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Research Article

Expression Levels of LINC01296 and LINC00152 in Breast Cancer Tissue: Association with the Use of Oral Contraceptives

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Abstract

Objectives: Breast cancer is among the most commonly diagnosed cancers and one of the main causes of cancer-related deaths in females. Long noncoding RNAs (lncRNA) are vital regulators of both oncogenesis and tumor suppression involved in crucial cancer pathways. Recent studies have demonstrated that LINC01296 and LINC00152 were aberrantly upregulated in different tumor types. In this study, we aimed to assess the expression levels of LINC01296 and LINC00152 in breast cancer and its adjacent tissues.

Methods: Sample tissues were collected from 49 women with breast cancer referred to Shahid Faghihi Hospital in Shiraz for surgery from 2017 to 2018. Total RNA was extracted from fresh tumor and normal breast cancer tissues and expression of lncRNAs was assessed using real-time polymerase chain reaction.

Results: A significant upregulation of LINC01296 and LINC00152 in breast cancer tissues compared with the adjacent normal tissues was observed ($p<0.01$). The Mann–Whitney analysis showed a significant relationship between the upregulation of LINC01296 and the use of oral contraceptives in luminal B breast cancer subjects ($p=0.028$). No significant relationship was found between the expression of LINC01296 and quantitative variables.

Conclusion: This study showed an upregulation of LINC01296 and LINC00152 in breast cancer tissues compared with the adjacent normal tissues.

Keywords: Breast cancer, Long noncoding RNA, LINC00152, LINC01296, Oral contraceptives.

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Cancer is a major burden of disease worldwide and tens of millions of people are diagnosed with cancer each year globally.[1–4] A status report about the global burden of cancer utilizing the data of GLOBOCAN with an emphasis on geographic variation among 20 regions of the world showed 18.1 million new cases of cancer and 9.6 million

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cancer deaths in 2018.[5, 6] Among females, breast cancer is the most commonly diagnosed cancer and is the main cause of cancer-related deaths. A review of public health data demonstrates that the global burden of breast cancer in women, evaluated by occurrence, economic costs, and mortality, is significant and on the rise.[7–10] Risk factors for breast cancer are divided into two different groups. The first group consists of intrinsic factors such as age, race, gender, and genetic makeup that promote the family-related incidence of the disease. All of them are independent parameters and do not undergo modification during an individual's life.[11] The second group includes external factors associated with lifestyle, diet, or long-term medical interventions such as consumption of oral hormonal birth control drugs, and their effect on the tumor progression may be changed to a certain degree.[12–14] Breast cancer is more commonly associated with environmental, reproductive, and lifestyle factors, and less than 10% of breast cancers can be attributed to an inherited genetic mutation.[15, 16]

The exact molecular mechanisms of the effect of these factors and their association with breast cancer have not yet been clearly identified. RNAs have been considered as a mediator between protein and DNA that translate genetic data into a diverse set of biological processes. Although up to 70% of the genome of humans is transcribed, only 2% of them are interpreted into protein. Later on, the detection of noncoding RNAs (ncRNAs) revealed that intermediate RNAs (mRNAs) were only a small fraction of the total population of RNA.[17, 18] Rather than encoding proteins, these ncRNAs directly operate as regulatory, catalytic, or structural RNAs. With regard to their sizes, the regulatory ncRNAs can be categorized as small ncRNAs (<200 bps) and long ncRNAs (>200 bps).[19, 20] Due to the significant progress of high-throughput sequencing methods and genomic profiling combined with the robust bioinformatics tools, a considerable amount of data about long noncoding RNAs (lncRNAs) has been accumulated in the recent decade.[21] As most lncRNAs are expressed in a tissue-specific manner, they may play contributing roles in physiological and biological functions such as controlling cell cycle, apoptosis, and differentiation. Moreover, lncRNAs are vital regulators of both oncogenesis and tumor suppression, and previous studies demonstrated that lncRNAs are involved in the main cancer-driving crosstalks and pathways at transcriptional, posttranscriptional, and epigenetic levels.[22] Recently, both LINC00152 and LINC01296 have attracted great attention because of their participation in several cancers. It has been shown that these lncRNAs are upregulated in different types of cancers, such as pancreatic cancer, gastric cancer, gallbladder cancer, hepatocellular carcinoma, renal cell carcinoma, and breast cancer. In addition, they have been linked to cell immigration, invasion, proliferation, cell cycle arrest, and apoptosis in vitro. Increased expression of these lncRNAs was also considerably associated with clinicopathological features including overall survival (OS), low differentiation, lymph node metastasis (LNM), and high tumor node metastasis (TNM) stage. In this study, we assessed the expression levels of LINC01296 and LINC00152 in breast cancer tumor and its adjacent normal tissue. Moreover, the relationship between the expression levels of these RNAs with reproductive risk factors was evaluated.

