The Possible Roles of Long Non-Coding RNAs FOXD2-AS1 and LINC00968 in Breast Cancer: Case- Control Study and in Silico Analysis

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Abstract

Background: Breast Cancer (BC) is the most common cancer in women worldwide. Long non-coding RNAs (lncRNAs) are regulatory non-coding transcripts and longer than 200 nucleotides. lncRNAs can affect many biological and pathological processes and dysregulation of them is related and studied in many human diseases like, cancers. We performed this study to evaluate the probable functions of lncRNAs FOXD2-AS1 and LINC00968 in breast cancer.

Methods: The tumor tissue and adjacent non-tumor tissue specimens of luminal A and B breast cancer (the most frequent subtypes of breast cancer) were used to analyze the expression of these two lncRNAs, using the qRT-PCR technique. Furthermore, two luminal A breast cancer cell lines, MCF7 and T47D, were used to evaluate the expression of FOXD2-AS1 and LINC00968 compared with control breast cell line, MCF10A. Because of the luminal subtypes are the most frequent subtypes of breast cancer and also the most common subtypes among breast cancer patients, the present study was generally focused on the luminal and breast cancer. Also, some *in silico* analyses were done according to some databases and software for better understanding about the potential functions of mentioned lncRNAs in breast cancer.

Results: Our data indicated the significant upregulation and downregulation of FOXD2-AS1 and LINC00968, respectively, in tumor tissues and breast cancer cell lines (MCF7, T47D). The bioinformatic analyses confirmed the experimental results. FOXD2-AS1 expression was positively associated with p53 protein and LINC00968 expression was negatively associated with tumor stage and lymph node metastasis. According to our findings, LINC00968 might be function as a tumor suppressor gene in breast cancer and this lncRNA might function in some cellular signaling pathways, like PI3K/Akt and Ras signaling pathways based on the co-expressed genes, but more investigations are required.

Conclusions: The two mentioned lncRNAs might play roles in breast cancer pathogenesis but more experimental studies are needed to explore the mechanisms of the functions of these two lncRNAs in breast cancer.

1. Introduction

Breast Cancer (BC) is the most common cancer in women worldwide and the leading cause of cancer death in female patients (1). The incidence of breast cancer is increasing in many developing countries (2). North America, Australia, New Zealand, Northern and Western Europe have the highest incidence of breast cancer (1). Breast cancer is the most frequent malignancy in Iranian women with an average age of 46-49 years old (3). The etiology of breast cancer is multifactorial, and some genetic and environmental factors play key roles in breast cancer risk (1). Breast cancer is a heterogeneous disease and is classified into five intrinsic/molecular subtypes, according to the expression of ER (Estrogen Receptor), PR (Progesterone receptor), and HER2 (ERBB2) genes including, luminal A, luminal B, HER2 enriched, triple-negative (basal-like), and normal-like, that display different biological behavior and progression. Luminal A is the most frequent molecular subtype, which expresses ER and PR but not HER2 (1). Luminal A and B constitute approximately 70% of all breast cancers (2).

Recent advances in sequencing technologies revealed that less than 2% of the human genome is encoded into proteins and approximately 90% of the human genome is transcribed actively (4). Most of the human RNA transcripts are noncoding RNAs ($\geq 80\%$) (4, 5), which play important functions in the different biological processes (4). Long noncoding RNAs (lncRNAs) are noncoding regulatory RNAs and have 200nt or more in length (4-6). About 4-9 % of the mammalian genome is transcribed into lncRNAs, which is higher than protein-coding mRNAs, but they have lower expression levels (5). Long noncoding RNAs have diverse roles in the regulation of tumor suppressor genes and oncogenes through epigenetic, transcriptional, post-transcriptional and translational mechanisms, and the regulation of some signaling pathways in cancers (7). Noncoding RNAs, like some long noncoding RNAs show differential expression between tumor tissues and normal tissues (6). lncRNAs can be categorized into tumor suppressor genes and oncogenes according to their functions and the expression patterns in the tumor tissues (8). lncRNAs can interact with DNA, RNAs and protein macromolecules in the cells and can lead to the cancer phenotypes (9). Some lncRNAs are expressed differentially in the molecular subtypes of breast cancers (6).

FOXD2 Adjacent Opposite Strand RNA 1 (FOXD2-AS1) is located on chromosome 1p33 and has one exon. FOXD2-AS1 was upregulated and increased the proliferation and progression in bladder cancer, esophageal squamous cell carcinoma, hepatocellular carcinoma, non-small cell lung cancer (NSCLC), gastric cancer, papillary thyroid cancer, nasopharyngeal carcinoma, and glioma, through several mechanisms (10-16).

