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ARTICLE

Identification of differentially expressed miRNAs and mRNAs associated with the regulation of breast cancer via in silico and in vitro methods

Pelin Telkoparan-Akillilar · Dilek Cevik

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Abstract miRNA expressions are altered during development of breast cancer (BC). The aim of this study is to identify novel cancer-related miRNAs and pathways to understand the mechanisms of BC subtypes. GSE59247 dataset was downloaded from gene expression omnibus (GEO) database and analyzed with GEO2R software. The differential miRNA expressions in BC cells were evaluated by miRNome PCR array. Venn diagram was used to reveal co-differentially expressed miRNAs between GSE59247 dataset and miRNome array. Clinical prognostic significance of selected miRNAs was evaluated via Kaplan Meier curve. KEGG pathway enrichment analysis was performed to find miRNA targets and results were validated by TNM plot analysis and q-RT-PCR. TargetScan database was used to predict the association of miRNAs and 3′-untranslated regions of target genes and their expressions were visualized by human protein atlas database. Venn diagram analysis showed overlap of 11 miRNAs from in silico and in vitro analysis. KEGG analysis revealed 'Lysine Degradation Pathway' as the most significantly enriched targeted pathway. q-RT-PCR results confirmed that Lysine degradation pathway related genes SETD7, SETDB2, EHHADH, SETMAR, KMT2A and SUV39H2 were differentially expressed in BC cells. Target prediction analysis identified binding sites between miR-1323-5p and 3′-UTR of SETD7, miR-129-5p and 3′-UTR of EHHADH and miR-628-5p and 3′-UTR of SETDB2 mRNA. Notably, miR-1323-5p, miR-129-5p, and miR-628-5p are differentially expressed in BC and they bind to 3'UTR of critical genes of Lysine degradation pathway, namely SETD7, SETDB2 and EHHADH. These miRNAs might serve as potential diagnostic and prognostic biomarkers for progression.

Keywords Breast cancer · miRNA · Biomarker · Lysine degradation pathway

Introduction

Despite the developments in early diagnosis methods and advances in treatments, Breast Cancer (BC) still has the highest incidence and mortality in women all over the world (Silvestris et al. 2020). 2.2 million women were diagnosed with BC and 684,996 women died due to BC in 2020 (Sung et al. 2021). Today, prognosis of BC is evaluated based on the expressions of hormone receptors such as estrogen receptor (ER) alpha, progesterone receptor (PR), human epidermal growth factor receptor 2 (HER2) in the tumor tissue together with factors like tumor size, tumor grade, histopathological subtype, tumor cell invasion and axillary lymph node (X. Dai et al.

P. Telkoparan-Akillilar (✉) · D. Cevik
Department of Medical Biology, Faculty of Medicine, Yuksek Ihtisas University, No.18A, 1505. Street, Ankara 06530, Turkey
e-mail: pelinta@yiu.edu.tr

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2017; Prat et al. 2015). Different combinations of these factors produce subtypes such as Luminal A (ER positive, PR positive, HER2-negative), Luminal B (ER positive and HER2 negative), Luminal B-like (ER positive and HER2 positive), and triple- negative BC (TNBC) in which none of these hormones are expressed (X. Dai et al. 2017). TNBC is a specific BC subtype without ER, PR, or HER2 expression, comprising an increased metastatic potential, tendency to relapse, and poor prognostic clinical features (Perou et al. 2000; Faulkes WD et al., 2010). Since TNBC tumors lack receptors, they are not sensitive to endocrine therapy or molecular targeted therapies. In this case, chemotherapy is the main systemic therapy, but the efficacy of conventional postoperative adjuvant chemoradiotherapy is poor. Therefore, development of novel TNBC treatment strategies is an urgent clinical need.

MicroRNAs (miRNAs) are evolutionarily conserved, small, non-coding, endogenous, universal RNA regulators of important biological processes with 19 to 25 nucleotides long (Ha and Kim 2014). In several cancer types, dysregulation of the miRNA profile has been associated with mechanisms of disease development, including invasiveness and metastasis (Shuibin Lin and Richard I. Gregory, 2015). In *vitro* and in vivo studies in TNBC identified several miRNAs likely to be related to the aggressive phenotype (Chang et al. 2015a; Zhu et al. 2017). miRNAs have great potential in therapeutic design due to their variable functions in cell homeostasis, showing that miRNAs could be a potential therapeutic tool as well as promising biomarkers in personalized therapy. Therefore, more miRNA-target pathway information needs to be uncovered for the development of novel targeted molecules and identification of biomarkers to prolong the lifespan of BC patients.

In silico bioinformatics platforms can support the analysis of miRNA expressions using microarrays and RNA sequencing data uploaded by different researchers to identify novel disease-related biomarkers for use in cancer diagnosis and treatment. In previous studies, large volumes of microarray data, and several gene expression profiling studies on BC identified differentially expressed miRNAs, as well as novel miRNAs in various pathways, molecular functions, and biological processes (Chang et al. 2015b; Hu et al. 2018; Wu et al. 2010). In this study, we first determined differentially expressed miRNAs between normal breast tissue and BC tissue data with different clinicopathological features using GSE59247 data set (Lesurf et al. 2016). Afterwards, we compared global miRNA levels of MDA-MB-231 and MCF10A cell lines by using human miRNA arrays. Finally, we identified co-differentially expressed miRNAs in the intersection of in silico and in vitro data and analyzed their targeted pathways.

Our findings are notable in terms of revealing BC-specific miRNA biomarkers and their targeted pathways obtained from clinical and in vitro studies. And these findings may serve as prognostic and predictive targets for future studies, specifically for reducing mortality of BC patients.

Materials and methods

Differential expression analysis of miRNAs

The Gene Expression Omnibus database (GEO, http://www.ncbi.nlm.nih.gov/geo) was used for miRNA expression profiling studies in BC samples and adjacent normal breast tissue from BC patients. GEO database serves as a public platform providing high-throughput gene expression data. Above all, miRNAs in BC samples that are differentially expressed between BC patients (n = 44) and normal breast tissue (n = 4) were identified by GEO2R from the GSE59247 dataset (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE59247, accessed on 14 January 2022). This dataset was obtained from a study that performed gene expression analysis using Agilent SurePrint G3 Human GE 8 × 60 K Microarrays and Human miRNA Microarray Release 16.0, 8 × 60 K, respectively (Lesurf et al. 2016).

Cell culture

Human BC cells (MDA-MB-231, MCF7) and human normal breast cell (MCF10A) were obtained from the ATCC (Manassas, VA, USA). MDA-MB-231 and MCF-7 cells were cultured in DMEM included 10% FBS, 1% penicillin/streptomycin. MCF10A cells were cultured in DMEM (Lonza) supplemented with 20 ng/ml epidermal growth factor (EGF), 0.01 mg/ml insulin, 500 ng/ml hydrocortisone, and 20% FBS, 1% penicillin/streptomycin. All cells were maintained at 37 °C in a 5% CO2 air atmosphere.

miRNA isolation and human miScript miRNA PCR array

miRNAs were isolated from MDA-MB-231 and MCF10A cells using a miRNeasy Mini kit (Qiagen) and cDNA synthesis was performed using miScript II RT (Qiagen), according to the manufacturer's instructions. Qiagen 100-well rotor discs (MIHS-216ZR-4) were used to determine the expression profiles of 1008 miRNAs between MDA-MB-231 and MCF-10A cell lines via array analysis. To perform the miRNome PCR array cDNA was diluted and combined with miScript SYBR Green PCR components according to the manufacturer's protocol and then it was added to a miScript miRNA PCR Array 100 well Rotor-Disc. The thermocycling conditions were as follows: 15 min at 95° followed by 40 cycles of 94 °C for 15 s, 55 °C for 30 s, and 70 °C for 30 s. A cycle threshold (CT) value of 0.02 was used to analyze the data according to the protocol. CT values were inserted into the miScript miRNA PCR Array Data Analysis Excel Template available at https://dataanalysis.qiagen.com/mirna/templates/miScript_miRNA_PCR_Array_Template.xls and the quantification of differentially expressed miRNAs was performed with the ΔΔCt method by using Qiagen miScript miRNA PCR Arrays & Assays Data Analysis Tool of the manufacturer's website (https://login.qiagen.com/login?service=https://dataanalysis.qiagen.com/mirna/arrayanalysis.php). This tool automatically performed relative quantification of the expression of miRNAs of interest in MDA-MB-231 cell line and compared their levels with those of MCF-10A cells.

