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ORIGINAL ARTICLE

The clinical significance of circulating DSCAM-AS1 in patients with ER-positive breast cancer and construction of its competitive endogenous RNA network

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Abstract

Long Non-Coding RNAs (lncRNAs), with diagnostic and therapeutic applications in malignancies, are newly described tumour-related molecules. Here, we reported the importance of circulating DSCAM-AS1 as the biomarker to detect Estrogen Receptor (ER)-positive breast cancer (BC) cases. Moreover, the expression of a BC-associated lncRNAs, namely DSCAM-AS1, was measured in tumoural and Paired Adjacent Non-Tumoral (PANT) tissue, as well as plasma, using Real-Time Polymerase Chain Reaction (RT-PCR). Besides, the correlations between gene expression and the clinicopathological features were analyzed. The diagnostic power of circulating DSCAM-AS1 in BC was estimated using the Area Under the Curve (AUC) value. Furthermore, we studied the DSCAM-AS1 associated with the network of competitive endogenous RNA (ceRNA) in BC using the literature review and in silico analysis. We found a significant increase in the expression levels of lncRNA in the tumour ($P < 0.001$) and in plasma ($P < 0.001$) of ER-positive BC patients. The sensitivity and specificity of DSCAM-AS1 in plasma for detection of BC from healthy controls were 100 and 97%, respectively ($AUC = 0.98$, $P < 0.001$). Accordingly, we suggest an elevated level of circulating DSCAM-AS1 as a candidate biomarker of ER-positive BC patients. Moreover, perturbation of DSCAM-AS1, as a ceRNA, acts in the tumor progression and drug resistance by affecting different cell signaling.

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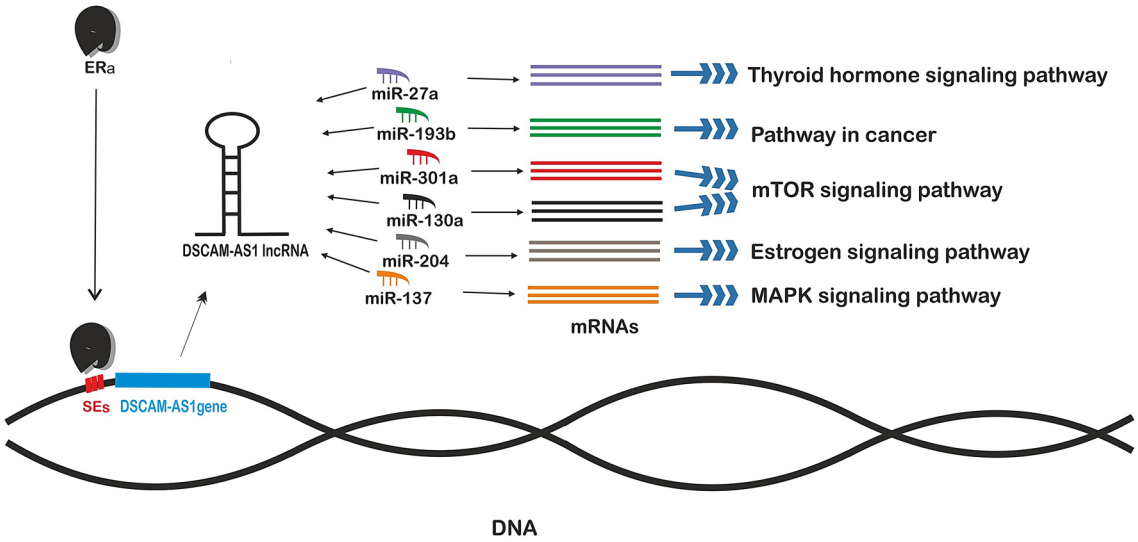
Graphic abstract



Keywords Biomarker · ceRNA · DSCAM-AS1 · Estrogen receptor · lncRNA

Abbreviations

BC	Breast cancer
ceRNA	Competing endogenous RNA
DSCAM-AS1	Down syndrome cell adhesion molecule anti-sense 1
EMT	Epidermal-mesenchymal transition
ER	Estrogen receptor
lncRNAs	Long noncoding RNAs

Introduction

According to the report of the Non-Communicable Diseases Research Center (NCDRC) of Iran, breast cancer (BC) is the most prevalent type of cancer among females in Kermanshah in western Iran [1].

The main drawbacks of BC therapy are the delayed diagnosis, tumour metastasis, and recurrence. Accordingly, early detection of BC could be a key factor in improving the prognosis and reducing the mortality rate. Blood-based testing is considered as an ideal strategy for discovering cancer biomarkers due to its easiness and non-invasive nature [2]. Though, measuring conventional serum biomarkers for BC diagnoses, such as measuring Carbohydrate Antigen (CA125) and Carcino-Embryonic Antigen (CEA), could not be employed for early diagnoses [3, 4]. That is why the identification of biomarkers is essential for early diagnosis and monitoring the BC progression.

Long Non-Coding RNAs (lncRNAs), with a length longer than 200 nucleotides, are known to be connected with various biological functions and known to have regulatory roles in the progression of multiple cancers, either as oncogenes or tumour suppressor genes [5, 6]. Evidence proposed lncRNAs could behave like a miRNA-sponge by competing with the endogenous circulatory RNAs (ceRNAs) activity [7]. Uncovering the crosstalk between this novel class of RNAs with other components of the gene regulatory system would bring insight into their role in development and disease [8].

DSCAM-AS1 is an estrogen receptor (ER)-dependent lncRNA that is located within the sequence of Down Syndrome Cell Adhesion Molecule (DSCAM) gene and expresses as an antisense intronic transcript. Knockdown of the DSCAM-AS1 gene shows similar effects as silencing of ER, which includes an increase in apoptosis, reduction of cell growth, and also induction of Epidermal-Mesenchymal-Transition (EMT) markers without effect on the ER expression [9].

In normal condition, estrogen receptor alpha (ERa) is expressed in about 10–15% of luminal epithelial breast cells. The activity of estrogen is mediated by ERa and ERb. Evaluation of ER levels has been used as a predictive and prognostic marker in the luminal BC subtype, which includes 75% of BC cases [10–12].

Currently, the most comprehensive endocrine therapy of BC is the prescription of Aromatase Inhibitors (AIs), which block the estrogen biosynthesis [13]. Despite its efficacy,

therapeutic failure occurs in about 25% of breast cancer patients with positive ERa. The reason might be the emergence of de novo or acquired endocrine resistance [11]. Interestingly, a Super Enhancer (SE) has been located in the proximity of the DSCAM-AS1 which is occupied by apoERα. Mostly, the estrogen therapy does not affect the apoERα activity, though E2 (17β-estradiol) enhances the binding of ERα to the DSCAM-AS1 promoter [11].

