	-0.38	-0.9174	Associated with BRCA1
TGFBR1	Transforming growth factor, beta receptor 1	0.8438	-0.21	-0.1888	Metastasis and invasiveness
KIF2A	Kinesin heavy chain member 2A	0.8019	-0.19	-1.2142	Proliferation and cell cycle
TNKS2	Tankyrase	0.7955	-0.15	-0.7630	Cell growth and invasiveness
TNFRSF10B	Tumor necrosis factor receptor superfamily, member 10b	0.7763	-0.20	-0.1971	Cell death and differentiation
IGF1R	Insulin-like growth factor 1 receptor	0.7714	-0.24	-0.1377	Survival, cell growth and migration
GNB3	Guanine nucleotide binding protein, beta polypeptide 3	0.7681	-0.26	-0.8943	Polymorphism
KLF11	Kruppel-like factor 11	0.7411	-0.26	-0.9554	EMT, invasion and metastasis
MCM6	Minichromosome maintenance complex component 6	0.7198	-0.20	-0.8081	Independent predictor of survival
RRAS2	Related RAS viral (v-ras) oncogene homolog 2	0.7095	-0.33	-0.3065	Malignant transformation
AHR	Aryl hydrocarbon receptor	0.6780	-0.23	-0.8462	EMT, invasion and invasive potential
STAT1	Signal transducer and activator of transcription 1	0.6461	-0.14	-0.8471	Pro- or antiapoptotic signaling molecule
NCOA3	Nuclear receptor coactivator 3	0.6364	-0.12	-1.0889	Cancer initiation and progression, drug resistance
MBL2	Mannose-binding lectin (protein C) 2	0.6296	-0.25	-0.5422	Polymorphisms, key genes of the innate immunity
CDK4	Cyclin-dependent kinase 4	0.6150	-0.32	-0.9499	Cell proliferation
TNFRSF10D	Tumor necrosis factor receptor superfamily 10d	0.6144	-0.10	-0.4242	Cell apoptosis
ZWINT	ZW10 interacting kinetochore protein	0.6108	-0.12	-0.5552	Cell growth
FCGR2B	Fc fragment of igg, low affinity IIb, receptor	0.6090	-0.30	-0.2650	Polymorphisms and chemotherapy
TBP	TATA box binding protein	0.6038	-0.28	-0.5106	Cellular transformation
DIRAS3	DIRAS family, GTP-binding RAS-like 3	0.6037	-0.41	-1.1800	Tumor suppressor
DPYD	Dihydropyrimidine dehydrogenase	0.6036	-0.24	-1.3142	Biomarker of toxicity

*Prediction score from DIANA-microT v5.0: the higher the score, the higher the probability of targeting with high precision; value more than 0.6. †Score from TargetScan 7.2: cumulative weighted context++ score cutoff level less than -0.1. ‡Score from miRANDA (microRNA.org): predicted targets with less than -0.1 score refers as "good" score.