Long Intergenic Non-Protein Coding RNA 968 (LINC00968) is located on chromosome 8q12.1 and includes 3 exons. Studies indicated that this lncRNA acts as an oncogene in osteosarcoma (17). Also, this gene was upregulated in lung squamous cell carcinoma (LUSC) and played roles in tumorigenesis of this carcinoma (18).

Exploring diverse functions of lncRNAs in cancers like, breast cancer may lead to the new ideas about novel biomarkers and targeted therapies and can be useful in early detection. In this study, we evaluated the expression levels of FOXD2-AS1 and LINC00968 lncRNAs in luminal A and B breast cancer tissues and cell lines, compared with adjacent non-tumor tissues and non-malignant breast cell line, respectively, using the qRT-PCR technique. Because of the luminal subtypes are the most frequent subtypes of breast cancer and also the most common subtypes among breast cancer patients, the present study was generally focused on the luminal and breast cancer. In addition to the experimental study, some bioinformatic analyses were performed to decipher the possible roles of these two novel lncRNAs in breast cancer. We found that these two lncRNAs were dysregulated in luminal A and B breast cancer and combination with in silico analysis, revealed that these two lncRNAs might be contributed to the tumorigenesis of breast cancer.

2. Materials And Methods

2.1 Screening for differentially expressed lncRNAs in breast cancer

We used Lnc2Cancer 3.0 database (<http://www.bio-bigdata.com> (19)) to define a list of novel lncRNAs associated with all cancers. Also, a TCGA invasive breast carcinoma (BRCA) RNA sequencing dataset (dbGaP Study Accession: phs000178.v11.p8) (Additional file 1) obtained from the GDC data portal (<https://portal.gdc.cancer.gov> (20)) was analyzed using DESeq2 package in R statistical software. This

analysis was performed to determine differentially expressed lncRNAs (DElncRNAs) between fifty pairs of the tumor and normal breast tissues across mentioned RNA seq dataset. The thresholds of selection for DElncRNAs obtained from Lnc2Cancer and GDC databases were set at adj p-value < 0.05 and $|\log_2FC| > 1$. Subsequently, we selected DElncRNAs that there is little or no previous research on them in breast cancer according to the PubMed search.

2.2 Experimental study

2.2.1. Patients and tissue samples

Seventy-one pairs of luminal A and luminal B breast tumor tissues and adjacent non-tumor tissues were obtained from Breast cancer Bio-bank (BCRC-BB) (Tehran, Iran) (21). Most of the tumor tissues (86%) were luminal A subtype. This study was approved by 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. All seventy-one tumor tissues and adjacent non-tumor tissues were immediately snap-frozen in liquid nitrogen and stored at -80°C.

2.2.2 Cell culture

The luminal A breast cancer cell lines, named MCF7 (ATCC® HTB-22™) and T47D (ATCC® HTB-133™) 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% CO₂ and 95% humidity. The non-tumorigenic epithelial breast cell line, named MCF10A (ATCC® CRL-10317™) 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.

2.2.3 RNA isolation and quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was extracted from tumor and adjacent non-tumor tissue samples and also cell lines, using RiboEx™ reagent (GeneAll). The extracted RNAs were treated with DNase I (EN0521, Thermo Fisher scientific, United States). Then, one µg RNA was reverse transcribed into cDNA by 5X All-In-One RT MasterMix kit (Applied Biological Materials). Real time PCR was performed in duplicate, using RealQ Plus 2x master mix (AMPLIQON) in Roche LightCycler® 96 instrument. The β2M housekeeping gene was used as normalizer. The primer sequences of FOXD2-AS1 were 5'-CTGTTCTCGGCTCTGGGAAAG-3' (forward) and 5'-GTGCAATCGTTCCGCTGTG-3' (reverse) and the primer sequences of LINC00968 were 5'-CCAGACTCCTCAGCCTGAAAT-3' (forward) and 5'-GTCCCAAACGCAGACCATTTT-3' (reverse). The relative expression was calculated using $2^{-\Delta\Delta CT}$ method.

2.3 In silico analysis

2.3.1 Gene expression analysis in normal breast tissue

Some databases and software were used to obtain FOXD2-AS1 and LINC00968 expression in the normal breast tissue. The gene expression atlas database (<https://www.ebi.ac.uk/gxa/home> (22)) provided the

representation of FOXD2-AS1 and LINC00968 expression levels across breast tissues using RNA-seq and microarray data. Also, GENEVESTIGATOR software (<http://www.genevestigator.com> (23)) provided visualization of FOXD2-AS1 and LINC00968 expression in normal breast tissues based on Affymetrix Human Genome U133 Plus 2.0 Array platform.