The present study aimed to investigate the levels of circulating DSCAM-AS1 in plasma and tumour tissue of BC patients to find the related potential role as a novel biomarker. Furthermore, we constructed the DSCAM-AS1 associated ceRNA network and its possible role in breast cancer.

Materials and methods

Participants and sample collection

All the participants were female with Kurdish background from Kermanshah, the west of Iran. The mean age of BC patients was 49 years (range 30–68 years). Also, healthy volunteer women with the mean age of 48 years (26–64 years, $P = 0.14$) consisted of our control group. The BC cases were selected at the time of the diagnosis at the Bistoon Hospital, Kermanshah Province, Iran, from 2016 to 2017. This study was approved by the ethics committee of Kermanshah University of Medical Sciences, and informed consent was obtained from all subjects. Only histologically confirmed breast cancer cases by an expert pathologist, without any previously diagnosed malignancies, were entered in the study. The first group of samples consisted of 20 tumoural and Paired Adjacent Non-Tumoural (PANT) tissue of BC patients, which was taken at the time of BC diagnosis (from frozen samples). The second group was 40 patients who peripheral blood was taken from them during the period of diagnosis (before surgery and any therapy). Accordingly, the blood samples were provided from a control group that consisted of 40 healthy women (third group). Immunohistochemical tests were done to determine the Estrogen Receptor (ER) or Progesterone Receptor (PR) in the clinical laboratory of Bistoon hospital, then read and confirmed by an expert pathologist. Breast tumors were classified as ER-positive or PR-positive if staining was present in 1% or more of tumor nuclei.

Stock of plasma samples

The blood samples were kept in EDTA containing tubes. To separate cellular fraction from plasma, samples were centrifuged at 3500×*g* for 20 min, within about one hour after blood collection.

RNA extraction and complementary DNA (cDNA) synthesis

Fresh frozen tissues were powdered in liquid nitrogen by mortar and pestle and then total RNA was isolated using Qizol reagent (QIAGEN, US) according to the manufacturer's procedure. Also, total RNA was extracted from 200 µl plasma using the miRNeasy Serum/Plasma Kit (QIAGEN, US) and was eluted into 14µl of elution solution. The concentration and purity of isolated RNA were measured by a Nanodrop spectrophotometer (Thermo Scientific, Waltham, MA). cDNA was synthesized from total extracted RNA using a QuantiTect Reverse Transcription Kit (QIAGEN, US) and the synthesized cDNA was stored at − 20 °C until further processing.

Quantitative Real-Time PCR (qRT-PCR)

The primers were chosen from the literature and then checked for their accuracy and specificity at the NCBI data bank (https://www.ncbi.nlm.nih.gov/). The sequences of selected primers used for qRT-PCR were as follow, AGA GCGAAACCCCATCTCAA (sense) and CTGAGAGAT CCCCTGTAGCG (antisense) for DSCAM-AS1 [14], and CTCGCTTCGGCAGCACA (sense) and AACGCTTCA CGAATTTGCGT (antisense) for U6-snRNA [15] as an internal control. The qRT-PCR assay was performed on a Rotor-gene machine (Corbett Research) with SYBR Premix Ex Taq II (TaKaRa, China). The PCR reaction conditions were: denaturation at 95 °C for the 30s as the initial step, followed by 45 PCR cycles at 95 °C for 10s, 60 °C for 15s and 72°C for 30s. All reactions were done in duplicate. The relative expression of the DSCAM-AS1 gene in each tumoural tissue was compared with its PANT. The cycle threshold (Ct) level is inversely proportional to the target gene expression. Relative expression of the genes was assessed by ΔCt values ($Ct_{\text{reference gene}} - Ct_{\text{target gene}}$). Fold changes of the gene expression in the cancer samples relative to the healthy controls were determined by the "$2^{-\Delta\Delta Ct}$ method".

Bioinformatics analysis and literature review

For finding miRNAs related to the DSCAM-AS1, we conducted an *in silico* search in the LncBase database (v.2) [16]. The prediction modules were set using the following search strategies: Filters: Threshold = 0.7, Tissue: mammary glands; Category: cancer/malignant and Experimental module were as follow: Filters: Tissue: mammary glands; miRNA Species: Homo sapiens; Validation type: direct; Validated as: positive; Source: LncBase v.2. Then, mRNAs targeted by the detected miRNAs were identified in the miRDB database [17, 18]. The prediction was limited to human species and gene targets with 'target prediction score'

less than 90 were excluded. The predicted mRNAs then further enriched using the Enrichr database [19].

Furthermore, in order to add more data about DSCAM-AS1: miRNAs: mRNAs connections, we conducted a literature review in the PubMed and Scopus with these keywords: (TITLE-ABS-KEY (*DSCAM-AS1*) AND TITLE-ABS-KEY (*"breast cancer"*) OR TITLE-ABS-KEY (*"breast carcinoma"*)) for finding the relation between DSCAM-AS1 and breast cancer, and then separately we searched for the relation between breast cancer and each DSCAM-AS1 related miRNA with this pattern, for instance: (TITLE ("breast cancer") AND TITLE (mir-27a) OR TITLE (mirna-27a) OR TITLE (mir-27a-3p)). For more assurance, all searches were performed by two researchers, blindly.

KEGG pathway and gene ontology (GO) analysis

Functional enrichment can be useful to find out the functions of genes. The GO functional annotation and KEGG pathway analysis for miRNA target mRNAs was performed by the web-based tool, Enrichr (https://amp.pharm.mssm.edu/Enrichr). Gene ontology categorizes genes based on cellular component, biological process, and molecular function. Potential biological pathways were identified through the analysis of the enriched genes in the KEGG database (https://www.genome.jp/kegg/). Gene ontology terms and KEGG pathways with $p < 0.05$ set as the cut-off to pick out significantly enriched terms and pathways.

