Identification of Tumor-Suppressive miRNA-1275 as a Novel Marker for Breast Cancer (BC) by MACE-Sequencing and RT-qPCR Techniques

Sevan Omer Majed (sevan.majed@su.edu.krd)
Salahaddin University- Hawler https://orcid.org/0000-0002-3636-7723
Suhad Asad Mustafa
Salahaddin University- Hawler

Research article

Keywords: Breast cancer, miRNA, miR-1275 and its target genes, differential expression, pathogenesis, tumor suppressor

Posted Date: November 5th, 2020

DOI: https://doi.org/10.21203/rs.3.rs-101189/v1

Abstract

Introduction

Disruption of cellular processes in the breast by abnormally expressed miRNA is characterized to develop cancer. We aimed to determine the differential expression of coding and non-coding RNAs in formalin fixed paraffin embedded (FFPE) blocks of breast cancer (BC) tissue and normal adjacent tissue (NAT). Another aim is to determine differential expression of *has-miR-1275* as novel biomarker for BC and identify its target genes using prediction sites and experimentally expression level of them via the MACE-sequencing technique.

Methods

MACE-sequencing technique was utilized to analyze differential expression of coding RNAs and small RNAs (sRNAs). Among small RNAs, *miRNA-1275* expression was focused and confirmed using RT-qPCR technique in 20 Kurdish cases with BC . Moreover, clinical significance of *miR-*1275 and its target genes was studied in a large number of patients with BC using the data obtained from The Cancer Genome Atlas database.

Results

The MACE-seq findings showed that 1400 sRNAs and 26843 coding RNAs were differentially expressed in FFPE of BC tissue compared to NAT. Among these sRNAs, *miRNA-1275* expression was found to be decreased in BC tissue compared to NAT. The decreased expression level of which was then confirmed via RT-qPCR technique to farther prove in 20 Kurdish cases with BC . Furthermore, the correlation between the expression level of *miRNA-1275* and clinical data were evaluated to be highly corrected in cases with BC (overall survival rate: P = 0.0401). However, putative target genes (*DVL3,* PPP *2R2D, THSD4, CREB1, SYT7,* and *PRKACA)* were computationally identified as direct targets of *miRNA-1275* in several target predicted sites. Among coding RNAs, the expression level of these targets was increased in BC tissue compared to NAT. The levels of these targets were negatively associated with *miRNA-1275* expression. Finally, the role of down-expressed *miRNA-1275* and its targets in BC cells were identified to attenuated biological mechanisms; including cell growth, proliferation, movement, invasion, metastasis, and apoptosis.

Conclusion

down-expressed *miR-1275* , a tumor suppressor, is as a novel biomarker for early detection of breast cancer. *DVL3,* PPP *2R2D, THSD4, CREB1, SYT7,* and *PRKACA* are novely identified to be targeted by *miR-1275* in BC cells.

1. Introduction

Breast malignant cell is a prominent type of cancers mostly diagnosed in females, and the second most frequent malignancy-associated deaths worldwide, especially in the US and Asian countries (1, 2). Approximately two million females are annually diagnosed and more than 620,000 deaths are newly recorded every year (3, 4). Frequently, BC is developed as a result of a genomic mutation. However, about 10% of BCs is inheritably come down from parents to their generations; whereas, more than 85% of BCs is developed in their lifetime (4, 5). Inherited abnormalities in *TP53* and *PTEN* genes were studied to result in the high risk of the breast malignant cell progression (6, 7).

Gene expression profiling has recently played a critical role in medicinal selection for BC subtypes. The analysis of BC gene expression can be used for molecular category of BC subtypes (8-10). Two studies reported that this classification facilitates the determination of the cure doses. The molecular subtypes of BC can be categorized into luminal-A, luminal-B (including HER2+ /-), HER2+, and triple negative (TN) (11, 12). These subtypes are pivotal for cure choice and are correlated to the biological characteristics of BC.

MicroRNA (miRNA), which is a type of untranslated sRNAs, is synthesized from eukaryotic genomes, consisting of a single stranded RNA of about 19-22nt in length (13, 14). These short non-coding miRNAs are described as regulators of coding and non-coding (nc) RNAs in eukaryotic cells because they are involved in silencing RNA transcripts and in regulating the stability of their targeted mRNAs (14, 15). MiRNAs play also several regulatory roles in several cellular processes; cell development, proliferation, migration, invasion and death (16). Because more than 50% of RNA molecules has been detected to be controlled by miRNA, these mRNAs were damaged because of the effect of aberrant miRNA in malignant cells.

MiRNAs in human malignancies were found to act as oncogenes or ant-oncogenes (tumor suppressors). Oncogenic miRNAs in tumor progress play a negative role in stimulating genes which regulate cell development and apoptosis process. Tumor suppressive miRNAs in human tumor have a key role in silencing genes which modulate cell development and apoptosis (17, 18). When normal cells do not undergo normal growth and apoptosis process, they normally cause tumor creation. Numerous recent experiments show that numerous miRNAs are directly implicated in modulating cell growth, proliferation as well as apoptosis (19, 20). They play major roles in the pathogenesis of a number of human

malignancies; such as breast, colorectal cancer, lung, leukemia liver, and brain(16, 18). The miRNA expression level may be either down- or up-regulated in these cancers. Several molecular techniques, such as RNA sequencing, miRNA microarray, RT-PCR and northern blot are applied to determine the expression level of them.

Numerous miRNAs have been recognized to be implicated in the pathogenesis of human breast cancer. It was found that the expression patterns of *miR-145*, *-125b*, *-155*, and *-21*were significantly downregulated. In breast malignant cells, these miRNAs were observed to be associated with pathologic properties; cell proliferation, expression of progesterone and estrogen receptors (21). A recent study revealed that tumor suppressive *miRNA-204-5p* plays a key role in targeting several oncogenic genes which are closely connected to BC pathogenesis(20). Complete information on *miR-1275* expression level and its targets in BC have not been available; whereas, the expression profile of which has be analyzed in some human cancer. A study reported that down-expressed *miR-1275* leads to overexpression *claudin11* in cancer stem cells or tumor-initiating cells (CSCs/TICs) (22). According to a study carried out on young women with BC, 6 miRNAs; including *miR-1275, miR-1228*, miR-139*, *miR-92b*, *miR-1207*, and *miR-3196,* were involved in the processes of cell movement, proliferation and invasion (23). It was also found that this miRNA was found to be downregulated in gastric cancer (24). Another study found that *miRNA-1275* expression level was significantly abnormally deregulated in Ewing's Sarcoma (ES) (25). The significance of a large number of miRNAs have been reported to become an appropriate biomarker for human cancer diagnostics. However, the significance of *miRNA-1275* in BC is not reported. The objective of this study was to determine the expression level of *miRNA-1275* as a biomarker for BC diagnostics. Another objective is to identify the potential targeted genes of this miRNA.

2. Materials And Methods

2.1 Collection of FFPE-blocks of BC samples

Formalin Fixed Paraffin Embedded (FFPE)-Block of 21 Kurdish cases with BC were collected at clinicopathological laboratories, called as Al Mufti and Luay. For each patient, two paraffin blocks (one adjacent normal tissue (NAT) and one breast cancer tissue) were collected. The normal tissues were histologically taken nearly 2 cm away from the tumor area. Clinical features of 21 cases were obtained using a questionnaire. The features were displayed in table 1. Permission was accomplished from all cases by signing the confirmed consent. any pre-operative chemotherapy or radiotherapy was taken by none of these cases

2.2 Generation of the mRNA and sRNA expression profiles for BC by MACE-sequencing

To determine differential expression of protein-coding RNAs (mRNA transcripts) and non-coding RNAs (ncRNAs), analyzing two paraffin blocks (cancerous and normal) of a BC patient, Massive Analysis of cDNA Ends (MACE)-seq technique was performed (GenXpro GmbH, Frankfurt, Germany). cDNA synthesis, NGS-library and sequencing were subsequently prepared after isolation of sRNAs from mRNA transcripts. The raw sequencing data was bioinformatically analyzed and solved. The raw sequencing data is demultiplexed based on the different barcodes (GenXpro GmbH, Frankfurt, Germany). For removing adapter, the organized reads were cut out for high-quality sequences. Bowtie 2 tool was later used for aligning the sorted reads to the nominated reference sequences and annotating with corresponding properties. The numbers of aligned reads were subsequently normalized to account for distinct sequencing depths. Finally, the normalized and original read numbers were considered during statistical analysis.

2.3 Total RNA purification and cDNA synthesis

miR-1275 was selected as a candidate from the MACE result and confirmed from 20 cases using Real Time-quantitative polymerase chain reaction (RT-qPCR) technique at Salahuddin University Research Center (SURC). Differential expression of *miRNA-1275* was measured in 40 block specimens (20 adjacent normal tissues (NATs) and 20 breast cancer tissues). The total RNA molecules including miRNA were extracted using FFPE RNA/DNA Purification Plus kit (Cat. No. 54300, NORGEN BIOTEK CORP, Canada). Complementary DNA (cDNA) was synthesized using miRNA All-In-One cDNA Synthesis Kit (Cat. No. G898, abmgood company, US).

2.4 Total RNA purification and cDNA synthesis

miR-1275 was selected as a candidate from the MACE result and confirmed from 20 cases using Real Time-quantitative polymerase chain reaction (RT-qPCR) technique at Salahuddin University Research Center (SURC). Differential expression of *miRNA-1275* was measured in 40 block specimens (20 adjacent normal tissues (NATs) and 20 breast cancer tissues). The total RNA molecules including miRNA were extracted using FFPE RNA/DNA Purification Plus kit (Cat. No. 54300, NORGEN BIOTEK CORP, Canada). Complementary DNA (cDNA) was synthesized using miRNA All-In-One cDNA Synthesis Kit (Cat. No. G898, abmgood company, US).

2.5 Most common putative targeted genes regulated by *miR-1275*

Eleven databases were searched for finding the most common putative targets of *miR-1275* (Table 3). Six putative targets (*DVL3, PPP2R2D, THSD4, CREB1, SYT7,* and *PRKACA*) were determined to possess binding sequence to *miR-1275* (Table 4)., Graphpad prsim, version 8.0.1 was used to show the differential expression of these selected target genes were performed by MACE-seq.

2.6 Analysis of clinicopathological data associated with BC

Association between *miR-1275* and its target genes was computationally analyzed to determine the clinical significance using databases of cBioPortal (http://www.cbioportal.org/) and OncoLnc (http://www.oncolnc.org/). Clinical data and expression levels of the *miR-1275* and its target genes gained from these sites and then were downloaded on 10 September 2020.

Table 1. Clinicopathological features of 21 cases with BC.