Prognostic value of co-differentially miRNAs in BC

To identify the prognostic values of co-differentially expressed miRNAs obtained from in silico and in vitro analyses, we used a web-tool established tool The KaplanMeier Plotter Database (KMPD) (http://kmplot.com/analysis/) by combining data from clinical BC patients (Lánczky et al. 2016). Kaplan Meier survival plots, hazard ratio HR with 95% confidence intervals CI and log rank P were obtained to analyze the correlation of hsa-miR-1323, hsa-miR-542-3p, hsa-miR-331-5p, hsa-miR-129-5p, hsa-miR-628-5p, hsa-miR-342-3p, hsa-miR-1280, hsa-miR-339-5p, hsa-miR-615-3p, hsa-miR-421, and hsa-miR-662 to the overall survival in patients with BC. P-value less than 0.05 (typically ≤ 0.05) was considered statistically significant.

Real time quantitative PCR

Total RNA was extracted from human BC cell lines (MDA-MB-231 and MCF7) and normal breast cell line (MCF10A) by using High Pure RNA Isolation Kit (Roche). cDNA synthesis was performed with High-Capacity cDNA Reverse Transcription Kit (Thermo) following the manufacturer's protocol. Q-RT-PCR was performed using WizPure qPCR SYBR Green Master Mix (Wizbio) in a Rotor-Gene Q (Qiagen). The following forward (Frw) and reverse (Rev) primers were used: *SETMAR*-Frw 5′ TGGAGG AAAGTATGCAGAGC 3′, Rev 5′ CCTTTCTGG ACCACTCTGTTTC 3′; *SETD7*-Frw: 5'CCTGGT GGAAGTTAGGTGCTA 3′, Rev 5′ CGGTGTCTC TAATGCCTCTGA 3′; *SETDB2*-Frw: 5′ GTGGAC CAAAAGATGGAGTTGG 3′, Rev: 5′ TCAGAC AAGCACCAGAGCAG 3′; *KMT2A*-Frw: 5′ CGA TCAAATGCCCGCCTAAA 3′, Rev 5′ TCAGCT GTACCTCCTCCTCT 3′; *EHHADH*-Frw: 5′ CCT GGGCTGTCACTATAGGATT 3′, Rev: 5′ AGAAGC TGGGTTCCTCTTGC 3′; *SUV39H2*-Frw: 5′ ATC CCACCTGGTACTCCCATCT 3′, Rev: 5′ ATCCCA CCTGGTACTCCCATCT 3′; *EHMT1*, Frw: 5′ CAG GACTTCCAAGGAGAGCA 3′, Rev: 5′ ACTCAG GTCAGACTCGTCAC 3′; *GAPDH*-Frw; 5′GACCAC AGTCCATGC CATCACT-3′, Rev 5′TCCACCACC CTGTTGCTGTAG-3′. Real Time PCR reaction conditions**:** 94 °C for 15 min, 45 cycles at 94 °C for 15 s, 54.5 °C for 30 s and 70 °C 30 s. Fold-changes were calculated according to the ΔΔCT method (Livak and Schmittgen 2001).

Bioinformatic analysis of miRNAs

Volcano plots of differentially expressed miRNAs were generated with the GEO2R analysis tool based on the GSE59247 dataset. Venn diagrams of overlapping differentially expressed miRNAs (between GSE59247 data set and miRNome array results) were produced by online tools (https://bioinformatics.psb.ugent.be/cgi-bin/liste/Venn/calculate_venn.htpl). Differentially expressed miRNAs were analyzed using mirPath V.3 online tool which is online available at DIANA-Tools (http://www.microrna.gr/

miRPathv2) (Vlachos and Hatzigeorgiou 2017). This tool performs an enrichment analysis of miRNAs and matches them to the signaling pathways in the KEGG database (Kyoto Encyclopedia of Genes and Genomes) to determine biological pathways regulated by the expression of miRNA queries. Differential expressed genes targeted by co-expressed miRNAs were analyzed by in silico web based TNMplot tool (https://tnmplot.com/analysis/). TNMplot tool compares gene expression changes between tumor and normal samples among different types of platforms across all genes by either grouping all specimens of the same category and running a Mann–Whitney U test by running a paired Wilcoxon statistical test. The results can be visualized by both boxplots and violin plots (Bartha et al. 2021). The analysis for the prediction of binding sites between miRNAs within the 3′UTR of mRNAs was performed using TargetScan database available online at http://www.targetscan.org (Bartel 2009). Protein Atlas (HPA) database (https://www.proteinatlas.org) was used to visualize the protein levels of miRNA target genes in BC compared to normal tissues.

Statistical Analysis

Statistical analyses were performed using the bioinformatic tools mentioned above where applicable. During differential expression analysis, only miRNAs with log2 FC > 1.5 and $p < 0.05$ were considered as statistically significant. For mRNA expressions, differences between groups were determined via student's t -test. $P < 0.05$ was considered as significant. All experiments were performed in triplicates.

Results

Workflow of the study design

In this study, the miRNA microarray GSE59247 dataset which included 44 samples from BC tissue and 4 samples from normal breast tissue was used for the analysis. After data preprocessing, differentially expressed miRNAs were compared with in vitro miRNA analysis results that are obtained from MDA-MB-231 and MCF10A miScript miRNA PCR array data using Venn diagram analysis tool. Afterwards, survival analysis was performed using miRpower software to determine the prognostic values of co-differentially expressed miRNAs (http://kmplot.com/analysis/index.php?p=service&cancer=breast_mirna). We then applied bioinformatic functional analysis via KEGG pathway analysis for common miRNAs of in silico analyses and in vitro cell culture experiments. Subsequently, we determined the most significantly regulated biological pathway and target genes regulated by miRNA queries. Then, differential target gene expressions in BC and normal tissues were visualized via TNMplot in silico tool (https://tnmplot.com/analysis/) (Bartha et al. 2021). We further investigated the expressions of these genes by q-RT PCR in different types of BC cell lines (MDA-MB-231 and MCF7) and normal epithelial BC cell line MCF10A. Target prediction of Lysine degradation pathway related miRNAs was conducted by TargetScan tool (http://www.targetscan.org). Finally, immunohistochemical assessment was performed in BC and normal tissues for genes expression that we differentially expressed according to q-RT PCR by using HPA database (https://www.proteinatlas.org) (Fig. 1).

