Clinical Values of two Novel Estrogen Receptor Signaling Targeted lncRNAs in Invasive ductal breast carcinoma

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Abstract

Invasive ductal carcinoma (IDC) is the most frequent type of breast cancer (BC) in women, with a high clinical burden due to its high invasive properties. Despite of new data regarding the molecular heterogeneity of invasive cancers is quickly emerging; far less is known about the molecular patterns among cases of IDC. An expanding body of evidence has demonstrated that dysregulation of long noncoding RNAs (lncRNAs) is involved in heterogeneity feature of the BC. In this study, we analyzed the expression levels of two novel lncRNAs LOC100288637 and RP11-48B3 in 51 IDC tissues in comparison with adjacent non-cancerous tissues (ANCTs). And finally, bioinformatic evaluation has been done.The qPCR results showed that LOC100288637 and RP11-48B3 were significantly overexpressed in tumor tissues compared to normal samples (P= 0.0085 and P= 0.0002, respectively). Also, the two lncRNAs were overexpressed in both MDA-MB-231 and MCF-7 BC cell lines, nevertheless, with a higher expression pattern in MDA-MB-231 than MCF7 cell line. Furthermore, LOC100288637 had an elevated expression level in HER-2 positive tumors compared to HER-2 negative tumors (P= 0.031). Interestingly, the lncRNA RP11-48B3.4 was upregulated in IDC subjects with age at menarche <14 compared to patients with age at menarche ≥14 (P= 0.041). It was observed in another result that lncRNA RP11-48B3.4 is significantly upregulated in tumors with a lower histological grade compared to tumor samples with higher grades (P= 0.047). And finally, using bioinformatic evaluation, we found a predicted interaction between RP11-48B3.4 and mRNA Zinc Finger and BTB Domain Containing 10 (ZBTB10).Altogether, our findings suggest that these lncRNAs with potential oncogenic roles involved in pathogenesis of IDC with clinical significance, and thus, they may serve as novel markers for diagnosis and treatment of IDC.

Introduction

Breast cancer (BC), as the most prevalent cancer in women, accounts for the second leading cause of the death among women worldwide. [1] From the molecular and clinical point of views, the public health concern of BC largely rises from its heterogeneous and complex nature that is characterized by a wide variety of clinical outcomes and multiple pathological subtypes and thus different prognostic statuses and variable responses to therapeutic approaches. [2] Two main invasive variants of the BC are invasive ductal carcinoma (IDC) and invasive lobular carcinoma (ILC) [3]. IDC, also known as infiltrating carcinoma, is the most common histological type of the BC, representing about 80% of all invasive BC diagnoses [4]. Although there are various therapeutic options for this type of BC tumor, its diagnosis and therapeutic management is challenging because it has a high cellular invasion whose development is arisen through a very complex molecular phenomenon.[5]. Thus, the molecular mechanisms behind the pathogenesis of IDC remains to be elucidated and a top biomedical research priority is needed for the identification of its novel prognostic and therapeutic targets.

Long noncoding RNA (lncRNA) transcripts are important class of non-coding RNAs (ncRNAs) with a structure longer than 200 nucleotides in length and a huge number of varieties [6]. LncRNAs play key roles in the expression regulation of their target genes at various levels including, transcriptional, post-transcriptional, and epigenetic levels. Available data revealed that lncRNAs in addition to their role in

multiple physiological processes, they also play critical roles in guiding pathological conditions, such as tumorigenesis through oncogenic and tumor suppressive functions [7, 8]. More importantly, lncRNAs also show great potential as the prognostic, diagnostic, and therapeutic targets for BC [9]. Consequently, current research on BC have focused on the role of lncRNAs in the biopathology of the disease. In this regards, the emerging advances in new generation sequencing and microarray techniques have revealed aberrant expression of several lncRNAs in BC, however, their underlying molecular mechanisms and clinicopathological importance, especially in IDC type, remains to be determined for most cases. [10-12]