Cases	Age	Tumor Size	Stage	Lymph node metastasis	Lymphatic Invasion	Venous Invasion	E.R.	Pg.R.	Her2	Ki-67	Technique
Case1	74	4cm	IIIA	Yes	1	1	Negative	Negative	Negative	60	MACE-seq.
Case2	35	4cm	IIB	Yes	1	0	Negative	Negative	Positive	35	RT-PCR
Case3	64	4.5cm	IIIA	Yes	1	0	Positive	Positive	Negative	5	RT-PCR
Case4	71	1.9cm	I	No	0	0	Positive	Positive	Negative	Unavailable	RT-PCR
Case5	40	2.3cm	IIB	Yes	1	0	Positive	Positive	Negative	4	RT-PCR
Case6	44	3.5cm	IIIC	Yes	1	0	Positive	Positive	Negative	13	RT-PCR
Case7	53	2cm	IIA	No	1	1	Positive	Negative	Positive	20-30	RT-PCR
Case8	46	1.8cm	IIA	Yes	1	1	Positive	Negative	Positive	10	RT-PCR
Case9	33	1.4cm	IIA	No	0	0	Positive	Positive	Positive	8	RT-PCR
Case10	49	5cm	IIIC	Yes	1	0	Negative	Positive	Negative	13	RT-PCR
Case11	45	1.9cm	IIIB	No	0	0	Negative	Negative	Negative	80-90	RT-PCR
Case12	64	1.5cm	IIA	No	0	0	Negative	Negative	Negative	80-90	RT-PCR
Case13	49	1.7cm	IIA	No	0	1	Negative	Negative	Negative	70-80	RT-PCR
Case14	35	1.3cm	IIA	No	0	0	Negative	Negative	Negative	90	RT-PCR
Case15	52	3cm	IIA	No	0	0	Negative	Negative	Negative	60	RT-PCR
Case16	18	2.5cm	IIA	No	0	0	Positive	Negative	Negative	50	RT-PCR
Case17	63	1.5cm	I	No	0	0	Negative	Negative	Positive	12	RT-PCR
Case18	19	2.5cm	IIB	Yes	1	0	Positive	Positive	Negative	24	RT-PCR
Case19	30	1.3cm	IIA	No	1	0	Positive	Positive	Negative	15-20	RT-PCR
Case20	45	1.5cm	IIA	No	0	0	Positive	Positive	Negative	15-20	RT-PCR
Case21	44	6cm	IIIC	Yes	0	0	Positive	Positive	Positive	7	RT-PCR

3. Results

3.1 Construction of expression profile of sRNAs for BC by sRNA-sequencing

SRNA sequencing was carried out to construct the differentia expression of non-coding RNAs in BC compared to NAT. Two small libraries were sequenced for two paraffin blocks (NAT and BC tissue). Histopathological properties of this specimen were shown in table 1 (Case1). By comparing non-coding RNA expression profiles of BC and NAT, 1400 sRNAs (p<0.05) were filtered out by a SAM software. The raw data were then standardized and log2-transformed to show on a scatter plot (Fig. 1A). Among 1400 sRNAs, 723 non-coding RNAs were downregulated, but 678 sRNAs were upregulated. Each dot on the scatter plot represents the sRNA. Among 1400 sRNAs, 520 microRNAs were differentially expressed. 185 microRNAs were down-expressed, but 335 were overexpressed. The x-axis denotes the data of the NAT and y-axis denotes the data of the BC. Correlation plot was constructed to show expression levels of non-coding RNAs between BC and NAT (Fig. 1B). Blue color

denoted the correlation of sRNAs between the BC and NAT. Heat map was designed to show 29 miRNAs which were markedly downregulated in BC compared to NAT (Fig. 1C). Table 2 shows the information on these 29 miRNAs which are notably downregulated. In this study, *has-miR-1275*, which underlined with red color in heat map, was focused to identify sequence and expression level.

3.2 Confirmation of *miR-1275* expression level by RT-qPCR

The *miR-1275* expression level in RNA-sequencing was observed to be significantly downregulated in BC tissue, as compared to NAT. The *p*.value of this miRNA was 0.614 (Fig. 2A). Then, this miRNA was selected to confirm. RT-qPCR machine was used to confirm the differential expression of *miR-1275* in the laboratory. 40 FFPE blocks for 20 cases was used in this experiment, including 20 BC tissues and 20 normal tissues next to tumoral tissues (2cm away from tumoral tissues). The clinical properties of these cases were briefly explained in table 1. The *miR-1275* expression level was detected to be markedly decreased in BC cells compared to adjacent normal cells and the *P*-value of this was 0.001** (Fig.2B). The mature sequence of which in the BC and NAT was made up of 17 nucleotides and also identical (Fig.2C). Then, Kaplan–Meier overall survival curve was designed to show the effect of *miR-1275* expression on the prognosis of cases with BC. Data was used from The Cancer Genome Atlas (TCGA) database and analyzed. Kaplan–Meier overall survival curve displayed that cases were separated into two classes according to its expression. The decreased *miR-1275* (*P*-value=0.0401) was related to overall survival in cases with BC (Fig.2D).

3.3 Differentially expressed genes for adjacent normal and BC tissue by MACE-seq.

Differentially expressed genes in BC and NATs was displayed (Fig. 3A). 26843 differentially expressed genes ($P \leq 0.05$) were filtered out by a SAM software. In order to show genes that were more significantly different in their expressions, 7041 genes were standardized and log2-transformed to show on a scatter plot. 3624 genes were significantly overexpressed and 3417 genes were significantly down-expressed in tumoral cells compared to adjacent normal cells. The *P* value for that was ranged from smaller (Blue) to greater (Red). Each point on the scatter plot represents the gene. the x-axis denotes the data of the NAT and y-axis denotes the data of the cancerous tissue.

3.4 Candidate target genes regulated in BC by *miRNA-1275*

Table 3 showed that eleven computational prediction programs were applied for discovering the strongest candidate genes possessed *miR-1275* binding sites in the 3' -UTR. Six predicted genes genes (*DVL3*, *PPP2R2D, THSD4, CREB1, SYT7,* and *PRKACA*) were selected to have binding site to *miRNA-1275*. The information on these six predicted genes were summarized in table 4. Eleven databases showed that DVL3 and *PPP2R2D* possess the binding site to *miRNA-1275*; whereas, *THSD4*, and *CREB1* were confirmed in ten prediction programs to be targeted by *miRNA-1275,* but *SYT7* and *PRKACA* were confirmed by six tools to be predicted targets. These putative target genes are important for biological analysis of the BC tissues because the over- or down-expression of which can play a damaging role in several cellular processes and contribute to the cancer progression and tumorigenesis.

3.5 Determination of expression level of candidate target genes by MACE-seq approach

Among 3624 upregulated genes, the six predicted genes (*DVL3, PPP2R2D, THSD4, CREB1, SYT7,* and *PRKACA*) were pointed and named in the BC cells as compared to NAT (Fig.3B). Then the differential expression of which and their binding sites to *has-miR-1275* were shown in figure 4. *SYT7* gene was more overexpressed in BC, as compared to *PRKACA* gene. Overexpression level of *THSD54* gene was higher than the up-regulation of *PPP2R2D* and DVL*3* genes. *CREB1* was upregulated but more over-expressed than *ST73* gene. Table 5 demonstrates the information on *P*.value, False Discovering Rate (FDR), and Fold Change (FC) of these six predicted genes possessed *miR-1275* binding sites in the 3' -UTR. They were identified as potentially modulated by *miR*-1275 using computational prediction databases and TCGA algorithm.

Next, the relationship between the expression level of these 8 candidates and histopathological significance were examined based on data from TCGA database. Among 204 target genes, *DVL3*: *P*=6.98E-04, *PPP2R2D*: *P*=1.53E-03, *THSD4*: *P*=2.28E-12, *CREB1*: *P*=3.12E-01, *SYT7*: *P*=1.28E-48, and *PRKACA*: *P*=5.68E-02 were markedly relationship with worse prognosis in cases with BC. The Kaplan–Meier overall survival curve analyses of cases with BC were designed to be separated into 2 classes according to their expression (Fig.5).

3.6 The role of *miR-1275* by targeting selected putative genes in BC

MiRNAs are implicated in silencing mRNA transcripts through matching or mismatching with target mRNAs. As hypothesis of microRNA biogenesis, major strands of *miR-1275* come from miRNA duplex are joined into the RISC protein and modulate the mRNA transcripts, but minor strands are broken down and cannot modulate gene expression. *miR-1275* can play an essential role in regulating several biological mechanisms; including cell growth, migration, differentiation, proliferation and apoptosis. In this study, the down-expression of which regulate a set of genes and regulators related with tumor development. Six genes (*DVL3,* PPP*2R2D, THSD4, CREB1, SYT7,* and *PRKACA*) were detected to be over-expressed in BC cells. Fig.6 shows the relationship between *miR-1275* and these target genes. *miR-1275* in breast cancer promotes cancer cell proliferation, cell differentiation, tumor growth, invasion and migration and also inhibits apoptosis through several gene targets. *PPP2R2D* acts as a tumor suppressor in signaling pathway in BC and is negatively regulated by *miR-1275*. The overexpression of which

decreases *AKT* and *RACK1* abilities. Then these regulators decrease cell survival and migration. *DVL3* is implicated in the breast cancer pathways and negatively controlled by *miR-1275*. The up-regulation of this gene increases the cancer cell proliferation, migration and invasion. The cancer cell proliferation ability is increased when *miR-1275* becomes overexpressed. another target gene. *CREB1* and *PRKACA* show also negative correlation with *miR-1275* level. Whereas *CREB1* was found to reduce apoptosis process and increase cell proliferation in breast cancer, *PRKACA* plays a key role in tumorigenesis and development of BC. However, the function of *THSD4* and *SYT7*, currently unidentified, may boost tumor growth in breast cancer.