2.3.2 Gene expression analysis in breast cancer

The expression levels of FOXD2-AS1 and LINC00968 in breast cancer tissues and samples were obtained from GENEVESTIGATOR software based on Affymetrix Human Genome U133 Plus 2.0 Array platform. We obtained FOXD2-AS1 and LINC00968 expression levels in invasive breast carcinoma dataset using the GEPIA2 web server (<http://www.GEPIA2.cancer-pku.cn> (24)).

2.3.3 Differential analysis

TANRIC (The Atlas of ncRNA in Cancer) database (<https://www.tanric.org> (25)) characterized the expression levels of FOXD2-AS1 across ER⁺/PR⁺ groups of TCGA invasive breast carcinoma (BRCA). In addition, the expression of FOXD2-AS1 and LINC00968 in different molecular subtypes of breast cancer were retrieved from the GEPIA2 web server.

2.3.4 Co-expression analysis

We used GENEVESTIGATOR software (Affymetrix Human Genome U133 Plus 2.0 Array platform) and GEPIA2 web server to obtain co-expressed genes ($R > 0.4$, $p\text{-value} < 0.05$) with FOXD2-AS1 and LINC00968 across breast cancer datasets. Pearson correlation analysis of these co-expressed genes has been computed by GENEVESTIGATOR and GEPIA2 web server using a standard method.

2.3.5 Mutations and copy number analysis

To identify the copy number alterations of FOXD2-AS1 and LINC00968 in invasive breast adenocarcinoma, the TCGA copy number portal (<http://www.broadinstitute.org> (26)) was utilized to represent GISTIC results. Also, copy number variations (CNVs) of FOXD2-AS1 and LINC00968 among nine invasive breast carcinoma studies (4618 samples), including TCGA and non-TCGA studies with no overlapping samples obtained from cBioPortal (<https://www.cbioportal.org> (27, 28)). The International Cancer Genome Consortium (ICGC) data portal (<https://dcc.icgc.org> (29)) provided the number of FOXD2-AS1 and LINC00968 mutations in three breast cancer projects.

2.3.6 Functional annotation analysis

Gene ontology (GO) term enrichment analysis was performed using DAVID version 6.8 (<https://david.ncifcrf.gov> (30, 31)). This web server was used to provide three GO term categories based on a large list of FOXD2-AS1 and LINC00968 co-expressed genes, including biological processes, cellular components and molecular functions. Also, GO terms obtained from DAVID were applied to the REVIGO (<http://revigo.irb.hr> (32)). REVIGO web server can take long lists of Gene Ontology terms and summarizes them by removing redundant GO terms. The files retrieved from REVIGO were imported into the Cytoscape version 3.8.0 to increase their visualization. BiNGO was used to determine which gene ontology terms were

significantly overrepresented in a set of genes (33). Besides, the protein-protein interaction (PPI) networks of FOXD2-AS1 and LINC00968 co-expressed genes were obtained by the STRING database (<https://string-db.org> (34)) and the hub genes were determined using cytoHubba application in the Cytoscape software (35). Also, Enrichr (<http://amp.pharm.mssm.edu/Enrichr> (36, 37)) was used to perform the pathway enrichment analysis of genes co-expressed with FOXD2-AS1 and LINC00968, using pathway databases such as KEGG, Reactome, WikiPathways.

2.4 Statistical analysis

The qPCR data analysis was performed using IBM SPSS version 24 software (IBM Co., Armonk, NY, USA). Data were expressed as mean $\pm$ standard deviation. Paired samples t-test was used to examine the significant differences between gene expression in tumoral tissues and adjacent non-tumor tissues. The association between gene expression and clinicopathological data was performed by χ^2 test and independent t-test. The Pearson correlation coefficient >0.4 and p-value <0.05 were considered as significant.

3. Results

3.1 FOXD2-AS1 and LINC00968 as differentially expressed lncRNAs in breast cancer

A list of novel lncRNAs associated with cancers were obtained from Lnc2Cancer database. Also, approximately 913 different DElncRNAs in fifty paired tumor and normal tissue samples of TCGA BRCA RNA-seq dataset achieved from the GDC data portal were analyzed (Additional file 2). Based on the analysis of RNA-seq data and the results of literature review, FOXD2-AS1 ($\log_2FC = 1.04$, adj.P-value = $4.88079E-07$) and LINC00968 ($\log_2FC = -3.3$, adj.P-value = $9.85436E-30$) were chosen for more investigations in the present study.