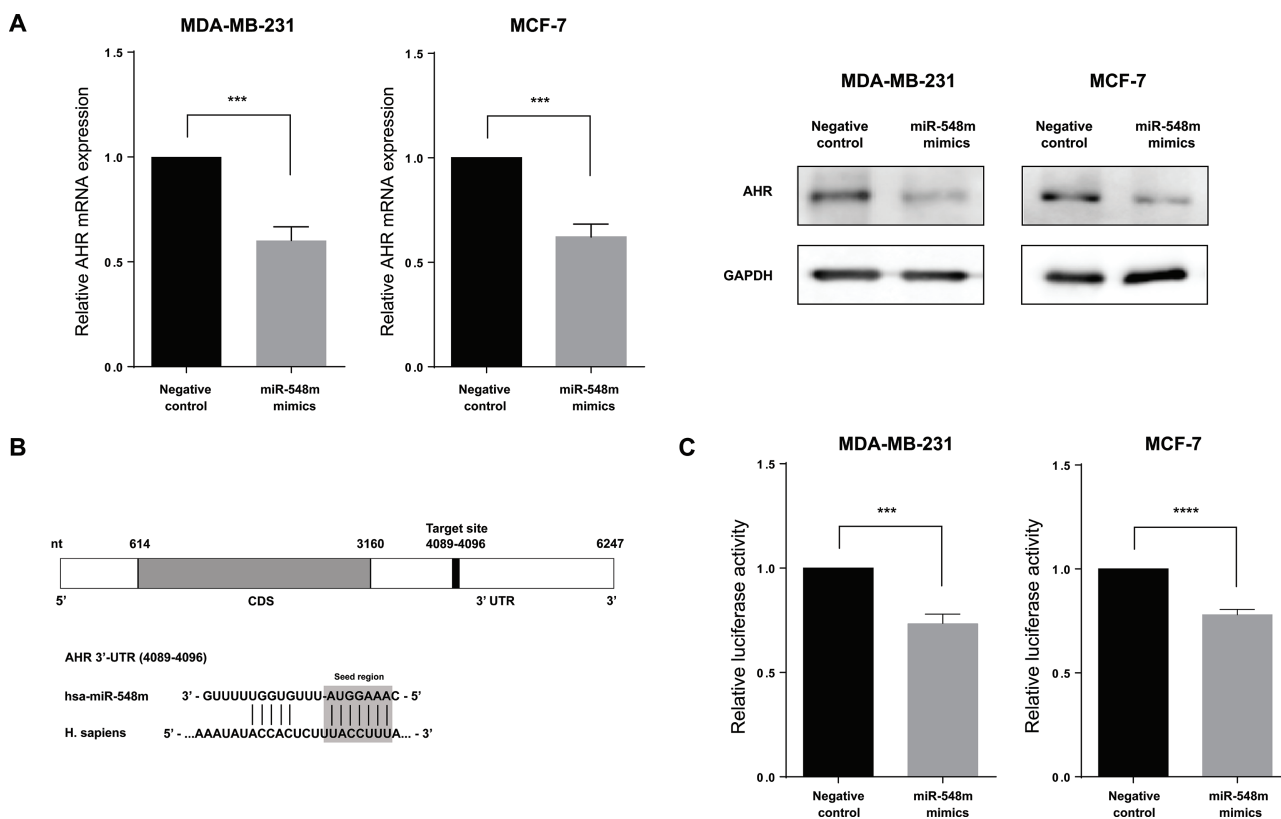


Figure 6. Validation of aryl hydrocarbon receptor (AHR) as a direct target of miR-548m. (A) MDA-MB-231 and MCF-7 cell lines were transfected with miR-548m mimics and its negative control and then subjected to RT-qPCR and Western blot analysis for the detection of AHR mRNA and protein expression, respectively. GAPDH was used as an internal control, respectively, to normalize the results to 1. (B) The predicted binding site of miR-548m and 3'-UTR of AHR as well as the potential binding sites of AHR with the seed region of miR-548m. (C) The luciferase reporter vector containing AHR 3'-UTR was cotransfected with miR-548m mimics or negative control into MDA-MB-231 and MCF-7 cells. The firefly luciferase activity was measured and normalized to *Renilla* luciferase activity. GAPDH was used as an internal control, respectively, to normalize the results. Graphs are displayed as mean \pm SEM, $n = 3$. *** $p < 0.001$; **** $p < 0.0001$.

identified miR-548m as a tumor suppressor miRNA in breast cancer cell lines, and its inhibitory effect on migration and invasion can be mediated through AHR.

During EMT, polarized epithelial cells undergo multiple biochemical and morphological changes, altering their phenotype from epithelial to mesenchymal. EMT processes are marked by the loss of epithelial markers such as E-cadherin and acquisition of mesenchymal markers such as vimentin. Following overexpression of miR-548m, we observed an increased expression of E-cadherin, while no significant changes were seen in vimentin expression in both MDA-MB-231 and MCF-7 cells. Indeed, in partial mesenchymal-epithelial transition (MET), studies showed that upregulation of epithelial markers were seen in metastatic tumor samples, whereas expression of mesenchymal markers such as vimentin was mostly unaltered⁴¹. However, the increase in E-cadherin expression alone could be insufficient to induce epithelial-like morphological transition in both cell lines. In our studies,

downregulation of ZEB2, but not ZEB1, in both breast cancer cells upon transfection of miR-548m mimics might lead to the increase in E-cadherin expression. This is consistent with earlier reports by Comijn et al., which suggested direct downregulation of E-cadherin by ZEB2 alone⁴². Other genes frequently associated with EMT in breast cancer are SNAI1 and SNAI2, from the *Snail* family of transcription factors. Expression of SNAI1, but not SNAI2, was downregulated in MDA-MB-231 following overexpression with miR-548m. By contrast, in MCF-7 cells, the expression of SNAI2 but not SNAI1 was downregulated. This disparity could be attributed to intrinsic regulatory network modulating SNAI1 and SNAI2, despite both being E-cadherin repressors. This is especially the case of the miR-548m/AHR axis, since activation of AHR has been shown to induce the transcriptional activation of its direct target, SNAI2⁴³. In addition, protein expression analysis could be done to detect proteins of EMT markers in breast cancer cells.