Table 1 Some clinicopathological characteristics of patients

Variable	Patients (%)
Histological type	
Invasive ductal carcinoma	40 (100)
Tumor stage	
I	11 (27.5)
II	19 (47.5)
III	10 (25)
ER	
Negative	9 (32.5)
Positive	31 (77.5)
PR	
Negative	12 (30)
Positive	28 (70)
HER2	
Negative	21 (52.5)
Positive	19 (47.5)
Ki67	
Negative	0 (0)
Positive	40 (100)

ER estrogen receptor; *PR* progesterone receptor; *HER2* human epidermal growth factor receptor 2; *Ki67* a cellular marker for proliferation

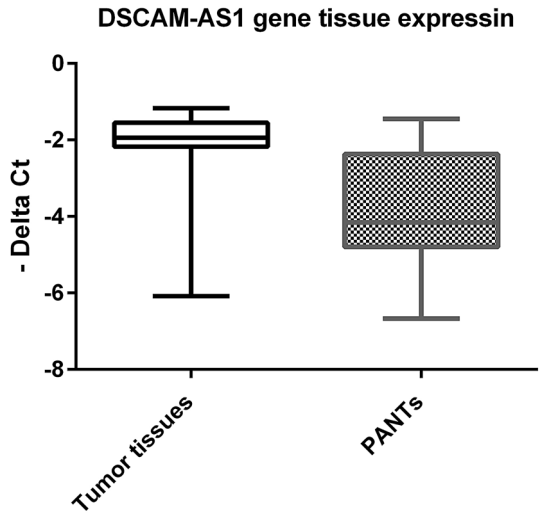


Fig. 1 The relative expression of the DSCAM-AS1 gene in tumoural tissue was compared with Paired Adjacent Non-tumoural (PANT) tissue. Relative expression was calculated by Delta Ct values (Ct reference gene − Ct target gene). Ct (Cycle threshold) level was inversely proportional to the amount of the target gene in the sample

Statistical analysis

T-test was used to assess the significance of the difference in the expression of DSCAM-AS1 between tumoural vs.

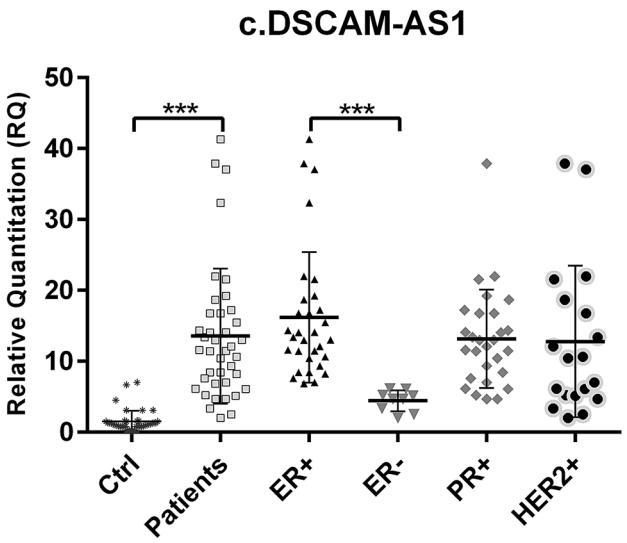


Fig. 2 Comparisons of circulating DSCAM-AS1 levels in the controls (Ctrl), all breast cancer patients (patients), Estrogen receptor-positive patients (ER+), Estrogen receptor-negative patients (ER-), progesterone receptor-positive patients (PR+), and human epidermal growth factor receptor 2-positive patients (HER2+). Relative Quantitation (RQ) was calculated by $2^{-\Delta\Delta Ct}$ method

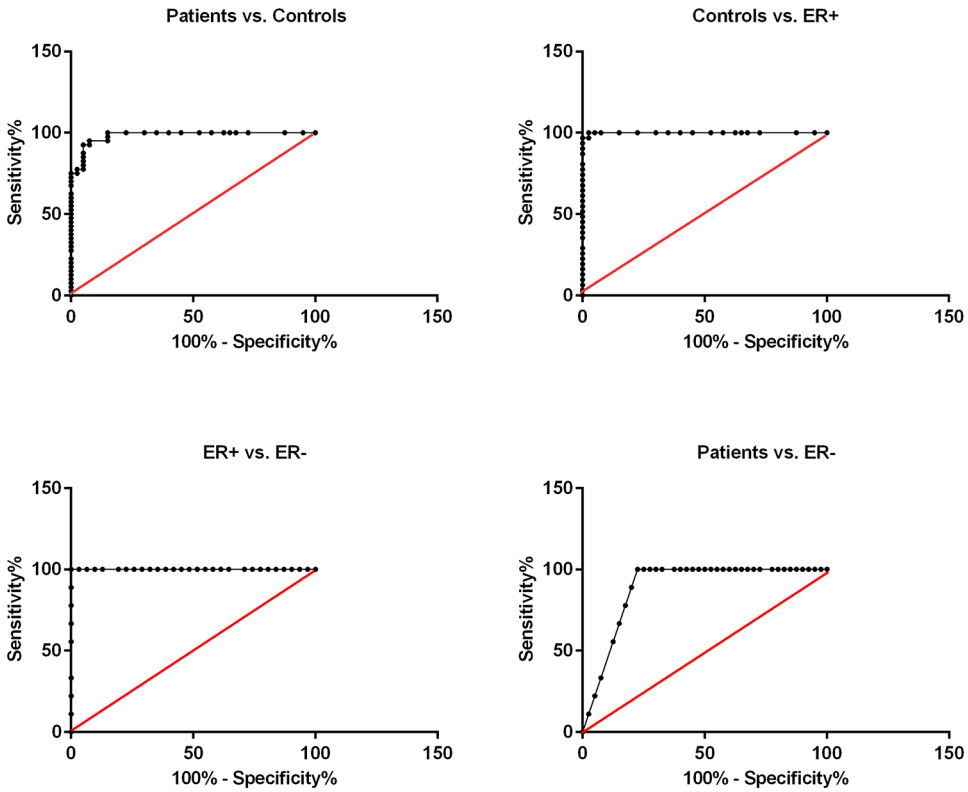


Fig. 3 The ROC curve was plotted for assessment of the diagnostic power of circulating DSCAM-AS1 as a candidate biomarker in breast cancer patients (regardless of ER status) versus healthy controls/ ER-negative patients, and also ER-positive patients versus healthy controls/ ER-negative patients. *ROC* receiver operating characteristic, *ER* estrogen receptor

Table 2 Value of the AUC, sensitivity, and specificity of DSCAM-AS1 as a biomarker for breast cancer were compared between studied groups: The cancer-free control (Ctrl) group with the all breast cancer patients (BC) group; The Ctrl group with ER-positive breast cancer (ER+) group; The BC group with ER-negative breast cancer (ER−) group; and ER+ with ER− groups

Groups	AUC	*P*-value	Sensitivity %	Specificity %
Ctrl vs. BC	0.982	< 0.01	92.5	95
Ctrl vs. ER+	0.997	< 0.01	100	95
BC vs. ER−	0.887	< 0.01	100	77.5
ER+ vs. ER−	0.993	< 0.01	100	96.77

AUC area under the Roc curve; *ER* estrogen receptor

paired-adjacent tissues or between plasma of patients vs. healthy controls. The relation among tumour features and expression of DSCAM-AS1 was appraised using the Chi-square test. P-values <0.05 were assumed as significant. The diagnostic power of circulating DSCAM-AS1 was estimated using the Receiver Operating Characteristic (ROC) curve. The Area Under the Curve (AUC) value were used for the decision about if the proposed biomarker is suitable or not. SPSS software (version 16) was applied for statistical analysis.