3.2. The expression levels of FOXD2-AS1 and LINC00968 in luminal breast cancer tissues and MCF7 and T47D cell lines

The quantitative real time PCR results indicated that the significant upregulation of FOXD2-AS1 (p-value = 0.002) and the highly significant downregulation of LINC00968 (p-value <0.001) were observed in luminal breast cancer tissues compared with adjacent non-tumor tissues (Fig. 1a, b). Also, the qRT-PCR results in T47D and MCF7 cell lines revealed that FOXD2-AS1 (p-value < 0.05) was highly expressed in these breast cancer cell lines compared with MCF10A control cell line (Fig. 1c, d). Furthermore, LINC00968 expression (p-value <0.001) was remarkably lower in breast cancer cell lines than MCF10A. Normal distributions of ΔCt values of tumor and adjacent non-tumor tissue samples for FOXD2-AS1 and LINC00968 were approved using the Kolmogorov-Smirnov Test.

3.3 The association of FOXD2-AS1 and LINC00968 expression with clinicopathological data

The clinicopathological characteristics of FOXD2-AS1 and LINC00968 were shown in Table 1. According to the median expression levels of FOXD2-AS1 and LINC00968 in luminal A and B breast cancer tissues, patients were classified into high and low expression groups.

Table 1. The association of FOXD2-AS1 and LINC00968 expression with clinicopathological characteristics in breast cancer patients HER2= human epidermal growth factor receptor 2. *Analyzed by independent t-test

Clinicopathological characteristic		Number of cases	LINC00968 expression		p-value (χ^2 test)	Number of cases	FOXD2-AS1 expression		p-value (χ^2 test)
			Low	High			Low	High	
Group	Luminal A	61	32	29	0.188	61	29	32	0.465
	Luminal B	10	3	7		10	6	4	
Estrogen receptor	Negative	0	0	0	-	0	0	0	-
	Positive	71	35	36		71	35	36	
Progesterone receptor	Negative	7	4	3	0.662	8	4	4	0.966
	Positive	64	31	33		63	31	32	
HER2	Negative	66	34	32	0.174	66	32	34	0.620
	Positive	5	1	4		5	3	2	
Tumor size	<2cm	18	11	7	0.458	20	10	10	0.861
	2-5cm	36	16	20		35	18	17	
	>5cm	14	8	6		14	6	8	
Lymph node metastasis	No	16	4	12	0.022	17	11	6	0.123
	Yes	52	30	22		51	22	29	
Tumor stage	I	5	3	2	0.021	4	2	2	0.498
	II	35	11	24		38	19	19	
	III	26	17	9		23	8	15	
Grade	1	8	5	3	0.555	9	3	6	0.500
	2	50	25	25		52	26	26	
	3	13	5	8		10	6	4	
P53	Negative	8	5	3	0.835	8	7	1	0.040
	Positive	24	14	10		24	11	13	
		Number of cases	Mean SD	MeanSD	p-value	Number of cases	Mean SD	Mean SD	p-value
Ki67*		51	12.4	15.8	0.056	51	14.8	12.7	0.249
			6.6	5.7			6.7	6	
Age at diagnose*		67	46.4	49	0.408	67	48.5	47.4	0.724
			13.3	12.2			12.6	12.2	

Low expression of LINC00968 in luminal A and B was significantly associated with advanced tumor stage ($P=0.021$) and lymph node metastasis ($P=0.022$). The expression level of LINC00968 was lowest in stage III (Fig. 1e). High expression of FOXD2-AS1 in luminal A and B was positively associated with p53 protein ($P=0.04$).

3.4 FOXD2-AS1 and LINC00968 expression levels in normal breast tissue and breast cancer according to the bioinformatic analyses

According to the Gene Expression Atlas, low baseline expression of FOXD2-AS1 and medium baseline expression of LINC00968 were shown in breast tissue (Additional file 3). The expression levels of FOXD2-AS1 and LINC00968 in breast tissue (mammary gland) are medium and low to medium levels, respectively, obtained from the GENEVESTIGATOR software (Additional file 4). The expression of FOXD2-AS1 and LINC00968 across 35 breast cancer categories, using GENEVESTIGATOR software indicated that FOXD2-AS1 is expressed at medium levels in most categories, while LINC00968 is expressed at low levels in most categories (Additional file 5). Also, analysis of 3933 breast cancer samples with GENEVESTIGATOR software indicated that FOXD2-AS1 express at medium levels, but LINC00968 is expressed at low levels in most samples (Additional file 6, 7). Combination of these results showed that the expression levels of FOXD2-AS1 and LINC00968 are different between tumor and normal categories. The analysis of the gene expression in breast cancer, using the GEPIA2 web server revealed that FOXD2-AS1 is upregulated and LINC00968 is downregulated in tumor tissues than that of normal tissues across BRCA (Invasive Breast Carcinoma) dataset (Fig. 2a, b).