Table 2. Comparison of marked down-expressed miRNAs in BC with NAT

miRNA	miRBase accession	Location	Log2FC	*P*. value	FDR
Hsa-miR-1	*MIMAT0031892*	20q13.33	-2.6291	0.3109	1.0
Hsa-miR-100-5p	*MIMAT0004512*	11q24.1	-1.2349	0.2824	1.0
Hsa-miR-10b-5p	*MIMAT0000254*	2q31.1	-1.2852	0.261	1.0
Hsa-miR-125a-5p	*MIMAT0000443*	19q13.41	-1.4153	0.213	1.0
Hsa-miR-125b-5p	*MIMAT0000423*	11q24.1	-2.0041	0.083	1.0
Hsa-miR-1275	*MIMAT0005929*	6p21.31	-1.4150	0.6 14	1.0
Hsa-miR-130a-3p	*MIMAT0004593*	11q12.1	-2.0168	0.104	1.0
Hsa-miR-133a-5p	*MIMAT0026478*	18q11.2	-2.3885	0.220	1.0
Hsa-miR-143-5p	*MIMAT0004599*	5q32	-1.2823	0.308	1.0
Hsa-miR-204-5p	*MIMAT0000265*	9q21.12	-4.0627	0.086	1.0
Hsa-miR-21-3p	*MIMAT0004494*	17q23.1	-0.0365	0.995	1.0
Hsa-miR-214-3p	*MIMAT0000271*	1q24.3	-0.8746	0.440	1.0
Hsa-miR-25-5p	*MIMAT0004498*	7q22.1	-1.2630	0.793	1.0
Hsa-miR-30a-3p	*MIMAT0000088*	6q13	-0.2358	0.838	1.0
Hsa-miR-30b-5p	MIMAT0000420	8q24.22	-1.3254	0.245	1.0
Hsa-miR-30d-5p	*MIMAT0000245*	8q24.22	-0.9730	0.389	1.0
Hsa-miR-374b-5p	*MIMAT0004955*	Xq13.2	-0.2420	0.8435	1.0
Hsa-miR-410-5p	*MIMAT0026558*	14q32.31	-0.6780	0.989	1.0
Hsa-miR-423-5p	*MIMAT0004748*	17q11.2	-0.5727	0.614	1.0
Hsa-miR-451a	*MIMAT0001631*	17q11.2	-0.41900	0.711	1.0
Hsa-miR-455-5p	*MIMAT0003150*	9q32	-2.5081	0.305	1.0
Hsa-miR-505-5p	*MIMAT0004776*	Xq27.1	-1.5956	0.611	1.0
Hsa-miR-532-3p	*MIMAT0004780*	Xp11.23	-0.7496	0.612	1.0
Hsa-miR-624-5p	*MIMAT0003293*	14q12	-0.0931	1.0	1.0
Hsa-miR-664a-3p	*MIMAT0005949*	1q41	-0.9249	0.539	1.0
Hsa-miR-664b-5p	*MIMAT0022271*	Xq28	-0.5081	0.899	1.0
Hsa-miR-92a-1-5p	*MIMAT0004507*	13q31.3	-0.2085	0.972	1.0
Hsa-miR-934	*MIMAT0004977*	Xq26.3	-3.0931	0.417	1.0
Hsa-miR-99b-5p	*MIMAT0000689*	19q13.41	-0.1667	0.883	1.0

Table 3. Brief information on target predicted databases was shown to find putative targets possessing binding sequence to *miR-1275*.

Target predicted sites	Species	Tool properties	Website Websites
miRTarBase	Human, Mouse, Rat	Conservation, seed location	http://mirtarbase.mbc.nctu.edu.tw/php/index.php
Target scan	Human, Mouse, Fly, Fish, and Worm	Conservation, seed location	http://www.targetscan.org/
TargetMiner	Human, Mouse, Rat, Fly	Conservation, seed location	https://www.isical.ac.in/~bioinfo_miu/TargetMiner.html
MirTar2	Human, Mouse, rat, Dog and Chicken	Conservation, seed location	http://www.mirdb.org/
DIANA	Any	Conservation, seed match, and free energy	http://www.microrna.gr/microT-CDS
miRWalk	Human, Mouse, and Rat	Conservation, seed match and free energy	http://mirwalk.uni-hd.de/
miRmap	Human, Chimpanzee, Mouse, Rat, Cow,Chicken, Zebrafish, and Opossum	Conservation, seed match, and free energy	https://mirmap.ezlab.org/
RNA22	Human, Fruit Fly, Mouse, and Worm	Seed match and free energy	https://cm.jefferson.edu/rna22/
PicTar - Tools4miRs	Human, Mouse, Rat, Fly	Conservation, seed location	https://tools4mirs.org/software/target_prediction/pictar/
mirPath	Human, Mouse, D. melanogaster, C. elegans, R. norvegicus, D. rerio and G. gallus	Conservation, seed match and free energy	http://snf-515788.vm.okeanos.grnet.gr/index.php?r=mirpath/geneList
Microrna. org	Human, mouse, Fruit Fly, and rat	Conservation, seed match, free energy	http://www.microrna.org/

Table 4. candidate target genes possessing binding sequence to *miR-1275* was shown.

Target gene	Ensemble ID	Position on Chromosome	No. of sites predicted the gene as *miR-1275* target
DVL3	ENST00000313143.3	3q27.1	11
PPP2R2D	ENST00000422256.2	10q26	11
THSD4	ENST00000355327.3	15q23	10
CREB1	ENST00000432329.2	2q34	10
SYT7	ENST00000263846.4	11q12.2	6
PRKACA	ENST00000308677.4	17q24.2	6

Table 5. Experimentally validated target genes of *miR-1275* in BC

Targets	Gen ID	Description	*P*.value	FDR	Log2fc
DVL3	ENSG00000161202	Dishevelled segment polarity protein 3	6.98E-04	3.48E-03	0.58975
PPP2R2D	ENSG00000175470	Protein phosphatase 2, regulatory subunit B, delta	1.53E-03	6.95E-03	0.616981
THSD4	ENSG00000187720	Thrombospondin type 1 domain containing 4	2.28E-12	3.63E-11	0.938081
CREB1	ENSG00000118260	cAMP responsive element binding protein 1	3.12E-01	5.13E-01	0.269971
SYT7	ENSG00000011347	Synaptotagmin 7	1.28E-48	8.14E-47	1.84905
PRKACA	ENSG00000072062	Protein kinase cAMP-activated catalytic subunit alpha	1.77E-02	5.68E-02	0.296759

4. Discussion

There is evidence that a single microRNA modulates multiple protein-coding and non-coding genes in different ordinary cells and cancerous cells. In human cancer cells, new RNA groups can be detected using the specific nature of microRNA from relevant microRNA analysis. Multiple high-throughput approaches, such as DNA microarrays, MACE-sequencing, PCR-based arrays, and RNA-sequencing, are now available and have made microRNA expression profiles of BC, showing the irregular expression of numerous miRNAs (26-29). One approach to detect the most essential miRNA from numerous miRNAs is to detect differential expression of miRNAs which have been shown in numerous experiments. Several researches have reported that multiple miRNAs, such as *miR-100, miR-107, miR-205-3p, miR-122* and *miR-99a-5p*, are continuously down-expressed and act as tumor-suppressive miRNA in BC cells (26, 28-33). In this study, these miRNAs were found to be downregulated in tumor cells but only *miR-1275* was focused and their putative target genes were newly explored in paraffin embedded BC tissues. Several researches have reported that this miRNA is down-expressed in gastric and nasopharyngeal carcinoma and function as a tumor suppressor (34-36); whereas, this miRNA is overexpressed in non-small lung cell cancer, squamous carcinoma and chronic myelogenous leukemia (37-39). Some recent studies revealed that this downregulated miRNA was detected to have an essential effect on cancer cell proliferation, migration, invasion, metastasis, and angiogenesis through targeting multiple oncogenic genes *HOXB5*, *WNT7B* and *LncRNA* HAND2-AS1(34, 36, 39). One previous study showed that *miR-1275* regulates *IGF1, NFIX*, *Claudin11* in very young women with BC (23). Whereas, down-expression of *miR-1275* in all subtypes of paraffin BC tissues was not fully investigated. In this study, down-expression of miRNA was observed in all subtypes of paraffin embedded BC tissues of 21 cases with different ages.

After that, the *miR-1275*-modulated putative targets and their pathways were aimed to explain in the cells of BC. Six genes (*DVL3*, *PPP2R2D*, *TSHD4*, *CREB1*, *SYT7*, and *PRKACA*) were experimentally observed to be overexpressed in the cells of BC. Based on the databases of miRNA target prediction, they were selected and closely correlated with poor prognosis. Among these candidate genes, four genes (*PPP2R2D*, *DVL3*, and *CREB1*) were shown to be strongly targeted by the *miR-1275* in the BC cells. Studies showed that these regulators were found to reduce cell survival and migration in cancer cells (40, 41). *DVL3* is observed to be implicated in the breast cancer pathways (34) and negatively regulated by *miR-1275*. The up-regulation of this gene can increase the cancer cell proliferation, migration and invasion in BC cells. The overexpression of which increase cancer cell proliferation ability (42). Another target gene, *CREB1* and *PRKACA* show also negative correlation with *miR-1275* level. Whereas *CREB1* was found to reduce apoptosis process and increase cell proliferation in breast cancer (43), *PRKACA* plays a key role in tumorigenesis and development of BC (44). However, the role of *THSD4* and *SYT7*, currently unidentified, may enhance tumor growth in a variety of cancers, especially breast cancer (45, 46).

5. Conclusion

In the present study, differential expression profiles of total mRNA transcripts and sRNAs were identified in BC paraffin tissue (NAT and tumoral tissue) by MACE- sequencing. Decreased *miRNA-1275* expression develops breast cancer by increasing the activity of biological processes; such as growth, migration, invasion and metastasis. Upregulated *miRNA-1275* prevented BC development by modulating direct expression of *DVL3*, *PPP2R2D*, *TSHD4*, *CREB1*, *SYT7*, and *PRKACA*. This is the first study revealing that *miR-1275* function as a tumor-suppressive miRNA in BC cells, regulating numerous targets which were closely related with BC pathogenesis and oncogenesis.

Abbreviations

FFPE: Formalin Fixed Paraffin Embedded; BC: Breast Cancer; NAT: Normal adjacent tissue; sRNA: small RNA; MACE: Massive Analysis of cDNA Ends; E.R.: Estrogen Receptor; Pg. R.: Progesterone Receptor; HER2: Human epidermal growth factor receptor 2; TCGA: The Cancer Genome Atlas; FDR: False Discovering Rate; FC: Fold Change.

Declarations

Acknowledgement

I wish to thank the patients and their families. I would like to express my specially thanks of gratitude to my supervisor for her frequent support and encouragement. I also offer my sincere appreciation for Al mufti and Luay laboratories which helped and supported me through collecting FFPE blocks of BC and clinical data.

Author contribution

Suhad and Sevan were responsible for the experimental design. They led to the execution of the experiments. Although analyses of MACE-sequencing and sRNA sequencing were done in Genxpro company, in Germany. Data analysis and bioinformatic tasks were done by Sevan. Sevan also discussed and interpreted the data. He also did the manuscript mapping and submission, but Suhad supervised the project.

Funding

This study was supported by Sevan Majed who is a Ph.D. student.

Availability of data and materials

Although row data of MACE-sequencing and sRNA may be available in the database of GenXpro, at https://genxpro.net/, These data will be further studied for another research in the future. The findings described in this manuscript were provided by the Co-author.

Ethics approval and consent to participate

This study was followed and approved by Human Research Ethics Committee at Science College in Salahuddin University-Erbil (Approval no.4c/132). Informed consent was received from all patients in accordance with the requirements of the Human Research Ethics Committee.