Results

Some characteristics of the patients' baseline profiles are indicated in Table 1.

The expression levels of DSCAM-AS1 was significantly higher in paired tumoural tissues compared with PANTs

Using qRT-PCR, the expression of DSCAM-AS1 was measured in 20 paired breast tumoural and adjacent non-tumoural tissues. Differences between each pair of the tumour and its normal margin tissues were significant by paired t-test ($P < 0.001$, Fig. 1).

The plasma levels of circulating DSCAM-AS1 was significantly higher in BC patients

The relative level of circulating DSCAM-AS1 was determined in plasma samples. We found that in the plasma samples of patients, the level of DSCAM-AS1 was significantly higher than in the cancer-free donors ($P < 0.001$). Moreover, it was higher in ER-positive BC patients compared to that of ER-negative patients ($P < 0.001$, Fig. 2).

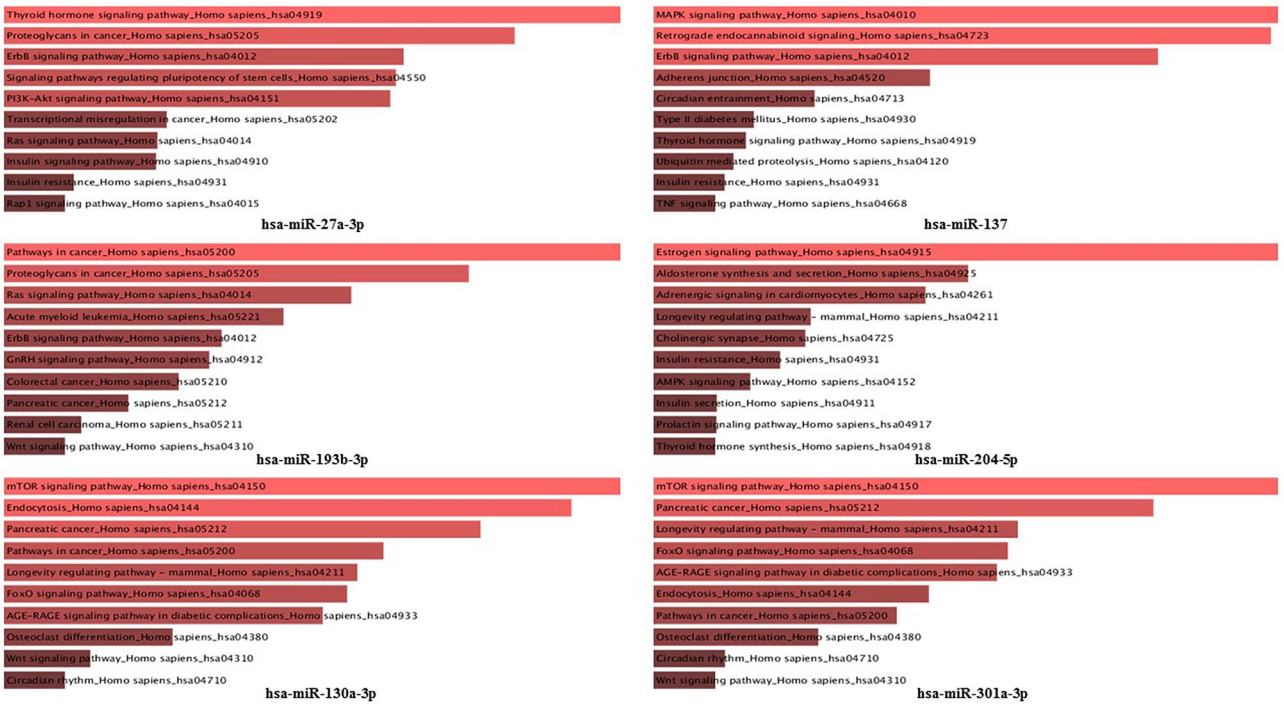


Fig. 4 Bar graph of the six miRNAs related to DSCAM-AS1 enrichment in KEGG (2016). Enrichment of the predicted genes in the KEGG database showed the top enriched pathways, probably through which the DSCAM-AS1 performs its function. Abbreviation: has-mir, homo sapiens – microRNA

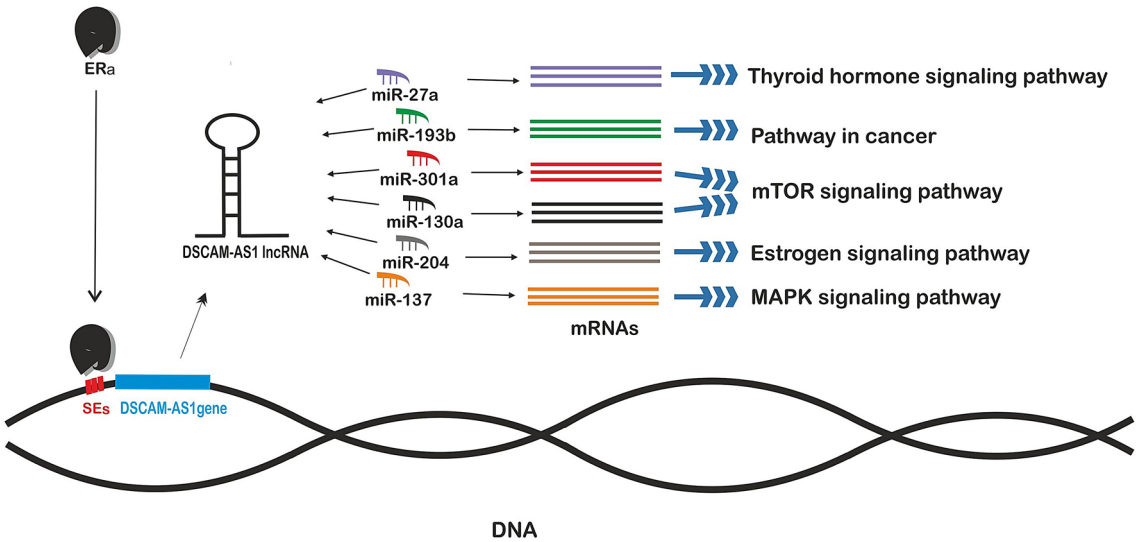


Fig. 5 ERα binds to super enhancers (SEs) upstream of the DSCAM-AS1 gene to regulate the expression of this gene. This study revealed six miRNAs which have binding sites on DSCAM-AS1. Each of these miRNAs has binding sites on some mRNAs which involved in a specific signaling pathway. Abbreviation: ERa, estrogen receptor alpha; SE, super enhancer; miR, micro RNA

DSCAM-AS1 as a diagnostic tool to discriminate ER-positive from ER-negative BC patients