3.5 The expression levels of FOXD2-AS1 and LINC00968 according to the ER and PR status and the molecular subtypes of breast cancer

According to the TANRIC database, FOXD2-AS1 expression levels were higher in the ER⁻ and PR⁻ groups of breast cancers compared to the ER⁺ and PR⁺ groups (ER-status p-value=0.007989) (PR-status p-value=0.015833) as shown in figure 3a and 3b, respectively. Furthermore, the expression of FOXD2-AS1 is highest in the luminal B and is lowest in the HER2 subtype based on the GEPIA2 web server (Fig. 3c) that is concordant to the results of TANRIC database. Also, the GEPIA2 web server (similar to TANRIC database) showed that the LINC00968 expression is lowest in luminal B subtype (Fig.3d).

3.6 The co-expressed genes with FOXD2-AS1 and LINC00968 lncRNAs

The co-expression analysis of FOXD2-AS1 and LINC00968, using the GENEVESTIGATOR software and the GEPIA2 web server, indicated that these lncRNAs are significantly co-expressed with 404 and 420 genes ($R>0.4$) according to the breast cancer datasets, respectively (Additional file 8). The expression analysis of FOXD2-AS1 and LINC00968 co-expressed genes, using the GEPIA2 web server indicated concordant results with the dysregulation analysis of FOXD2-AS1 and LINC00968 across BRCA cancer dataset (Fig. 4a, b).

3.7 Mutations and copy number variations of FOXD2-AS1 and LINC00968 in breast cancer

Results obtained from analysis of somatic copy number alterations (SCNAs) of FOXD2-AS1, using TCGA copy number portal (analysis version: 2015-06-01 stddata__2015_04_02 regular peel-off) indicated that FOXD2-AS1 is not significantly focally amplified and deleted in invasive breast carcinoma. LINC00968 is significantly focally amplified in invasive breast carcinoma, but is not deleted in this cancer. Based on the cBioPortal database, copy number variations (CNVs) of FOXD2-AS1 and LINC00968 among nine invasive breast carcinoma studies exist in four studies including 4046 samples, respectively. CNVs in FOXD2-AS1 are amplifications in these four studies (Fig. 5a). Also, LINC00968 is mostly amplified in these four studies and deletion exists only in the MBC project (Fig. 5b).

According to the ICGC data portal, there are 18 substitution mutations in FOXD2-AS1 across three breast cancer projects (BRCA-EU, BRCA-FR, BRCA-UK) and 206 mutations in LINC00968 across mentioned three breast cancer projects (Additional file 9). About 193 of 206 mutations occur in luminal subtype of breast cancer (BRCA-EU project) (Fig. 5c). Most of the LINC00968 mutations in breast cancer are substitutions. Most substitution mutations in FOXD2-AS1 occur in the upstream, but the number of mutations is low across thousands of samples in breast cancer projects. Most mutations of LINC00968 take place in the intron site (Fig. 5d).

3.8 Functional roles of FOXD2-AS1 and LINC00968 in breast cancer

A list of GO terms for three categories, including biological process, cellular component, and molecular function was retrieved from DAVID database. The top 10 significant GO terms ($p\text{-value} < 0.05$) for biological processes, molecular functions and cellular components are based on FOXD2-AS1 (Additional file 10) and LINC00968 (Additional file 11) co-expressed genes. FOXD2-AS1 co-expressed genes are mostly involved in some biological processes like, chemical synaptic transmission, trans-synaptic and anterograde trans-synaptic and synaptic signaling, and ion transmembrane transport. Also, some GO cellular component terms of FOXD2-AS1 co-expressed genes were included in synapse part, transmembrane transporter complex, and integral component of plasma membrane. Additionally, FOXD2-AS1 co-expressed genes are mostly enriched in some molecular functions like, cation and ion channel activity, and substrate-specific channel activity. Furthermore, LINC00968 co-expressed genes are involved in some biological processes like, nervous system development, response to external stimulus, and cell surface receptor signaling pathway. Also, some GO cellular component terms of LINC00968 co-expressed genes were included in neuron projection, intrinsic and integral component of plasma membrane. In addition, LINC00968 co-expressed genes are mostly enriched in some molecular functions like, receptor binding, substrate-specific channel activity, and passive transmembrane transporter activity. Summarization of Gene Ontology terms and removing redundant GO terms using REVIGO indicated GO terms similar to the GO term enrichment analysis retrieved from DAVID based on the co-expressed genes with FOXD2-AS1 and LINC00968 (Fig. 6a-f).

The overrepresentation of gene ontology (GO) terms related to FOXD2-AS1 and LINC00968 using BINGO application (a Cytoscape app) indicated results similar to the DAVID and REVIGO. Except similarities of some gene ontology terms with DAVID and REVIGO, developmental process, signaling, transporter activity, and several GO terms were significantly associated with FOXD2-AS1, as illustrated in Additional file 12, 13. Also, some GO terms including, biological regulation, developmental process, multicellular organismal process, and

other GO terms that were not similar to the terms obtained from DAVID and REVIGO, were significantly associated with LINC00968 and illustrated in Additional files 14, 15.

