

Differential microRNA expression is associated with androgen receptor expression in breast cancer

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Abstract. The androgen receptor (AR) is frequently expressed in breast cancer; however, its prognostic value remains unclear. AR expression in breast cancer has been associated with improved outcomes in estrogen receptor (ER)-positive breast cancer compared with ER-negative disease. Eliminating AR function in breast cancer is critically important for breast cancer progression. However, the mechanism underlying AR regulation remains poorly understood. The study of microRNAs (miRNAs) has provided important insights into the pathogenesis of hormone-dependent cancer. To determine whether miRNAs function in the AR regulation of breast cancer, the present study performed miRNA expression profiling in AR-positive and -negative breast cancer cell lines. A total of 153 miRNAs were differentially expressed in AR-positive compared with AR-negative breast cancer cells; 52 were upregulated and 101 were downregulated. A number of these have been extensively associated with breast cancer cell functions, including proliferation, invasion and drug-resistance. Furthermore, through pathway enrichment analysis, signaling pathways associated with the prediction targets of the miRNAs were characterized, including the vascular endothelial growth factor and mammalian target of rapamycin signaling pathways. In conclusion, the results of the present study indicated that the expression of miRNAs may be involved in the mechanism underlying AR regulation of breast cancer, and may improve understanding of the role of AR in breast cancer.

Introduction

Breast cancer remains a major health problem for women worldwide; accounting for one in three newly diagnosed cases of cancer in women. Targeting the estrogen receptor (ER) has achieved marked progress in the treatment of patients with ER-positive breast cancer. Although the ER is critical for breast cancer carcinogenesis, the most commonly expressed hormone receptor in invasive breast cancer is the androgen receptor (AR), which is expressed in 72.9% of primary breast cancer cases (1). Furthermore, the AR has been characterized in terms of its role as a predictive factor, and the clinical significance of its expression in breast cancer. The AR is inhibitory in breast cancer, counteracting the oncogenic effect of the ER. Due to this crosstalk, AR expression is associated with improved prognosis in ER-positive breast cancer (2,3). However, the prognostic value of AR expression in triple negative breast cancer (TNBC) remains unclear, with certain studies suggesting reduced mortality (4), and others indicating poor prognosis (5). A recent systematic review of 19 separate studies investigated the association between AR expression, overall survival and disease free survival, and revealed that the expression of AR is a marker of good prognosis, with an approximate doubling of overall survival at 3 or 5 years (6). Therefore, further examination of the role of the AR in breast cancer is essential, and may contribute to the development of novel therapies for AR-positive tumors, particularly ER⁻, progesterone receptor (PR)⁻, AR⁺ breast cancer patients who derive limited benefit from current chemotherapeutic strategies.

MicroRNAs (miRNAs) are small non-coding RNAs, ~22 nucleotides in length, which regulate the expression of multiple genes by degrading messenger RNA (mRNA) or interrupting the translation process (7). Aberrant expression of miRNAs contributes to cancer pathogenesis by inducing oncogenes, inhibiting tumor suppressor genes or disrupting important signaling pathways. Dysregulation of miRNAs occurs in breast cancer pathogenesis. Sets of genes have been identified to be dysregulated in breast cancer using miRNA expression profiling studies (8,9). Furthermore, functional studies have confirmed miRNAs as tumor suppressors and oncogenes in breast cancer (10).

miRNAs impact the pathobiology of hormone-regulated cancers. The potential association between AR expression and

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miRNA signature in prostate cancer has been examined. A miRNA profiling study of 6 prostate cancer (PCa) cell lines, 9 prostate cancer xenograft models and 9 prostate carcinoma samples identified a significant link between miRNAs and AR expression (11), and revealed a potential correlation for further investigation. Similarly, in 2007, Shi *et al.* (12) observed differential expression of miR-125b in androgen-dependent and -independent PCa cells, as well as in benign and malignant prostate tissues. This study suggested that androgen-AR signaling may regulate the differential expression of miR-125b. Furthermore, a study in 2011 described 71 miRNAs that influenced the expression levels of AR in human PCa cells, and 13 miRNAs were validated to regulate the long AR 3'untranslated region (UTR) (13). Taken together, these findings indicate a potential link between AR expression and miRNAs. In breast cancer, miRNAs have been studied primarily with regard to ER and human epidermal growth receptor 2. AR-associated miRNAs in breast cancer have been less well investigated.