Differential expression of miRNAs in BC samples and BC cell lines

We initially compared miRNA expression profiles of BC tissue samples with normal breast tissue samples by analyzing GSE59247 public data set obtained from the GEO database (Lesurf et al. 2016). Clinicopathologic characteristics of the tissue samples could be found in the original data (Lesurf et al. 2016). For global miRNA expression profiling of BC and control samples GEO2R analysis tool was used. Totally, 197 miRNAs, with a threshold of $p < 0.05$ and log2 FC > 1.2, were found significantly differentially expressed and visualized via volcano plot (Fig. 2). The list of top 20 significantly differentially expressed miRNAs are shown in Table 1.

To determine the differentially expressed miRNAs in TNBC, we performed a q-RT-PCR based global miRNome miRNA PCR Array experiment covering 1008 human miRNAs in MDA-MB-231 and MCF10A cell lines. A total of 172 miRNAs were identified as differentially expressed (log2 FC ≥ 1.5) in BC cell line MDA-MB-231 with compared to epithelial breast cell line MCF10A. The list of top 10 up-regulated and top 10 down-regulated

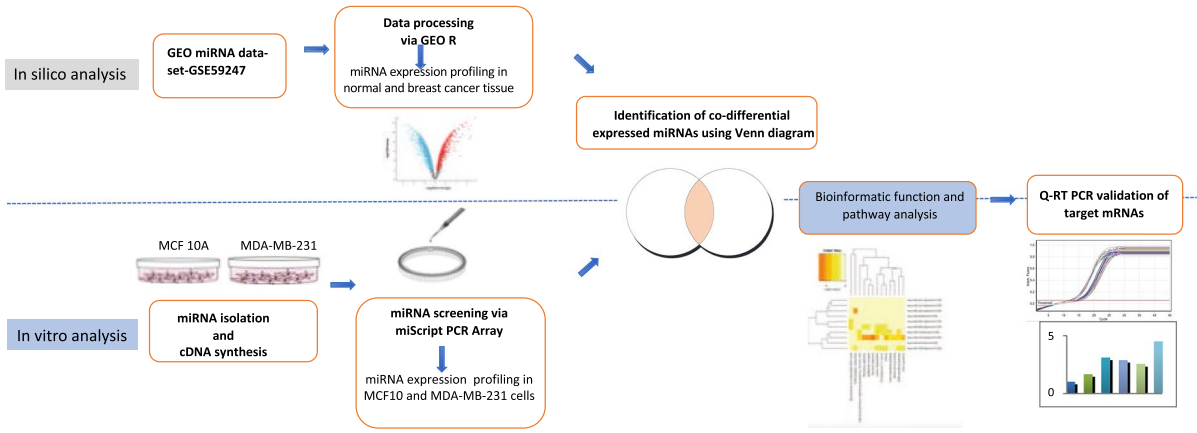


Fig. 1 Workflow of the study design

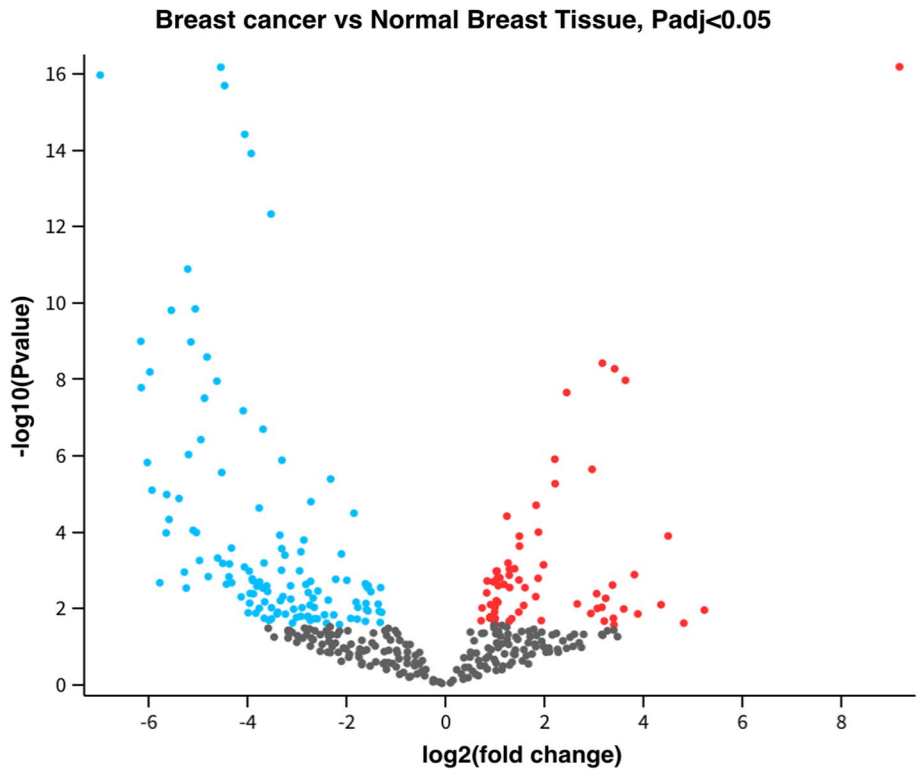


Fig. 2 Volcano plot analysis of differentially expressed miRNAs between BC and normal breast tissue (GSE59247). The blue circles indicate downregulated miRNAs (log2 FC ≤ -1.2) and the red circle's indicate upregulated miRNAs (log2 FC ≥ 1.2, p-value of 0.05)

miRNAs in MDA-MB-231 cell line compered to MCF-10A, defined by miScript microRNA PCR array analysis was shown in Table 2.

Afterwards, the list of co-differentially expressed miRNAs as prognostic biomarkers between in silico miRNA analysis and in vitro miRNA analysis

Table 1 The list of top 20 differentially expressed miRNAs in GSE59247 data set

microRNAs	*P value*	*Fold Change*
hsa-miR-517c	4.46E-14	4.546
hsa-miR-1254	4.79E-14	6.984
hsa-miR-190	6.79E-14	4.470
hsa-miR-520c-3p	1.03E-12	4.062
hsa-miR-519b-3p	2.74E-12	3.932
hsa-miR-520 h	9.06E-11	3.531
hsa-miR-329	2.2E-09	5.215
hsa-miR-383	2.12E-08	5.062
hsa-miR-3925-5p	2.12E-08	5.547
hsa-miR-517b	1.2E-07	6.163
hsa-miR-3200-5p	1.2E-07	5.151
hsa-miR-488	2.75E-07	4.826
*hsa-miR-21**	3.69E-07	− 3.164
hsa-miR-210	4.86E-07	− 3.413
hsa-miR-323-3p	5.48E-07	5.981
hsa-miR-4286	8.5E-07	− 3.632
hsa-miR-298	8.5E-07	4.624
hsa-miR-517a	1.19E-06	6.154
hsa-miR-1280	1.52E-06	− 2.444
hsa-miR-520e	2.03E-06	4.876

Table 2 The list of top 20 differentially expressed miRNAs in MDA-MB-231 compared to MCF10A

	microRNAs	*Fold change*	*Accession*
Up regulated	hsa-miR-10a-5p	290.2	MI0000266
	hsa-miR-139-5p	92.47	MI0000261
	hsa-miR-18a-5p	56.53	MI0000072
	hsa-miR-424-5p	49.55	MI0001446
	hsa-miR-19b-3p	36.78	MI0000074
	hsa-miR-662	36.27	MI0003670
	hsa-miR-126-5p	32.92	MI0000471
	hsa-miR-615-3p	29.06	MI0003628
	hsa-miR-126-3p	28.07	MI0000471
	hsa-miR-100-5p	27.49	MI0000102
Down regulated	hsa-miR-205-5p	− 3954.06	MI0000285
	hsa-let-7b-5p	− 94.95	MI0000063
	hsa-miR-26b-5p	− 20.38	MI0000084
	hsa-miR-34a-5p	− 12.37	MI0000268
	hsa-miR-215-5p	− 8.51	MI0000291
	hsa-miR-1323	− 6.86	MI0003786
	hsa-miR-200c-3p	− 5.73	MI0000650
	hsa-miR-493-3p	− 5.24	MI0003132
	hsa-miR-326	− 5.24	MI0000808
	hsa-miR-342-3p	− 3.73	MI0000805

