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RESEARCH ARTICLE

The potential roles of lncRNAs DUXAP8, LINC00963, and FOXD2-AS1 in luminal breast cancer based on expression analysis and bioinformatic approaches

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Abstract

Numerous studies have demonstrated that lncRNAs participate in regulatory networks of different cancers. Dysregulation of various lncRNAs such as DUXAP8, LINC00963, and FOXD2-AS1 has been reported in the development of various cancers. The aim of this study was investigation of the importance and potential roles of DUXAP8, LINC00963, and FOXD2-AS1 in ER^+ breast cancer (BC). We examined the expression levels of DUXAP8, LINC00963, and FOXD2-AS1 in 71 luminal A and B tumor tissues and two luminal A cell lines (MCF7 and T47D) compared with adjacent non-tumor tissues and MCF10A cell line by qRT-PCR assay, respectively. For identifying the relation between three lncRNAs and luminal BC, bioinformatic analyses were performed using some databases and software including GENEVESTIGATOR software, GEPIA2, DAVID, REVIGO, STRING, lncATLAS, Kaplan–Meier plotter, starBase, and miRNet tool. The results showed the significant upregulation of all three lncRNAs in luminal A and B tumor specimens and cell lines. Upregulation of DUXAP8 and FOXD2-AS1 was significantly associated with progesterone receptor-positive (PR^+) and p53 protein expression in luminal BC patients, respectively. Based on bioinformatic analyses, DUXAP8 can be considered as a prognostic biomarker for patients with luminal BC. DUXAP8, LINC00963, and FOXD2-AS1 are involved in several cancer-associated signaling pathways and multiple cancer-related processes. In addition, bioinformatic analyses indicated that LINC00963/hsa-mir-130a-3p/HSPA8 axis might have potential regulatory role in BC. In conclusion, dysregulation of DUXAP8, LINC00963, and FOXD2-AS1 can play roles in the development of luminal BC. They may exert their functions through involvement in some cancer signaling pathways and processes. In addition, they may interact with miRNAs like predicted interaction of LINC00963 with miR-130a-3p.

Keywords Breast cancer · DUXAP8 · FOXD2-AS1 · LINC00963 · Luminal subtypes

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Abbreviations

BC	Breast cancer
BRCA	Invasive breast carcinoma
CeRNA	Competing endogenous RNA
DUXAP8	Double homeobox A pseudogene 8
ER	Estrogen receptor
FOXD2-AS1	FOXD2 adjacent opposite strand RNA 1
GO	Gene ontology
HER2	Human epidermal growth factor receptor 2
LncRNA	Long noncoding RNAs
LINC00963	Long intergenic non-protein coding RNA 963
PR	Progesterone receptor
BCRC-BB	Breast Cancer Research Center Bio-bank
BP	Biological process
CC	Cellular component

MF	Molecular function
qRT-PCR	Real-time quantitative reverse transcription-polymerase chain reaction

Introduction

Breast cancer (BC), the main cause of cancer-related death in women, is a heterogeneous disease and the most frequently diagnosed neoplasm in female patients worldwide. Thus, BC represents a priority for research works. BCs are classified into five intrinsic/molecular subgroups based on the presence or absence of three hormone receptors [estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor 2 (HER2)] and a proliferation index marker, Ki67 [1]. Subgroups of BC include luminal A, luminal B, HER2 overexpression, basal, and normal like tumors [2]. The most frequent BC subtypes are luminal A (ER^+/ $PR^{\pm}$/ $HER2^-$) and luminal B (ER^+/$PR^{\pm}$/$HER2^{\pm}$) tumors (around 70%) [3]. ER-positive breast tumors have less heterogeneity than ER-negative ones. The prognosis of luminal B subtype is worse than luminal A and shows more aggressive phenotype [4]. Luminal B has higher levels of Ki67 than luminal A and its growth is faster [4].

LncRNAs are a class of non-coding RNA molecules with more than 200 base pairs in length which lack open reading frames [5]. LncRNAs can exert diverse functions in cells and play important roles in different cellular processes [6]. Most of the lncRNAs have organ, cell, and state-specific expression which indicates their potential biological and physiological roles [7]. A lot of lncRNAs are associated with some of the human diseases like cancers and are prominent regulators in oncogenic or tumor suppressor pathways in the cells through interaction with DNA, RNA, protein, and other molecules [5, 8]. Recent studies have reported that some lncRNAs are abnormally expressed in various types of cancers and can impact the occurrence and development of malignant tumors via cellular signaling pathways [9]. The molecular mechanisms of BC have been extensively elucidated in previous studies and some molecules such as lncRNAs and miRNAs can improve diagnosis and prognosis of BC. In addition, these molecules can be developed as new targets in molecular targeted therapies [10].

Double homeobox A pseudogene 8 (DUXAP8) is a pseudogene-derived lncRNA and some diseases have been associated with this lncRNA. Previous studies have reported that DUXAP8 was upregulated in bladder cancer [11, 12], esophageal squamous cell carcinoma (ESCC) [13], renal cell carcinoma (RCC) [14, 15], non-small cell lung cancer (NSCLC) [16], and gastric cancer [17]. In addition, studies on Long Intergenic Non-Protein Coding RNA 963 (LINC00963; MetaLnc9) have indicated that the expression levels of this lncRNA significantly increased in non-small cell lung cancer (NSCLC) [18] and hepatocellular carcinoma (HCC) [19]. This lncRNA was able to activate PI3K/Akt/mTOR oncogenic pathway in two studied cancers. Furthermore, FOXD2 Adjacent Opposite Strand RNA 1 (FOXD2-AS1) was upregulated in bladder cancer, esophageal squamous cell carcinoma, hepatocellular carcinoma (HCC), non-small cell lung cancer (NSCLC), gastric cancer, papillary thyroid cancer, nasopharyngeal carcinoma, and glioma [20–25]. This lncRNA increased the proliferation and progression of mentioned cancers through several mechanisms and pathways [20–25].

Many attempts were made to identify lncRNAs as novel functional genes in carcinogenesis and to understand their roles as potential new biomarkers in diagnosis and prognosis of BC. In the present study, we have focused on three novel lncRNAs (DUXAP8, LINC00963, and FOXD2-AS1) which their potential roles have not been identified in luminal subtypes of BC. In addition, literature review and Lnc2Cancer database have shown that these novel lncRNAs have impact on the tumorigenesis of other human cancers. We examined the expression levels of lncRNAs DUXAP8, LINC00963, and FOXD2-AS1 in luminal A and B BC tissues and luminal A cell lines (MCF7 and T47D) compared with adjacent non-tumor tissues and non-malignant breast cell line (MCF10A), respectively. Furthermore, various bioinformatic analyses were done alongside the experimental analysis for understanding the importance and roles of mentioned lncRNAs in luminal subtypes of BC.

Materials and methods

LncRNA selection, tissue samples, and clinical data collection

We used Lnc2Cancer 3.0 database (http://www.bio-bigdata.com [26]) to select novel lncRNAs that no experiments have been conducted on their roles in the luminal subtypes of BC. This database provides associations between 2659 human lncRNAs and 216 human cancer subtypes. In addition, literature review was performed to confirm the importance of selected novel lncRNAs in the pathology of human cancers.

Seventy-one pairs of luminal A and B tumor, and matched adjacent non-tumor tissues were collected from Breast cancer Bio-bank (BCRC-BB) (Tehran, Iran) [27]. All tissues were immediately frozen in liquid nitrogen and subsequently stored at – 80 °C until RNA extraction. The present study received approval from the Ethics Committee of Tehran University of Medical Sciences (TUMS) (approval no. IR.TUMS.MEDICINE.REC.1398.659), and informed consent was obtained from all patients. Clinicopathological information was retrieved from the medical documents.