To determine the possibility of using plasma levels of DSCAM-AS1 as a diagnostic tool, ROC curves were used. As shown in Fig. 3, the value of the AUC was 0.98 for the cancer-free group compared to that of the BC group. In this model, optimal cutoff points of DSCAM-AS1 were 6.76 (with 100% sensitivity and 97% specificity). Comparing cancer-free group with ER-positive BC group, AUC was 0.99

Table 3 The mRNA targeted by the miRNAs (literature review)

miRNA name	Gene name	Description	Ref.
miR-193b-3p	ESR1	Estrogen-induced growth of BC cells is inhibited by the targeting of ERa by miR-193b	21,512,034
	DDAH1	miR-193b by targeting DDAH1 regulates BC cell migration and vasculogenic mimicry. DDAH1 is over-expressed in aggressive BC cell lines	29,070,803
	MCL1	miR-193b makes MCF-7/DOXR cells sensitive to doxorubicin through the downregulation of MCL-1 gene	26,526,790
	RAB22A	mir-193b by targeting the RAB22A gene restrict growth and metastasis mediated by exosome in breast	30,272,274, 25,550,792
	HSP40	miR-193b promotes BC progression via targeting HSP40 (DNAJC13)	25,550,792
	YWHAZ	miR-193b targets YWHAZ by binding to its 3-UTR region	21,512,034
	SHMT2	miR-193b targets SHMT2 by binding to its 3-UTR region	21,512,034
	AKR1C2	miR-193b by targeting 5-UTR of AKR1C2 could inhibit the local production of steroid hormones such as estrogen	21,512,034
miR-301a-3p	ESR1	miR-301a-3p by targeting ESR1 could play a part in the estrogen independence of cells	29,763,890
	PTEN	miR-301a by targeting PTEN retains activated Wnt/β-catenin signaling	24,315,818
miR-130a-3p	RAB5B	miR-130a-3p by regulating RAB5B inhibits migration and invasion of human BC stem cell-like cells	29,746,865
	FOSL1	miR-130a targets FOSL1 and suppresses the inhibition of ZO-1, thus inhibiting cancer cell migration and invasion in TNBCs	29,384,218
	PTEN	miR-130a targets PTEN to drive malignant cell survival and tumor growth	28,935,812
	TAC1	miR130a competes with Msi1 for interaction with TAC1 mRNA, to stabilize and enhance its translation	26,373,800
	HOXA5	miR-130a, a c-Myc responsive miRNA, suppresses HOXA5 gene expression	23,528,537
miR-27a-3p	CDC-27	miR-27a by targeting CDC-27 interfere with the radioresistance of the cells	25,943,633
	SFRP1	miR-27a by modulating SFRP1 activates the Wnt/β-catenin pathway which leads to promoting the invasion phenotype of BC cells	28,099,945
	ZBTB10	miR-27a by repression of ZBTB10 indirectly controls E2-responsiveness in MCF-7 cells, thereby upregulates ERa	20,382,698, 18,006,846
	Myt-1	miR-27a facilitates cancer cell proliferation through suppression of Myt-1	18,006,846
	FOXO1	A transcription factor that has a target site in 3-UTR for miR27a, take parts in the regulation of genes in the cellular metabolism, apoptotic response, and cell cycle checkpoints	19,574,223
	AMPKa2	miR-27a-mediated metformin anti-proliferative effects on the MCF7 cell line by targeting of AMPKa2	27,779,715
	ESR1	miR-27a make BC cells sensitive to SERM treatments through a positive feedback loop with ERa	30,415,143
	SPRY2	miR-27a downregulate SPRY2 and activate p44/42 MAPK, thereby promotes cell migration and invasion	23,649,631
	FBXW7	miR-27a promotes BC cell migration in an FBXW7 suppression-dependent manner	30,365,154
mir-137	EPS8	EPS8 triggers cell proliferation and represses apoptosis in Tamoxifen resistance -BC	30,203,615
	FSTL1	Stemness and chemoresistance in BC cells control by the axis of miR-137/FSTL1/integrin β3/Wnt/β-catenin. Moreover, it promotes proliferation, metastasis, and blocks apoptosis in BC cells	30,336,071
	ERRA	miR-137 negatively regulates ERRA, and also through cyclin E1 and WNT11 suppresses the growth and migration of BC cells	22,723,937
mir-204-5p	RRM2	DSCAM-AS1 promotes tumor growth by reducing miR-204-5p and up-regulating RRM2	30,457,164
	AP1S3	mir-204 with the regulation of AP1S3 is involved in TNBC	30,228,364
	Six1	miR-204-5p/Six1 feedback loop promotes EMT in BC	26,408,179

ERa Estrogen Receptor alpha, *ESR1* Estrogen Receptor 1, *BC* breast cancer, *DDAH1* Dimethylarginine Dimethylaminohydrolase 1, *MCL1* Myeloid Cell Leukemia Sequence 1, *RAB22A* Ras-related protein Rab-22A, *YWHAZ* Tyrosine 3-Monooxygenase/Tryptophan 5-Monooxygenase Activation Protein Zeta, *UTR* Untranslated region, *SHMT* Serine hydroxymethyltransferase, *SERM* Selective Estrogen Receptor Modulators, *TAC1* oncotachykinin 1, *MSi1* Musashi 1, *EPS8* Epidermal growth factor receptor Pathway Substrate 8, *ERRA* Estrogen Related Receptor Alpha, *RRM2* Ribonucleotide Reductase M2, *TNBC* Triple Negative Craeast Cancer, *AP1S3* Adaptor-related Protein complex 1 sigma 3 subunit, *Six1* Sineoculis homeobox homolog 1

and optimal cutoff points were found to be 6.76 (with 100% sensitivity and 97% specificity). In the comparison between ER-positive and ER-negative groups, the value of the AUC for DSCAM-AS1 was 1.00 and the cutoff point was 6.76, where both sensitivity and specificity were 100% (Table 2).

Bioinformatics analysis and literature review revealed six miRNAs related to DSCAM-AS1

The LncBase software (v.2) prediction module identified 145 miRNAs with binding sites on the DSCAM-AS1 transcript (Supplementary File 1). The Experimental module of LncBase software (v.2) showed that DSCAM-AS1 is a verified target for four miRNAs (hsa-miR-130a-3p, hsa-miR-27a-3p, hsa-miR-301a-3p, and hsa-miR-193a-3p). Pilli et al. (2014) experimentally validated these four miRNAs as potential targets of DSCAM-AS1 in the mammary gland tissue and MCF7 cell line, using an immunoprecipitation method, HITS-CLIP [20]. Although, in the literature review we found only a report for hsa-mir-137 [21] and hsa-mir-204-5p [22] as direct regulators of DSCAM-AS1 in breast cancer.