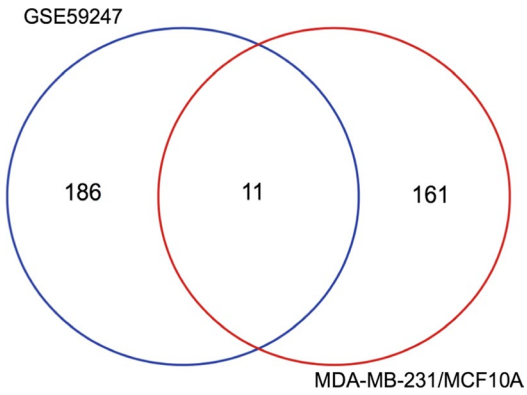
were obtained by constructing Venn diagram. We detected 11 differentially expressed miRNAs namely, hsa-miR-1323, hsa-miR-542-3p, hsa-miR-331–5, hsa-miR-129-5p, hsa-miR-628-5p, hsa-miR-342-3p, hsa-miR-1280, hsa-miR-339-5p, hsa-miR-615-3p, hsa-miR-421, hsa-miR-662 (Fig. 3).

Survival analysis

To identify prognostic values of 11 selected miRNA in BC, we constructed Kaplan Meier plots. Kaplan Meier survival analysis indicated significantly reduced overall survival BC patients with miR-542-3p (HR 0.68, P: 0.00013), miR-628-5p (HR 0.59, P: 1e-05), miR-339-5p (HR 0.79, P: 0.018) expressing, and increased overall survival in BC patients with miR-331-3p (HR 0.2.64, P: 0.014), miR-129-5p (HR 1.28, P: 0.022), miR-421 (HR 1.28, P: 0.017) expressing. However, no significant prognostic effect of miR-1323, miR-342-3p, miR-1280, miR-615-3p was found for BC (Fig. 4).

Regulatory networks of co-differentially expressed miRNA

To assess the biological role of co-differentially expressed miRNAs obtained from in silico and in vitro analysis, we used DIANA mirPath V.3 web-based tool for pathway enrichment analysis (www.microrna.gr) (Vlachos and Hatzigeorgiou 2017). This analysis included the 11 miRNAs that are co-differentially expressed in BC tissues compared to normal breast samples and MDA-MB-231 cell line compared to MCF10A cells. hsa-miR-1280 was not recognized by Diana Tools and it was removed from the analysis. In total, 13 signaling pathways related to co-differentially expressed miRNAs were revealed. These pathways were shown in Fig. 5 as a Heat Map. Lysine degradation, ErbB signaling pathway, ECM-receptor interaction, Proteoglycans in cancer, FoxO signaling pathway came forward with very significant p values in all enriched the pathways (Table 3). The Lysine degradation pathway contained 4 common miRNAs' (hsa-miR-129-5p,



Co-differentially expressed miRNAs
hsa-miR-1323
hsa-miR-542-3p
hsa-miR-331-5p
hsa-miR-129-5p
hsa-miR-628-5p
hsa-miR-342-3p
hsa-miR-1280
hsa-miR-339-5p
hsa-miR-615-3p
hsa-miR-421
hsa-miR-662

Fig. 3 Identification of co-differentially expressed miRNAs in the GSE59247 data set and in vitro miRNA array. **a** Venn diagram displaying the number of overlapping differentially expressed miRNAs. miRNAs differentially expressed in GSE59247 data set are compared with miRNAs differentially expressed in MDA-MB-231 versus MCF10A in miRNA PCR array. **b** The list of eleven co-differentially expressed miRNAs

hsa-miR-1323, hsa-miR-339-5p, hsa-miR-628-5p) whose target genes are also shown in Table 3.

Activation of lysine degradation pathway impaired cancer cell proliferation in a previous study (Z. Dai et al. 2019). To determine the relevance of target genes of 4 common miRNAs that are associated with Lysine degradation pathway, we used an in silico TNM plot analysis tool consisting of 8666 tumor tissue samples and 645 adjacent control tissue samples (Bartha and Győrffy 2021). The results were visualized by violin plots, and expressions of SET Domain and Mariner Transposase Fusion Gene (SETMAR), SET Domain Containing 7, Histone Lysine Methyltransferase (SETD7), SET Domain Bifurcated Histone Lysine Methyltransferase 2 (SETDB2), Lysine Methyltransferase 2A (KMT2A), and Enoyl-CoA Hydratase And 3-Hydroxyacyl CoA Dehydrogenase (EHHADH) were diminished and SUV39H2 Histone Lysine Methyltransferase (SUV39H2) and Euchromatic Histone Lysine Methyltransferase 1 (EHMT1) were increased in BC tissues compared to adjacent normal tissues (Fig. 6a and b).

Validation of selected miRNA target genes in the lysine degradation pathway by q-RT PCR

To validate expressions of Lysine degradation pathway related target genes of miR-129-5p, miR-1323, miR-339-5p, miR-628-5p resulting from in silico TNM plot analysis, we performed qRT-PCR analysis of SETMAR, SETD7, SETDB2, KMT2A, EHHAD, SUV39H2 and EHMT1 genes in MCF-10A, MCF7 and MDA-MB-231 cells (Fig. 7a and 7b). SETD7, SETDB2, and EHHADH genes are significantly downregulated in MCF7 cell line compared to MCF10A (Fig. 7a). When we compared MDA-MB-231 with MCF10A for the same set of genes; SETMAR, KMT2A and SUV39H2 genes were significantly upregulated in MDA-MB-231 (Fig. 7b). The rest of the genes were not changed significantly.

Prediction of binding sites of miRNAs on target genes

TargetScan tool predicts mRNA targets of interested miRNAs through searching for the presence of matching sites of each miRNA (Bartel 2009). Once we detected critical changes mRNA levels of SETD7, SETDB2, EHHAD, SETMAR, KMT2A and SUV39H2 genes in MDA-MB231 and MCF7 cell lines compared to MCF10A, we further investigated the connection between co-differentially expressed miRNAs obtained from miRNome array and GEO data set using TargetScan. Our bioinformatics analysis identified that there are predicted binding sites between miR-1323-5p and 3′-UTR of SETD7 mRNA, miR-129-5p and 3′-UTR of EHHADH mRNA and miR-628-5p and 3′-UTR of SETDB2 mRNA (Fig. 8).