A recent study revealed that abnormal expression of two novel lncRNAs LOC100288637 and RP11-48B3 is linked to abnormal regulation of the estrogen receptor (ER) signaling pathway in BC. Dysregulation of ER signaling pathway plays key roles in the development and pathophysiology of the BC. Wu et al, provided evidence that LOC100288637 and RP11-48B3 has maximum accuracies for predicting resistance-free survival and distant metastasis-free survival in BC [13]. thus, expression analysis of these lncRNAs in BC samples is noteworthy. Biologically, the RP11-48B3 encodes an antisense lncRNA with a length of 1080 nucleotides. The lncRNA LOC100288637, located in the region of 15q13, is derived from an important protein coding sequence (isoform) of mRNA ARHGAP11A. Moreover, expression analysis through next generation sequencing and bioinformatic techniques have revealed that he dysregulation of LOC100288637 had a high positive correlation with HER-2 in HER-2-enriched subtype BC[14]. Therefore, such findings highlight the importance of dysregulated lncRNAs LOC100288637 and RP11-48B3 in BC development. However, their roles and relations in IDC remain unclear. The aim of the present study was to focus on expression levels of LOC100288637 and RP11-48B3 in IDC tumor and matched BC cell lines as well as their association with clinicopathological parameters.

Materials And Methods

Study population and breast tissue specimens

A total of 51 invasive ductal carcinoma samples from BC patients were selected. Fresh cancerous and their adjacent noncancerous tissue (ANCT) samples were taken from patients in Faghihie hospital. ANCT was the normal breast tissue diagnosed by the pathologists through H.E. staining. None of the patients had received chemotherapy or radiotherapy before surgery. Clinical and pathological data of patients were collected. The BC tissues were excised and then snap frozen in liquid nitrogen, and stored at - 80°C until RNA experimental analyses. Written informed consent was obtained from each individual, and the local Ethics Committee of Shiraz University of Medical Sciences approved the study protocol.

Estrogen receptor (ER), progesterone receptor (PR), and Her2/neu status of the tumor samples

In this study, the different markers of BC, including ER, PR, Her2/neu were determined according to the patients' histopathological data, which were carried out through immunohistochemistry (IHC) assay. The ER and PR were considered positive if more than 1% of tumor cells revealed positive reaction. For Her2/neu a test result of 3+ was regarded as positive.

BC cancer cell lines

In addition, we used BC cancer cell lines to reveal more details about the link of expression patterns of the lncRNAs LOC100288637 and RP11-48B3 with the BC malignancy such as metastasis hallmark. In this case, human BC cell lines MDA-MB-231 and MCF-7 BC were used. Of note, the MCF-7 is a widely used BC cell line to study estrogen signaling [15, 16]. Besides, the key features of the MDA-MB-231 cell line are highly aggressive, invasive and poorly differentiated triple-negative breast cancer (TNBC) for ER and PR expression as well as HER2 amplification [17]. In the present study, these cell lines were maintained form cell bank of Pasteur Institute of Iran ,and cultured in RPMI-1640 medium (Sigma 42 Aldrich, St. Louis, MO, USA) supplemented with %10 fetal bovine serum (Gibco, Carlsbad, CA, USA), 100 U/ml penicillin, and 100 µg/ml streptomycin. The cells were incubated in %5 CO2/% 95 humidity at 37°C. Expression analyzes of target lncRNAs were then performed on them.

Total RNA extraction and complementary DNA (cDNA) synthesis

Total RNA was extracted from tissues samples as well as BC cell lines using the TRIzol reagent (Life Technologies, Carlsbad, CA). The purity and concentration of the extracted RNA were determined by Thermo Scientific Nano Drop 1000 Spectrophotometer (Thermo Scientific, Germany) and the RNA integrity was confirmed by gel electrophoresis. For removal of the DNA contamination, the total RNA was treated with DNase (Takara Bio Inc, Ostu, Japan) according to the manufacturer's instruction. 1 mg RNA was then used for cDNA synthesis, using random hexamers as primers and Prime Script-RT kit (Takara, Japan).

Quantitative gene expression determining through quantitative real-time PCR (qPCR)

Real-time qPCR was carried out using lncRNA-specific primers and SYBR Premix Ex Taq II kit (Takara, Japan) according to the manufacturer's instruction. QuantStudio™ 3 system ((Applied Biosystems, USA by Thermo Fisher Scientific) was used for amplification. The thermal cycling condition was set as follows: an initial hold at 95°C for 30 s, followed by 40 cycles of 95°C for 05 s and 60°C for 30s. No template controls (NTCs) were included in each run. To verify the reaction efficiency for each primer set, standard curves were prepared using data from serially diluted samples. Melting curve analyses was performed for each primer set. In addition, PCR products were electrophoresed on 2% agarose gel to verify the product sizes. *B2M* gene was used as a normalizer. The relative expression was calculated as fold changes by the comparative Ct (ΔΔCt) method. The sequence of primers was as follows ;(RP11-48B3-forward: CAAGCCCTGATCAACTAGGAATA; RP11-48B3.4-revers: GGAAAGTTGGTTGCTGTGTAAG), (LOC100288637-forward: CTAAGCCCTGCTTCTGGTATG; LOC100288637-revers: GGAGGCAGATCCAGTTCATTAG). *B2M*- forward: AGATGAGTATGCCTGCCGTG, B2M- revers: GCGGCATCTTCAAACCTCCA