Supplementary file 2 contains the predicted genes targeted using these six miRNAs (*miR-130a-3p, miR-27a-3p, miR-301a-3p, miR-193a-3p, miR-137, and miR-204-5p*) according to the miRDB databank. In total, 910 unique genes are potential targets of these six miRNAs. Enrichment analysis for the predicted genes (with a prediction score > 60) conducted in the KEGG database (2016), showed that they are involved in different signaling pathways (Figs. 4, 5). In brief, mRNAs targeted by *miR-130a-3p* and *miR-301a-3p* are involved in the mTOR signaling pathway, and mRNAs targeted using *miR-204-5p* are involved in the estrogen signaling pathway. It was predicted that some proteins of the ErbB signaling pathway are targeted by *miR-137, miR-27a-3p*, and *miR-193b-3p*. Table 3 illustrates experimentally validated mRNAs targeted using the six miRNAs in the literature. In brief, ESR1 (estrogen receptor 1 which encodes Era) is the target of 3 miRNAs related to DSCAM-AS1 (*miR-193b-3p, miR-301a-3p, and miR-27a-3p*). In addition, *miR-193b, miR-27a-3p*, and *miR-137* are involved in estrogen signaling through affecting AKR1C2, ZBTB10, and ERRA, respectively. Phosphatase and tensin homolog (PTEN) is another important gene in the development of breast cancer, which is targeted by *miR-301a-3p* and *miR-130a-3p*. Many of the proteins affected through miRNAs related to DSCAM-AS1 are involved in tumour growth and metastasis such as PTEN, RAB5B, FOSL1, SFRP1, SPRY2, and FSTL1.

KEGG pathways and GO classification of miRNA target genes

The 910 target genes which had a prediction target score equal 90 or more were subjected to the KEGG pathway and GO analysis (Supplementary File 3). In the KEGG analysis, two significantly enriched pathways were including 'ErbB signaling pathway' (Combined Score (CS) = 81.5, $P = 1.5 \times 10^{-7}$) and 'Proteoglycans in cancer' (CS = 49.8, $P = 3.7 \times 10^{-7}$). The top 15 enriched KEGG pathways and GO items, based on the Enrichr combined score (CS), are displayed on Table 4. Through GO annotation and enrichment analysis, the roles of gene products from the cellular component, biological process, and molecular function were identified. The most significant term in GO cellular component was 'RISC complex' (GO:0016442, CS = 115.7 $P = 1.4 \times 10^{-4}$) and RNAi effector complex (GO:0031332, CS = 115.7 , $P = 1.4 \times 10^{-4}$), that in GO biological process was 'positive regulation of transcription, DNA-templated' (GO:0045893, CS = 68.7, $P = 1.3 \times 10^{-13}$), and that in GO molecular function was 'transcription regulatory region DNA binding' (GO:0044212, CS = 33.4 , $P = 1.7 \times 10^{-6}$). To better illustrate gene enrichment in the three mentioned GO categories, a clustergram was constructed consisting of the top 20 enriched GO terms, according to combined score (Fig. 6).

Discussion

The DSCAM-AS1 levels in tissue and plasma of BC patients were measured. The possible correlations between circulating DSCAM-AS1 and clinicopathological features were analyzed. Furthermore, the potential ceRNA network of DSCAM-AS1-miRNAs-mRNAs was investigated. According to our best knowledge, these results, for the first time, showed that the circulating level of DSCAM-AS1 might be useful as a potential novel biomarker for identifying ER-positive BC.

It is suggested that lncRNAs play an important role in cell differentiation and development [23, 24]. Significant correlations have been reported between malignancies and aberrant expression of lncRNAs [25, 26]. Since circulating lncRNAs are somehow stable in plasma/serum and their transcripts are the final functional production, assessing the lncRNA level could directly indicate the levels of the active product. Consequently, lncRNAs have a potential value as a bio-marker [23].

"Subtypes of breast tumour are defined using the immunohistochemical expression of Estrogen Receptor (ER), Progesterone Receptor (PR), and HER2" [27]. ERα-positive breast tumours have a good response to endocrine therapies. However, 25% of these cases show primary or acquired resistance which motivates researchers to develop novel biomarkers and therapeutic targets [28].

There are pieces of evidence indicating that the increased level of DSCAM-AS1 as a lncRNA is highly specific for luminal BC and, therefore, can be used as a biomarker for

Table 4 KEGG pathways and GO terms related to the mRNAs which are targets of the 6 miRNAs