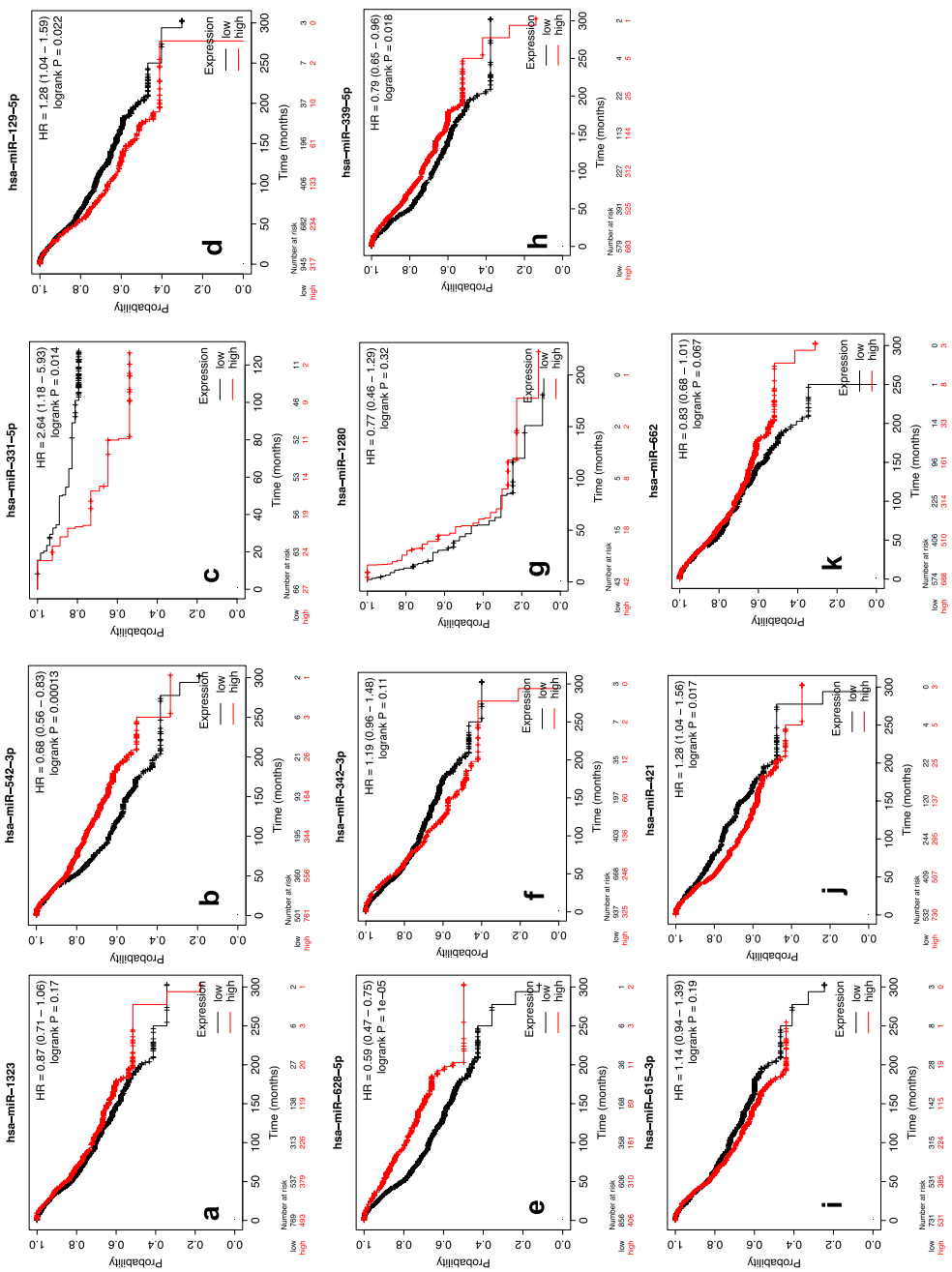


Fig. 4 KaplanMeier survival curves. KaplanMeier survival curves for 11 miRNAs. **a** miR-1323, **b** miR-542-3p, **c** miR-331-5p, **d** miR-129-5p, **e** miR-628-5p, **f** miR-342-3p, **g** miR-1280, **h** miR-339-5p, **i** miR-615-3p, **j** miR-421, and **k** miR-662 associated with the overall survival of patients with BC. Log-rank p values and hazard ratios (HRs; 95% confidence interval in parentheses) are shown

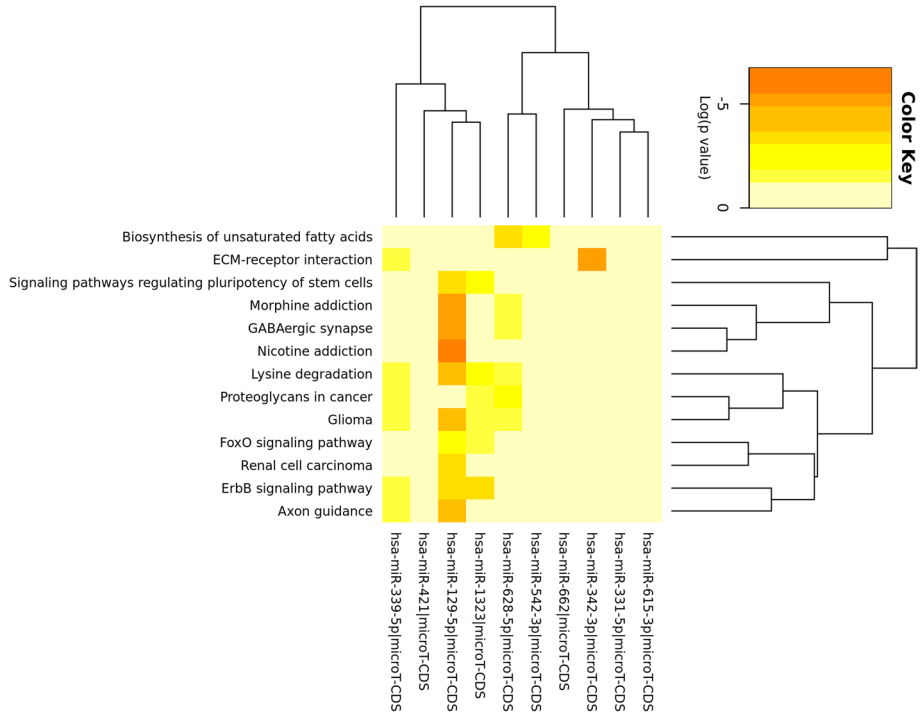


Fig. 5 KEGG Pathway Heat Map of the Differentially expressed miRNAs. Heat Map was created by DIANA-miRPath v3.0. miRNA target enrichment analysis. Orange and red color represent lower enrichment p -values while shades of yellow represent higher enrichment p-values

Validation of protein expressions in breast tissues by human protein Atlas databases

We finally investigated the protein levels of target genes obtained from TargetScan analysis in BC tissue samples. Consistently with qRT-PCR, SETD7, SETDB2 and KMT2A genes were highly expressed in BC tissues than in normal tissues, according to the immunohistochemical results of HPA database (Fig. 9). However, EHHAD and SUV39H2 expression data were not observed in HPA.

Discussion

Our understanding of the pathogenesis and clinical treatment of BC has made significant progress. However, the overall mortality rate of BC is still concerning. This is attributed to the lack of molecular markers for effective diagnosis and treatment. For this reason, it is important to search for novel molecular markers of breast carcinoma to improve the survival rate of patients. TNBC initially tends to respond well to chemotherapy, it tends to recur more often compared to other BCs. TNBC tumor type has a heterogeneous clinical behavior and patients with this disease generally have a poor prognosis. Thus, it is very important to understand the molecular basis of this heterogeneity.