Bioinformatic analysis

In current study, we also conducted different bioinformatic analysis, mainly by using data of TCGA, (https://www.cancer.gov/about-nci/organization/ccg/research/structural-genomics/tcga) to get more information about RP11-48B3.4 and LOC100288637. In this regard, we investigated the expression correlation between these two lncRNAs and mRNAs in TCGA-BRCA dataset through using TANRIC webserver (https://bioinformatics.mdanderson.org/public-software/tanric/). Subsequent, we used the possible correlated mRNAs for any possible interactions between these mRNAs and lncRNA using LncRRIsearch webserver (http://bioinfo.life.hust.edu.cn/lncRNASNP#!/lncrna_info?lncrna=NONHSAT127417.2.).

Statistical analysis of the data

The data are presented as mean and standard deviation. qPCR data were analyzed using unpaired t-test and Mann-Whitney tests. The comparison of gene expression among the subgroups was done using t-test or ANOVA. Then, the expression level of lncRNAs was compared between the subgroups through nonparametric tests using Mann-Whitney and Kruskal-Wallis. The correlation assessment between the expression level and variables in our study was performed through the spearman correlation coefficient. All statistical analyses were performed using SPSS version 20.0 software (IBM, Carlsbad, CA, USA). P value <.05 values were considered to be statistically significant.

Results

Demographics, reproductive and clinical data of the patients

The average patient age was 45.7 with a range from 27 to 68 years. The 51 percent of the subjects had a family history for BC and other cancers while 49 percent were negative for this variable. Among the 51 enrolled women, 36 participants were parous and 15 cases were nulliparous. The 37 women had experienced breastfeeding in their life and the rest were negative for breastfeeding experience. Besides, 47 patients were in postmenopausal status and 4 patients were in premenopausal condition. Other demographics and reproductive characteristics of the patients are shown in Table 1. Clinically, all 51 tumor samples from BC patients had invasive ductal breast carcinoma. The most tumors were positive for ER (+45 vs -6) and PR (+34 vs -17) receptors, but negative for HER-2/neu marker (-30 vs +21). The %33.3 of the tumor samples had a size <2 cm, %64.7 with 2-5 cm, and %2 with >5 cm. The tumor size was measured as the largest dimension of the microscopic invasive component in pathologic sections. Data on other tumor features including histological grade and lymph node metastasis (TNM) are indicated in Table 2. The tumor grade and TNM stage of the cells were determined based on the standard of WHO [18].

Expression levels of LOC100288637 and RP11-48B3 in BC tissues and different subgroups of the samples regarding to the clinicopathological, demographic, and reproductive characteristics

In the next step, we investigated the expression profile of these two lncRNAs in 51 BC tissues and their adjacent normal tissues. The qPCR results showed that LOC100288637 (P= 0.0085) was significantly

upregulated in tumor tissues compared to normal samples. Furthermore, RP11-48B3 had similar significant overexpression pattern in tumor tissues versus normal tissues (P= 0.0002). Figure 1 represents the results on relative expression levels of both LOC100288637 and RP11-48B3 in tumor tissues compared to normal samples. In next step, we compared the expression levels of the two lncRNAs in different subgroups of the patients in relation to the various demographic, reproductive, and clinicopathological features of the subjects (Table 3 and Table 4). The findings showed that the lncRNA RP11-48B3.4 is upregulated in patients with age at menarche <14 compared to patients with age at menarche ≥14 (P= 0.041). The expression of this lncRNA was not significantly difference among the different subgroups of the demographic and reproductive variables of the participants. In addition, it was not observed any significant data on difference in expression of the lncRNA LOC100288637 among various levels of the demographic and reproductive characteristics. Clinicopathologically, our analyses indicated that lncRNA RP11-48B3.4 was significantly upregulated in tumors with a lower histological grade (grade 1) compared to tumor samples with higher grades including grade 2 and 3 (P= 0.047).