EMT is also largely discussed as a promoter of metastasis, enabling motility and invasion of epithelial cells in cancer progression. In this study, miR-548m significantly reduces migration and invasion of breast cancer cells with no effect on cell proliferation. However, there have been a limited number of researches that directly address the biological role of miR-548m specifically. Lwin et al. showed the biological function of miR-548m in lymphoma progression, where miR-548m was found acting as a tumor suppressor by directly targeting HDAC6 and formed a feed-forward loop with c-Myc, which later contributed to stroma-mediated c-Myc activation and miR-548m downregulation in a lymphoma microenvironment⁴⁴. HDAC6 is related to several cell functions, including tubulin stabilization, cell motility, and cell cycle progression⁴⁵. Interestingly, recent studies have shown that miR-548 family members are generally involved in regulating cell proliferation, apoptosis, migration, and invasion, where they act as tumor suppressor miRNA^{20,22,46} or oncogenic miRNA^{21,47} in different types of cancers. These suggested that miR-548 family members might take on different functions in different types of cancers. The differential effects could have been contributed by the ability of a particular miRNA to target multiple mRNAs, thus consequently different pathways and functions in tumorigenesis^{48–51}. Indeed, in a screening for EMT modulators in bladder cancer, miR-548m was identified as an inducer of mesenchymal phenotype⁵². This is in contrast with other conserved miRNA families, such as let-7, that have an exact “seed sequence” that leads to

similar targets, functions, and molecular mechanisms⁵³. Taken together, these results indicate that the inhibitory effect of miR-548m on migration and invasion in breast cancer cells might be associated with EMT.

A number of studies reported the involvement of miRNAs to promote or suppress tumor metastasis^{54,55}, where some miRNAs are consistently downregulated or eliminated^{56,57}, whereas other miRNAs are significantly accumulated in metastatic breast cancer⁵⁸. miRNAs perform biological functions by directly binding to the 3'-UTR of mRNAs and by impairing protein translation⁵⁹. Screening putative target genes via bioinformatic prediction demonstrates that the miR-548m target sequence within the 3'-UTR of several gene mRNAs is closely related to cell migration, cell invasion, and EMT in breast cancer. We established that AHR mRNA and protein expression were significantly downregulated upon the introduction of miR-548m in the MDA-MB-231 and MCF-7 cell lines and validated AHR as a direct target gene of miR-548m.

AHR is a member of the basic helix-loop-helix-ARNT-SIM (bHLH-PAS) subgroup of the bHLH superfamily of transcription factors⁶⁰. AHR is known to be involved in transforming growth factor- β (TGF- β), receptor tyrosine kinases, Notch, and Wnt signaling pathways, which associates AHR with cell proliferation, differentiation, and migration⁶¹. AHR is a ligand-activated transcription factor that could be mainly induced by aromatic hydrocarbons such as 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and benzo(a)pyrene (BaP)⁶². Even without the presence of exogenous or environmental

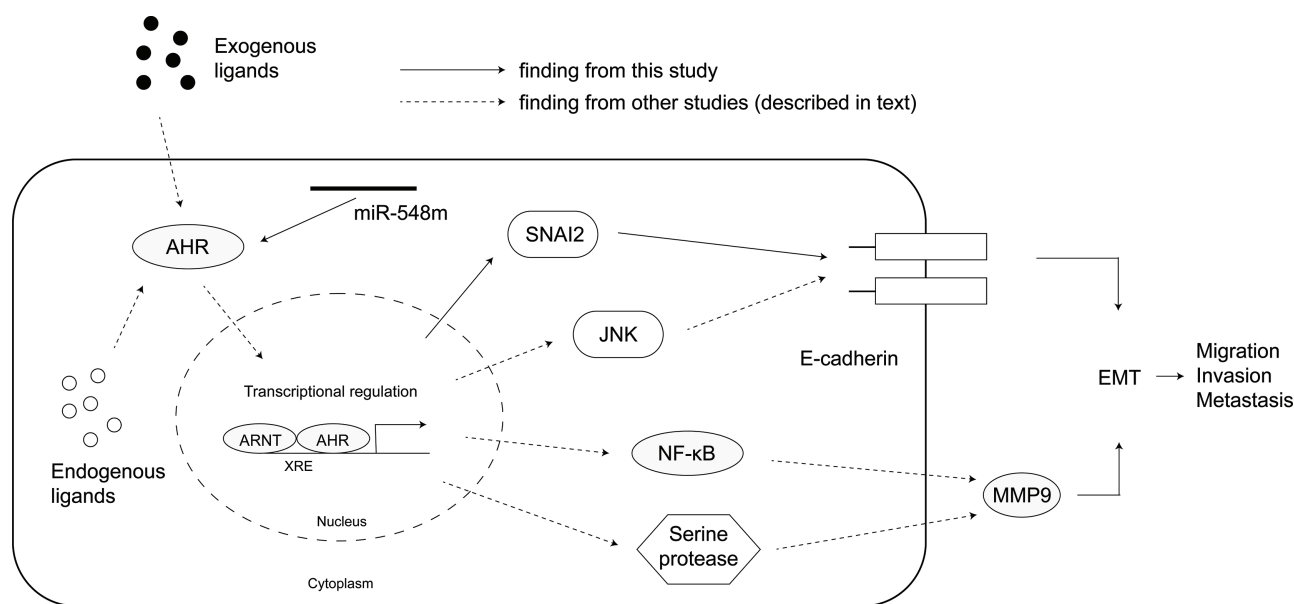


Figure 7. Potential role of miR-548m in regulating EMT in breast cancer. The transcriptional upregulation of E-cadherin may either be mediated by downregulation of AHR-dependent activation of SNAI2 and/or JNK. Downregulation of matrix metalloproteinase 9 (MMP9) may be mediated by serine protease and/or NF- κ B, which are also targets of the AHR pathway.