Consent for publication

Not applicable.

Competing interest

The author announces no conflict of interest. Mr. Sevan is a teacher of Salahaddin University-Erbil, a subsidiary of ministry of higher education in Kurdistan region government (KRG).

Author details

[1]Biology Department, College of Education, Salahaddin University-Erbil. [2]Research Center, Molecular genetics lab, Salahaddin University-Erbil.

References

1. Control CfD, Prevention. Cancer survivors–United States, 2007. MMWR Morbidity and mortality weekly report. 2011;60(9):269.
2. Jiang X, Tang H, Chen T. Epidemiology of gynecologic cancers in China. J Gynecol Oncol. 2018;29(1).
3. Adhami M, Haghdoost AA, Sadeghi B, Malekpour Afshar R. Candidate miRNAs in human breast cancer biomarkers: a systematic review. Breast Cancer. 2018;25(2):198-205.
4. Toda H, Seki N, Kurozumi S, Shinden Y, Yamada Y, Nohata N, et al. RNA-sequence-based microRNA expression signature in breast cancer: tumor-suppressive miR-101-5p regulates molecular pathogenesis. Mol Oncol. 2020;14(2):426-46.
5. DeSantis C, Howlader N, Cronin KA, Jemal A. Breast cancer incidence rates in US women are no longer declining. Cancer Epidemiology and Prevention Biomarkers. 2011;20(5):733-9.
6. Campeau PM, Foulkes WD, Tischkowitz MD. Hereditary breast cancer: new genetic developments, new therapeutic avenues. Human genetics. 2008;124(1):31-42.
7. Economopoulou P, Dimitriadis G, Psyrri A. Beyond BRCA: new hereditary breast cancer susceptibility genes. Cancer treatment reviews. 2015;41(1):1-8.
8. Perou CM, Sørlie T, Eisen MB, Van De Rijn M, Jeffrey SS, Rees CA, et al. Molecular portraits of human breast tumours. Nature. 2000;406(6797):747-52.
9. Rouzier R, Perou CM, Symmans WF, Ibrahim N, Cristofanilli M, Anderson K, et al. Breast cancer molecular subtypes respond differently to preoperative chemotherapy. Clinical cancer research. 2005;11(16):5678-85.
10. Eroles P, Bosch A, Pérez-Fidalgo JA, Lluch A. Molecular biology in breast cancer: intrinsic subtypes and signaling pathways. Cancer treatment reviews. 2012;38(6):698-707.
11. Park S, Koo JS, Kim MS, Park HS, Lee JS, Lee JS, et al. Characteristics and outcomes according to molecular subtypes of breast cancer as classified by a panel of four biomarkers using immunohistochemistry. The Breast. 2012;21(1):50-7.
12. Prat A, Pineda E, Adamo B, Galván P, Fernández A, Gaba L, et al. Clinical implications of the intrinsic molecular subtypes of breast cancer. The Breast. 2015;24:S26-S35.
13. Duttagupta R, Jiang R, Gollub J, Getts RC, Jones KW. Impact of Cellular miRNAs on Circulating miRNA Biomarker Signatures. PloS one. 2011;6(6):e20769.
14. Tomimaru Y, Eguchi H, Nagano H, Wada H, Kobayashi S, Marubashi S, et al. Circulating microRNA-21 as a novel biomarker for hepatocellular carcinoma. Journal of hepatology. 2012;56(1):167-75.
15. Gebert LF, MacRae IJ. Regulation of microRNA function in animals. Nature reviews Molecular cell biology. 2019;20(1):21-37.

16. Treiber T, Treiber N, Meister G. Regulation of microRNA biogenesis and its crosstalk with other cellular pathways. Nature Reviews Molecular Cell Biology. 2019;20(1):5-20.
17. Zhang B, Pan X, Cobb GP, Anderson TA. microRNAs as oncogenes and tumor suppressors. Developmental biology. 2007;302(1):1-12.
18. Rufino-Palomares EE, Reyes-Zurita FJ, Lupiáñez JA, Medina PP. MicroRNAs as oncogenes and tumor Suppressors. MicroRNAs in Medicine. 2014:223-43.
19. Cheng AM, Byrom MW, Shelton J, Ford LP. Antisense inhibition of human miRNAs and indications for an involvement of miRNA in cell growth and apoptosis. Nucleic Acids Research. 2005;33(4):1290-7.
20. Toda H, Kurozumi S, Kijima Y, Idichi T, Shinden Y, Yamada Y, et al. Molecular pathogenesis of triple-negative breast cancer based on microRNA expression signatures: Antitumor miR-204-5p targets AP1S3. Journal of human genetics. 2018;63(12):1197-210.
21. Iorio MV, Ferracin M, Liu C-G, Veronese A, Spizzo R, Sabbioni S, et al. MicroRNA gene expression deregulation in human breast cancer. Cancer research. 2005;65(16):7065-70.
22. Kwon MJ. Emerging roles of claudins in human cancer. International journal of molecular sciences. 2013;14(9):18148-80.
23. Peña-Chilet M, Martínez MT, Pérez-Fidalgo JA, Peiró-Chova L, Oltra SS, Tormo E, et al. MicroRNA profile in very young women with breast cancer. BMC cancer. 2014;14(1):529.
24. Liu D, Hu X, Zhou H, Shi G, Wu J. Identification of aberrantly expressed miRNAs in gastric cancer. Gastroenterology research and practice. 2014;2014.
25. Parafioriti A, Bason C, Armiraglio E, Calciano L, Daolio PA, Berardocco M, et al. Ewing's Sarcoma: An Analysis of miRNA Expression Profiles and Target Genes in Paraffin-Embedded Primary Tumor Tissue. Int J Mol Sci. 2016;17(5).
26. Kurozumi S, Yamaguchi Y, Kurosumi M, Ohira M, Matsumoto H, Horiguchi J. Recent trends in microRNA research into breast cancer with particular focus on the associations between microRNAs and intrinsic subtypes. Journal of human genetics. 2017;62(1):15-24.
27. Adhami M, Haghdoost AA, Sadeghi B, Afshar RM. Candidate miRNAs in human breast cancer biomarkers: a systematic review. Breast Cancer. 2018;25(2):198-205.
28. Gupta I, Sareyeldin RM, Al-Hashimi I, Al-Thawadi HA, Al Farsi H, Vranic S, et al. Triple negative breast cancer profile, from gene to microRNA, in relation to ethnicity. Cancers. 2019;11(3):363.
29. Khordadmehr M, Shahbazi R, Ezzati H, Jigari-Asl F, Sadreddini S, Baradaran B. Key microRNAs in the biology of breast cancer; emerging evidence in the last decade. Journal of cellular physiology. 2019;234(6):8316-26.
30. Gebeshuber CA, Martinez J. miR-100 suppresses IGF2 and inhibits breast tumorigenesis by interfering with proliferation and survival signaling. Oncogene. 2013;32(27):3306-10.
31. Li XY, Luo QF, Wei CK, Li DF, Li J, Fang L. MiRNA-107 inhibits proliferation and migration by targeting CDK8 in breast cancer. International journal of clinical and experimental medicine. 2014;7(1):32-40.
32. Wang B, Wang H, Yang Z. MiR-122 inhibits cell proliferation and tumorigenesis of breast cancer by targeting IGF1R. PloS one. 2012;7(10):e47053.
33. Toda H, Seki N, Kurozumi S, Shinden Y, Yamada Y, Nohata N, et al. RNA-sequence-based microRNA expression signature in breast cancer: tumor-suppressive miR-101-5p regulates molecular pathogenesis. Molecular oncology. 2020;14(2):426-46.
34. Wang Y-W, Zhang W, Ma R. Bioinformatic identification of chemoresistance-associated microRNAs in breast cancer based on microarray data. Oncology reports. 2018;39(3):1003-10.
35. Mei JW, Yang ZY, Xiang HG, Bao R, Ye YY, Ren T, et al. MicroRNA-1275 inhibits cell migration and invasion in gastric cancer by regulating vimentin and E-cadherin via JAZF1. BMC cancer. 2019;19(1):740.
36. Sun KY, Peng T, Chen Z, Huang J, Zhou XH. MicroRNA-1275 suppresses cell growth, and retards G1/S transition in human nasopharyngeal carcinoma by down-regulation of HOXB5. J Cell Commun Signal. 2016;10(4):305-14.
37. He J, Yu L, Wang CM, Zhou XF. MiR-1275 promotes non-small cell lung cancer cell proliferation and metastasis by regulating LZTS3 expression. European review for medical and pharmacological sciences. 2018;22(9):2680-7.
38. Liu MD, Wu H, Wang S, Pang P, Jin S, Sun CF, et al. MiR-1275 promotes cell migration, invasion and proliferation in squamous cell carcinoma of head and neck via up-regulating IGF-1R and CCR7. Gene. 2018;646:1-7.
39. Yang JR, Shi MX, Zeng Y. LncRNA HAND2-AS1 inhibits proliferation and promotes apoptosis of chronic myeloid leukemia cells by sponging with micRNA-1275. European review for medical and pharmacological sciences. 2019;23(5):2103-11.
40. Kuo Y-C, Huang K-Y, Yang C-H, Yang Y-S, Lee W-Y, Chiang C-W. Regulation of phosphorylation of Thr-308 of Akt, cell proliferation, and survival by the B55α regulatory subunit targeting of the protein phosphatase 2A holoenzyme to Akt. Journal of Biological Chemistry. 2008;283(4):1882-92.

41. Kiely PA, O'Gorman D, Luong K, Ron D, O'Connor R. Insulin-like growth factor I controls a mutually exclusive association of RACK1 with protein phosphatase 2A and β1 integrin to promote cell migration. Molecular and cellular biology. 2006;26(11):4041-51.
42. Jiang H, Cheng L, Hu P, Liu R. MicroRNA-663b mediates TAM resistance in breast cancer by modulating TP73 expression. Molecular medicine reports. 2018;18(1):1120-6.
43. Friedrich M, Heimer N, Stoehr C, Steven A, Wach S, Taubert H, et al. CREB1 is affected by the microRNAs miR-22-3p, miR-26a-5p, miR-27a-3p, and miR-221-3p and correlates with adverse clinicopathological features in renal cell carcinoma. Scientific Reports. 2020;10(1):6499.
44. Tang W, Li GS, Li JD, Pan WY, Shi Q, Xiong DD, et al. The role of upregulated miR-375 expression in breast cancer: An in vitro and in silico study. Pathology, research and practice. 2020;216(1):152754.
45. Cohen H, Ben-Hamo R, Gidoni M, Yitzhaki I, Kozol R, Zilberberg A, et al. Shift in GATA3 functions, and GATA3 mutations, control progression and clinical presentation in breast cancer. Breast cancer research : BCR. 2014;16(6):464-.
46. Liu X, Li C, Yang Y, Liu X, Li R, Zhang M, et al. Synaptotagmin 7 in twist-related protein 1-mediated epithelial – Mesenchymal transition of non-small cell lung cancer. EBioMedicine. 2019;46:42-53.