Cell lines and culture

Two luminal A human BC cell lines (MCF7 and T47D) were obtained from Breast cancer Bio-bank (BCRC-BB) (Tehran, Iran) [27]. MCF7 cell line expresses wild-type p53; however, this cell line displays amplification of MDMX gene that suppresses p53 protein [28]. T47D cell line has a mutation in TP53 gene (c.580C > T) [28]. MCF7 and T47D cell lines were cultured in DMEM (Sigma-Aldrich, St. Louis, MO, USA) with 10% fetal bovine serum (Gibco, Carlsbad, CA, USA), 100 U/ml penicillin and streptomycin (Sigma-Aldrich, St. Louis, MO, USA) in a cell culture incubator at 37 °C, 5% CO_2, and 95% humidity. The non-tumorigenic epithelial breast cell line (MCF10A) was cultured in DMEM containing 5% horse serum, 10 µg/ml insulin, 20 ng/ml EGF, 100 ng/ml cholera toxin, and 0.5 µg/ml hydrocortisone.

RNA extraction, cDNA synthesis, and qRT-PCR analysis

The total RNAs were isolated from cell lines and tissue samples by RiboEx™ reagent (GeneAll, Korea). The extracted RNAs were treated with DNase I (EN0521, Thermo Fisher scientific, United States) and reverse transcription was performed with 5X All-In-One RT MasterMix kit (Applied Biological Materials, CA). The quantitative real-time PCR assays were carried out in duplicate and were implemented with RealQ Plus 2 × Master Mix Green (AMPLIQON, Denmark) on LightCycler® 96 system (Roche Diagnostics, Mannheim, Germany). The β2M housekeeping gene was used as normalizer. The primer sequences of target genes in this study are shown in Table 1. The expression levels of DUXAP8, LINC00963 and FOXD2-AS1 were calculated using $2^{-\Delta\Delta Ct}$ method [29].

Prognosis analysis of DUXAP8, LINC00963, and FOXD2-AS1 expressions in luminal BC patients

Overall survival analysis was performed using GEPIA2 web server (http://www.GEPIA2.cancer-pku.cn [30]) to predict prognostic values of DUXAP8, LINC00963, and FOXD2-AS1 in patients with luminal subtypes of BC. Log-rank p values < 0.05 were considered as statistically significant.

Co-expression analysis of DUXAP8, LINC00963, and FOXD2-AS1

We obtained co-expressed genes with DUXAP8, LINC00963, and FOXD2-AS1 across multiple BC datasets using Affymetrix Human Genome U133 Plus 2.0 Array platform in GENEVESTIGATOR software. GENEVESTIGATOR is a web-based software tool to analyze the curated transcriptomic data from several repositories like GEO and Array Express. Calculation of Pearson correlation coefficient for the co-expressed genes was carried out by GENEVESTIGATOR using a standard method.

Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis

The co-expressed genes with DUXAP8, LINC00963, and FOXD2-AS1 were used for the gene set enrichment analyses. For this purpose, Gene Ontology (GO) enrichment and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses were done using The Database for Annotation, Visualization and Integrated Discovery (DAVID) version 6.8 (http://david.ncifcrf.gov [31, 32]). Three GO term categories were included in biological process (BP), cellular component (CC), and molecular function (MF). Then, we used REVIGO web server (http://revigo.irb.hr [33]) to summarize long lists of GO terms retrieved from DAVID and visualize non-redundant GO term sets.

Protein–protein interaction (PPI) network analysis

The protein–protein interaction (PPI) networks for the co-expressed genes with DUXAP8, LINC00963, and FOXD2-AS1 were downloaded from STRING database (https://string-db.org [34]) and the hub genes of these networks were ascertained by cytoHubba plugin [35] in the Cytoscape software.

Development of a lncRNA–miRNA interaction in breast cancer

We evaluated the subcellular localization of DUXAP8, LINC00963, and FOXD2-AS1 in MCF7 cell line using lncATLAS database (https://lncatlas.crg.eu [36]). This database provides a resource of 6768 lncRNAs localization in 15

Table 1 The primer sequences of DUXAP8, LINC00963, FOXD2-AS1, and β2M

Gene symbol	Forward primer sequence (5′–3′)	Reverse primer sequence (5′–3′)
DUXAP8	5′-TGTTGCTCTAAATGACACTGCT-3′	5′-TGACTAGCCTGTTCATCCACA-3′
LINC00963	5′-TCCACGCCTGAACACTTCTG-3′	5′-AGGAAAACAACGCTGCAAAAGA-3′
FOXD2-AS1	5′-CTGTTCTCGGCTCTGGGAAAG-3′	5′- GTGCAATCGTTCCGCTGTG-3′
β2M	5′-AGATGAGTATGCCTGCCGTG-3′	5′-GCGGCATCTTCAAACCTCCA-3′

human cell lines based on RNA-sequencing datasets. Then, the potential lncRNA–miRNA interactions were predicted using starBase version 3 database in invasive breast carcinoma (http://starbase.sysu.edu.cn [37]). The interactions with $R < -0.1$ and p value < 0.05 were considered as possible interactions. The basis of ceRNA mechanism is negative correlation between miRNA expression and lncRNA expression levels in BC. The expression and prognostic values of miRNAs in BRCA were determined using starBase and Kaplan–Meier plotter databases (http://kmplot.com/analysis [38]), respectively.

Prediction of potential target gene of miRNA in breast cancer

We can predict the target genes of potential miRNAs using miRNet tool (http://www.mirnet.ca [39]) for further investigation of DUXAP8, LINC00963, and FOXD2-AS1 functions. The protein–protein interaction networks of the potential target genes were retrieved from STRING database and the hub genes were defined using cytoHubba plugin of Cytoscape software. The prognostic values of hub genes were ascertained by Kaplan–Meier plotter, and the correlations between hub genes and lncRNA were obtained by GEPIA2 web server. The expression of the selected potential target genes was defined by GEPIA2 and starBase.

Statistical analysis

The IBM Statistical Package for the Social Sciences (SPSS) version 24 software (IBM Co., Armonk, NY, USA) was utilized for the paired samples t test to analyze the qRT-PCR data. The experimental results were shown as mean ± SD. Paired samples t test was used to estimate the significance of differences between gene expression in tumor and adjacent non-tumor tissues. The Kolmogorov–Smirnov test was applied to approve the normal distribution of ΔCt values of DUXAP8, LINC00963, and FOXD2-AS1 expression levels in tumor and adjacent non-tumor tissue samples. The associations of DUXAP8, LINC00963, and FOXD2-AS1 expression levels with the clinicopathological characteristics were evaluated by χ^2 test. The Pearson correlation coefficient > 0.7 was set for the correlation between DUXAP8/LINC00963/FOXD2-AS1 and co-expressed genes, and p values < 0.05 were considered as significant.

Results

Upregulation of DUXAP8, LINC00963, and FOXD2-AS1 in luminal breast tumor tissues and cell lines

The results of qRT-PCR technique showed that DUXAP8 ($p = 0.002$), LINC00963 ($p = 0.016$), and FOXD2-AS1 ($p = 0.002$) were significantly upregulated in luminal BC tissues compared with adjacent non-cancerous tissues (Fig. 1a–c). In addition, qPCR analysis revealed that DUXAP8 ($p < 0.001$), LINC00963 ($p < 0.05$), and FOXD2-AS1 ($p < 0.05$) were remarkably upregulated in MCF7 and T47D cell lines versus that in non-tumorigenic epithelial breast cell line (MCF10A) (Fig. 1d–f).

Overall survival analysis was conducted to investigate the correlation between DUXAP8 expression and the prognosis of 599 patients with luminal invasive breast carcinoma using GEPIA2 web server. Results revealed that the overall survival rate of patients with low levels of DUXAP8 expression is significantly higher in luminal BRCA (logrank $p = 0.015$) compared with patients with high levels of DUXAP8 expression (Fig. 1g). According to the GEPIA2 web server, we cannot predict the prognosis of luminal BC patients based on LINC00963 and FOXD2-AS1 expression levels (logrank $p_{\text{LINC00963}} = 0.57$, logrank $p_{\text{FOXD2-AS1}} = 0.067$).