ligands such as TCDD and BaP in our study, which is commonly known to increase AHR activities⁶³, excessive production of endogenous ligands in breast cancer cells is also known to contribute to the overexpression and constitutive activation of AHR^{64,65}. Several studies have proposed the correlation between endogenous ligand-activated AHR and breast cancer progression including growth and cell motility^{64,66}. Activation of the AHR pathway in response to TCDD has been shown to modulate the expression and activity of MMP9 in prostate cancer⁶⁷ and melanoma cells⁶⁸, while silencing of AHR expression decreased cell invasion and MMP9 expression in urothelial cancer⁶⁹. In breast cancer, AHR expression in human breast cancer tissues was correlated with expression of MMP genes, including MMP1, MMP2, and MMP9, and is involved in signaling pathways related to cell motility⁷⁰. This supported our findings whereby overexpression of miR-548m downregulates AHR and MMP9 in both MDA-MB-231 and MCF-7 cells, and thus suggesting that AHR signaling might regulate EMT through MMP9 in our cell lines. MMPs are predominantly expressed by stromal cells and has the ability to degrade and modify the ECM components and facilitate detachment of epithelial cells from the surrounding tissue⁷¹. Alteration of serine proteases and NF- κ B signaling, directly associated with the AHR pathway, might be able to modify the proteolytic activation of MMP9⁷².

AHR activation has also been shown to potentially trigger the breakdown or downregulation of E-cadherin^{73,74}. This transcriptional inhibition might be mediated by AHR-dependent activation of transcriptional factor SNAI2 and/or activation of JNK. In breast cancer cells, activation of AHR induces the transcriptional activation of its direct target, SNAI2⁴³. Constitutive expression and activation of AHR have been found to lead the activation of JNK genes, which led to loss of cell–cell adhesion and increased migration in MCF-7 cells⁷⁵. This supports our findings in which SNAI2 was downregulated in MCF-7 cells, while E-cadherin expression was upregulated in both MDA-MB-231 and MCF-7 upon miR-548m overexpression. Although we did not examine whether AHR is a substantial target of miR-548m in regulating the migration and invasion of breast cancer cell lines, previous reports have shown the inhibition of breast cancer cell line migration, invasion, and metastasis following AHR knockdown⁶⁴. Thus, decrease in AHR expression exerted by miR-548m could inhibit EMT by upregulating E-cadherin, leading to reduced cell migration and invasion of breast cancer. It is clearly shown that regulated activities of miRNAs are able to alter or change the target gene expression program to control metastatic processes in breast cancer cells.

Taken together, this study identified miR-548m to be a potential tumor suppressor miRNA in breast cancer by

inhibiting cell invasion and migration. We have shown that AHR is a direct downstream target of miR-548m in breast cancer cells, and other studies have correlated the AHR pathway with migration and invasion^{64,70,74,75} (Fig. 7). To the best of our knowledge, published studies in breast cancer^{27,28} only detect miR-548m expression through miRNA array analysis without looking at their potential functions and mechanisms. Thus, our current study provides a novel insight into molecular mechanisms of miR-548m in regulating breast cancer progression and new understanding into potential therapeutic strategies and clinical classification markers for metastatic breast cancer.

ACKNOWLEDGMENTS: *This study was supported by the University of Malaya Research Grant (RG360-15AFR). Laboratory works were performed at the Translational Core Laboratory, Faculty of Medicine, University of Malaya. The authors declare no conflicts of interest.*