Figures

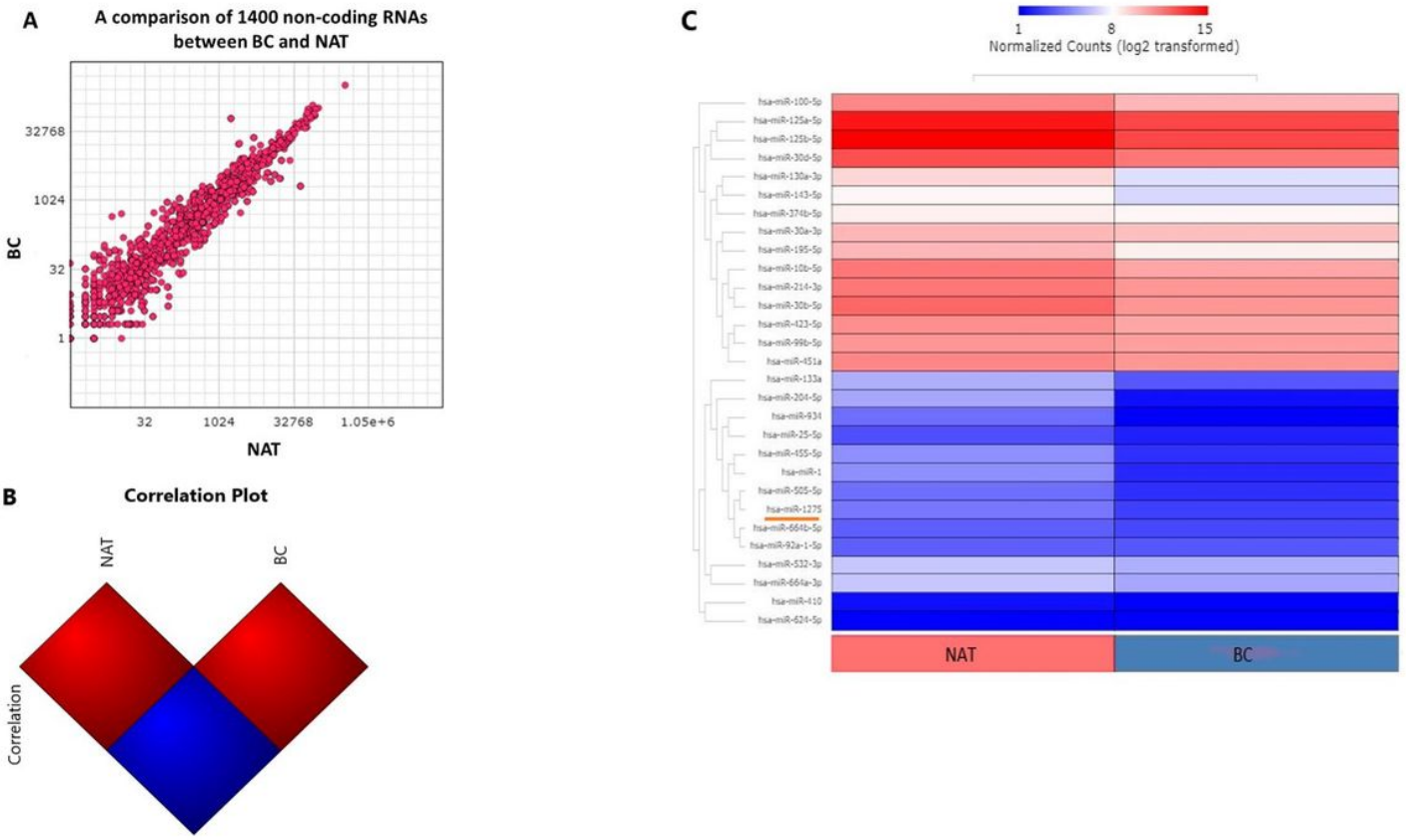

Figure 1

Differential expression analysis of non-coding RNAs by MACE-sequencing. A The expression profile of 1400 sRNAs in BC compared to NAT is plotted. Red dots represent the sample sRNAs. A light green dot represents miRNA-1275 expression level in BC tissue compared to NAT. B Heat-map based clustering of several downregulated miRNAs in BC compared to NAT. Has-miRNA-1275 is underlined. C correlation between BC and NAT in differential expression of sRNAs.

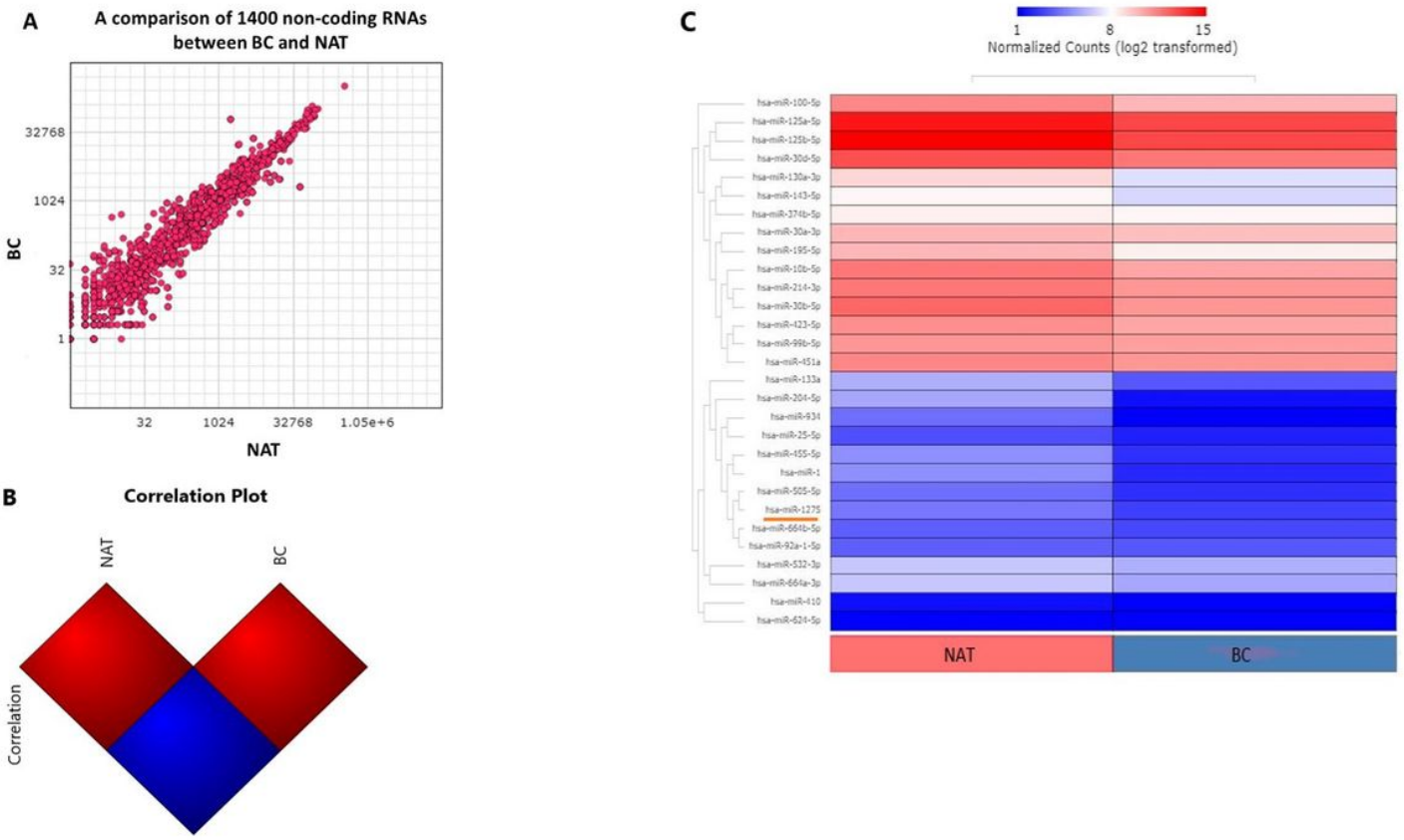

Figure 1

Differential expression analysis of non-coding RNAs by MACE-sequencing. A The expression profile of 1400 sRNAs in BC compared to NAT is plotted. Red dots represent the sample sRNAs. A light green dot represents miRNA-1275 expression level in BC tissue compared to NAT. B Heat-map based clustering of several downregulated miRNAs in BC compared to NAT. Has-miRNA-1275 is underlined. C correlation between BC and NAT in differential expression of sRNAs.