To reveal the association between miRNAs and AR in breast cancer, miRNA expression profiling was performed in breast cancer cell lines representative of various AR expressions. Further target prediction was conducted on the significantly dysregulated miRNAs. Target genes were classified into different pathways according to their biological functions, as determined by the Gene Ontology (GO) system. Vascular endothelial growth factor (VEGF) and mammalian target of rapamycin (mTOR) signaling pathways were demonstrated to correlate with the significantly dysregulated miRNAs. The results of the present study revealed a correlation between differential miRNA expression and AR expression levels in breast cancer, and described a putative interaction between the AR, VEGF and mTOR signaling pathways. These results may improve understanding of the role of the AR in breast cancer.

Materials and methods

Cell culture. The Hs578T, MDA-MB-231, MCF-7 and SK-BR-3 human breast cancer cell lines were purchased from the American Type Culture Collection (Manassas, VA, USA). Hs578T and MCF-7 cells were cultured in Dulbecco's modified Eagle's medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.), 1% penicillin/streptomycin and 4 mg/ml insulin, whereas MDA-MB-231 and SK-BR-3 cells were cultured in RPMI 1640 medium (Gibco; Thermo Fisher Scientific, Inc.) containing 10% FBS and 1% penicillin/streptomycin. All cells were cultured at 37°C, in an atmosphere of 5% CO₂.

RNA preparation. Samples were harvested from Hs578T, MDA-MB-231, MCF-7 and SK-BR-3 human breast cancer cells. Total RNA was prepared using TRIzol® (Invitrogen; Thermo Fisher Scientific, Inc.), and the quality and quantity of the RNA were assessed using a NanoDrop ND-1000 (Thermo Fisher Scientific, Inc.). Optical density (OD) 260/280 \geq 1.6 and OD 260/230 \geq 1 indicated acceptable RNA purity, whereas acceptable RNA integrity (RNA integrity number \geq 5) was determined using an Agilent RNA 6000 Nano assay (Agilent Technologies, Inc., Santa Clara, CA, USA). Gel electrophoresis was used to evaluate genomic DNA contamination.

miRNAs and gene expression analysis. RNA samples were subjected to Human OneArray® version 6 (Phalanx Biotech Group, Hsinchu, Taiwan). Data were analyzed with Rosetta Resolver System software (Rosetta Biosoftware, Seattle, WA, USA). Standard selection criteria were used to identify differentially expressed genes: i) Absolute log₂ fold change \geq 0.585; absolute fold change \geq 1.5 and ii) false discovery rate $<$ 0.05, which were subsequently categorized into up and downregulated genes for AR-positive vs. -negative breast cancer cells.

miRNA target prediction and miRNA-gene interaction analysis. The miRNAs whose expression was significantly dysregulated between AR-positive and -negative breast cancer cells were selected for target prediction. Predicted target genes were identified by at least three of the seven well-established databases: DIANA (diana.imis.athena-innovation.gr/DianaTools/index.php), miRanda (www.microna.org/microna/home.do), miBridge (sitemaker.umich.edu/mibridge/home), PicTar (pictar.mdc-berlin.de/), PITA (genie.weizmann.ac.il/pubs/mir07/mir07_data.html), RNA22 (cm.jefferson.edu/rna22/) and TargetScan (www.targetscan.org/vert_71/). Pathway enrichment analysis was performed using the GO system to gain insight into the molecular networks and canonical pathways associated with differentially expressed miRNA. Of the enriched pathways with a P-value of $<$ 0.05, the associations between the differentially expressed miRNAs and their candidate target genes were visualized with an interaction network, for the VEGF and mTOR signaling pathways.

Results

miRNAs are differentially expressed in AR-positive and -negative breast cancer cell lines. Previous reports have indicated that AR protein expression levels may be affected by a set of miRNAs in PCa cells (13); another 25 miRNAs have been reported to be differentially expressed in PCa compared with matched benign tissues (14). This evidence suggests that there is an association between miRNA expression and the AR signaling pathway. However, the mechanism underlying AR-mediated regulation of miRNAs in PCa and TNBC remains unclear. To further identify differentially expressed miRNAs in breast cancer cell lines with varying AR expression, miRNA expression profiling was performed in breast cancer cell lines representative of AR-negative (Hs578T: ER⁻/PR⁻/HER2⁻/AR⁻) and AR-positive (MDA-MB-231: ER⁺/PR⁺/HER2⁺/AR⁺; MCF-7: ER⁺/PR⁺/HER2⁺/AR⁺; SK-BR-3: ER⁺/PR⁺/HER2⁺/AR⁺) molecular subtypes. A total of 153 miRNAs were identified as significantly altered in AR-positive vs. AR-negative cell lines, with at least a threefold change in expression (P $<$ 0.05; Fig. 1). Of the 52 upregulated miRNAs, miR-933 and miR-5793 had the most significant increases, with miR-933 expression undergoing the greatest upregulation (2.83-fold; P=0.026). Of the 101 downregulated miRNAs, miR-4792 expression underwent the greatest decrease (5.93-fold; P=0.002).