REFERENCES

1. de Sanjose S, Tsu VD. Prevention of cervical and breast cancer mortality in low- and middle-income countries: A window of opportunity. *Int J Womens Health* 2019;11:381–6.
2. Siegel RL, Miller KD, Jemal A. Cancer statistics, 2020. *CA Cancer J Clin*. 2020;70(1):7–30.
3. Polyak K. Heterogeneity in breast cancer. *J Clin Invest*. 2011;121(10):3786–8.
4. McGuire A, Brown JA, Kerin MJ. Metastatic breast cancer: The potential of miRNA for diagnosis and treatment monitoring. *Cancer Metastasis Rev*. 2015;34(1):145–55.
5. Martin TA, Ye L, Sanders AJ, Lane J, Jiang WG. Cancer invasion and metastasis: Molecular and cellular perspective. *Madame Curie Bioscience Database [Internet]: Landes Bioscience*; 2013.
6. Lamouille S, Xu J, Derynck R. Molecular mechanisms of epithelial–mesenchymal transition. *Nat Rev Mol Cell Biol*. 2014;15(3):178–96.
7. Liu H. MicroRNAs in breast cancer initiation and progression. *Cell Mol Life Sci*. 2012;69(21):3587–99.
8. Pileczki V, Cojocneanu-Petric R, Maralani M, Neagoe IB, Sandulescu R. MicroRNAs as regulators of apoptosis mechanisms in cancer. *Clujul Medical* 2016;89(1):50.
9. Bueno MJ, Malumbres M. MicroRNAs and the cell cycle. *Biochim Biophys Acta* 2011;1812(5):592–601.
10. Yu Z, Tozeren A, Pestell RG. Function of miRNAs in tumor cell proliferation. In Alahari, S. (ed.), *MicroRNA in cancer*. Springer; 2013. p 13–27.
11. Ding XM. MicroRNAs: Regulators of cancer metastasis and epithelial–mesenchymal transition (EMT). *Chin J Cancer* 2014;33(3):140–7.
12. Jansson MD, Lund AH. MicroRNA and cancer. *Mol Oncol*. 2012;6(6):590–610.
13. Garzon R, Marcucci G, Croce CM. Targeting microRNAs in cancer: Rationale, strategies and challenges. *Nat Rev Drug Discov*. 2010;9(10):775–89.
14. Zhang J, Liu D, Feng Z, Mao J, Zhang C, Lu Y, Li J, Zhang Q, Li Q, Li L. MicroRNA-138 modulates metastasis and EMT in breast cancer cells by targeting vimentin. *Biomed Pharmacother*. 2016;77:135–41.
15. Stinson S, Lackner MR, Adai AT, Yu N, Kim HJ, O'Brien C, Spoeck J, Jhunjhunwala S, Boyd Z, Januario T, Newman RJ, Yue P, Bourgon R, Modrusan Z, Stern HM, Warming S,