The association of DUXAP8, LINC00963, and FOXD2-AS1 expression levels with the clinicopathological features

To get better understanding of the clinical values of all three lncRNA expression levels in luminal BC patients, the associations between lncRNA expression and clinicopathological characteristics of patients were analyzed. According to the median expression levels of DUXAP8, LINC00963, and FOXD2-AS1 in luminal BC tissues, patients were assigned into high and low expression groups. The clinicopathological features of BC patients are demonstrated in Tables 2 and 3. High expression levels of DUXAP8 and FOXD2-AS1 in luminal BC patients were significantly associated with progesterone receptor-positive ($p = 0.002$) and p53 protein expression ($p = 0.04$), respectively. There were not any significant associations between LINC00963 expression and the clinicopathological data.

GO functional annotation and KEGG pathway enrichment analyses

At the first step, the co-expressed genes with DUXAP8, LINC00963, and FOXD2-AS1 were predicted using

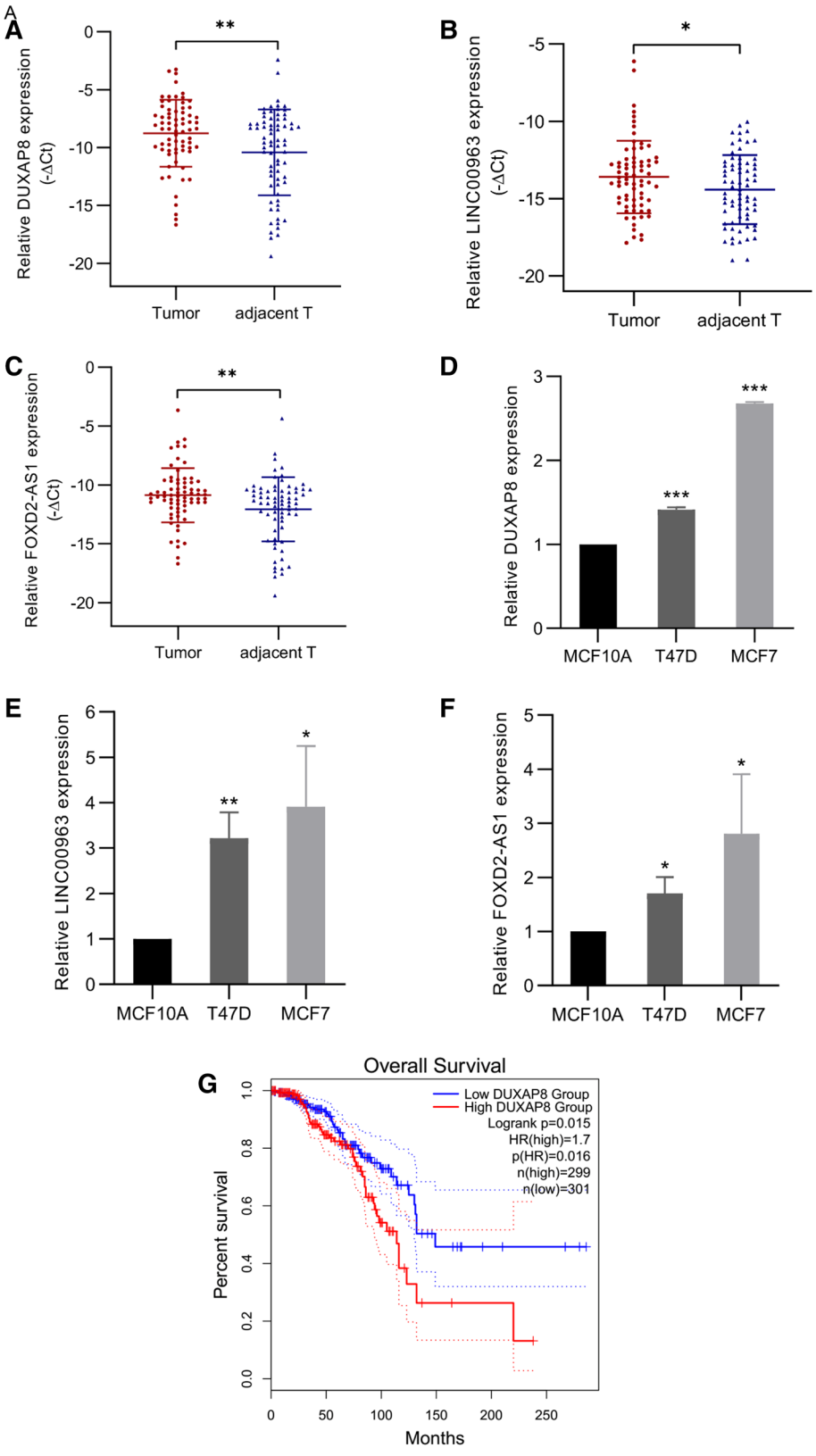

Fig. 1 DUXAP8, LINC00963, and FOXD2-AS1 were upregulated in luminal BC. **a** DUXAP8 was significantly upregulated in 71 luminal breast tumor tissues compared with adjacent non-cancerous tissues. **b** Higher expression level of LINC00963 in 71 luminal breast tumor tissues compared with adjacent non-tumor tissues. **c** The significant upregulation of FOXD2-AS1 in 71 luminal BC tissues in comparison with adjacent non-tumor tissues. **d** Significant upregulation of DUXAP8 in luminal BC cell lines. **e** LINC00963 expression is higher in luminal BC cell lines than in non-malignant epithelial breast cell line. **f** Upregulation of FOXD2-AS1 in MCF7 and T47D compared with MCF10A. **g** The prognostic values of DUXAP8 in patients with luminal BC. The results are presented as the mean ± SD, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$

Table 2 The associations of DUXAP8 and LINC00963 expression with clinicopathological characteristics in breast cancer patients

Clinicopathological characteristic	Number of cases	DUXAP8 expression level			LINC00963 expression level		
		Low *N* (%)	High *N* (%)	*p* value (χ^2 test)	Low *N* (%)	High *N* (%)	*p* value (χ^2 test)
Group							
Luminal A	61	28 (80.0)	33 (91.7)	0.158	32 (91.4)	29 (80.6)	0.188
Luminal B	10	7 (20.0)	3 (8.3)		3 (8.6)	7 (19.4)	
Estrogen receptor							
Negative	0	0 (0.0)	0 (0.0)		0 (0.0)	0 (0.0)	
Positive	71	35 (100.0)	36 (100.0)		35 (100.0)	36 (100.0)	
Progesterone receptor							
Negative	8	8 (22.9)	0 (0.0)	**0.002****	4 (11.4)	4 (11.1)	0.966
Positive	63	27 (77.1)	36 (100.0)		31 (88.6)	32 (88.9)	
HER2[a]							
Negative	66	31 (88.6)	35 (97.2)	0.154	33 (94.3)	33 (91.7)	0.666
Positive	5	4 (11.4)	1 (2.8)		2 (5.7)	3 (8.3)	
Tumor size							
< 2 cm	20	9 (26.5)	11 (31.4)	0.176	8 (22.9)	12 (35.3)	0.472
2–5 cm	35	15 (44.1)	20 (57.1)		20 (57.1)	15 (44.1)	
> 5 cm	14	10 (29.4)	4 (11.4)		7 (20.0)	7 (20.6)	
Lymph node metastasis							
No	17	9 (27.3)	8 (22.9)	0.674	9 (26.5)	8 (23.5)	0.779
Yes	51	24 (72.7)	27 (77.1)		25 (73.5)	26 (76.5)	
Stage							
I	4	1 (3.3)	3 (8.6)	0.603	2 (6.4)	2 (5.8)	0.298
II	38	19 (63.3)	19 (54.3)		21 (67.7)	17 (50.0)	
III	23	10 (33.3)	13 (37.1)		8 (25.8)	15 (44.1)	
Grade							
1	9	4 (11.4)	5 (13.9)	0.750	3 (8.6)	6 (16.7)	0.588
2	52	27 (77.1)	25 (69.4)		27 (77.1)	25 (69.4)	
3	10	4 (11.4)	6 (16.7)		5 (14.3)	5 (13.9)	
P53							
Negative	8	6 (35.3)	2 (12.5)	0.152	5 (35.7)	3 (16.7)	0.217
Positive	24	11 (64.7)	13 (87.5)		9 (64.3)	15 (83.3)	
Age at diagnose							
< 40	16	10 (30.3)	6 (17.6)	0.542	10 (29.4)	6 (18.2)	0.281
> 40	51	23 (69.7)	28 (82.4)		24 (70.6)	27 (81.8)	

[a]*HER2* human epidermal growth factor receptor 2

**$p < 0.01$

GENEVESTIGATOR software in multiple BC datasets containing 1910 luminal samples. As shown in Data S1, a total of 403, 400, and 400 potential co-expressed genes with DUXAP8, LINC00963, and FOXD2-AS1 ($R > 0.7$) were obtained, respectively. Next, the co-expressed genes were utilized to retrieve the GO functional annotations using DAVID tool. The top 5 enriched GO terms ($p < 0.05$) of BP, CC, and MF categories for the co-expressed genes with DUXAP8, LINC00963, and FOXD2-AS1 are shown in Fig. 2a–c.