Predicted targets of the differentially expressed miRNAs and their involvement in signaling pathways. As a single miRNA may target multiple mRNA transcripts, the dysregulation of

sets of miRNAs may impact significantly on cancerogenesis via the regulation of multiple signaling pathways. To determine the probable biological functions of the differentially expressed miRNAs, the potential targets of the 153 differentially expressed miRNAs were predicted in seven online databases: DIANA, miRanda, miRBridge, PicTar, PITA, RNA22 and TargetScan. A total of 5,576 target genes were predicted, and classified into different pathways according to their basic biological functions by the GO system. Multiple effectors of pathways involved in tumor cell proliferation and invasion were characterized, for example in the VEGF and mTOR signaling pathways (Fig. 2). Due to the importance of the VEGF and mTOR signaling pathways in breast cancer, the VEGF-miRNA-target gene network (Fig. 3A) and mTOR-miRNA-target gene network (Fig. 3B) were constructed to reveal the association between AR-associated miRNAs and their target effectors in the two signaling pathways. The present study revealed a putative role of AR in the VEGF and mTOR signaling pathways, suggesting that AR may regulate the pathways through up or down-regulating miRNAs. Further work is required to improve the understanding of the role of AR in these signaling pathways.

Discussion

The present study performed a screening to identify AR-associated miRNAs in four breast cancer cell lines. By focusing on miRNAs that were associated with AR in three separate cell lines, cell-dependent bias was reduced. A total of 153 miRNAs were identified as differentially expressed. Of these, 52 were upregulated and 101 were downregulated. By predicting the targets of differentially expressed miRNAs, interactions between AR, mTOR and VEGF signaling pathways were indicated, suggesting that the AR may interact with miRNAs and contribute to breast cancer carcinogenesis via VEGF or mTOR signaling pathways. To date, studies validating the association between the AR and miRNA expression have primarily been conducted in PCa. Few studies have reported the role of AR-associated miRNAs in breast cancer. Thus, the present study increases the understanding of the role of AR in breast cancer.

miRNAs have previously been implicated in the regulation of AR signaling in prostate cancer. Chromatin immunoprecipitation analysis confirmed AR binding to putative androgen responsive elements (AREs) within the promoter regions of these miRNAs, thereby regulating miRNA expression (15). Porkka *et al* (11) in 2007 were the first to reveal an association between the AR and miRNAs. Following miRNA expression profiling of PCa cell lines and carcinoma samples, hierarchical clustering of the aberrantly expressed miRNAs accurately separated the carcinomas according to AR status, indicating that the AR may regulate the expression of miRNAs. Furthermore, a number of studies have described the roles of miRNAs as regulators of AR activity. Östling *et al* (13) demonstrated that the majority of the miRNAs regulating the AR targeted an extended 6 kb 3'UTR. A total of 71 unique miRNAs were identified to influence the level of AR expression in PCa cell lines. Therefore, there exists a potential link between AR signaling and miRNAs, with implications for the development of strategies to inhibit AR function via miRNAs-associated pathways.