Regarding the LOC100288637, its expression showed a higher level in HER-2 positive tumors than HER-2 negative tumors (P= 0.031). For other different subgroups of the clinical characteristics, any noteworthy results were not found among them in terms of difference in expression of the two studied lncRNAs (Table 4). In another our statistic evaluations, we determined the correlation of LOC100288637 and RP11-48B3 with clinicopathological features of the BC patients. The Spearman's correlation analysis disclosed that the expression level of RP11-48B3 was negatively correlated with histological grade (r= -282, P= 0.045). The Spearman's analysis did not found any other significant correlation between the expression level of these lncRNAs and the other studied variables (Table 5).

Expression levels of the LOC100288637 and RP11-48B3 in MDA-MB-231 and MCF-7 cell lines

For getting more information about involving the lncRNAs LOC100288637 and RP11-48B3 in the pathogenesis of IDC, especially metastasis, their expression levels were compared in human BC cell lines MDA-MB-231 and MCF-7 BC. The qPCR data showed that the two lncRNAs were overexpressed in both MDA-MB-231 and MCF-7 BC cell lines, nevertheless, both lncRNAs showed a higher expression pattern in MDA-MB-231 than MCF7 cell line (P= 0.0013 and P= 0.0003, respectively) (Figure 2).

Bioinformatic evaluations

Through expression correlation analysis, we found that there are some correlations between RP11-48B3.4 and mRNA expression in TCGA-BRCA, with negative correlation for mRNA MR1 (r: -.409, P: 0) and positive correlations for mRNAs MRPS28 (r: 0.426, P: 0), NSMCE2 (r: 0.444, P: 0), POLR2K (r: 0.436, P: 0), TCEB1 (r: 0.476, $P< 10^{-47}$), UQCRB (r:0.418, P: 0), YWHAZ (r: 0.464, P: 0), ZBTB10 (r: 0.567, P: 0), and ZNF706 (r: 0.471, P: 0) (Figure 3). Regarding interaction between the correlated mRNAs and RP11-48B3.4, we found that only Zinc Finger and BTB Domain Containing 10 (ZBTB10) was predicted to have interaction with this lncRNA (Figure 4). It was not any observable interaction between lncRNA LOC100288637 and evaluated mRNAs.

Discussion

Invasive ductal carcinoma (IDC), as the most frequent form of the BC in women, has a high clinical burden due to its high invasive properties. Although recent investigations have made significant advances in revealing some key molecular mechanisms regarding its pathogenesis, it shows a very heterogeneous and complex etiology with many unknown aspects [19, 20]. Thus, it is extremely expedient to clarify the underlying molecular mechanisms through which IDC develops. LncRNA transcripts are emerging as key players in cancer initiation and pathobiology of BC, with both oncogenic and tumor suppressive roles. In this regard, new experimental studies have revealed some novel molecular mechanisms by which lncRNAs involved in BC malignancy, providing a new avenue of investigation for characterizing the different hallmarks of BC. [21]. Moreover, these small noncoding RNAs not only play an important role in BC development, but also have some links with BC risk factors in breast tissue of healthy women [22, 23]. And interestingly, lncRNAs have been shown to exhibit diagnostic and prognostic biomarker properties as well as therapeutic targets for BC [24].

In the current study, we determined the expression levels of two novel ER signaling pathway-targeted lncRNAs LOC100288637 and RP11-48B3 in clinical samples of BC tumors type IDC. To the best of our knowledge, this is the first study to explore the links of expression signatures of these two lncRNAs to IDC type of BC as well as its clinicopathological characteristics. Our results showed a significant overexpression of these two ER-related lncRNAs in IDC tumor tissues compared to normal breast samples. Emerging studies have reveal that dysregulation of ER expression and its signaling pathway is intensely linked to development and pathophysiology of the BC. Furthermore, provided evidence from different investigations are available for involving some lncRNAs in regulating the ER signaling and their aberrant expressions play key roles in development of BC malignancy, especially the progression and endocrine-resistance of ER positive subtype. The findings of present study are in agreement with the results from previous array-based study that revealed dysregulation of the LOC100288637 and RP11-48B3.4 through regulating signaling pathway in ER $^{+}$ BC patients. They indicated that expression pattern of these two lncRNAs were significantly correlated with endocrine resistance-free survival and distant metastasis-free survival as well as disease free survival of ER+ BC patients. Of note, the most of samples included in current study were ER+ that is consistent with this observation that the majority of the BC tumors are molecularly fallen into estrogen receptor-positive (ER+) subtype. Therefore, these observations highlight the clinical significance of these lncRNAs in ER+ BC subtype via regulating ER signaling pathway.