The results of REVIGO web server showed Gene Ontology terms similar to the GO terms obtained from DAVID (Figure S1, 2, 3).

KEGG pathway analysis for the co-expressed genes with DUXAP8 and LINC00963 indicated that they were significantly enriched in several cancer-associated pathways and human cancers as are reported in Table 4. However, KEGG pathway analysis did not show any significant pathway related to the co-expressed genes with FOXD2-AS1.

Table 3 The association of FOXD2-AS1 expression with clinicopathological characteristics in breast cancer patients

Clinicopathological characteristic	Number of cases	FOXD2-AS1 expression level		p value (χ^2 test)
		Low *N* (%)	High *N* (%)	
Group				
Luminal A	61	29 (82.9)	32 (88.9)	0.465
Luminal B	10	6 (17.1)	4 (11.1)	
Estrogen receptor				
Negative	0	0 (0.0)	0 (0.0)	–
Positive	71	35 (100.0)	36 (100.0)	
Progesterone receptor				
Negative	8	4 (11.4)	4 (11.1)	0.966
Positive	63	31 (88.6)	32 (88.9)	
HER2[a]				
Negative	66	32 (91.4)	34 (94.4)	0.620
Positive	5	3 (8.6)	2 (5.6)	
Tumor size				
< 2 cm	20	10 (29.4)	10 (28.6	0.861
2–5 cm	35	18 (52.9)	17 (48.6)	
> 5 cm	14	6 (17.6)	8 (22.9)	
Lymph node metastasis				
No	17	11 (33.3)	6 (17.1)	0.123
Yes	51	22 (66.7)	29 (82.9)	
Tumor stage				
I	4	2 (6.9)	2 (5.6)	0.498
II	38	19 (65.5)	19 (52.8)	
III	23	8 (27.6)	15 (41.7)	
Grade				
1	9	3 (8.6)	6 (16.7)	0.500
2	52	26 (74.3)	26 (72.2)	
3	10	6 (17.1)	4 (11.1)	
P53				
Negative	8	7 (38.9)	1 (7.1)	**0.040***
Positive	24	11 (61.1)	13 (92.9)	
Age at diagnosis				
< 40	16	6 (17.6)	10 (30.3)	0.224
> 40	51	28 (82.4)	23 (69.7)	

Bold numbers indicates the significant p value

*$P < 0.05$

[a]*HER2* human epidermal growth factor receptor 2

Protein–protein interaction (PPI) network construction

It has been extensively acknowledged that genes exert their biological functions through interaction with each other. According to the STRING database, we found that 273/403, 270/400, and 127/400 co-expressed genes with DUXAP8, LINC00963, and FOXD2-AS1 have strong interaction with each other (interaction score > 0.4), respectively (data were not shown). The hub genes of PPI networks for the co-expressed genes with DUXAP8 and LINC00963 were selected using degree method (node degree ≥ 10) in cytoHubba plugin of Cytoscape software (Table 5). The sub-PPI networks containing the top 40 and 24 hub genes were constructed for the co-expressed genes with DUXAP8 and LINC00963 as shown in Fig. 3a, b, respectively. Furthermore, the hub genes of the PPI network for FOXD2-AS1 co-expressed genes were ranked by degree metric (degree ≥ 6) and the top 7 hub genes were achieved using the CytoHubba plugin in Cytoscape software (Fig. 3c). The hub genes of PPI network for FOXD2-AS1 co-expressed genes included KIF5A (node

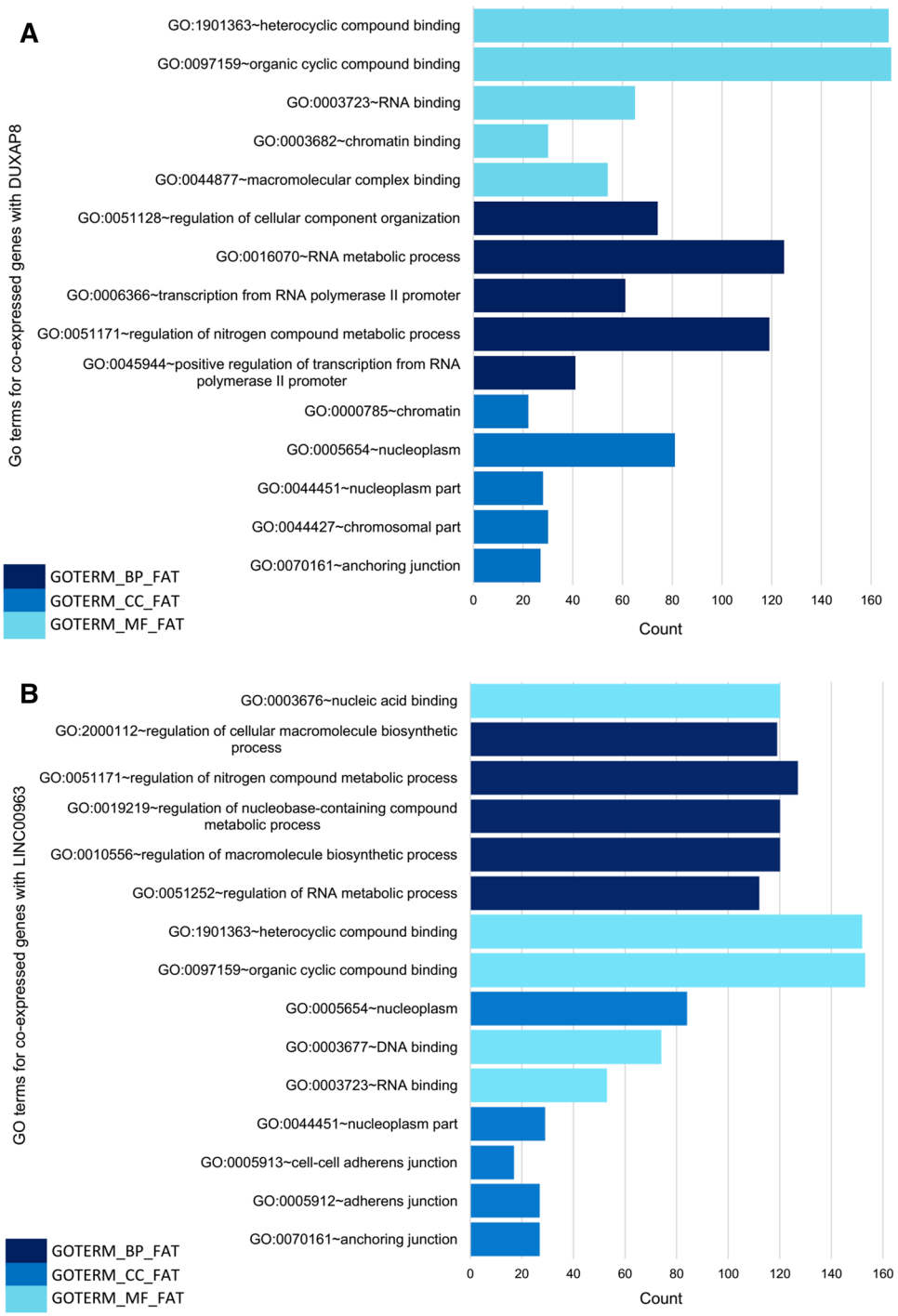

Fig. 2 GO functional annotations for the co-expressed genes with DUXAP8, LINC00963, and FOXD2-AS1 retrieved from DAVID. **a** The top 5 GO terms of BP, CC, and MF categories associated with the co-expressed genes with DUXAP8. **b** The top 5 GO terms of BP, CC, and MF categories associated with the co-expressed genes with LINC00963. **c** The top 5 GO terms of BP, CC, and MF categories associated with the co-expressed genes with FOXD2-AS1. GOTERM_BP_FAT, GOTERM_CC_FAT, and GOTERM_MF_FAT is representative of BP, CC, and MF terms, respectively. The highest bar represents the lowest p value and the lowest bar represents the highest p value

degree = 11), NCAN, BSN, CPLX2, RHO, REEP2, and NANOG.