Also, the results retrieved from the STRING database revealed that 103 of 404 co-expressed genes with FOXD2-AS1 have strong interaction (interaction score > 0.4) with each other. This network indicates three hub genes with highest degree including NPTX1, POMC, DRD4 obtained from the cytoHubba application in the Cytoscape software (Fig. 7a). The protein-protein interaction (PPI) network of the LINC00968 co-expressed genes indicated that 240 of 420 co-expressed genes have interaction score > 0.4 with each other (Fig. 7b). The top seven hub genes with highest degree are included in BDNF, SNAP25, GNG2, GNG11, HTR2A, IGF1, TAC1 retrieved from the cytoHubba application.

The results of the pathway enrichment analysis (KEGG, WikiPathways, Reactome) of the co-expressed genes using Enrichr indicated the potential mechanisms of the roles of FOXD2-AS1 and LINC00968 (Fig. 8a-f). FOXD2-AS1 is involved in the activation of matrix metalloproteinases and cell differentiation. Also, LINC00968 might have a role in some cellular signaling pathways, such as PI3K/Akt and Ras signaling pathways, and etc.

4. Discussion

Extensive studies reported that most of the lncRNAs are abnormally expressed in tumor tissues, and each lncRNA exerts its function through diverse mechanisms in different cancers. According to the previous studies, FOXD2-AS1 was upregulated in some cancers like, bladder cancer, gastric cancer, non-small cell lung cancer, and esophageal squamous cell carcinoma, while LINC00968 was upregulated in osteosarcoma. There are little data about the expression levels and the roles of FOXD2-AS1 and LINC00968 in breast cancer. Thus, the combination of the gene expression experimental and bioinformatics analyses can be useful for deciphering a part of the probable roles of FOXD2-AS1 and LINC00968 in breast cancer.

In the present study, FOXD2-AS1 is significantly up-regulated in luminal A and B breast cancer tissues and T47D and MCF7 cell lines compared with adjacent non-tumor tissues and MCF10A control cell line, but LINC00968 is significantly downregulated in the breast cancer tissues and cell lines. As mentioned above, FOXD2-AS1 and LINC00968 were upregulated in other cancers, and upregulation of them contributed to the progression and tumorigenesis of these cancers. *In silico* analysis indicate that FOXD2-AS1 expression levels are low in almost all normal breast tissues and mostly high in breast cancer tissues and samples. Additionally, LINC00968 expression levels are medium in almost all normal breast tissues and low in most breast cancer tissues and samples. These two mentioned lncRNAs are thought to have similar expression pattern with co-expressed genes based on GEPIA2 web server. Thus, these two lncRNAs might be involved in the breast cancer carcinogenesis.

Studies on human tissues indicated that the expression of lncRNAs are variable among human tissues from different persons, and the inter-individual expression variations of lncRNAs are more than mRNAs (38). Upregulation of FOXD2-AS1 and downregulation of LINC00968 in a great fraction of tumor tissues (62% and 79% of tumor tissues, respectively) may be due to the inter-individual expression variability of these two lncRNAs in breast cancer tissues.

The expression levels of FOXD2-AS1 in the tumor samples are positively associated with p53 protein and low expression levels of LINC00968 in tumor samples are significantly associated with advanced tumor stage and lymph node metastasis. TP53 mutations are often associated with the production of a stable protein that is detectable by immunohistochemistry. Accumulation of the p53 protein is associated with the malignant disease, and DNA damage (39) and the overexpression of the p53 protein is a marker of poor prognosis in breast cancer. Also, it is considered that LINC00968 may act as a tumor suppressor gene in the luminal breast cancer based on tissue-specific manner and dysregulation of this lncRNA may play role in the progression of breast cancer.

Studies demonstrated that dysregulation of some lncRNAs can lead to the resistance of cancer cells to some anti-cancer drugs like tamoxifen (40). Tamoxifen is an antagonist of estrogen and the most widely used adjuvant endocrine therapy in the estrogen receptor-positive breast cancer patients (41). Evaluation of the probable roles of FOXD2-AS1 and LINC00968 in acquired tamoxifen-resistant breast cancer cell lines can be done in the future studies to investigate the potential roles of mentioned lncRNAs in tamoxifen resistance breast cancer.