Furthermore, our study demonstrated a significant overexpression of the lncRNAs LOC100288637 and RP11-48B3.4 in human BC cell lines, including MDA-MB-231 (TNBC for ER and PR expression, as well as HER2 amplification) and MCF-7 (positive for ER, PR expression,). However, the results represented a higher expression of the both lncRNAs in MDA-MB-231 cells than MCF7 cells. Thus, it could be thought that they might have oncogenic roles in BC tumorigenesis through dysregulating the ER signaling pathway and also give invasiveness and metastatic properties to BC tumor cells.

Notably, our analyses further showed a significantly increased level of the LOC100288637 in HER-2 positive tumors compared to HER-2 negative samples. This finding is consistent with the results of previous work by Fan and colleagues that indicated the elevated expression level of LOC100288637 was strongly correlated with Her2/neu positive status in BC through next generation sequencing and bioinformatics. Accordingly, we supposed that this lncRNA may play crucial roles in pathogenesis of IDC via oncogenic functions. However, its exact mechanism need more investigation by future studies. Furthermore, we observed a higher expression of the RP11-48B3 in lower grade tumors in comparison with the higher grade tumors which also correlatively indicated by correlation analysis. Although this may inconsistent with above mentioned observation that this lncRNA had a higher expression in MDA-MB-231 cell line with high aggressive, invasive, and poorly differentiated properties, such a finding may occur due to our relatively small sample size or unknown complex nature of RP11-48B3 function during BC tumorigenesis which requires more investigation to disclose.

Interestingly, another result of the current study was that the lncRNA RP11-48B3.4 had an elevated expression pattern in BC patients with age at menarche <14 in comparison to patients with age at menarche ≥14. It has been reported that lower age at menarche increases the risk of BC through estrogen-related mechanisms [25, 26] however, little is known about its molecular mechanisms behind the risk of BC. Therefore, it can be suggested that lower age at menarche may increase the risk of IDC partly through affecting the expression level of estrogen-linked lncRNA RP11-48B3.4. Although, confirmation of such assumptions require conducting more functional studies.

Lastly, current study also bioinformatically demonstrated some correlations between lncRNA RP11-48B3.4 and expression of several mRNAs in TCGA-BRCA, including negative expression correlation for mRNA MR1 and positive correlations for mRNAs MRPS28, NSMCE2, POLR2K, TCEB1, UQCRB, YWHAZ, ZBTB10, and ZNF706. However, only the mRNA zinc finger and BTB Domain Containing 10 (ZBTB10) was predicted to have interaction with the lncRNA RP11-48B3.4. Regarding the LOC100288637, the results were not detected any evident interaction with given mRNAs. This highlights that the novel lncRNA RP11-48B3.4 may via interacting with some encoding genes by different mechanisms involved in pathogenesis of the IDC. There is evidence that lncRNAs through influencing expression of other coding and non-coding genes play key roles in development of BC [27]. In this way, one suggested mechanism is competing endogenous RNA (ceRNA) function through which lncRNAs via sponging miRNAs regulate expression of mRNAs in regulatory molecular networks whose roles in cancer development, especially in BC, are emerging with clinical significance [28, 29].

In conclusion, the present study showed the elevated expression levels of LOC100288637 and RP11-48B3 lncRNA in IDC breast tumors as well as BC cell lines and with some important significant on its clinical outcome. This suggested them as a putative oncogenic marker in molecular level in IDC. However, it is important to analyze the correlation between their expression as well as progression-free survival time in order to conclude these lncRNAs as prognostic biomarkers. In our study, it was not possible to do such evaluation, since we used samples from newly diagnosed cancers. These findings can also be useful to candidate these two lncRNAs as targets for BC treatment. Future studies are warranted to analysis the

expression of these two lncRNAs in various type of cancers in order to propose them as a candidate of tumor biomarker in combination with biomarker panels.

Declarations

Acknowledgment

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CONFLICTS OF INTEREST

The authors declare that there are no conflict of interests.

DATA AVAILABILITY

The data that support the findings of this study are available from the corresponding author upon reasonable request.

AUTHOR CONTRIBUTIONS

A.D, Y.M, S.I contributed to the conception and design of the research. F.F,S.I, M.D.F and M.R.M performed the experiments. F.F. and S.I, H.D .M.D.F ,Z.A interpreted the results of the experiments, analyzed data, and prepared the figures. S.I, M.R.M ,A.D drafted the manuscript. A.D and H.D, M.B.T ,S.I edited and revised the manuscript. M.B.T and Y.M, Study supervision and technical or material support. All authors read and approved the final manuscript.