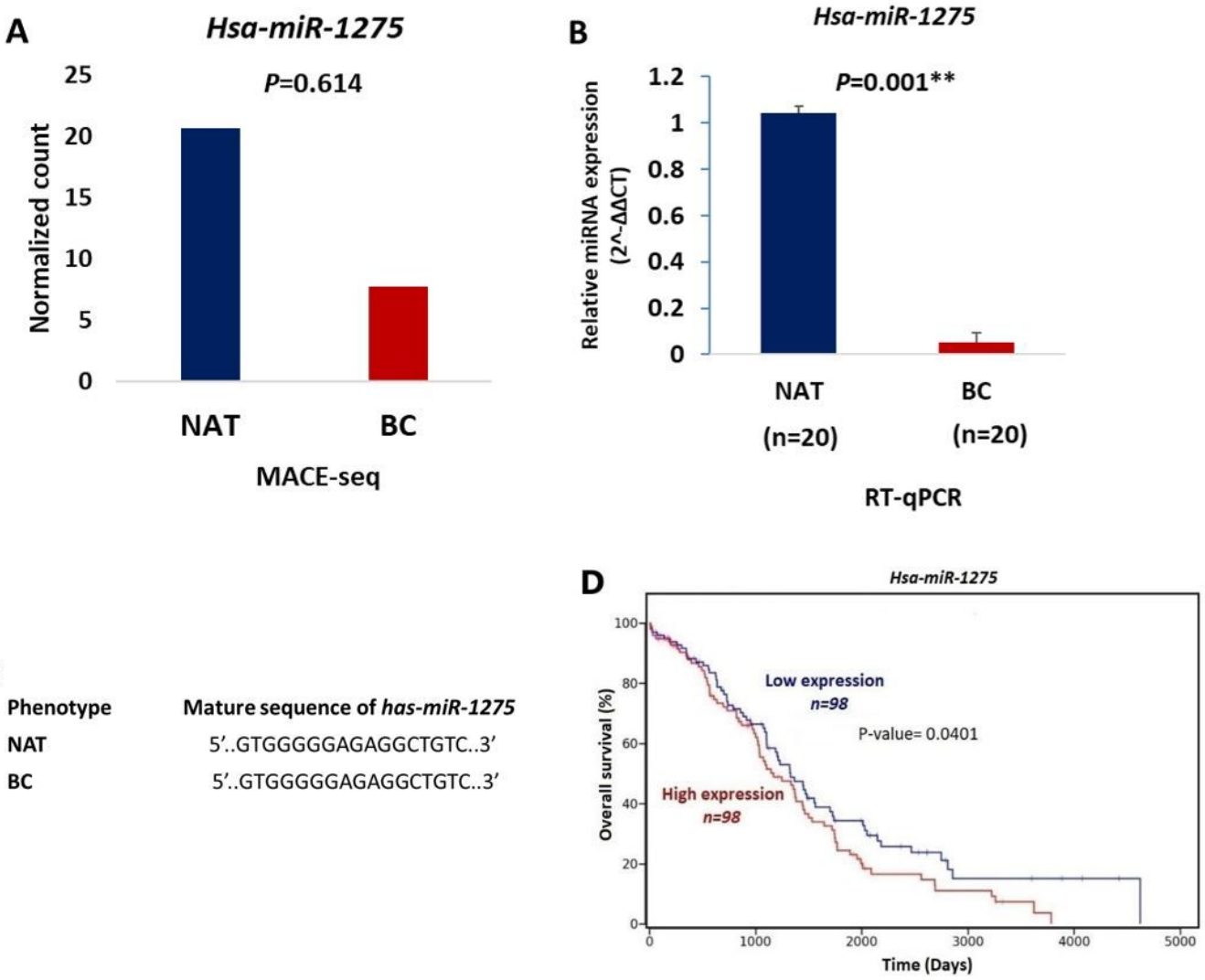

Figure 2

A Comparison of differential expression of miR-1275 by MACE-seq approach. B Comparison of differential expression of miR-1275 by RT-qPCR approach. C mature sequence of miR-1275 in NAT and BC was the same. D Kaplan–Meier overall survival curve designed to show the differential expression of miR-1275 related to overall survival in the patients with the BC.

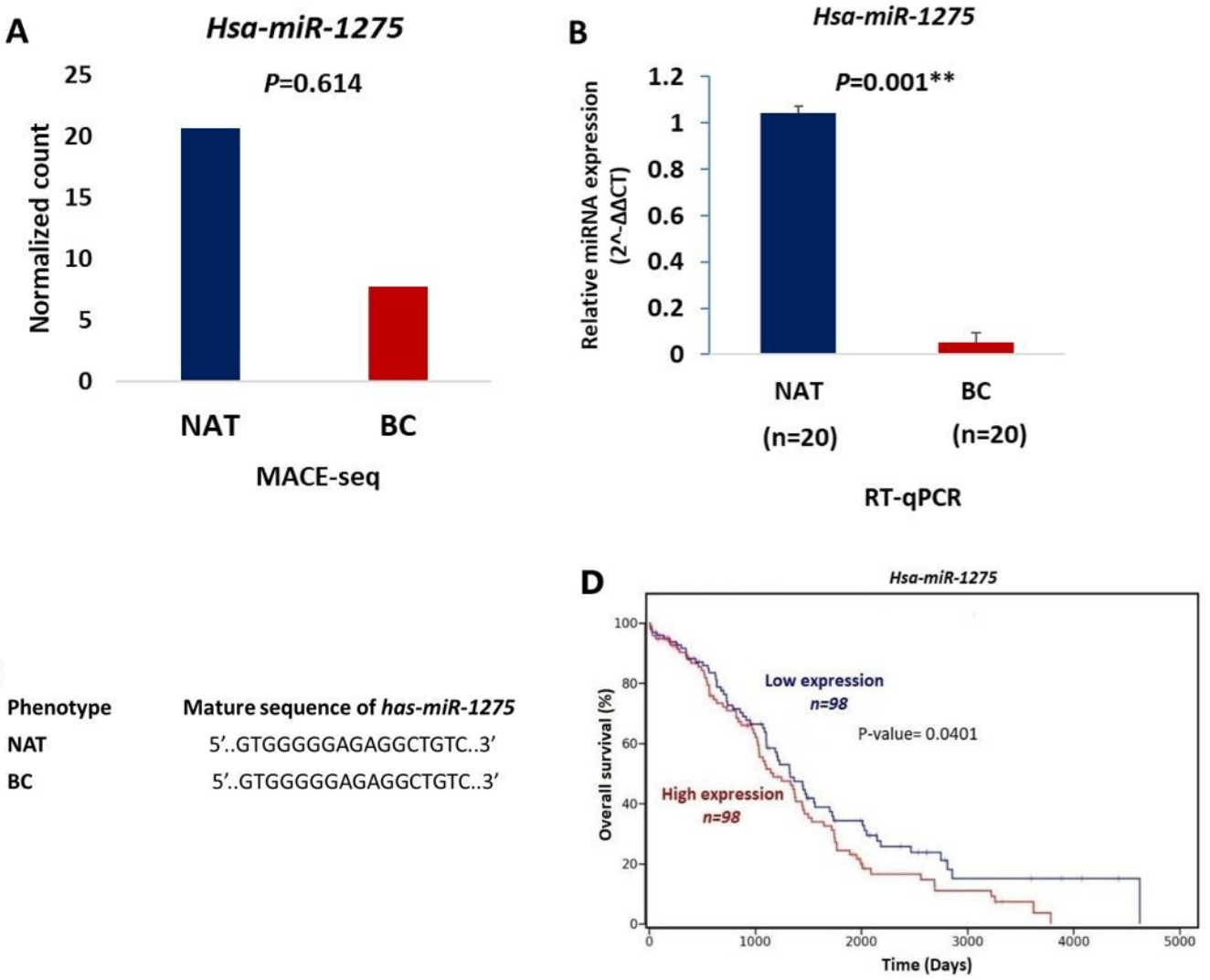

Figure 2

A Comparison of differential expression of miR-1275 by MACE-seq approach. B Comparison of differential expression of miR-1275 by RT-qPCR approach. C mature sequence of miR-1275 in NAT and BC was the same. D Kaplan–Meier overall survival curve designed to show the differential expression of miR-1275 related to overall survival in the patients with the BC.

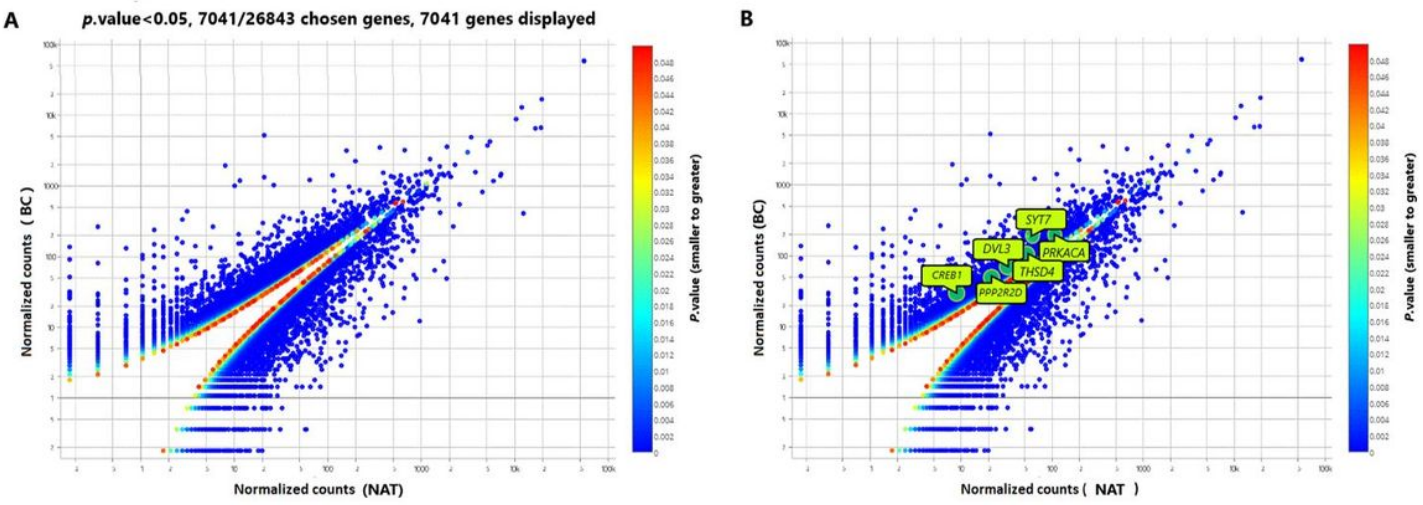

Figure 3

A Scatter plot analysis of gene expression profile displays up- or down-regulation of genes in BC tissue, compared to NAT. Each point denotes the average value of one transcript in the experiment. The expression difference is taken account of significance for a Pvalue (0.05). B Outlined points and names denote the selected target genes for has-miR-1275.

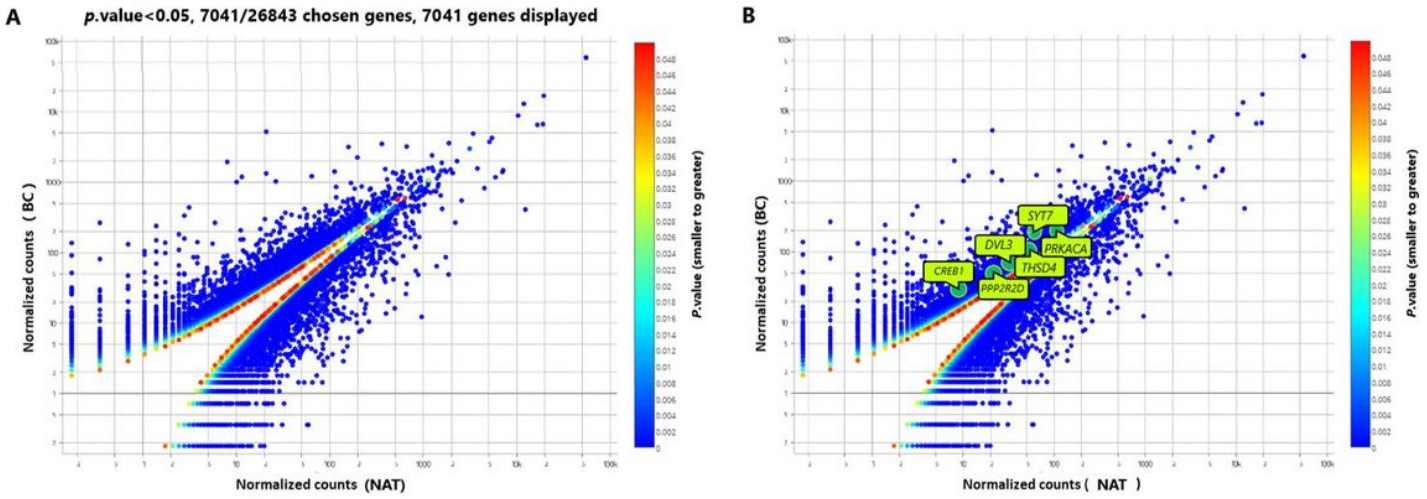

Figure 3

A Scatter plot analysis of gene expression profile displays up- or down-regulation of genes in BC tissue, compared to NAT. Each point denotes the average value of one transcript in the experiment. The expression difference is taken account of significance for a Pvalue (0.05). B Outlined points and names denote the selected target genes for has-miR-1275.