The data retrieved from the ICGC data portal report that a few mutations occur in FOXD2-AS1 across breast cancer projects and most of them take place in upstream region. The higher mutations occur in LINC00968 than FOXD2-AS1 across three breast cancer projects. Most of the LINC00968 mutations are substitution type and mostly take place in intronic regions. The somatic mutations directly or indirectly change gene expression (42) and also lncRNA expression profiles in cancers. Some somatic mutations located on lncRNA transcription factor binding sites which affect the lncRNA expression in cancers (42). Somatic mutations can affect RNA secondary structure and the regulation of gene expression (43). Somatic mutations in introns can disrupt splice sites or intronic splicing regulatory sequences, activate cryptic splice sites, and can change transcriptional enhancer or silencer binding sequence. Thus, the intronic mutations in LINC00968 may affect transcription level and RNA splicing and may lead to the carcinogenesis of breast cancer. More investigations of FOXD2-AS1 and LINC00968 mutations are required to provide novel insights into the effects of mutations on the functions and the transcription levels of these two molecules in cancers like breast cancer. The data obtained from the cBioPortal database illustrate that FOXD2-AS1 and LINC00968 are mostly amplified in breast cancer studies. On the other hand, the analysis of SCNAs in FOXD2-AS1 and LINC00968 show that FOXD2-AS1 is not significantly focally amplified or deleted, but LINC00968 is significantly focally amplified in invasive breast carcinoma. Amplification is a genetic mechanism of overexpression that is a well-accepted concept in cancer genetics (44). Amplification of FOXD2-AS1 might be played a role in the breast cancer tumorigenesis, but it is thought that amplification might not relate to the low expression of LINC00968 in breast cancer.

According to the “guilt by association” principle, which states one gene might show the same regulatory mechanisms and roles with its co-expressed genes in the related process and function (45). Thus, considering the guilt by association principle and according to the gene ontology (GO) terms enrichment analyses, FOXD2-AS1 might be involved in some biological processes such as chemical synaptic transmission, trans-synaptic and anterograde trans-synaptic and synaptic signaling, and ion transmembrane transport that are considered as mechanisms related to cancer process and breast to brain metastasis.

Furthermore, nerves as a part of tumor microenvironment constitute a complex network of interaction that promote tumor progression (46). Also, the analysis indicated that FOXD2-AS1 might be involved in cation and ion channel activity, and substrate-specific channel activity. The dysregulation of ion channels activity is involved in the carcinogenesis processes of some cancers like, breast cancer. Cation channels like, calcium, potassium and sodium channels might play a key role in sustaining the proliferative signaling in luminal breast cancer cells (47). LINC00968 might be involved in some biological processes like, nervous system development, response to external stimulus, and cell surface receptor signaling pathway that all of them are cancer related processes. Also, the functional analyses indicate that LINC00968 is mostly involved in receptor binding, substrate-specific channel activity, and passive transmembrane transporter activity. The dysregulation of different signaling pathways through improper functions of receptors and transducer molecules is important in cancer progression. According to the Enrichr database and breast cancer related co-expressed genes, LINC00968 is involved in some signaling pathways, like neuroactive ligand-receptor interaction pathway, PI3K/Akt signaling pathway, Ras signaling pathway, and some cancer signaling pathways.

According to the STRING database, about 25% of the co-expressed genes with FOXD2-AS1 have medium to high interaction with each other. Among hub genes of FOXD2-AS1 co-expressed genes in the protein-protein interaction network, DRD4 is significantly upregulated in breast cancer tissues compared with normal tissues based on GEPIA2 and UALCAN databases (<http://ualcan.path.uab.edu> (48)) (data not shown). Also, based on STRING database, about 56% of the LINC00968 co-expressed genes have medium to high interaction with each other. In the network of the co-expressed genes with LINC00968 and considering GEPIA2 and UALCAN databases, all of the seven hub genes are significantly downregulated in breast cancer tissues compared with normal breast tissues (data not shown). These results confirm that the LINC00968 co-expressed genes and LINC00968 itself are involved in common biological processes.

The present study has some limitations and different future studies are required to accomplish other aspects of the functions of FOXD2-AS1 and LINC00968 in breast cancer tumorigenesis and other cancers. Also, the diverse mechanisms of actions for these two novel lncRNAs in breast cancer remain to be elucidated to design better therapeutic and diagnostic strategies.

5. Conclusion

Based on the data from previous research and our study, dysregulation of lncRNAs expression is thought to involve in carcinogenesis. Experimental and bioinformatic analyses performed in this study, show that FOXD2-AS1 and LINC00968 might participate in breast cancer carcinogenesis. LINC00968 might function as tumor suppressor gene in breast cancer and might have roles in some cancer signaling pathways. Thus, these two lncRNAs might have impacts on the tumorigenesis of breast cancer that further experimental tests are needed to confirm the validity of our conclusions and clarify the mechanisms of actions of these two novel lncRNAs in breast cancer.