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Tables

Table 1. Demographic and reproductive characteristics of the participant subjects.

Variables	Subgroup	Number	Valid percent
Age (year)	<40	15	29.4
	≥40	36	70.6
Family history for cancer	Positive	26	51
	Negative	25	49
Marital status	Married	43	84.3
	Single	8	15.7
Parity status	Paros	36	70.6
	Nulliparous	15	29.4
Age at first full term pregnancy (FFTP) (year)	< 25	38	74.5
	≥ 25	10	19.6
Abortion history	Positive	12	23.5
	Negative	37	72.5
Breastfeeding experiences	Positive	37	72.5
	Negative	14	27.5
Breastfeeding (month)	0-6	20	39.2
	6-24	20	39.2
	≥ 24	11	21.6
Age at menarche (year)	< 14	36	70.6
	≥14	15	29.4
Menstrual cycles	Regular	43	84.3
	Irregular	8	15.7
Menopausal status	Pre	4	7.8
	Post	47	92.2
OCP consumption	Positive	10	19.6
	Negative	41	80.4

Table 2. Pathological data of the evaluated tumor samples from BC patients.

Clinical characteristics	Subgroup	Number	Valid percent
Tumor size (cm)	<2 cm	17	33.3
	2-5 cm	33	64.7
	>5 cm	1	2
Estrogen receptor	Positive	45	88.2
	Negative	6	11.8
Progesterone receptor	Positive	34	66.7
	Negative	17	33.3
HER-2/neu status	Positive	21	41.2
	Negative	30	58.8
Histological grade	1	11	21.6
	2	25	49
	3	15	29.4
Lymph node metastasis	Involved	30	58.8
	Free	21	41.2

Figures

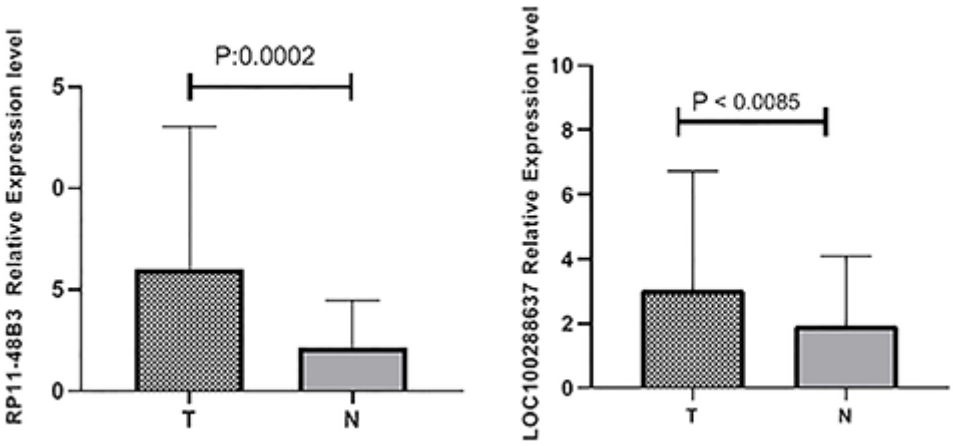

Figure 1

Expression levels of lncRNAs LOC100288637 and RP11-48B3 in IDC tumors and adjacent normal tissues. T and N denote tumor tissue and normal breast samples, respectively.