In the present study, we aimed to perform global profiling of miRNA expression from BC tissue and normal breast tissue samples. We initially analyzed miRNA data set obtained from GEO. In total, 197 miRNAs were differentially expressed including 143 upregulated and 54 downregulated. Then, we performed miRNome analysis in TNBC cell line MDA-MB-231 and epitel BC cells MCF10A by using the miScript miRNA PCR array covering 1008 human miRNAs. With a PCR array approach, we found a total of 172 miRNAs were differentially expressed, of which 150 were increased, while 22 miRNAs were decreased. Venn diagram result, which we created to identify common differentially expressed miRNAs

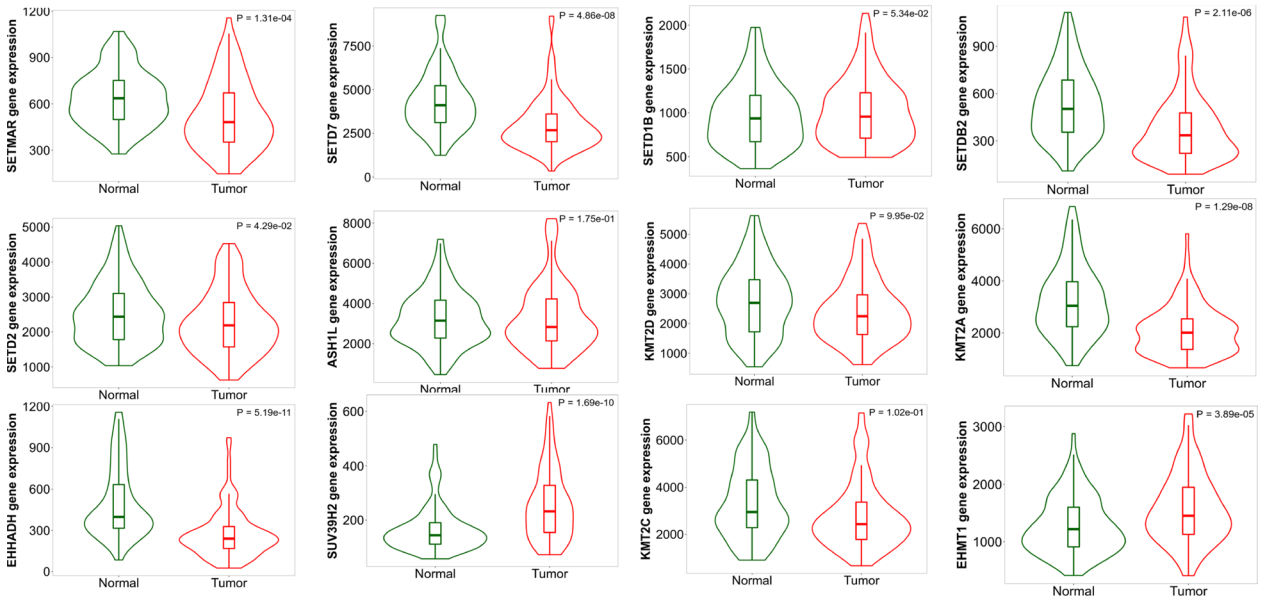
Table 3 Biological pathways enriched by overlapping differentially expressed miRNAs

KEGG pathway	p-value	genes	miRNAs
Lysine degradation	4.415689E-05	**SETMAR**, WHSC1L1, **SETD7**, SETD1B, **SETDB2**, SETD2, ASH1L, KMT2D, SUV420H1, WHSC1, **KMT2A, EHHADH**, **SUV39H2**, KMT2C, **EHMT1**	hsa-miR-129-5p hsa-miR-1323 hsa-miR-339-5p hsa-miR-628-5p
ErbB signaling pathway	0.001112681	CAMK2D, BRAF, GSK3B, SOS2, CBL, CRKL, NRG4, CRK, SHC1, PIK3CB, PAK2, NRG2, PAK7, EGFR, KRAS, PAK3, STAT5B, NCK1, PTK2, CBLB MAPK9, PIK3R3, NCK2, MAPK8, NRG3 PIK3R1, SOS1, PAK6, BTC, PRKCB, SHC4, CAMK2B, PAK6, PIK3CA, MAP2K1, MAP2K4, MAPK1, ABL2, ERBB4, RPS6KB1	hsa-miR-129-5p hsa-miR-1323 hsa-miR-339-5p
ECM-receptor interaction	0.01350386	COL6A5, SV2B, COL1A2, COL11A1, THBS3, COL4A6, COL5A2	hsa-miR-339-5p hsa-miR-342-3p
Proteoglycans in cancer	0.01445244	CAMK2D, BRAF, PDCD4, SOS2, SMAD2, CBL, CAMK2G, PIK3CB, PTCH1, ARHGEF12, FRS2, EGFR, FZD3, RRAS2, COL21A1, PTK2, CBLB, ITGAV, ANK3, FZD4, PPP1R12A, PIK3R3, FLNB, EIF4B, FLNA, PIK3R1, SOS1, DDX5, ANK1, PLCE1, PIK3CA, SDC2, MAP2K1, VEGFA, MAPK1, MDM2, ERBB4	hsa-miR-1323 hsa-miR-339 hsa-miR-628-5p
FoxO signaling pathway	0.02103311	IRS2, BRAF, RBL2, STAT3, SOS2, SMAD2, PRKAA2, SIRT1, KLF2, CCND2, IGF1R, TNFSF10, EGFR, KRAS, ATM, MAPK9, PIK3R3, CCND1, SKP2, MAPK8, PIK3R1, IRS1, BCL6, IGF1, TGFB2, EP300, SOD2, PIK3CA HOMER1, FOXO3, USP7, MAP2K1, ATG12, PTEN, SGK3, MAPK1, CREBBP, CCNG2, BCL2L11	hsa-miR-129-5p hsa-miR-1323

KEGG pathways, p-values, genes, and differentially expressed miRNAs are displayed

that could be prognostic biomarkers between in silico miRNA analysis outputs and in vitro miRNA analysis, showed that hsa-miR-1323, hsa-miR-542-3p, hsa-miR-331–5 found hsa-miR-129-5p, hsa-miR-628-5p, hsa-miR-342- 3p, hsa-miR-1280, hsa-miR-339-5p, hsa-miR-615-3p, hsa- miR-421, and hsa-miR-662 differentially expressed in both BC tissues and MDA-MB-231 cell lines compared to controls (Fig. 3). The dysregulation of these miRNAs was shown in previous studies on pathogenesis of many types of cancer, including breast carcinoma through various mechanisms. For example, miR-1323 was shown as an early prognostic biomarker lung cancer by promoting cell migration through targeting Cbl-b (Zhao et al. 2020). Wang et al. discovered that downregulation of miR-542-3p could promote cancer metastasis through activating TGF-β/Smad signaling in hepatocellular carcinoma (T. Zhang et al. 2018). In addition, miR-542-3p was found associated with poor prognosis in patients with gliomas (Kim et al. 2021). McAnena et al. demonstrated that patients with metastatic BC have a higher expression of miR-331 in patient blood with compared to healthy group (McAnena et al. 2019). miR-129-5p was found to suppresses BC proliferation by targeting CBX4 (Meng et al. 2018). Hsa-miR-628-5p, has been reported to be differentially expressed in various cancers, and its expression was found mostly associated with tumor suppression (Rios-Colon et al. 2019). BC patients with high miR-342 expression levels were shown to have significantly better survival compared to patients with low expression (Young et al. 2017). In another study, the miR-1280 expression was found higher in the NSCLC tissues samples than distal normal tissues (Xu et al. 2015). Different groups have observed the miR-339-5p inhibits BC cell migration and lung adenocarcinoma invasion (Li et al. 2018; Wu et al. 2010). The critical role of miR-615-3p was shown in epithelial-mesenchymal transition and metastasis of BC by targeting PICK1/TGFBRI axis (Lei et al. 2020). Hu et al. indicated that miR-421 promotes BC progression by inhibiting caspase-10 (Hu et al. 2018).