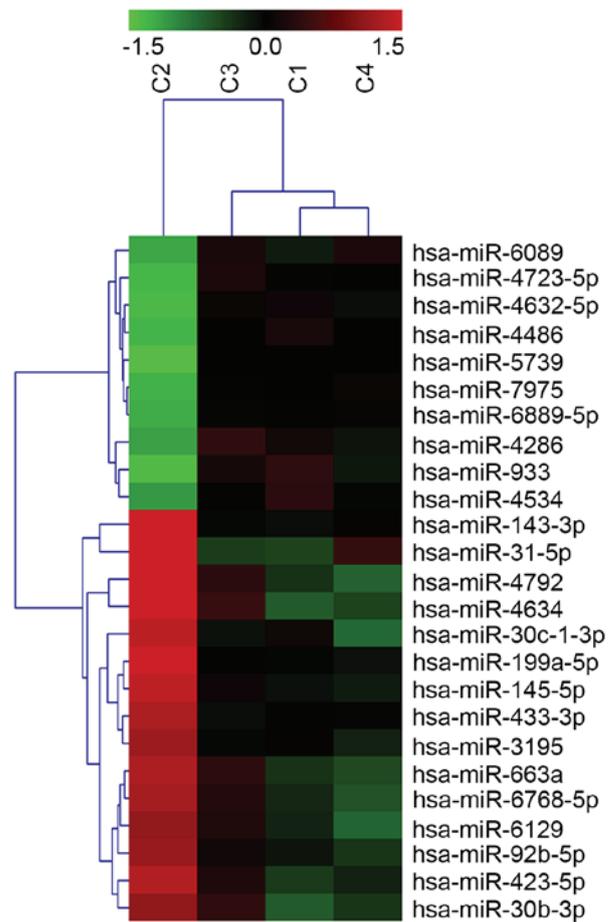


Figure 1. Differential expression of miRNAs. Of the 153 differentially expressed miRNAs in AR-positive vs. -negative breast cancer cell lines, hierarchical clustering of the 25 most variably expressed are presented. Rows, individual microRNAs; columns, individual breast cancer cell lines (C1, MDA-MB-231; C2, Hs578T; C3, MCF-7; C4, SK-BR-3). The colors in the heatmap represent the normalized expression values with downregulation represented by shades of green and upregulation by shades of red. miRNA, microRNA.

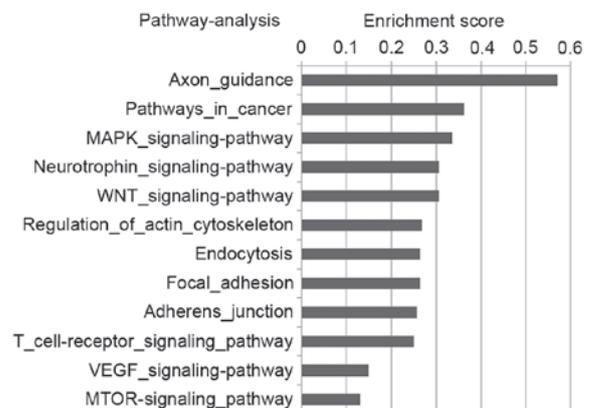


Figure 2. Signaling pathways of the target genes of differentially expressed miRNAs. The target genes of the differentially expressed miRNAs were classified into various signaling pathways. The vertical axis represents the pathway category, and the horizontal axis the enrichment score. miRNA, microRNA.

A differential miRNA expression pattern, involving 153 miRNAs, was detected in the present study, 52 upregulated

Table I. Significantly downregulated miRNAs in the present study are associated with breast cancer.