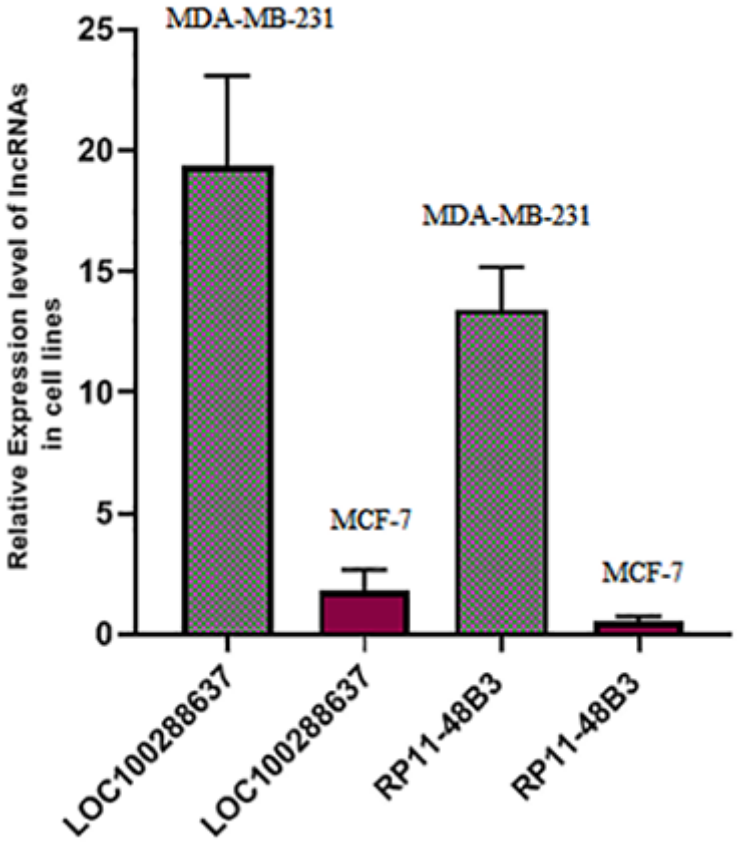

Figure 2

Relative expression of target lncRNAs in MDA-MB-231 and MCF-7 cell lines compared to normal cell lines.