Prediction of potential binding miRNAs for DUXAP8, LINC00963, and FOXD2-AS1 in invasive breast carcinoma

Competing endogenous RNAs (ceRNAs) can indirectly regulate mRNAs through binding to miRNAs competitively and play important roles in pathological processes such as cancers. According to the lncATLAS database, DUXAP8, LINC00963, and FOXD2-AS1 are localized in nucleus of MCF7 cell line. Based on starBase database, 22, 59, and 6 potential miRNAs were predicted to bind to DUXAP8, LINC00963, and FOXD2-AS1 in invasive breast carcinoma (BRCA), respectively ($p < 0.05$) (Data S2). Based on the ceRNA mechanism, DUXAP8 had significant negative relationship with

Fig. 2 (continued)

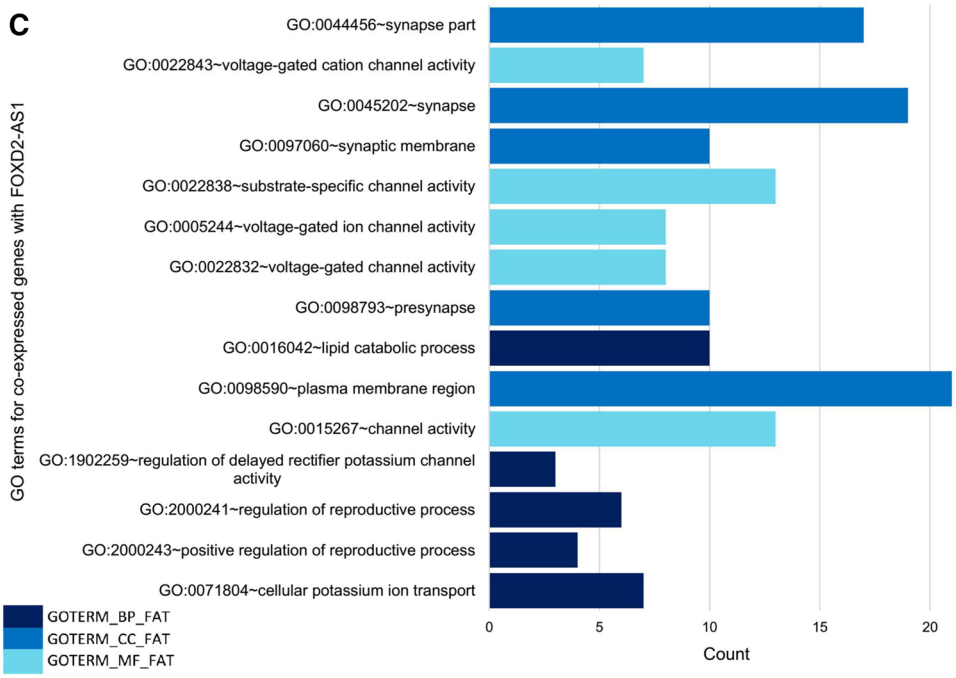

Table 4 KEGG pathway enrichment analysis for the co-expressed genes with DUXAP8 and LINC00963

Pathway ID	Description	Count	Genes	*p* value
For the co-expressed genes with DUXAP8				
hsa05203	Viral carcinogenesis	12	HDAC5, IRF3, STAT3, CCR8, EIF2AK2, CHD4, ACTN4, LTBR, TP53, RELA, HDAC6, NFKB2	0.00312
hsa04360	Axon guidance	9	NTNG1, SEMA4A, EFNB3, SEMA3D, PLXNA2, PLXNB3, EPHA1, EFNA5, EPHB3	0.00440
hsa05160	Hepatitis C	8	SCARB1, CLDN15, IRF3, STAT3, EIF2AK2, PPP2R2A, TP53, RELA	0.01914
hsa04310	Wnt signaling pathway	8	TCF7L1, VANGL2, CHD8, CSNK2B, LRP5, DVL3, CSNK1E, TP53	0.02293
hsa05221	Acute myeloid leukemia	5	TCF7L1, SPI1, STAT3, RELA, PML	0.02730
hsa05132	Salmonella infection	6	DYNC1H1, PLEKHM2, FLNA, PKN1, WASF2, RELA	0.02732
hsa05169	Epstein-Barr virus infection	7	IRF3, STAT3, EIF2AK2, TP53, RELA, RELB, NFKB2	0.03887
For the co-expressed genes with LINC00963				
hsa04520	Adherens junction	8	PTPRB, CREBBP, ACTN1, LEF1, CTNNA1, EP300, MAPK1, IQGAP1	1.67E-04
hsa05200	Pathways in cancer	17	FZD1, CREBBP, ARHGEF12, FZD2, F2R, LEF1, AXIN2, ADCY7, LPAR5, APC, TRAF3, CTNNA1, EP300, MAPK1, IKBKG, APPL1, PPARD	7.71E-04
hsa04310	Wnt signaling pathway	9	FZD1, CREBBP, FZD2, PPP3CC, APC, LEF1, EP300, AXIN2, PPARD	0.00208
hsa04390	Hippo signaling pathway	9	FZD1, LATS1, YAP1, FZD2, APC, LEF1, CTNNA1, SCRIB, AXIN2	0.00364
hsa04916	Melanogenesis	7	FZD1, CREBBP, FZD2, LEF1, EP300, MAPK1, ADCY7	0.00638
hsa05203	Viral carcinogenesis	10	HDAC4, CREBBP, SNW1, TRAF3, ACTN1, MRPS18B, EP300, MAPK1, SCRIB, IKBKG	0.00714
hsa04330	Notch signaling pathway	5	CREBBP, JAG1, SNW1, MAML1, EP300	0.00818
hsa05213	Endometrial cancer	5	APC, LEF1, CTNNA1, MAPK1, AXIN2	0.01082
hsa05217	Basal cell carcinoma	5	FZD1, FZD2, APC, LEF1, AXIN2	0.01233
hsa05210	Colorectal cancer	5	APC, LEF1, MAPK1, AXIN2, APPL1	0.01965
hsa04611	Platelet activation	7	GUCY1A2, GP9, ARHGEF12, F2R, MAPK1, ITPR2, ADCY7	0.02137
hsa04720	Long-term potentiation	5	CREBBP, PPP3CC, EP300, MAPK1, ITPR2	0.02414
hsa04550	Signaling pathways regulating pluripotency of stem cells	7	FZD1, ACVR1, FZD2, APC, MAPK1, AXIN2, JARID2	0.02944

Table 5 The hub genes in the PPI networks of the co-expressed genes with DUXAP8 and LINC00963 (node degree ≥ 10)