Term	Count	OR	CS	P-value	Adjusted P-value
KEGG 2019 human					
ErbB signaling pathway	15	5.20	81.47	1.5×10^{-7}	4.8×10^{-5}
Proteoglycans in cancer	23	3.37	49.85	3.7×10^{-7}	5.8×10^{-5}
Choline metabolism in cancer	15	4.46	60.83	1.2×10^{-6}	1.2×10^{-4}
Phospholipase D signaling pathway	18	3.58	45.70	2.9×10^{-6}	2.2×10^{-4}
Prolactin signaling pathway	12	5.05	63.12	3.7×10^{-6}	2.3×10^{-4}
Signaling pathways regulating pluripotency of stem cells	17	3.60	43.97	5×10^{-6}	2.6×10^{-4}
Autophagy	16	3.68	43.64	7.1×10^{-6}	3.1×10^{-4}
Relaxin signaling pathway	16	3.63	42.24	8.7×10^{-6}	3.3×10^{-4}
Neurotrophin signaling pathway	15	3.71	41.96	1.2×10^{-5}	4.2×10^{-4}
mTOR signaling pathway	17	3.29	36.25	1.7×10^{-5}	5.1×10^{-4}
TNF signaling pathway	14	3.75	40.30	2.1×10^{-5}	6×10^{-4}
FoxO signaling pathway	15	3.35	33.67	4.3×10^{-5}	0.001
Transcriptional misregulation in cancer	18	2.85	27.41	6.7×10^{-5}	0.001
PI3K-Akt signaling pathway	27	2.25	21.11	8.3×10^{-5}	0.002
Fc epsilon RI signaling pathway	10	4.33	40.18	9.4×10^{-5}	0.002
GO cellular compartment					
RISC complex (GO:0016442)	4	13.09	115.73	1.4×10^{-4}	0.05
RNAi effector complex (GO:0031332)	4	13.09	115.73	1.4×10^{-4}	0.03
Golgi subcompartment (GO:0098791)	32	1.97	16.49	2.3×10^{-4}	0.03
Golgi membrane (GO:0000139)	30	2.00	16.41	2.7×10^{-4}	0.03
Clathrin-coated vesicle membrane (GO:0030665)	10	3.64	28.39	4.1×10^{-4}	0.04
Trans-Golgi network (GO:0005802)	16	2.55	18.93	5.9×10^{-4}	0.04
Integral component of plasma membrane (GO:0005887)	72	1.45	10.12	9.3×10^{-4}	0.06
Nuclear body (GO:0016604)	36	1.72	11.50	0.001	0.06
Messenger ribonucleoprotein complex (GO:1990124)	3	12.62	84.58	0.001	0.07
Clathrin-coated vesicle (GO:0030136)	10	2.95	18.14	0.002	0.06
RISC-loading complex (GO:007578)	3	9.82	57.69	0.003	0.09
Chromatin (GO:0000785)	20	1.99	11.66	0.003	0.1
Early endosome membrane (GO:003901)	8	3.23	18.48	0.003	0.1
Clathrin-coated endocytic vesicle membrane (GO:0030669)	5	4.46	23.83	0.004	0.1
Trans-Golgi network membrane (GO:003588)	8	2.77	13.31	0.008	0.2
GO biological process					
Positive regulation of transcription, DNA-templated (GO:0045893)	88	2.31	68.72	1.3×10^{-13}	3×10^{-10}
Regulation of transcription from RNA polymerase II promoter (GO:0006357)	101	2.01	51.29	8.6×10^{-12}	2.2×10^{-8}
Positive regulation of transcription from RNA polymerase II promoter (GO:0045944)	68	2.36	56.48	4.1×10^{-11}	7×10^{-8}
Positive regulation of gene expression (GO:0010628)	63	2.41	55.31	1×10^{-10}	1.3×10^{-7}
Positive regulation of nucleic acid-templated transcription (GO:1903508)	48	2.82	64.55	3×10^{-10}	1.1×10^{-7}
Positive regulation of dendritic spine development (GO:0060999)	9	13.25	249.22	6.8×10^{-9}	5.8×10^{-6}
Regulation of transcription, DNA-templated (GO:0006355)	97	1.79	32.50	1.3×10^{-8}	9.3×10^{-6}
Regulation of Wnt signaling pathway (GO:0030111)	18	4.86	84.82	2.7×10^{-8}	1.7×10^{-5}
Negative regulation of transcription, DNA-templated (GO:0045892)	58	2.10	34.32	$8 \times \times 10^{-8}$	4.6×10^{-5}
Phosphorylation (GO:0016310)	35	2.67	42.07	1.4×10^{-7}	7.4×10^{-5}
Epidermal growth factor receptor signaling pathway (GO:0007173)	12	6.20	91.82	3.7×10^{-7}	1.7×10^{-4}
ERBB signaling pathway (GO:0038127)	14	5.15	75.34	4.5×10^{-7}	1.9×10^{-4}
Regulation of cell proliferation (GO:0042127)	52	2.07	29.60	6.2×10^{-7}	2.4×10^{-4}
Protein phosphorylation (GO:0006468)	38	2.38	33.50	7.8×10^{-7}	2.8×10^{-4}
Positive regulation of dendrite development (GO:1900006)	9	8.28	116.39	7.9×10^{-7}	2.7×10^{-4}
GO molecular function					
Transcription regulatory region DNA binding (GO:0044212)	32	2.52	33.40	1.7×10^{-6}	0.002

Table 4 (continued)

Term	Count	OR	CS	P-value	Adjusted P-value
Protein kinase activity (GO:0004672)	39	2.24	28.85	2.5×10^{-6}	0.001
Transcriptional activator activity, RNA polymerase II transcription regulatory region sequence-specific binding (GO:0001228)	26	2.70	33.00	4.8×10^{-6}	0.001
Transcriptional activator activity, RNA polymerase II core promoter proximal region sequence-specific binding (GO:0001077)	19	3.20	37.41	8.3×10^{-6}	0.002
Protein serine/threonine kinase activity (GO:0004674)	30	2.40	27.74	9.6×10^{-6}	0.002
Phosphatidylinositol-4,5-bisphosphate 3-kinase activity (GO:0046934)	11	4.76	52.39	1.7×10^{-5}	0.003
RNA polymerase II regulatory region sequence-specific DNA binding (GO:0000977)	34	2.18	23.55	2×10^{-5}	0.003
Phosphatidylinositol bisphosphate kinase activity (GO:0052813)	11	4.56	48.26	2.6×10^{-5}	0.004
Transcription factor activity, RNA polymerase II core promoter proximal region sequence-specific binding (GO:0000982)	24	2.52	26.05	3.3×10^{-5}	0.004
Transmembrane receptor protein kinase activity (GO:0019199)	10	4.75	47.93	4.1×10^{-5}	0.005
Low-density lipoprotein receptor activity (GO:0005041)	5	11.33	113.24	4.6×10^{-5}	0.005
Phosphatidylinositol 3-kinase activity (GO:0035004)	11	4.26	42.31	4.9×10^{-5}	0.005
Transcription regulatory region sequence-specific DNA binding (GO:0000976)	24	2.42	23.36	6.4×10^{-5}	0.006
Wnt-activated receptor activity (GO:0042813)	6	8.03	76.84	7×10^{-5}	0.006
Lipoprotein particle receptor activity (GO:0030228)	5	9.82	90.38	1×10^{-4}	0.008

OR odds ratio; *CS* Combined Score

this subtype [11, 28]. Furthermore, it has been reported that increased expression of DSCAM-AS1 is related to worse overall survival rates among BC patients [29]. We found that in addition to an increase in the expression of DSCAM-AS1 in the ER-positive breast tumour tissues, the circulating DSCAM-AS1 level increased in the plasma of ER-positive BC patients compared to those healthy controls and ER-negative patients. Next, the ROC curve was used to find out if DSCAM-AS1 could take a role as a tumour biomarker. The ROC curve demonstrated the sensitivity and specificity of DSCAM-AS1 (100% and 96.77%) for ER-positive BC vs. ER-negative patients, respectively.

To further investigate the function of this transcript in the development of breast cancer, we employed bioinformatics approaches. Our findings suggested that lncRNA DSCAM-AS1 might be involved in mTOR, MAPK, estrogen, and ErbB signaling pathways through at least six miRNAs (*hsa-miR-130a-3p, hsa-miR-27a-3p, hsa-miR-301a-3p, hsa-miR-193a-3p, has-miR137,* and *has-miR-204-5p*). Four of them (hsa-miR-130a-3p, hsa-miR-27a-3p, hsa-miR-301a-3p, and hsa-miR-193a-3p) were validated as potential targets of DSCAM-AS1 by an immunoprecipitation method, namely HITS-CLIP, in mammary gland tissue, and MCF7 cell line [20]. Moreover, hsa-mir-137 and hsa-mir-204-5p as direct regulators of DSCAM-AS1 in breast cancer were introduced by Ma et al. [21] and Liang et al. [22], respectively. According to the theory of ceRNA [30, 31], lncRNA DSCAM-AS1 as a ceRNA can attenuate the activity of the miRNAs that interfere in the signaling pathways involved in breast cancer development. ErbB receptors control key pathways which regulate cellular processes. In tumours, activated ErbB2 stimulates several intracellular pathways, e.g. the mTOR, PI3K/Akt, and MAPK pathways [32]. There are interactions between the ER and PI3K/mTOR. Activation of the mTOR and ER pathways up-regulates cyclin D to trigger cell cycle progression [32]. Since the ectopic expression of DSCAM-AS1 in ER-positive BC patients could decrease the level of related miRNAs, probably upregulation of the predicted mRNAs leads to activation of the ErbB signaling pathway among these patients.