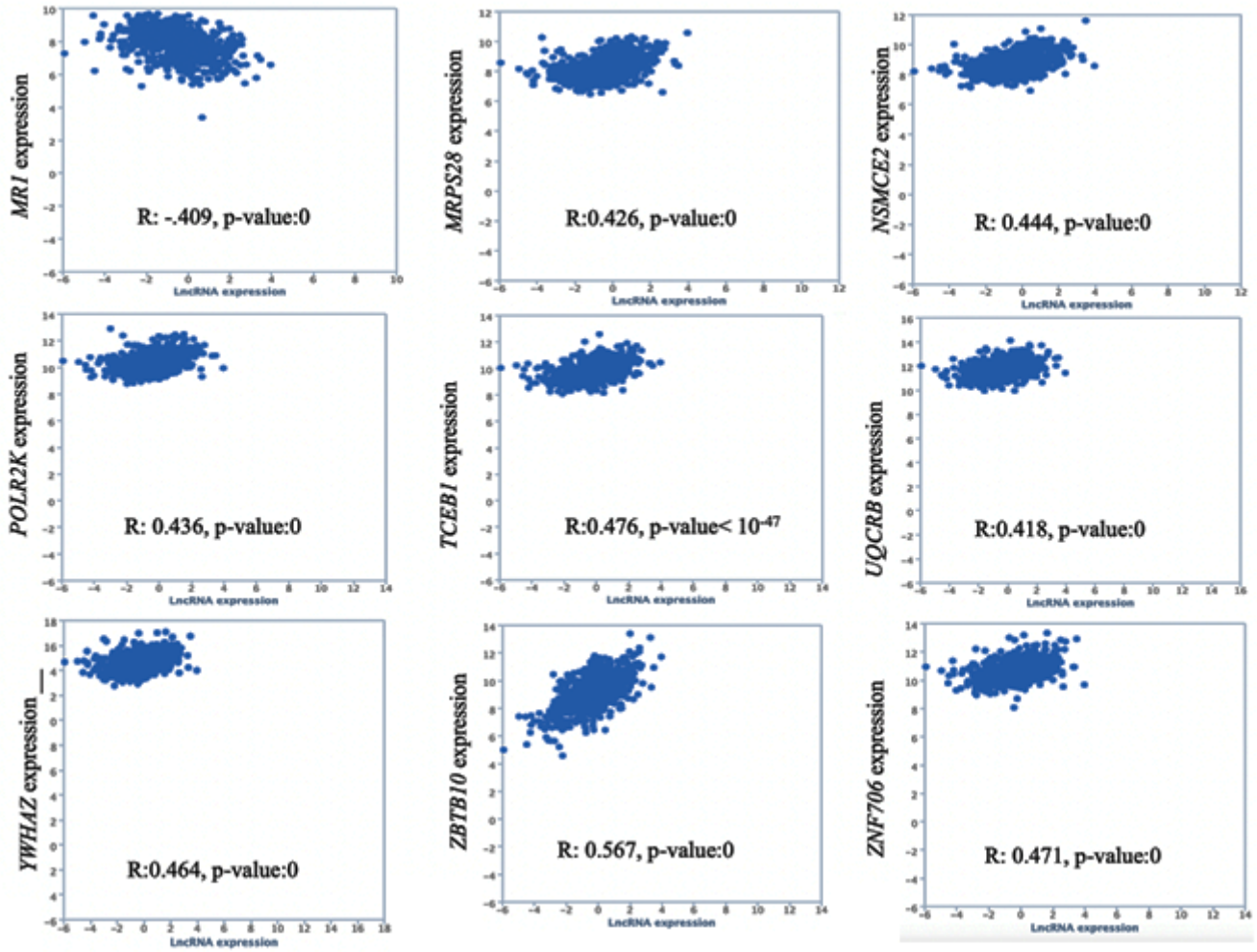

Figure 3

Correlation analysis between RP11-48B3.4 and mRNAs expression in TCGA-BRCA.

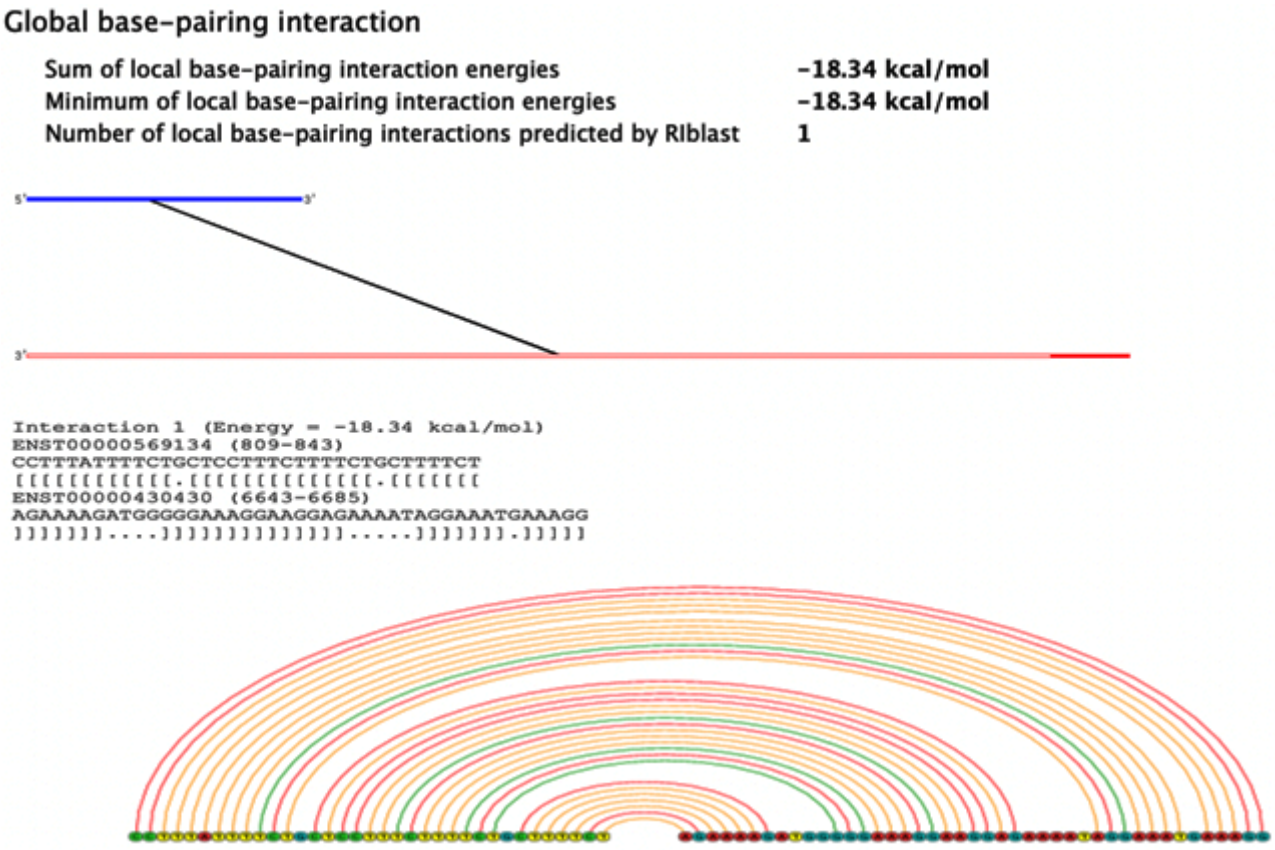

Figure 4

Prediction of possible interaction between RP11-48B3.3 and ZBTB10.