Author, year	miRNA	Cancer	Target gene	Clinical specimen/animal model/cell line	Function	Ref.
Ng, 2014	miR-143	Breast	DNMT3A	MDA-MB-231, T47D	Inhibit cell proliferation	16
Yan, 2014			ERBB3	Breast cancer tissues, MDA-MB-231, MCF-7, xenograft mouse model	Inhibit cell proliferation and invasion	17
Jiang, 2012			HK2	MDA-MB-231, ZR-75-30, xenograft mouse model	Inhibit cell proliferation and invasion	18
Yu, 2012			Survivin	MCF-7	Inhibit cell proliferation	19
Rasheed, 2015			GNA13	Breast cancer tissues, MDA-MB-231, MCF-10a	Inhibit cell invasion	20
Augoff, 2011	miR-31	Breast	β 1-Integrin	MDA-MB-231	Inhibit cell invasion	21
Sossey-Alaoui, 2011			Wave3	Breast cancer tissues, MDA-MB-231	Inhibit cell invasion	22
Körner, 2013			PKC ϵ	MDA-MB-231, MCF-10A	Inhibit cell invasion	23
Valastyan, 2010			RDX	MDA-MB-231, xenograft mouse model	Inhibit cell invasion	24
			ITGA5			
			RhoA			
Ben-Chetrit, 2015			SYNJ2	Breast cancer tissues, MD-MB-231, MCF-10a, xenograft mouse model	Inhibit cell invasion	25
Dobson, 2014	miR-30c	Breast	NOV/CCN3	MDA-MB-231	Promote cell invasion	26
Shukla, 2015			TRADD	Breast cancer tissues, MDA-MB-231	Inhibit cell proliferation and invasion	27
			CCNE1			
Tanic, 2012			KRAS	MDA-MB-436	Inhibit cell growth	28
Bockhorn, 2013			TWFI	MDA-MB-231, xenograft mouse model	Inhibit cell invasion	10
			VIM			
Bockhorn, 2013			TWFI	Breast cancer tissues, MDA-MB-231, xenograft mouse model	Inhibit chemo-resistance	29
Fang, 2014			YWHAZ	MCF-7, xenograft mouse model	Inhibit chemo-resistance	30
Yi, 2013	miR-199a	Breast	DRAM1	MDA-MB-231, MCF-7	Regulate apoptosis	31
			Beclin 1			
Shatseva, 2011			CAV-2	MDA-MB-231	Inhibit cell proliferation	32
Yan, 2014	miR-145	Breast	ERBB3	Breast cancer tissues, MDA-MB-231, MCF-7, xenograft mouse model	Inhibit cell proliferation and invasion	17
Eades, 2015			ARF6	Breast cancer tissues, MDA-MB-231	Inhibit cell invasion	33
Zou, 2012			N-RAS	Breast cancer tissues, MDA-MB-231, MCF-7, xenograft mouse model	Inhibit cell growth, Invasion, and angiogenesis	34
			VEGF-A			
Wang, 2009			RTKN	MFC-7	Inhibit cell growth	35
Spizzo, 2010			ER- α	MDA-MB-231, MCF-7	Inhibit cell growth	36
Gotte, 2010			JAM-A	MB-MDA-231, MCF-7, MDA-MB-468, SK-BR-3	Inhibit cell invasion and motility	37
			Fascin			

Table I. Continued.

Author, year	miRNA	Cancer	Target gene	Clinical specimen/animal model/cell line	Function	Ref.
Liu, 2014			Muc-1	Breast cancer tissues, MDA-MB-231, LM2-4142	Inhibit cell growth and invasion	38
Ichikawa, 2012	miR-30b-3p	Breast	CCNE2	SKBR3; BT474	Inhibit cell growth	39
miRNA, microRNA.						

and 101 downregulated, thus identifying those miRNAs that may be significant in breast cancer development. Certain significantly dysregulated miRNAs in the upregulated and downregulated groups have been previously associated with breast cancer progression. Notably, certain of the significantly downregulated miRNAs have been associated with breast cancer cell functions, including proliferation, invasion and drug-resistance (Table I; Fig. 4). For example, miR-143 and miR-145, components of the miR-143/145 cluster, were significantly downregulated. miR-143 and miR-145 have been demonstrated to possess anti-tumorigenic activity (Table I). Furthermore, expression of the miR-143/145 cluster was revealed to function as a tumor suppressor in breast cancer via the inhibition of ERBB3 (17). Stimulation of EGF pathways, including ERBB3, leads to enhanced AR stability and promotes AR binding to AREs. Therefore, the miR-143/145 cluster may be involved in the underlying mechanism of the AR in breast cancer. Furthermore, miR-31, an miRNA that was significantly downregulated in the present study, has been identified to directly target and destabilize the AR through its coding sequence. The mutual regulation between miR-31 and AR maintains prostate cellular homeostasis, and the loss of miR-31 contributes to PCa progression through unregulated AR expression. In breast cancer, miR-31 serves as a tumor suppressor via inhibition of guanine nucleotide binding protein alpha 13, WAS protein family member 3, β 1-Integrin and protein kinase C ϵ (Table I). miR-181, another dysregulated miRNA in the present study, has been demonstrated to indirectly target AR to function as a growth suppressive miRNA in prostate cancer (40). Taken together, the dysregulated miRNAs identified by the present study may regulate the expression of AR in breast cancer. It may be useful to determine whether the dysregulated pattern is implicated in the role of the AR in breast cancer. The majority of the upregulated miRNAs identified in the present study have not been widely reported as aberrantly expressed in other cancers, which suggests that AR-associated breast cancer may have a specific signature. Further studies are required to confirm the expression of the dysregulated miRNAs by polymerase chain reaction analysis.