a



b

	P value	Fold change
SETMAR	1.31e-04	0.76
SETD7	4.86e-08	0.68
SETD1B	5.34e-02	1.17
SETDB2	2.11e-06	0.69
SETD2	4.29e-02	0.90
ASH1L	1.75e-01	1.18
KMT2D	9.95e-02	0.94
KMT2A	1.29e-08	0.66
EHHADH	5.19e-11	0.56
SUV39H2	1.69e-10	1.67
KMT2C	1.02e-01	0.90
EHMT1	3.89e-05	1.31

Fig. 6 TNM plot analysis of differential gene expression analysis in Normal and BC Tissues. **a** Violin plots of Lysine degradation pathway related gene expressions in BC and normal breast tissues. The violin chart is plotting numerical data with the addition of a core density plot rotated to each side. This plot is representing the breast tumor samples showing higher expression of the selected gene compared to normal tissues at each region. **b** Fold changes of genes involved in the lysine degradation pathway, whose expression changes were displayed in violin plot analysis, according to Mann–Whitney analysis

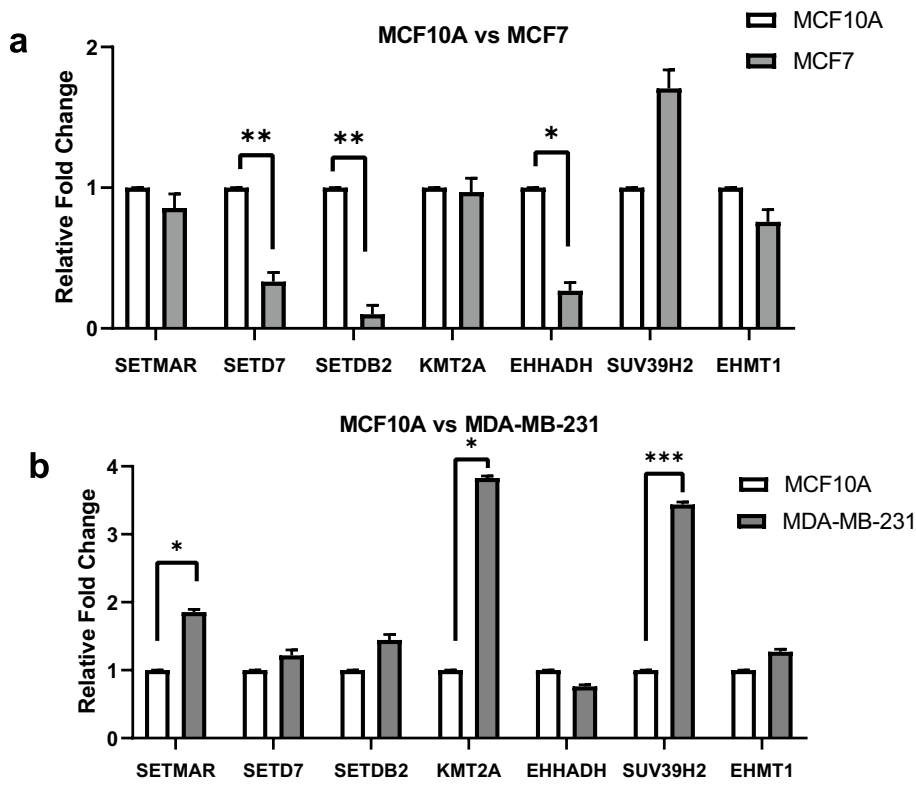


Fig. 7 Relative expression values of target genes SETMAR, SETD7, SETDB2, KMT2A, EHHAD, SUV39H2 and EHMT1 are determined via q-RT-PCR and analyzed by ΔΔCt method **a** Relative fold changes in MCF10A versus MCF7 cells **b** Relative fold changes in MCF10A versus MDA-MB231 cells. *means $P \leq 0.05$, **means $P \leq 0.01$

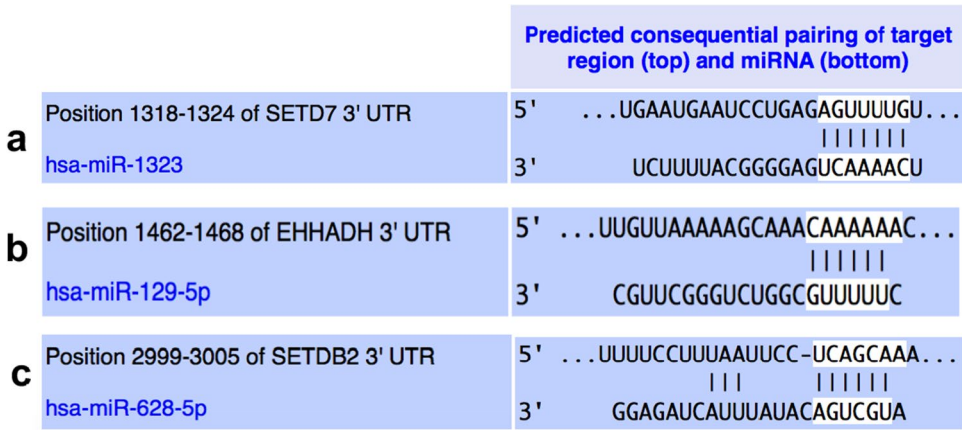


Fig. 8 TargetScan analysis predicts the association of 3 interest miRNAs and 3′-untranslated region (UTR) of some lysine degradation pathway related genes. Schematic representation of the miR-1323 recognition site in the SETD7 3′-UTR, schematic representation of the miR-129-5p recognition site in the EHHADH 3′-UTR, and schematic representation of the miR-628-5p recognition site in the SETDB2 3′-UTR

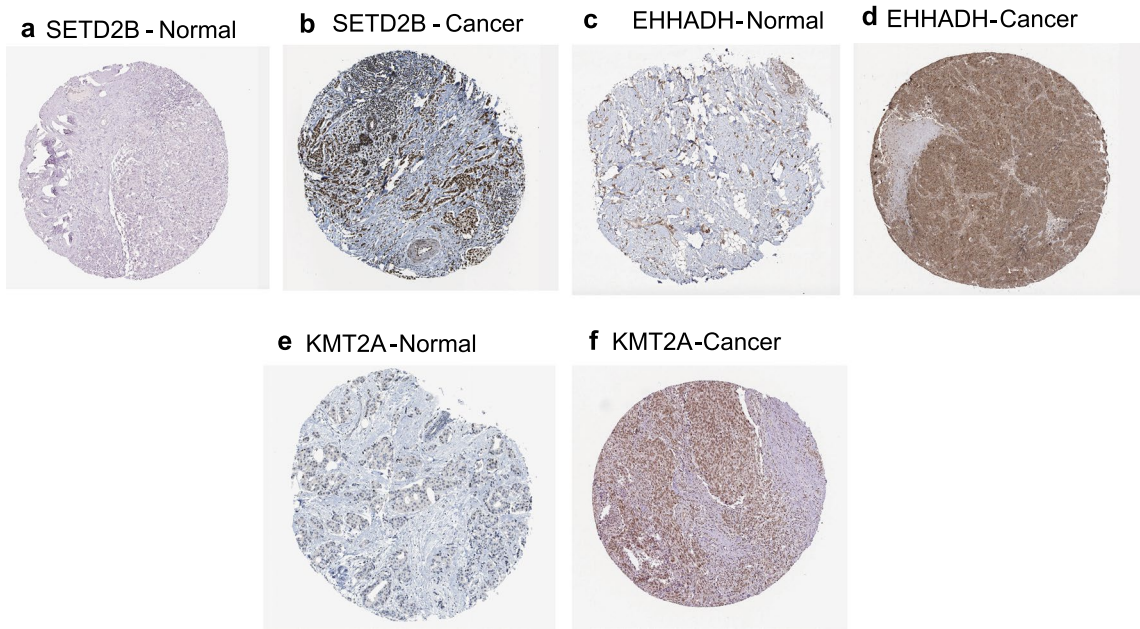


Fig. 9 Validation of some lysine degradation pathway related gene expressions in BC and normal tissue samples obtained from the human protein atlas (HPA) database (https://www.proteinatlas.org). Protein expression levels of SETDB2, EHHADH, KMTA2 were higher in BC samples (**b**, **d**, **f**) compared to normal samples (**a**, **c**, **e**) in immunohistochemistry data of HPA

miR-662 was found to enhance chemoresistance and invasiveness of squamous cell lung carcinoma (Filipska et al. 2018). According to the above-mentioned literature, in order to use miRNAs as biomarkers and potential therapeutic agents in the diagnosis and treatment of challenging diseases, including BC, it is necessary to elucidate how/by which cellular factors their expression is regulated.