- de Sauvage FJ, Amler L, Yeh RF, Dornan D. miR-221/222 targeting of trichorhinophalangeal 1 (TRPS1) promotes epithelial-to-mesenchymal transition in breast cancer. *Sci Signal* 2011;4(186).
16. Gwak JM, Kim HJ, Kim EJ, Chung YR, Yun S, Seo AN, Lee HJ, Park SY. MicroRNA-9 is associated with epithelial-mesenchymal transition, breast cancer stem cell phenotype, and tumor progression in breast cancer. *Breast Cancer Res Treat.* 2014;147(1):39-49.
 17. Han M, Wang Y, Liu M, Bi X, Bao J, Zeng N, Zhu Z, Mo Z, Wu C, Chen X. MiR-21 regulates epithelial-mesenchymal transition phenotype and hypoxia-inducible factor-1 α expression in third-sphere forming breast cancer stem cell-like cells. *Cancer Sci.* 2012;103(6):1058-64.
 18. Liang T, Guo L, Liu C. Genome-wide analysis of mir-548 gene family reveals evolution and functional implications. *J Biomed Biotechnol.* 2012;2012:679563.
 19. Lu J, Zhang M, Yang X, Cui T, Dai J. MicroRNA-548c-3p inhibits T98G glioma cell proliferation and migration by downregulating c-Myb. *Oncol Lett.* 2017;13(5):3866-72.
 20. Liu C, Yang H, Xu Z, Li D, Zhou M, Xiao K, Shi Z, Zhu L, Yang L, Zhou R. microRNA-548l is involved in the migration and invasion of non-small cell lung cancer by targeting the AKT1 signaling pathway. *J Cancer Res Clin Oncol.* 2015;141:431-41.
 21. Zhan Y, Liang X, Li L, Wang B, Ding F, Li Y, Wang X, Zhan Q, Liu Z. MicroRNA-548j functions as a metastasis promoter in human breast cancer by targeting Tensin1. *Mol Oncol.* 2016;10(6):838-49.
 22. Shi Y, Qiu M, Wu Y, Hai L. MiR-548-3p functions as an anti-oncogenic regulator in breast cancer. *Biomed Pharmacother.* 2015;75:111-16.
 23. Sha M, Huang X, Yin Q. MiR-548b-3p inhibits proliferation and migration of breast cancer cells by targeting MDM2. *Eur Rev Med Pharmacol Sci.* 2020;24(6):3105-12.
 24. Guo X, Lee S, Cao P. The inhibitive effect of sh-HIF1A-AS2 on the proliferation, invasion, and pathological damage of breast cancer via targeting miR-548c-3p through regulating HIF-1 α /VEGF pathway in vitro and vivo. *Onco Targets Ther.* 2019;12:825.
 25. Liang Y, Song X, Li Y, Su P, Han D, Ma T, Guo R, Chen B, Zhao W, Sang Y, Zhang N, Li X, Zhang H, Liu Y, Duan Y, Wang L, Yang Q. circKDM4C suppresses tumor progression and attenuates doxorubicin resistance by regulating miR-548p/PBLD axis in breast cancer. *Oncogene* 2019;38(42):6850-66.
 26. Said NA, Gould CM, Lackovic K, Simpson KJ, Williams ED. Whole-genome multiparametric screening to identify modulators of epithelial-to-mesenchymal transition. *Assay Drug Dev Technol.* 2014;12(7):385-94.
 27. Shah MY, Pan X, Fix LN, Farwell MA, Zhang B. 5-fluorouracil drug alters the microRNA expression profiles in MCF-7 breast cancer cells. *J Cell Physiol.* 2011;226(7):1868-78.
 28. Fix LN, Shah M, Efferth T, Farwell MA, Zhang B. MicroRNA expression profile of MCF-7 human breast cancer cells and the effect of green tea polyphenon-60. *Cancer Genomics Proteomics* 2010;7(5):261-77.
 29. Riffo-Campos Á, Riquelme I, Brebi-Mieville P. Tools for sequence-based miRNA target prediction: What to choose? *Int J Mol Sci.* 2016;17(12):1987.
 30. Wang J, Song C, Tang H, Zhang C, Tang J, Li X, Chen B, Xie X. miR-629-3p may serve as a novel biomarker and potential therapeutic target for lung metastases of triple-negative breast cancer. *Breast Cancer Res.* 2017;19(1):72.