According to the literature, ESR1 is targeted using three DSCAM-AS1-related miRNAs (miR-193b-3p, miR-301a-3p, and miR-27a-3p) [33–35]. Pillai et al. identified miR-193a/b-3p as a novel regulator of the ER pathway, which targets multiple genes involved in ER signaling [20]. The miR193b-3p could suppress the local production of estrogens and other steroid hormones using the direct targeting of 5′-Un-Translated Region (5′-UTR) of AKR1C2 [33]. The miR-27a-3p targets ESR1 as well as indirectly control E2-responsiveness in MCF-7 cells using the repression of ZBTB10, which increases the expression of ERα [36]. These results can explain the peculiar correlation between ERα and DSCAM-AS1 expression. On the other hand, miR-301a-3p and miR-130a-3p through targeting PTEN, and miR-137 could trigger the Wnt/β-catenin pathway and thereby promote BC invasion and metastasis through targeting PTEN and Follistatin Like Protein 1 (FSLT1), respectively. Moreover, it could regulate stemness and chemo resistance [37, 38].

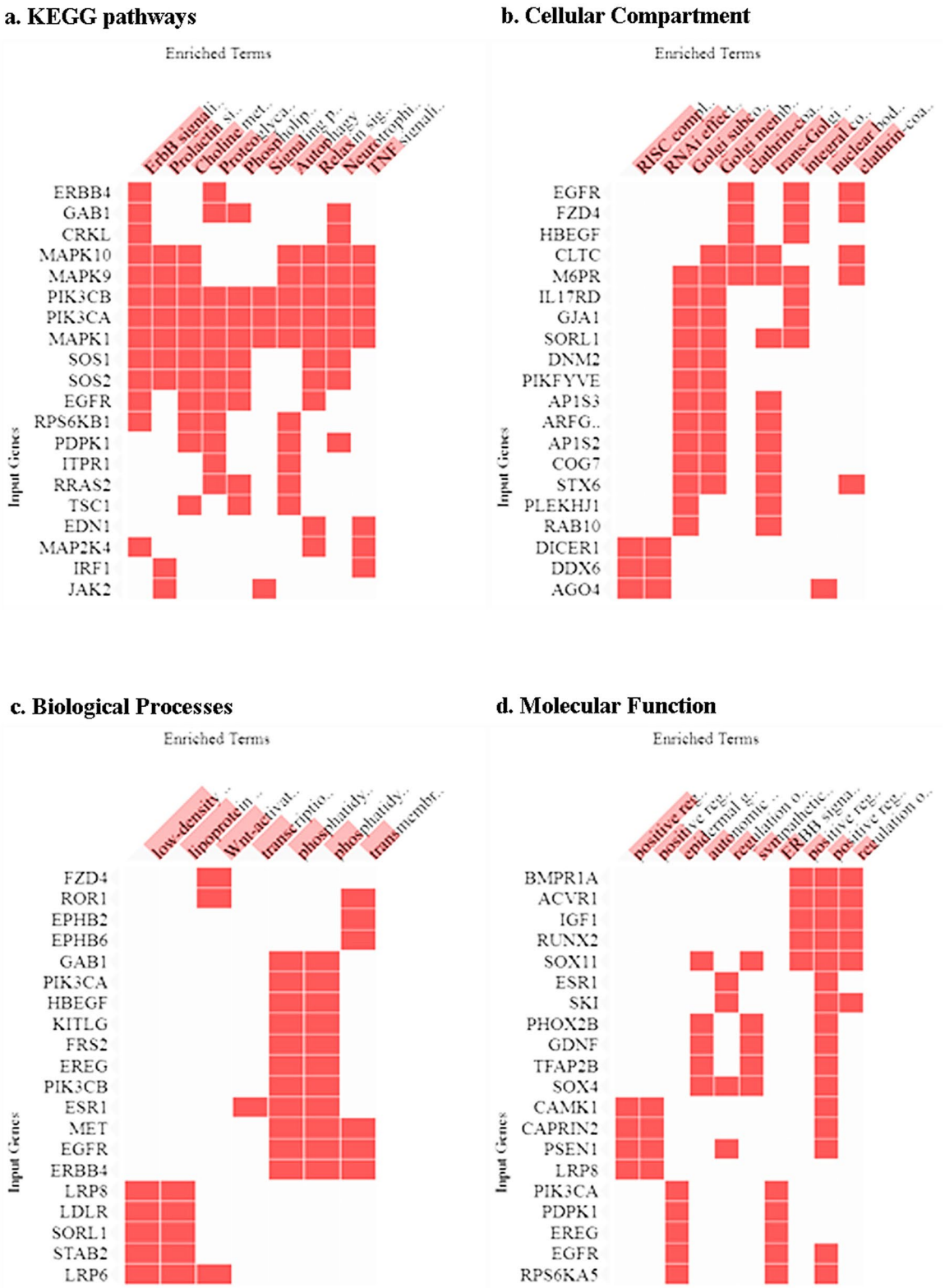


Fig. 6 The top 20 enriched terms based on Enrichr combined score visualized by clustergrams in KEGG human pathways (**a**), GO cellular compartment (**b**), GO biological processes (**c**), and GO molecular function (**d**). *KEGG* Kyoto Encyclopedia of Genes and Genomes, *GO* Gene Ontology

Our findings proposed a candidate circulating biomarker, namely DSCAM-AS1, for clinical assessment of ER-positive breast cancer patients. Presumably, perturbation of DSCAM-AS1, as a ceRNA, acts in the progression of breast cancer and drug resistance by affecting different cell signaling. However, to verify these results more studies with larger sample sizes and among other ethnic groups are necessary as a future suggestion.

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Author contributions M-TM: Performed all experiments, analyzed the data and wrote the initial draft of the manuscript. HF: Contributed to bioinformatic analysis and revised the manuscript. ZR: Contributed to concept and design, financial support, and revised the manuscript. All authors read and approved the final manuscript.

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest in this study.

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