However, studies on AR-associated miRNAs in breast cancer are limited. To the best of our knowledge, only two studies have been performed to date. Nakano *et al* (41) identified miRNAs induced by dihydrotestosterone (DHT) in an AR-positive breast cancer cell line. A total of 13 miRNAs were upregulated and 28 miRNAs were downregulated significantly. Of the aberrantly expressed miRNAs, miR-363, with the fold-change (8.15-fold) targets IQ motif- and WD repeats-containing 1 to influence the mechanism underlying the action of androgens. Furthermore, to identify critical miRNAs associated the androgen-induced AR signaling pathway in breast cancer, Lyu *et al* (42) examined global miRNA expression in DHT-treated MCF-7 and MDA-MB-453 cells. A total of 10 differentially expressed miRNAs were identified in MDA-MB-453 cells. Of these, 4 upregulated miRNAs (let-7a, b, c and d) were detected. Androgen-induced AR activating signal was revealed to directly upregulate let-7a expression, downregulate CMYC and KRAS expression and inhibit cell proliferation in ER⁻, PR⁻, AR⁺ breast cancer cells. miR-30b, another critical miRNA associated with the androgen-induced AR activating signal in breast cancer (42),

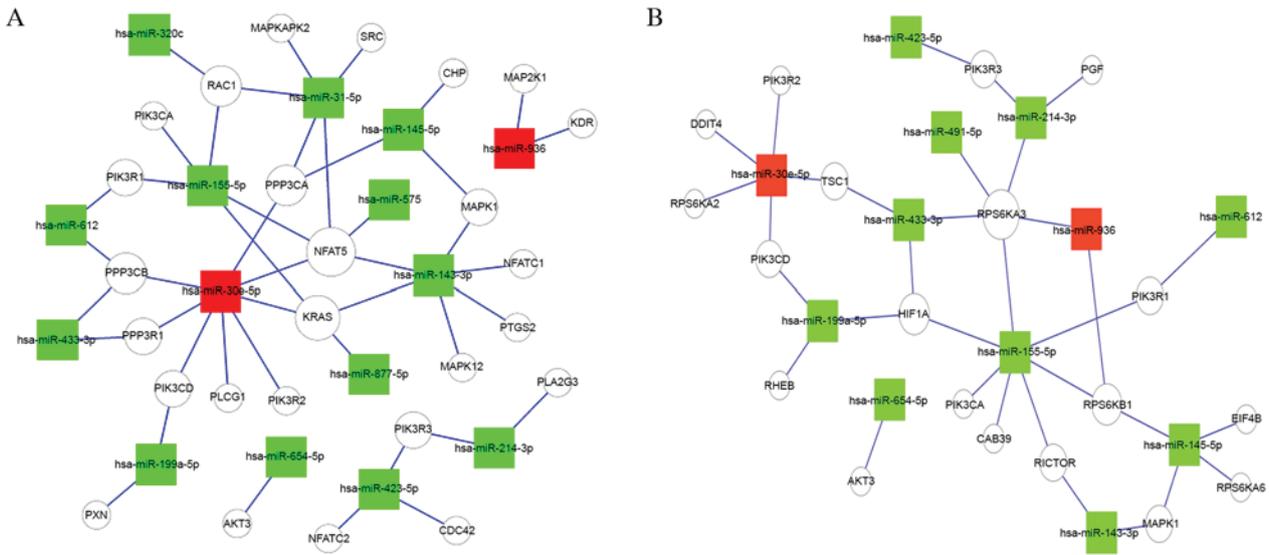


Figure 3. Signaling pathway networks. (A) VEGF-miRNA-target genes network. (B) mTOR-miRNA-target genes network. The red/green square nodes represent upregulated/downregulated miRNAs in AR-positive vs. -negative breast cancer cell lines. The hollow circles represent predicted target genes. VEGF, vascular endothelial growth factor; miRNA, microRNA; mTOR, mammalian target of rapamycin; AR, androgen receptor.