We also investigated prognostic values of 11 miRNAs by using miRpower: a web-tool expression data from 2178 BC patients (Fig. 4). Kaplan Meier survival analysis indicated that overall survival BC patients significantly affected with miR-542-3p miR-628-5p, miR-339-5p, miR-331-3p, miR-129-5p, and miR-421expressions. According to the survival analysis results, although miR-1323, miR-342-3p, miR-1280, miR-615-3p did not show a significant prognostic effect for BC, their expression levels differed in both in silico and experimental data, we did not exclude them for further analysis.

To facilitate a more in-depth understanding of the biological role of co- differentially expressed miRNAs obtained from in silico and in vitro analysis in BC, we performed KEGG pathway enrichment analysis via DIANA mirPath V.3 web- based tool (Fig. 5). Our results showed 11 co-differentially expressed miRNA participated in a various biological pathway including Lysine degradation, ErbB signaling pathway, ECM-receptor interaction, Proteoglycans in cancer, FoxO signaling pathway. The role of all of these pathways in cancer or BC was shown in previous studies by different researchers. Abnormalities in the Lysine degradation pathway could cause serious diseases including cancer (Dimitrova et al. 2015; Shen et al. 2017). The role of ErbB signaling was shown in EMT-associated invasion and migration in normal and malignant mammary epithelial cells (Hardy et al. 2010). ECM-receptor interaction pathways were also shown the most upregulated gene-enriched signaling pathway in BC development (Bao et al. 2019). Proteoglycans control many normal and pathological processes and during BC development proteoglycan expression is modified in the tumor microenvironment (Theocharis et al. 2015). Hornsveld et al. showed the critical role of Foxo transcription factors in both suppression and activation BC progression (Hornsveld et al. 2018). According to the KEGG analysis results Lysine degradation pathway have more common

miRNAs including, hsa-miR-129-5p, hsa-miR-1323, hsa-miR-339-5p, hsa-miR-628-5p in all miRNAs of our interest. Therefore, we used a TNM plot analysis tool to investigate expression of target genes of 4 common miRNAs that are associated with Lysine degradation pathway in BC. The violin plot results indicate that gene expressions of SETMAR, SETD7, SETDB2, KMT2A, and EHHADH decreased, while the expression of SUV39H2 and EHMT1 increased in BC compared to normal tissues (Fig. 6a, b). SETMAR is a protein methylase with a sequence-specific DNA binding domain, which was shown dysregulated in several cancers including glioblastoma, leukemia, hematologic neoplasms, breast, and colon cancer (Tellier 2021). Another Lysine degradation pathway genes SETD7 and KMT2A are well known Lysine Methyltransferases. SETD7 was shown as a potential serum biomarker for colon cancer (Duan et al. 2018). KMT2A regulated cervical cancer cell growth via targeting VDAC1 signaling (C. Zhang 2008). In addition, Chen et al. observed that SETD7 promotes cell proliferation by regulating cell cycle in hepatocellular carcinoma (Chen et al., 2016a). SETDB2 is a histone H3K9 methyltransferase, its novel function was revealed in cancer stem cell maintenance in BC (Ying et al. 2020). EHHADH was recognized as a target of miRNA-486-5p having a role in potential mechanisms of cisplatin resistance in BC (Okamura et al. 2021).

These previous findings suggest that Lysine degradation pathways related genes, which are regulated by 4 common miRNAs obtained from our in vitro and in silico analysis could have critical roles in different type of BC development. Therefore, we investigated the expression levels of these genes by q-RT-PCR in MDA-MB-231 and MCF-7 cell line with compered to control cells MCF10A. Similar to violin plots, SETD7, SETDB2, and EHHADH genes were significantly downregulated in both MCF7 cell lines and BC tissue samples compared to control groups (Fig. 7A**).** Moreover, SETMAR, KMT2A and SUV39H2 genes were significantly upregulated in MDA-MB-231 compared to MCF10A (Fig. 7B). SUV39H2 was also upregulated according to violin plot analysis. Unlikely, q-PCR results obtained from MDA-MB-231 cells, SETMAR and KMT2A expressions were decreased in Violin plot chart (Fig. 6). SETD7, SETDB2, and EHHADH expression could be specifically downregulated in Luminal type BC which are represented in this study with MCF7 cell lines and SUV39H2 gene expression changes could be specific for TNBC that represented by MDA-MB-231 in this study. Additionally, we performed TargetScan bioinformatic analysis to find binding site between 4 miRNAs and 3-UTR of target gene's mRNA. Our results demonstrated that there are predicted binding site between miR-1323-5p and 3′-UTR of SETD7 mRNA, miR-129-5p and 3′-UTR of EHHADH mRNA and miR-628-5p and 3′-UTR of SETDB2 mRNA (Fig. 8). According to our in silico GEO data set and in vitro miRNome assay results the expressions of miR-1323, miR-129-5p and miR-628 were increased and violin plot and q-RT PCR results demonstrated that SETD7, SETDB2 and EHHADH gene expression were downregulated (Fig. 6 and Fig. 7a) in BC tissue and cell lines. We further investigated the expressions of these key target genes in BC from HPA database and results showed that SETD7, SETDB2, KMT2A were significantly up regulated in BC tissue samples compared to normal tissue (Fig. 9). The critical role of these genes also previously shown in cancer progression (Chen et al. 2016; Duan et al. 2018; Okamura et al., 2021b; Ying et al. 2020). All of these findings suggest that if the expression of these miRNAs could be targeted by miRNA modulators, progression of BC may be prevented.

Conclusion

In conclusion, miRNA and mRNA interactions in in silico and in vitro BC samples were investigated by taking advantage of bioinformatic tools and GEO profiles. Our results revealed that miR-1323-5p, miR-129-5p, and miR-628-5p are differentially expressed in BC and they bind to the 3'UTR of critical genes SETD7, SETDB2 and EHHADH, which are also deregulated in BC. Therefore, these miRNAs might serve as potential diagnostic and prognostic biomarkers for BC progression. In the future, in vitro and in vivo experiments are required to confirm the underlying mechanism of miR-1323-5p, miR-129-5p, and miR-628-5p and their interactions with the target genes in BC models.

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Author contributions Design and implementation of the research: PTA. Performed the experiments: PTA and DC. Analyzed the data: PTA and DC. Wrote the paper: PTA. Review and editing: DC. Both authors have read and approved the final version of the manuscript and they have met the criteria for authorship.

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Data availability Data can be provided upon request by the corresponding author.

Declarations

Competing interests The authors declare no competing interests.

Ethical approval Not applicable.

Consent for publication Not applicable.

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