Network of DUXAP8 co-expressed genes				Network of LINC00963 co-expressed genes	
Gene name	Node degree	Gene name	Node degree	Gene name	Node degree
TP53	60	FLNA	12	EP300	33
STAT3	30	SF3A1	11	MAPK1	30
RBBP4	20	MED12	11	CREBBP	28
RELA	19	GATAD2B	11	ATM	21
CHD8	18	SPTAN1	11	DICER1	19
CHD4	16	PABPC1	11	SNW1	15
PML	16	TGFB1	11	RBM8A	14
GTF2F1	15	ACTN4	11	IKBKG	14
HUWE1	15	PHC2	11	LEF1	14
CDK9	14	SNRPA	10	SRRT	14
UBA1	14	EFNA5	10	HDAC4	13
IRF3	14	EFNB3	10	EXOSC10	13
HDAC6	14	MLLT3	10	APC	12
FBXL19	14	NTRK2	10	BCL6	11
UBXN7	14	RBM10	10	HNRNPM	11
CSNK2B	13	NES	10	TRAF3	11
HDAC5	13	FLNA	12	ARF6	11
MECP2	13	SF3A1	11	APP	11
SMARCB1	13	MED12	11	CHERP	11
SSRP1	12	GATAD2B	11	YAP1	10
EPRS	12	SPTAN1	11	AXIN2	10
TRIM28	12	PABPC1	11	RPS28	10
NUP214	12	TGFB1	11	PPP2R5C	10
LMNA	12	ACTN4	11	ALDH18A1	10

hsa-miR-29c-3p, and LINC00963 had significant negative interaction with several potential miRNAs including hsa-miR-505-3p, hsa-miR-92a-3p, hsa-miR-130a-3p, hsa-miR-532-3p, hsa-miR-526b-5p, hsa-miR-205-5p, and hsa-miR-378a-3p in BRCA ($R < -0.1$, $p < 0.05$). But, FOXD2-AS1 did not have any significant negative interaction among 6 potential binding miRNAs. Next, the expression of these miRNAs demonstrated that hsa-miR-130a-3p, and hsa-miR-532-3p were significantly downregulated, but the expression level of hsa-miR-29c-3p was not significantly changed ($p = 0.16$) in BRCA (Fig. 4a, c). According to the Kaplan–Meier plotter database, downregulation of hsa-mir-130a showed poor prognosis of BRCA patients, but BRCA patients with low expression of hsa-miR-532 had better prognosis (Fig. 4b, d). Taken together, hsa-mir-130a-3p might be potential binding miRNA of LINC00963 in BRCA. However, we did not find a potential binding miRNA for DUXAP8 and FOXD2-AS1 in BRCA according to databases. The consensus sequences of LINC00963 and hsa-mir-130a-3p are demonstrated in Fig. 5.

Prediction of the target gene of hsa-mir-130a-3p in invasive breast carcinoma

To more clarification of the functions of LINC00963 in BC, the potential target genes of hsa-mir-130a-3p were predicted using miRNet tool. A total of 399 potential target genes were obtained (Data S3). In the PPI network of target genes, about 67 hub genes were obtained based on degree method (node degree ≥ 10) (Data S4). For discovering the potential functional targets of LINC00963, the top 15 hub genes were selected and among them, upregulation of CUL3, HSPA8, and MAPK1 showed a poor prognosis of BRCA patients (Table 6). Next, correlation between LINC00963 and the top 15 hub genes were assessed by GEPIA2 (Table 7). According to the correlation and survival analyses, we suggested that CUL3, HSPA8, and MAPK1 may be potential functional targets of LINC00963. Among these three target genes, HSPA8 was significantly upregulated in BRCA and luminal subtypes based on GEPIA2 and starBase (Fig. 4e, f). The expression levels of CUL3 and MAPK1 did not

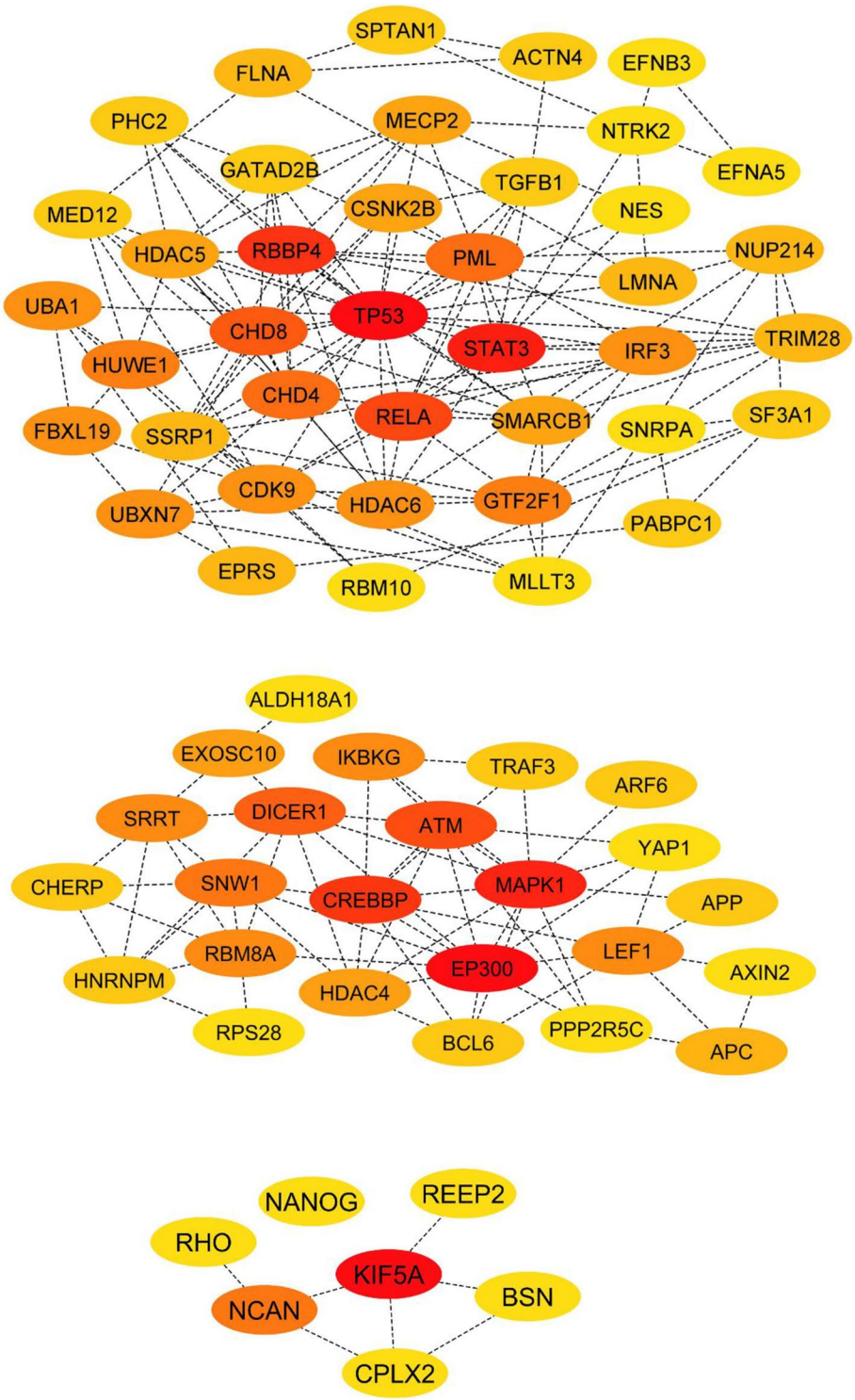

Fig. 3 PPI network analysis for the co-expressed genes with DUXAP8, LINC00963, and FOXD2-AS1. **a** The top 40 hub genes (node degree ≥ 10) in PPI network of the co-expressed genes with DUXAP8. **b** The top 24 hub genes (node degree ≥ 10) in PPI network of the co-expressed genes with LINC00963. **c** The top 7 hub genes (node degree ≥ 6) in PPI network of co-expressed genes with FOXD2-AS1

significantly change. It is suggested that the LINC00963/hsa-mir-130a-3p/HSPA8 axis might be contributed in BC pathology.