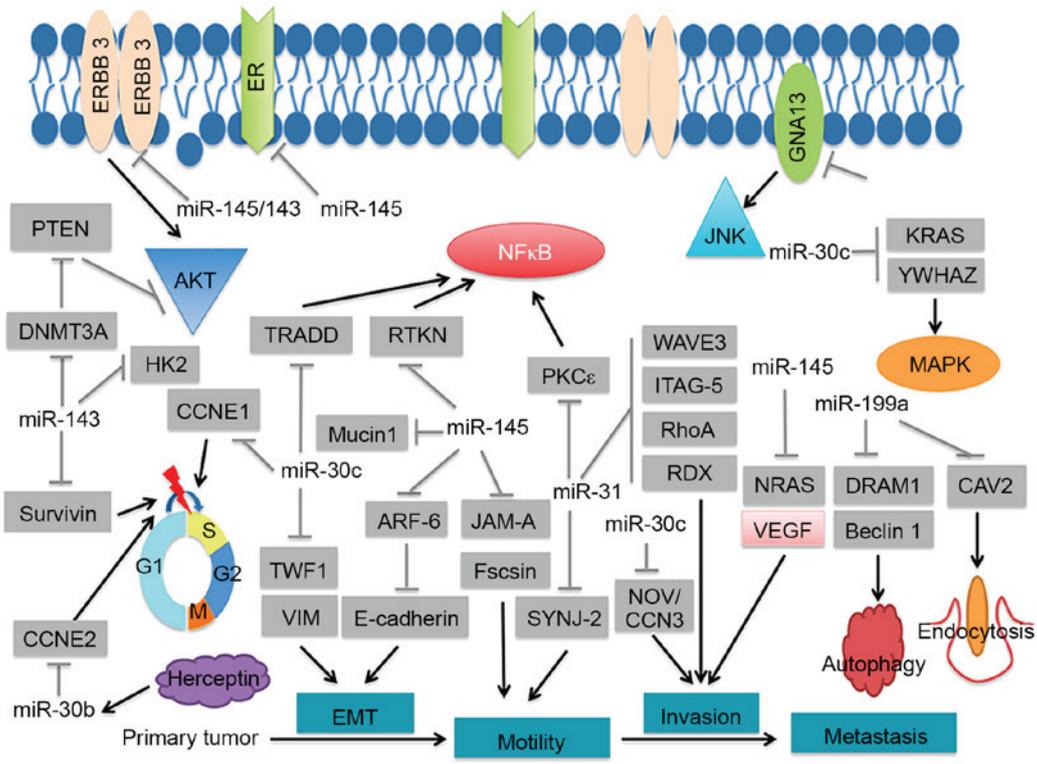


Figure 4. Involvement of the significantly dysregulated microRNAs in breast cancer signaling pathways.

was aberrantly expressed in the present study, which screened the AR 3'UTR for potential miRNA target sites. miR-30b was predicted by miRanda to target the 3'UTR of the AR. Taken together, these data suggested a potential link between AR signaling and miRNAs in breast cancer. To investigate the role of AR-associated miRNAs in breast cancer, further investigations are required. The present study has identified a distinct miRNA expression pattern in AR-positive breast

cancer cell lines, which indicates a critical role for miRNAs in AR-positive and -negative breast cancer.

Pathway enrichment analysis suggested that the differentially expressed miRNAs collectively targeted various signaling pathways associated with cell proliferation and invasion. Notably, VEGF and mTOR pathways, the key pathways of breast tumorigenesis, were identified as associated with the deregulated miRNAs of the present study (43). Previous studies

have revealed that the involvement of miRNAs was critical for VEGF-induced angiogenesis and mTOR-associated tumor proliferation (44,45). In the present study, a putative correlation was revealed between AR, VEGF and mTOR signaling pathways. The prognostic value of AR expression in TNBC remains elusive, with certain studies suggesting reduced mortality (4), and others indicating poor prognosis (5). Furthermore, our previous study identified a role for AR in tumorigenesis role and the inhibitory effect of AR antagonist in AR-positive mesenchymal stem-like TNBC *in vitro* and *in vivo*, suggesting that AR inhibition may be a potential therapeutic approach for AR-positive TNBC patients (46). AR may therefore be a tumorigenic factor in breast cancer. AR-associated miRNAs may be involved in the VEGF and mTOR signaling pathways to promote tumor cell proliferation.

In conclusion, the results of the present study indicated that there is an imbalance of miRNAs levels in AR-positive breast cancer cells compared with AR-negative cells, suggesting an important role for miRNAs in the function of the AR in breast cancer. Following classification of the miRNAs targets into different pathways, a potential interaction between AR, VEGF and mTOR signaling pathways was revealed. These results suggested a potential underlying mechanism of AR in breast cancer via the dysregulation of the expression pattern of miRNAs.

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