Abbreviations

BRCA, invasive breast carcinoma; lncRNA, long noncoding RNAs; qRT-PCR, real time quantitative reverse transcription- polymerase chain reaction; ER, estrogen receptor; PR, progesterone receptor; HER2, human epidermal growth factor receptor 2; CNV, copy number variation; GISTIC, Genomic Identification of Significant Targets in Cancer; GO, gene ontology.

Declarations

Ethics approval and consent to participate

The present study was approved by 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.

Consent for publication

Written informed consent for publication was obtained from all participants.

Availability of data and materials

The data generated or analyzed during this study, are included in this article and its additional files and also retrieved from mentioned databases and software.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

M.A. performed experimental study and data analysis and designed bioinformatics analysis and performed literature review and wrote the manuscript. S.M.L. contributed to experimental study and data analysis. K.M-A. designed the research strategy and performed literature review and edited the manuscript and reviewed the manuscript. J.T.B. designed the research strategy and performed literature review and edited the manuscript and reviewed the manuscript. M.M.N. performed literature review and performed data analysis and performed statistical analysis and edited the manuscript. A.B. supervised the whole project and designed the research strategy, performed literature review, edited the manuscript and reviewed the manuscript.

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Figures

Figure 1

FOXD2-AS1 and LINC00968 expression in luminal breast cancer. (A) The higher expression of FOXD2-AS1 (p-value<0.01) and (B) the lower expression of LINC00968 (p-value<0.001) in tumor tissues compared with adjacent non-tumor tissues. Upregulation of (C) FOXD2-AS1 (*P < 0.05) in MCF7 and T47D compared with MCF10A. D. Downregulation of LINC00968 (***P<0.001) in MCF7 and T47D compared with MCF10A. E. The significant association between low expression of LINC00968 and advanced tumor stage (stage III).

Figure 2

The expression level of FOXD2-AS1 and LINC00968 across BRCA cancer dataset based on the GEPIA2 web server. The upregulation of FOXD2-AS1 (A) and downregulation of LINC00968 (B) in breast cancer tissues (n=1085) compared with non-cancerous tissues (n=291). The red and blue boxes represent tumor tissues and normal tissues, respectively.

Figure 3

The expression levels of FOXD2-AS1 and LINC00968 in the breast cancer subtypes according to the TANRIC and GEPIA2 databases. The expression levels of FOXD2-AS1 in (A) ER $\pm$, and (B) PR $\pm$ groups based on the TANRIC database. The expression levels of (C) FOXD2-AS1 and (D) LINC00968 across different breast cancer subtypes based on the GEPIA2 web server.

A.

B.

Figure 4

The expression analysis of FOXD2-AS1 and LINC00968 co-expressed genes in breast cancer by GEPIA2 web server. The expression levels of the co-expressed genes with A. FOXD2-AS1 and B. LINC00968 across BRCA (Invasive Breast Carcinoma) cancer dataset.

Figure 5

Mutations and copy number variations of FOXD2-AS1 and LINC00968 in breast cancer using the cBioPortal database. The copy number variations of (A) FOXD2-AS1 and (B) LINC00968 across four invasive breast carcinoma studies. C. The number of LINC00968 mutations across three breast cancer projects. D. The distribution of LINC00968 mutations in different regions of this lncRNA across patients with breast cancer.

Figure 6

The summarization of gene ontology (GO) terms related to FOXD2-AS1 and LINC00968. The summarization of (A) GO biological process terms, (B) GO cellular component terms and (C) GO molecular function terms associated with FOXD2-AS1 retrieved from DAVID. The summarization of (D) GO biological process terms, (E) GO cellular component terms and (F) GO molecular function terms associated with LINC00968 retrieved from DAVID. The summarization was done using the REViGO web server. The visualization of networks performed using the Cytoscape software. Node color indicates p-value and node size indicates log size.

Figure 7

The protein-protein interaction (PPI) networks of FOXD2-AS1 and LINC00968 co-expressed genes according to the STRING database. (A) The PPI network of the FOXD2-AS1 co-expressed genes. (B) The PPI network of LINC00968 co-expressed genes. The thicker edge shows the higher STRING combined score and the stronger node color is indicative of the higher degree.

Figure 8

The pathway enrichment analysis of FOXD2-AS1 and LINC00968 co-expressed genes according to the Enrichr web server. The pathway analysis of FOXD2-AS1 co-expressed genes according to (A) the WikiPathways 2019, (B) the Reactome 2016. The pathway analysis of the LINC00968 co-expressed genes according to (C) the KEGG 2019, (D) the WikiPathways 2019, (E) the Reactome 2016. The length of bars shows the significance of that specific gene-set or term. Also, the brighter the color, the more significant that term is.

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