Discussion

Breast cancer has the highest incidence of diagnosed cancer and high mortality rate among women around the world. ER^+ breast cancers (luminal A and luminal B) consist the majority of BC patients. Studies have reported that lncRNAs

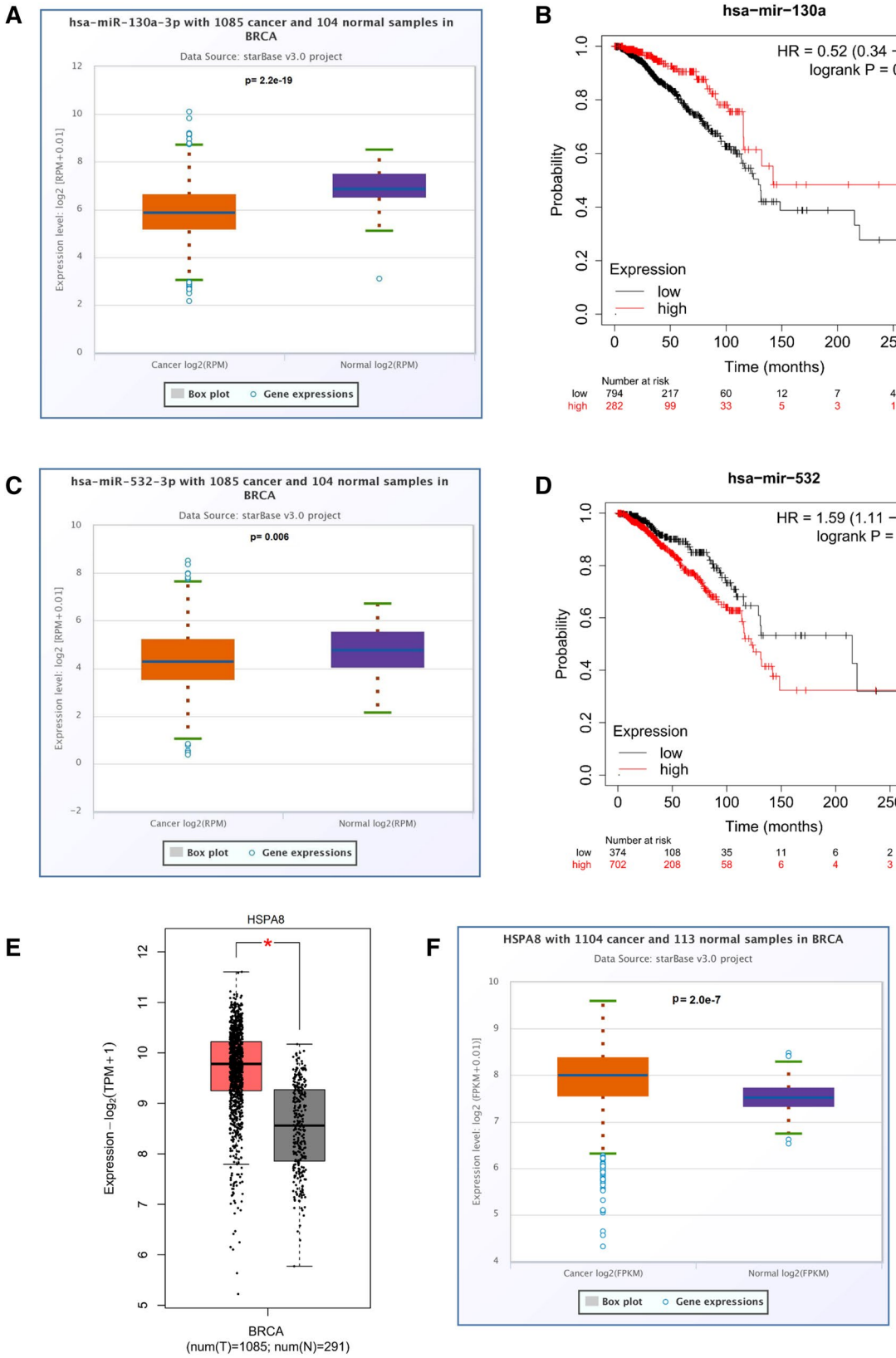

Fig. 4 Potential binding miRNAs and potential functional target of LINC00963. **a** Lower expression of hsa-mir-130a-3p in tumor tissues of BRCA than that in normal control. **b** Prognostic value of hsa-mir-130a-3p in BRCA. **c** Lower expression of hsa-mir-532-3p in tumor tissues than that in normal tissues. **d** Prognostic value of hsa-mir-532-3p in BRCA. Upregulation of HSPA8 in invasive breast carcinoma tissues compared with normal control tissues determined by **e** GEPIA2 and **f** starBase databases

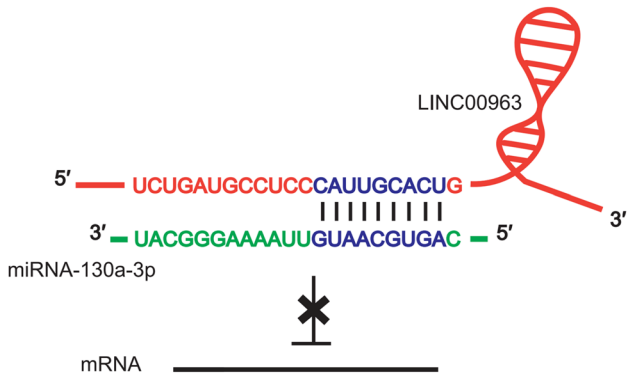

Fig. 5 Interaction of LINC00963 with hsa-mir-130a-3p and their consensus sequences

Table 6 The prognostic values of the top 15 hub genes in the PPI network of target genes across breast cancer using KM plotter

Gene name	*p* value	HR	Prognosis outcome
MYC	0.03	0.7	Good
UBC	0.074	0.75	Good
RPS27A	0.055	0.73	Good
UBB	0.091	1.35	Poor
PTEN	0.14	1.34	Poor
MAPK1	0.0044	1.6	Poor
ESR1	0.1	1.35	Poor
TNF	0.19	0.81	Good
HSPA8	0.0044	1.73	Poor
SMAD4	0.12	1.31	Poor
DICER1	0.43	0.87	Good
TGFB1	0.015	0.57	Good
CUL3	0.022	1.48	Poor
APP	0.36	0.86	Good
UBE2D2	0.11	1.3	Poor

Bold genes indicates genes with significant *p* value

Table 7 The correlation between LINC00963 and the expression of the top 15 hub genes in the PPI network of target genes across breast cancer by GEPIA2

Gene name	*R*	*p* value	Gene name	*R*	*p* value
MYC	− 0.12	5.8e−05	HSPA8	0.12	5.6e−05
UBC	0.23	1e−14	SMAD4	0.13	1.7e−05
RPS27A	− 0.19	6.8e−10	DICER1	0.24	3.1e−15
UBB	0.084	0.0058	TGFB1	0.074	0.014
PTEN	0.14	5.5e−06	CUL3	0.19	1.7e−10
MAPK1	0.16	4.9e−08	APP	− 0.068	0.025
ESR1	0.16	5.4e−08	UBE2D2	0.12	8.3e−05
TNF	− 0.13	2.2e−05			

are involved in the pathogenesis of cancers such as BC. It is proposed that imbalance expression of lncRNAs may play role in malignant transformation [7]. LncRNAs can be critical in clinical use for diagnosis, prognosis, and treating cancers [7, 40]. The probable roles of DUXAP8, LINC00963, and FOXD2-AS1 in the most prevalent subtypes of BC (luminal subtypes) have not been studied until now.

Previous studies have demonstrated that DUXAP8 was upregulated in several cancers and this lncRNA can increase the expression of Wnt/β-catenin pathway-related genes in esophageal squamous cell carcinoma (ESCC). It also can inhibit PTEN and activate PI3K/Akt signaling pathway in bladder cancer [12]. In addition, upregulation of LINC00963 in hepatocellular carcinoma (HCC) and non-small cell lung cancer (NSCLC) can lead to the activation of oncogenic PI3K/Akt signaling pathway [18, 19]. FOXD2-AS1 increased activity of AKT and PI3K/Akt signaling pathways in bladder cancer and glioma [20, 41]. In addition, this lncRNA enhanced proliferation of colorectal cancer and non-small cell lung cancer (NSCLC) through Notch/delta and Wnt/β-catenin signaling pathways, respectively [24, 42]. Moreover, Wnt/β-catenin and PI3K/Akt/mTOR signaling pathways are aberrantly activated during the tumorigenesis of BC [4]. Thus, these three novel lncRNAs play crucial roles in the pathogenesis of human cancers [4].