 31. Tanic M, Andrés E, Rodriguez-Pinilla SM, Marquez-Rodas I, Cebollero-Presmanes M, Fernandez V, Osorio A, Benítez J, Martínez-Delgado B. MicroRNA-based molecular classification of non-BRCA1/2 hereditary breast tumours. *Br J Cancer* 2013;109(10):2724-34.
 32. Lee CH, Kuo WH, Lin CC, Oyang YJ, Huang HC, Juan HF. MicroRNA-regulated protein-protein interaction networks and their functions in breast cancer. *Int J Mol Sci.* 2013;14(6):11560-606.
 33. Zhao XG, Hu JY, Tang J, Yi W, Zhang MY, Deng R, Mai SJ, Weng NQ, Wang RQ, Liu J, Zhang HZ, He JH, Wang HY. miR-665 expression predicts poor survival and promotes tumor metastasis by targeting NR4A3 in breast cancer. *Cell Death Dis.* 2019;10(7):479.
 34. Avery-Kiejda KA, Braye SG, Mathe A, Forbes JF, Scott RJ. Decreased expression of key tumour suppressor microRNAs is associated with lymph node metastases in triple negative breast cancer. *BMC Cancer* 2014;14:51.
 35. Peña-Chilet M, Martínez MT, Pérez-Fidalgo JA, Peiró-Chova L, Oltra SS, Tormo E, Alonso-Yuste E, Martínez-Delgado B, Eroles P, Climent J, Burgués O, Ferrer-Lozano J, Bosch A, Lluch A, Ribas G. MicroRNA profile in very young women with breast cancer. *BMC Cancer* 2014;14:529.
 36. Feliciano A, Castellvi J, Artero-Castro A, Leal JA, Romagosa C, Hernández-Losa J, Peg V, Fabra A, Vidal F, Kondoh H, Ramón YCS, Leonart ME. miR-125b acts as a tumor suppressor in breast tumorigenesis via its novel direct targets ENPEP, CK2- α , CCNJ, and MEGF9. *PLoS One* 2013;8(10):e76247.
 37. Bond DR, Kahl R, Brzozowski JS, Jankowski H, Naudin C, Pariyar M, Avery-Kiejda KA, Scarlett CJ, Boucheix C, Muller WJ, Ashman LK, Cairns MJ, Roselli S, Weidenhofer J. Tetraspanin CD9 is regulated by miR-518f-5p and functions in breast cell migration and in vivo tumor growth. *Cancers (Basel)* 2020;12(4):795.
 38. Kaur G, Dufour JM. Cell lines: Valuable tools or useless artifacts. *Spermatogenesis* 2012;2(1):1-5.
 39. Salvador E, Burek M, Förster CY. Tight junctions and the tumor microenvironment. *Curr Pathobiol Rep.* 2016;4(3):135-45.
 40. Zhou H, Huang S. Role of mTOR signaling in tumor cell motility, invasion and metastasis. *Curr Protein Pept Sci.* 2011;12(1):30-42.
 41. Chao Y, Wu Q, Acquafondata M, Dhir R, Wells A. Partial mesenchymal to epithelial reverting transition in breast and prostate cancer metastases. *Cancer Microenviron.* 2012;5(1):19-28.
 42. Comijn J, Berx G, Vermassen P, Verschueren K, van Grunsven L, Bruyneel E, Mareel M, Huylebroeck D, Van Roy F. The two-handed E box binding zinc finger protein SIP1 downregulates E-cadherin and induces invasion. *Mol Cell* 2001;7(6):1267-78.
 43. Ikuta T, Kawajiri K. Zinc finger transcription factor Slug is a novel target gene of aryl hydrocarbon receptor. *Exp Cell Res.* 2006;312(18):3585-94.
 44. Lwin T, Zhao X, Cheng F, Zhang X, Huang A, Shah B, Zhang Y, Moscinski LC, Choi YS, Kozikowski AP, Bradner JE, Dalton WS, Sotomayor E, Tao J. A microenvironment-mediated c-Myc/miR-548m/HDAC6 amplification loop in non-Hodgkin B cell lymphomas. *J Clin Invest.* 2013;123(11):4612.
 45. Li T, Zhang C, Hassan S, Liu X, Song F, Chen K, Zhang W, Yang J. Histone deacetylase 6 in cancer. *J Hematol Oncol.* 2018;11(1):1-10.