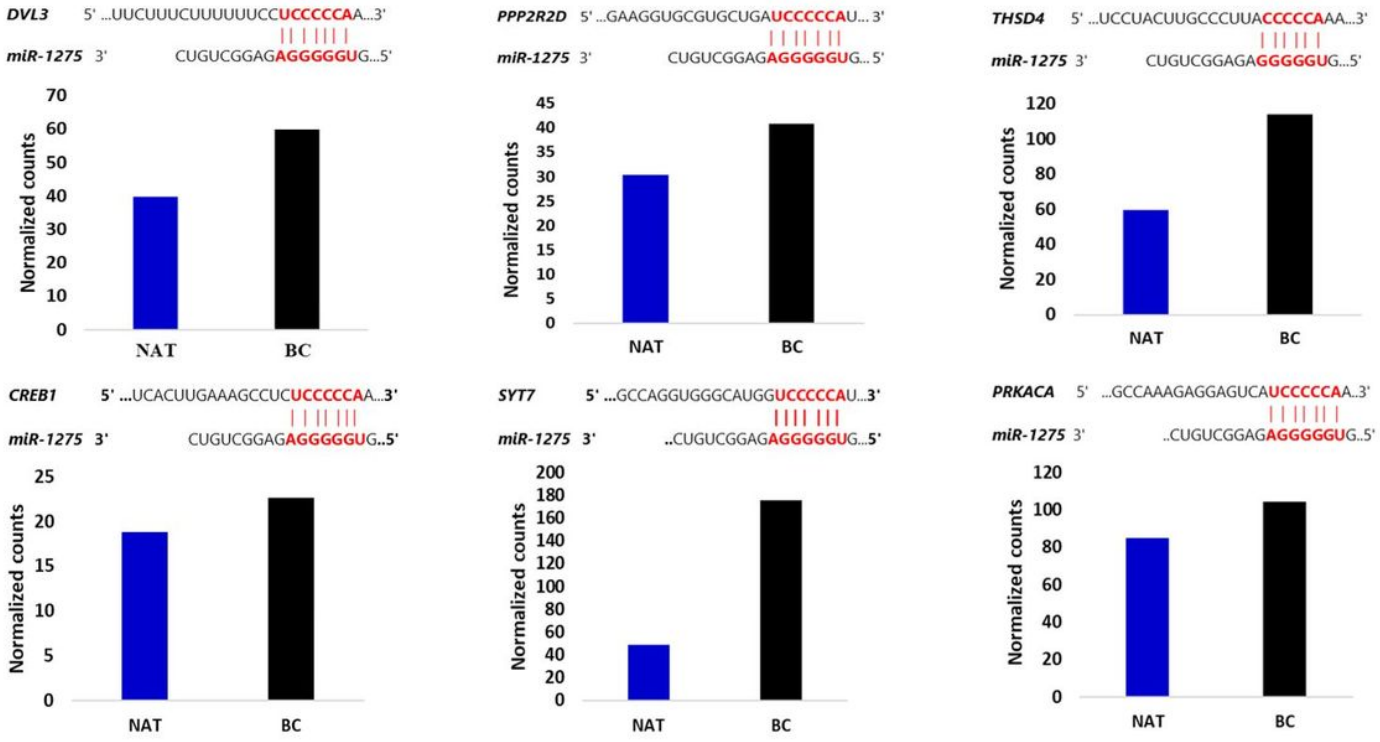

Figure 4

Target genes and has-miR-1275 are combined in the seed region, including 6 to 8 nucleotides in the 5' end, showing in red color. Differential expression level of target genes in BC compared to NAT.

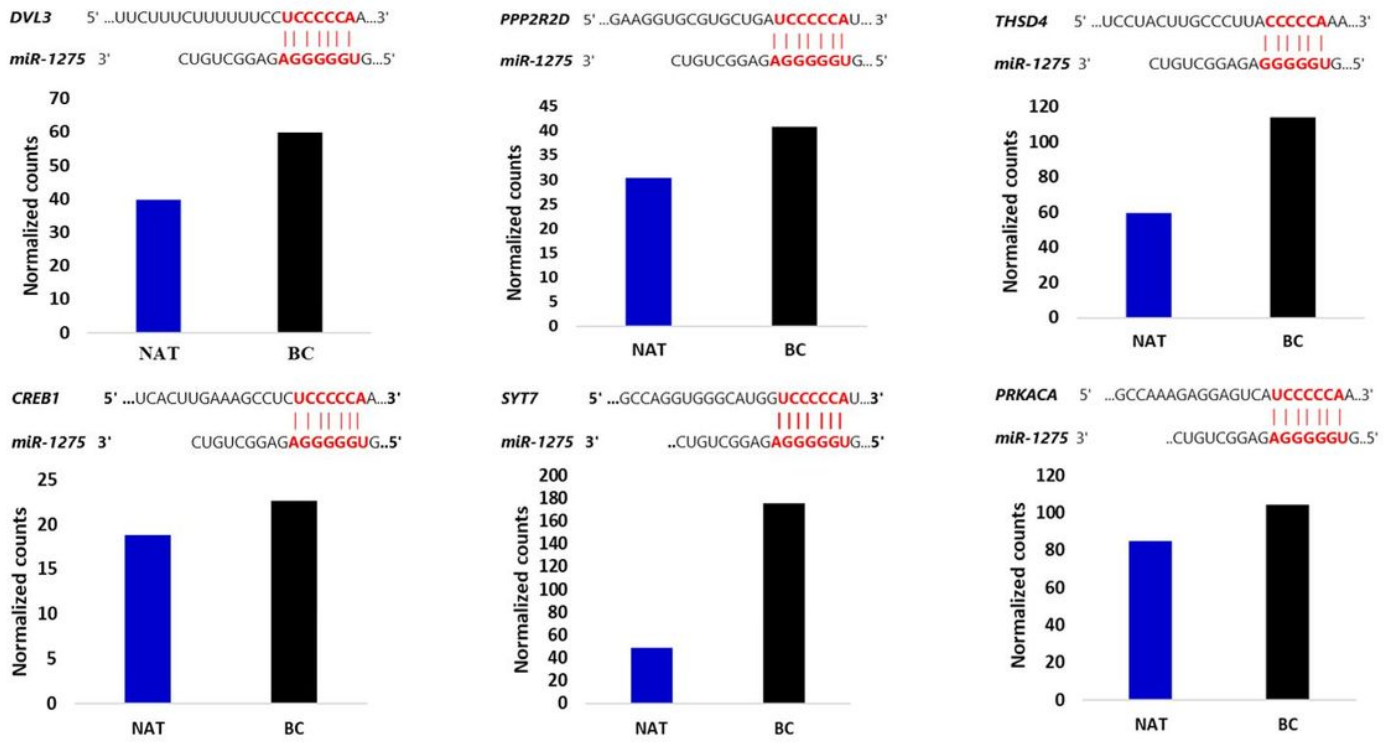

Figure 4

Target genes and has-miR-1275 are combined in the seed region, including 6 to 8 nucleotides in the 5' end, showing in red color. Differential expression level of target genes in BC compared to NAT.

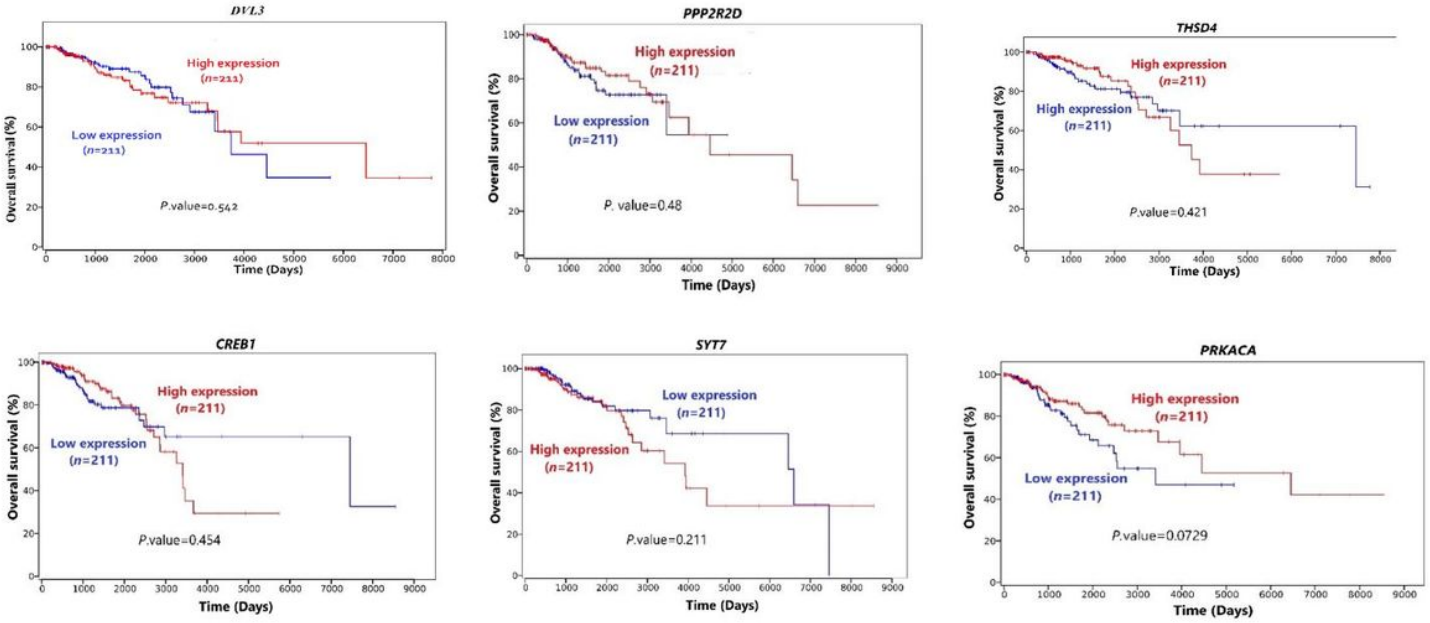

Figure 5

An association between the expression levels of six genes (DVL3, PPP2R2D, TSHD4, CREB1, SYT7, and PRKACA) and histopathological significance was shown using data from TCGA database. The Kaplan–Meier overall survival curves show that patients with BC were separated into 2 classes according to their expression levels.