In the present study, we assessed the expression and potential roles of three novel lncRNAs including DUXAP8, LINC00963, and FOXD2-AS1 in tissue samples of luminal BC patients and through some bioinformatic data analyses as well. Our findings illustrated that LINC00963, FOXD2-AS1, and DUXAP8 were more upregulated in luminal A and B BC tissues and luminal A cell lines (MCF7 and T47D) than that of the adjacent non-cancerous tissues and control cell line (MCF10A).

Besides, the association of DUXAP8, LINC00963, and FOXD2-AS1 expression levels with the clinicopathological features of patients indicated that high expression levels of DUXAP8 and FOXD2-AS1 had the significant positive correlation with progesterone receptor-positive (PR^+) and p53 protein expression, respectively. Accumulation of the p53 protein is associated with the malignant disease and DNA damage [43], and the overexpression of the p53 protein is a marker of poor prognosis in BC. In addition, survival analysis revealed that patients with higher DUXAP8 expression have poor overall survival, suggesting that DUXAP8 can be a candidate prognostic biomarker in luminal BC patients.

A widely validated principle named "guilt by association" identified and predicted the functions and regulatory mechanisms for gene of interest based on a set of genes with known function that were co-expressed with it in related biological processes [44]. GO enrichment analysis based on the co-expressed genes revealed that DUXAP8 might be involved in certain biological processes such as 'regulation

of cellular component organization', 'RNA metabolic process', and 'transcription from RNA polymerase II promoter'. RNA metabolic process included in dNTP biosynthesis and catabolism, and dNTP imbalances have critical roles in cancer initiation and progression [45]. In addition, functional annotation analysis uncovered that LINC00963 may be involved in a number of biological processes related to cell cycle including 'regulation of cellular macromolecule biosynthetic process', 'regulation of nitrogen compound metabolic process', and 'regulation of nucleobase-containing compound metabolic process'. In addition, FOXD2-AS1 might contribute to multiple biological processes and molecular functions, including 'lipid catabolic process', 'regulation of delayed rectifier potassium channel activity', 'regulation of reproductive process', 'voltage-gated cation and ion channel activity', and 'substrate-specific channel activity'. The dysregulation of ion channels activity involves in the carcinogenesis processes of some cancers like breast cancer. Cation channels might play a key role in sustaining the proliferative signaling in luminal BC cells [46].

KEGG pathway enrichment analysis elucidated that DUXAP8 might be involved in some cancer-associated pathways, including 'viral carcinogenesis', 'Wnt signaling pathway', and cancers like acute myeloid leukemia. In addition, LINC00963 might be involved in some signaling pathways related to cancers such as 'Wnt signaling pathway', 'Notch signaling pathway', 'Hippo signaling pathway', 'pathways in cancers', 'viral carcinogenesis', and several cancers such as endometrial cancer, basal cell carcinoma, colorectal cancer, and melanogenesis. It is thought that DUXAP8 and LINC00963 might function through interaction with some of the mentioned signaling pathways in luminal A and B BC.

The data retrieved from STRING database demonstrated that about 68% of the co-expressed genes with DUXAP8 and LINC00963, and 32% of FOXD2-AS1 co-expressed genes had strong interaction with each other. It shows that each lncRNA with its co-expressed genes is involved in the same biological processes. Most of the hub genes in the PPI network of the co-expressed genes with DUXAP8 such as TP53, RBBP4, CHD4, GTF2F1, and HUWE1 were upregulated in luminal BC based on GEPIA2 database (data were not shown). Moreover, among hub genes in the PPI network of the co-expressed genes with LINC00963, some were upregulated in luminal BC tissues compared to normal ones, including EP300, MAPK1, and CREBBP (data were not shown). KIF5A as the first hub gene in the network of FOXD2-AS1 co-expressed genes was downregulated in luminal BC. NANOG is a hub gene in the PPI networks of the co-expressed genes with FOXD2-AS1. Previous studies have demonstrated that NANOG has tumorigenic features in human cancers like BC [47, 48]. On the other hand, p53 can bind to the NANOG promoter and suppress it after DNA damage [47, 48]. P53 pathway can regulate NANOG expression in cancer stem cells [47]. In addition, RARA is a co-expressed gene with FOXD2-AS1 in this study. RARA is a p53 target gene and it is reported that binding of Sp1 to the p53 target genes is required for the p53-mediated pro-apoptotic transcriptional repression [49]. Due to the significant positive association between FOXD2-AS1 and p53 protein expression in luminal BC patients' samples, as well as the interaction between p53 and two genes (NANOG and RARA), FOXD2-AS1 may play a role in p53 pathway of luminal BC.

The competitive endogenous RNA (ceRNA) mechanisms regulate biological processes and these regulatory mechanisms are involved in the pathological status such as in the tumorigenesis. The combination results of correlation, survival, and expression analysis using several databases showed that hsa-mir-130a-3p was a potential binding miRNA for LINC00963 in BRCA. We did not find any binding miRNAs for DUXAP8 and FOXD2-AS1 in invasive breast carcinoma. miR-130a-3p as a tumor suppressor gene is downregulated in multiple human cancers such as breast cancer [50], gastric cancer [51], prostate carcinoma [52], and gefitinib resistant non-small cell lung cancer [53]. The potential functional target gene of hsa-mir-130a-3p was HSPA8 based on several analyses. HSPA8 as a cancer-related gene is a member of HSP70 family and is upregulated in endometrial carcinoma and is a candidate biomarker for early diagnosis and therapy of endometrial carcinoma [54]. HSPA8 functions as a positive regulator in growth of glioma cancer cells [55]. HSP70 family can increase malignancy and resistance to therapy and also inhibit apoptosis [56]. These findings indicated that LINC00963 may have function in development of BC by targeting HSPA8 through competitively binding to hsa-mir-130a-3p, and this axis deserves more investigations in BC and luminal subtypes at experimental level.

In conclusion, our findings revealed the significant upregulation of DUXAP8, LINC00963, and FOXD2-AS1 in luminal subtypes of BC. These lncRNAs might exert their functions in luminal subtypes of BC through their involvement in some pathways and processes related to cancer. Prediction of ceRNA mechanism in BC shows that LINC00963/hsa-mir-130a-3p/HSPA8 axis might have function in tumorigenesis of BC, and it is useful to investigate this axis in future experimental works on BC and its subtypes. Further studies are needed to discover the molecular mechanisms of mentioned lncRNAs in carcinogenesis of BC, especially luminal subtypes.

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Author contributions M.A. performed experimental study and data analysis, bioinformatic analysis, literature review and wrote the manuscript. S.M. contributed to experimental study and data analysis and edited the manuscript. J.T.B. designed the research strategy, performed literature review, edited the manuscript, and reviewed the manuscript. M.M.N. performed statistical analysis and edited the manuscript. K.M. designed the research strategy, performed literature review, edited the manuscript and reviewed the manuscript. A.S. supervised the whole project and designed the research strategy, performed literature review, edited the manuscript and reviewed the manuscript. All the authors read and approved the final manuscript.

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Data availability The datasets supporting the conclusions of this article are available in: [GEPIA2 web server] at (http://www.gepia.cancer-pku.cn); [GENEVESTIGATOR software] at (https://genevestigator.com); [DAVID database] at (https://david.ncifcrf.gov); [REVIGO database] at (http://revigo.irb.hr); [Cytoscape software] at (http://www.cytoscape.org); [STRING database] at (https://string-db.org); [lncATLAS] at (https://lncatlas.crg.eu); [starBase database] at (http://starbase.sysu.edu.cn); [miRNet tool] at (http://www.mirnet.ca); [Kaplan–Meier plotter database] at (http://kmplot.com/analysis). Citation of all data is provided in the references list. The datasets supporting the conclusions of this article are also included within the article and its additional files.

Declarations

Conflict of interest The authors declare that they have no conflicts of interest.

Ethical approval The present study was approved by the Ethics Committee of Tehran University of Medical Sciences (TUMS) and written informed consent was obtained from all the participants (Code of Ethics: IR.TUMS.MEDICINE.REC.1398.659).

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