46. Zhu S, He C, Deng S, Li X, Cui S, Zeng Z, Liu M, Zhao S, Chen J, Jin Y, Chen H, Deng S, Liu Y, Wang C, Zhao G. MiR-548an, transcriptionally downregulated by HIF1 α /HDAC1, suppresses tumorigenesis of pancreatic cancer by targeting Vimentin expression. *Mol Cancer Ther*. 2016;15(9):2209–19.
47. Song Q, Song J, Wang Q, Ma Y, Sun N, Ma J, Chen Q, Xia G, Huo Y, Yang L, Li B. miR-548d-3p/TP53BP2 axis regulates the proliferation and apoptosis of breast cancer cells. *Cancer Med*. 2016;5(2):315–24.
48. Humphreys KJ, McKinnon RA, Michael MZ. miR-18a inhibits CDC42 and plays a tumour suppressor role in colorectal cancer cells. *PLoS One* 2014;9(11):e112288.
49. Liang C, Zhang X, Wang HM, Liu XM, Zhang XJ, Zheng B, Qian GR, Ma ZL. MicroRNA-18a-5p functions as an oncogene by directly targeting IRF2 in lung cancer. *Cell Death Dis*. 2017;8(5):e2764.
50. Zaman MS, Thamminana S, Shahryari V, Chiyomaru T, Deng G, Saini S, Majid S, Fukuhara S, Chang I, Arora S, Hirata H, Ueno K, Singh K, Tanaka Y, Dahiya R. Inhibition of PTEN gene expression by oncogenic miR-23b-3p in renal cancer. *PLoS One* 2012;7(11):e50203.
51. Majid S, Dar AA, Saini S, Deng G, Chang I, Greene K, Tanaka Y, Dahiya R, Yamamura S. MicroRNA-23b functions as a tumor suppressor by regulating Zeb1 in bladder cancer. *PLoS One* 2013;8(7):e67686.
52. Said NA, Williams ED. Growth factors in induction of epithelial–mesenchymal transition and metastasis. *Cells Tissues Organs* 2011;193(1–2):85–97.
53. Lee H, Han S, Kwon CS, Lee D. Biogenesis and regulation of the let-7 miRNAs and their functional implications. *Protein Cell* 2016;7(2):100–13.
54. Wang H, Tan Z, Hu H, Liu H, Wu T, Zheng C, Wang X, Luo Z, Wang J, Liu S, Lu Z, Tu J. microRNA-21 promotes breast cancer proliferation and metastasis by targeting LZTFL1. *BMC Cancer* 2019;19(1):738.
55. Tavazoie SF, Alarcón C, Oskarsson T, Padua D, Wang Q, Bos PD, Gerald WL, Massagué J. Endogenous human microRNAs that suppress breast cancer metastasis. *Nature* 2008;451(7175):147–52.
56. Li J, Lai Y, Ma J, Liu Y, Bi J, Zhang L, Chen L, Yao C, Lv W, Chang G, Wang S, Ouyang M, Wang W. miR-17-5p suppresses cell proliferation and invasion by targeting ETV1 in triple-negative breast cancer. *BMC Cancer* 2017;17(1):745.
57. Long J, Ou C, Xia H, Zhu Y, Liu D. MiR-503 inhibited cell proliferation of human breast cancer cells by suppressing CCND1 expression. *Tumour Biol*. 2015;36(11):8697–702.
58. Korpál M, Ell BJ, Buffa FM, Ibrahim T, Blanco MA, Celià-Terrassa T, Mercatali L, Khan Z, Goodarzi H, Hua Y, Wei Y, Hu G, Garcia BA, Ragoussis J, Amadori D, Harris AL, Kang Y. Direct targeting of Sec23a by miR-200s influences cancer cell secretome and promotes metastatic colonization. *Nat Med*. 2011;17(9):1101.
59. Friedman RC, Farh KK, Burge CB, Bartel DP. Most mammalian mRNAs are conserved targets of microRNAs. *Genome Res*. 2009;19(1):92–105.
60. Bersten DC, Sullivan AE, Peet DJ, Whitelaw ML. bHLH–PAS proteins in cancer. *Nat Rev Cancer* 2013;13(12):827.
61. Roman AC, Carvajal-Gonzalez JM, Merino JM, Mulero-Navarro S, Fernández-Salguero PM. The Aryl hydrocarbon receptor in the crossroad of signalling networks with therapeutic value. *Pharmacol Ther*. 2018;185:50–63.
62. Safe S, Lee SO, Jin UH. Role of the aryl hydrocarbon receptor in carcinogenesis and potential as a drug target. *Toxicol Sci*. 2013;135(1):1–16.
63. Kolluri SK, Jin UH, Safe S. Role of the aryl hydrocarbon receptor in carcinogenesis and potential as an anti-cancer drug target. *Arch Toxicol*. 2017;91(7):2497–513.
64. Goode GD, Ballard BR, Manning HC, Freeman ML, Kang Y, Eltom SE. Knockdown of aberrantly upregulated aryl hydrocarbon receptor reduces tumor growth and metastasis of MDA-MB-231 human breast cancer cell line. *Int J Cancer* 2013;133(12):2769–80.
65. Li ZD, Wang K, Yang XW, Zhuang ZG, Wang JJ, Tong XW. Expression of aryl hydrocarbon receptor in relation to p53 status and clinicopathological parameters in breast cancer. *Int J Clin Exp Pathol*. 2014;7(11):7931.
66. Novikov O, Wang Z, Stanford EA, Parks AJ, Ramirez-Cardenas A, Landesman E, Lakloulou I, Sarita-Reyes C, Gusenleitner D, Li A, Monti S, Manteiga S, Lee K, Sherr DH. An aryl hydrocarbon receptor-mediated amplification loop that enforces cell migration in ER-/PR-/Her2-human breast cancer cells. *Mol Pharmacol*. 2016;90(5):674–88.
67. Haque M, Francis J, Sehgal I. Aryl hydrocarbon exposure induces expression of MMP-9 in human prostate cancer cell lines. *Cancer Lett*. 2005;225(1):159–66.
68. Villano C, Murphy K, Akintobi A, White L. 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin (TCDD) induces matrix metalloproteinase (MMP) expression and invasion in A2058 melanoma cells. *Toxicol Appl Pharmacol*. 2006;210(3):212–24.
69. Ishida M, Mikami S, Kikuchi E, Kosaka T, Miyajima A, Nakagawa K, Mukai M, Okada Y, Oya M. Activation of the aryl hydrocarbon receptor pathway enhances cancer cell invasion by upregulating the MMP expression and is associated with poor prognosis in upper urinary tract urothelial cancer. *Carcinogenesis* 2009;31(2):287–95.
70. Vacher S, Castagnet P, Chemlali W, Lallemand F, Meseure D, Pocard M, Bieche I, Perrot-Applanat M. High AHR expression in breast tumors correlates with expression of genes from several signaling pathways namely inflammation and endogenous tryptophan metabolism. *PLoS One* 2018;13(1):e0190619.
71. Radisky ES, Radisky DC. Matrix metalloproteinase-induced epithelial–mesenchymal transition in breast cancer. *J Mammary Gland Biol Neoplasia* 2010;15(2):201–12.
72. Kung T, Murphy K, White L. The aryl hydrocarbon receptor (AhR) pathway as a regulatory pathway for cell adhesion and matrix metabolism. *Biochem Pharmacol*. 2009;77(4):536–46.
73. Dietrich C, Kaina B. The aryl hydrocarbon receptor (AhR) in the regulation of cell–cell contact and tumor growth. *Carcinogenesis* 2010;31(8):1319–28.
74. Chen JY, Li CF, Kuo CC, Tsai KK, Hou MF, Hung WC. Cancer/stroma interplay via cyclooxygenase-2 and indoleamine 2,3-dioxygenase promotes breast cancer progression. *Breast Cancer Res*. 2014;16(4):410.
75. Diry M, Tomkiewicz C, Koehle C, Coumoul X, Bock KW, Barouki R, Transy C. Activation of the dioxin/aryl hydrocarbon receptor (AhR) modulates cell plasticity through a JNK-dependent mechanism. *Oncogene* 2006;25(40):5570–4.