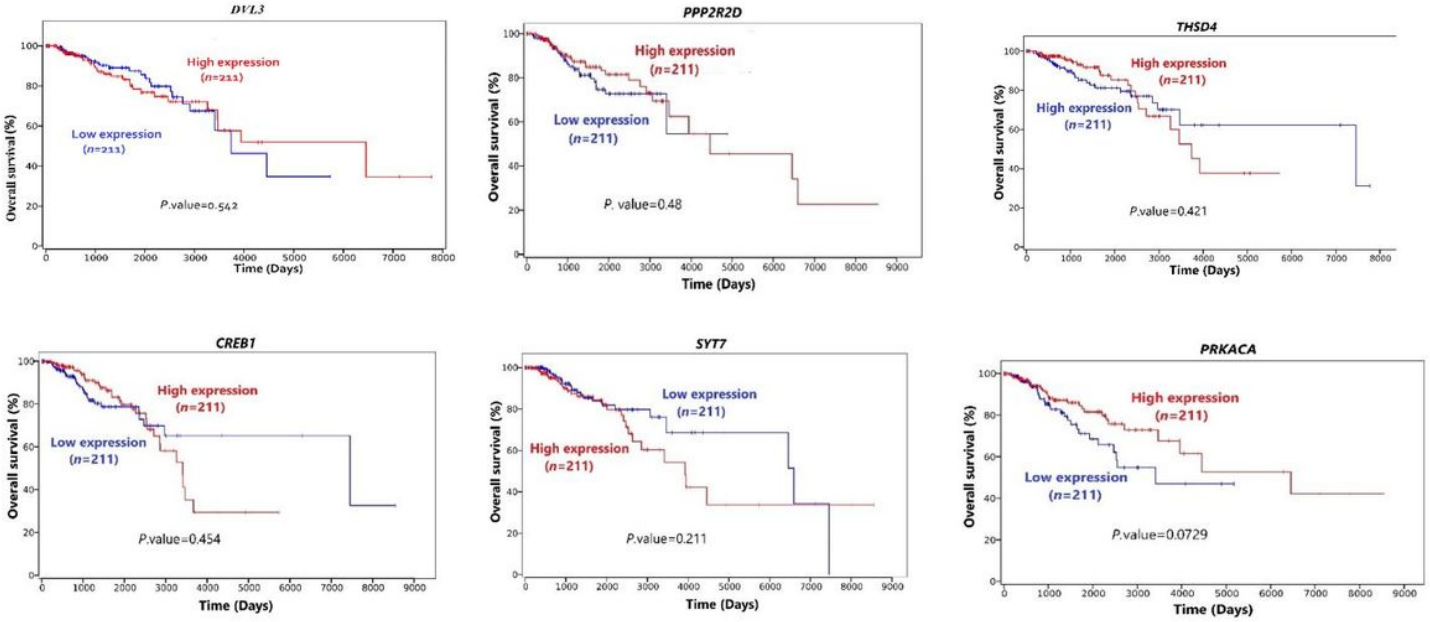

Figure 5

An association between the expression levels of six genes (DVL3, PPP2R2D, TSHD4, CREB1, SYT7, and PRKACA) and histopathological significance was shown using data from TCGA database. The Kaplan–Meier overall survival curves show that patients with BC were separated into 2 classes according to their expression levels.

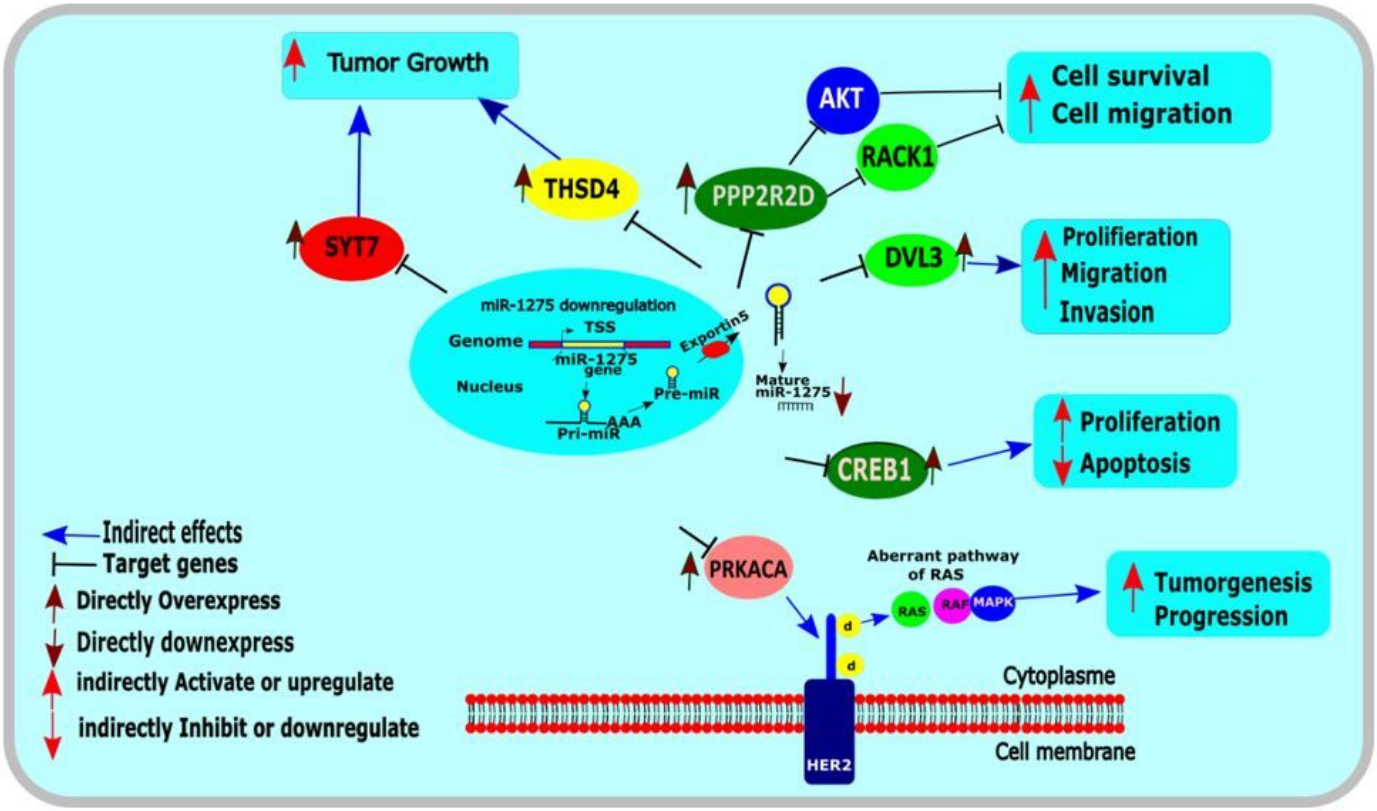

Figure 6

miR-1275 putative targets and their roles in the BC.

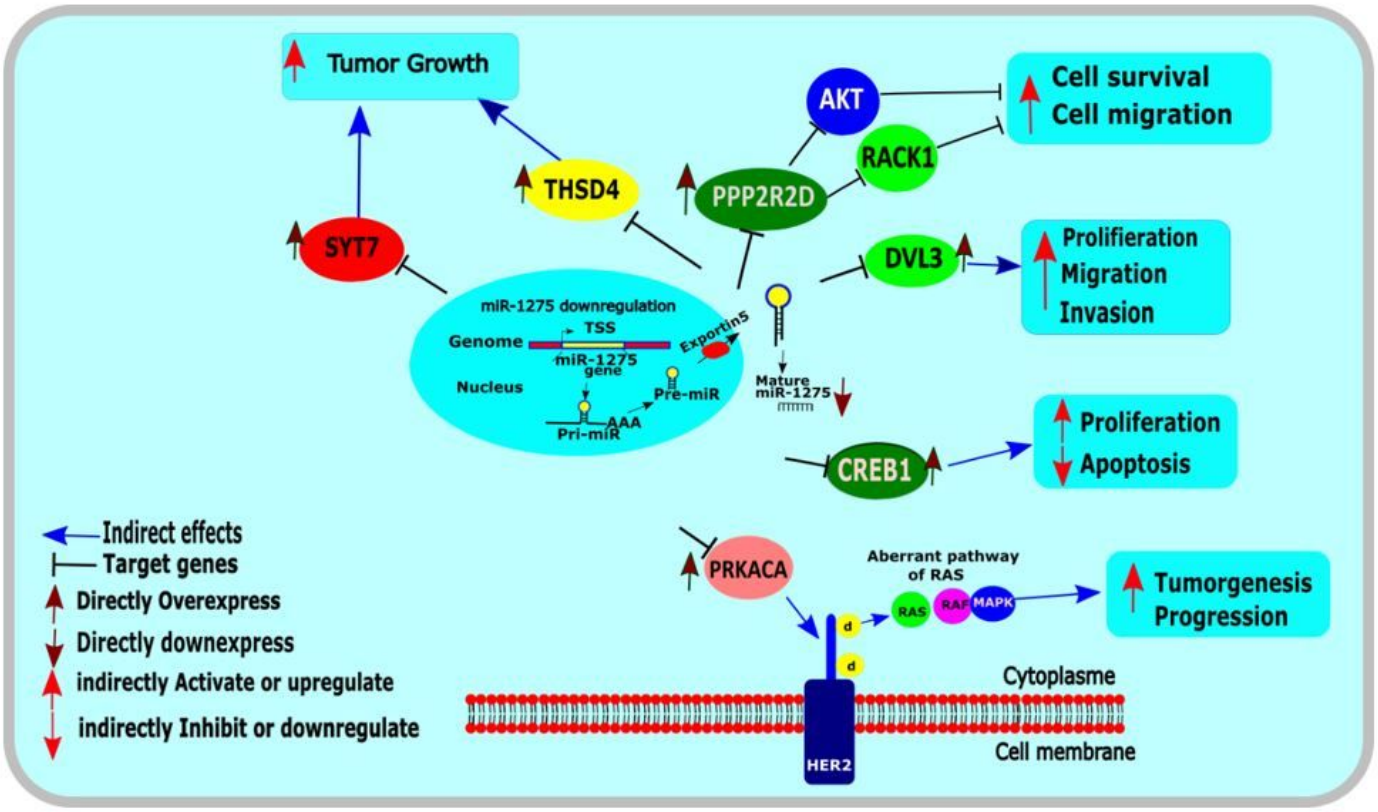

Figure 6

miR-1275 putative targets and their roles in the BC.