Methods

Study Population

Paired samples, including cancer tissues and their adjacent normal tissue, were collected from 49 women with breast cancer referred to Shahid Faghihi Hospital in Shiraz for breast surgery from 2017 to 2018. Breast cancer was confirmed using pathologic assessment and then classified into luminal A (27 cases), luminal B (17 cases), and TNBC (5 cases) groups based on estrogen and progesterone receptors. Informed consent was obtained from all participants and the confidentiality of patients' information was preserved. The study protocol was following the Helsinki Declaration and approved by the Ethics Committee of the Shiraz University of Medical Sciences.

RNA Extraction and cDNA Synthesis

Total RNA was extracted from fresh cancerous and normal breast tissues using Biozol solution (Hangzhou Bioer Technology, China). The quantity and integrity of extracted RNAs were assessed by gel electrophoresis and NanoDrop (Thermo Fisher Scientific, USA). To remove possible DNA contamination, the extracted RNAs were treated using DNase I (Takara Biomedical Technology, China) based on the manufacturer's instructions. The cDNA was constructed using treated RNAs and the Prime-Script RT kit (Takara Biomedical Technology, China) based on the manufacturer's instructions.

Real-Time PCR

The expression of LncRNAs was assessed using SYBR premix Ex Taq II (Takara Biomedical Technology, China) via QuantStudio real-time PCR system (Applied Biosystems, USA). The specific primers used were as follows: (F) GAG GCC AAG ACA GGT TGA TTA and (R) TCA AGT AAG GGC AGC AAG TAG for LINC01296; (F) GAT GGC TTG AAC ATT TGG TCT TC and (R) TCC TGT TTC ATC TCC CAG TTA TTC for LINC00152; and (F) AGA TGA GTA TGC CTG CCG TG and (R) GCG GCA TCT TCA AAC CTC CA for beta-2-microglobulin (B2M). The expression of LINC01296 and LINC00152 were normalized as

the internal control gene using the expression of the B2M housekeeping gene. The samples were analyzed in duplicate, and relative lncRNAs expressions were calculated using the formula $2^{-\Delta\Delta c}$.

Statistical Analysis

The Shapiro-Wilk test revealed a lack of normal distribution in data, and therefore the Mann–Whitney test was selected to investigate the association between the expression of RNA and clinical risk factors. The association between quantitative variables was also assessed using Spearman's correlation coefficient. All statistical analyses were performed using SPSS software version 20 (IBM, USA). A p-value of less than 0.05 was considered statistically significant.

Results

The mean±standard deviation of patients' age and body mass index (BMI) were 45.22±10.48 and 6.41±4.42, respectively. The evaluation of real-time PCR for LINC01296 and LINC00152 showed an increased expression of both these LncRNAs in cancerous tissues compared with the adjacent normal tissues (Fig. 1a, b). One sample with remarkably increased expression of LINC00152 and three samples with significantly increased expression level of LINC01296 were excluded from statistical analysis to prevent errors in data analysis.

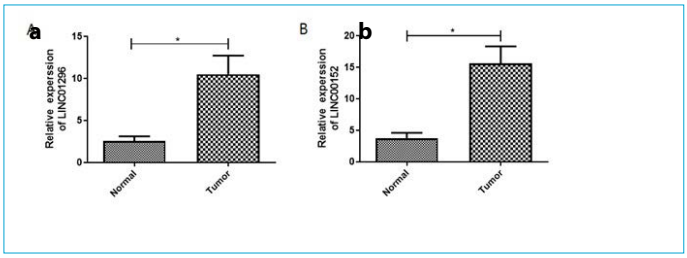

Figure 1. Relative expression of LINC01296 and LINC00152 in BC tissues and noncancerous tissue samples measured by qRT PCR. **(a)** The relative increase of LINC01296 expression in tumor tissues; **(b)** the relative increase of LINC00152 expression in tumor tissues.

Association of LncRNAs Expression with Clinicopathological Features and Reproductive Factors

The medium was determined as a cutoff value, and this threshold was used to divide the expression of LncRNAs into two groups of high and low expression (Table 1). Based on the Chi-squared test, higher expression of LINC00152 was closely correlated with the advanced TNM stage (p=0.029). Statistical analysis using the Mann–Whitney test showed a significant difference in expression levels of LINC01296 and not LINC00152, based on the use of oral contraceptives in patients with luminal B breast cancer type (p=0.028, Fig. 2). No significant difference was observed regarding the ex-

Table 1. Association of LncRNAs expression with clinicopathological features in breast cancer tissue.

Variables	LINC00152		p	LINC01296		p
	Low	High		Low	High	
Age (years)						
≤50	15	17	0.540	14	16	0.536
>50	9	7		9	7	
Tumor size (cm)						
≤2	8	9	0.917	9	7	0.608
>2	16	15		14	15	
TNM stage						
I + II	20	13	0.029*	17	14	0.345
III	4	11				
Lymph node metastasis						
Positive	12	17	0.140	15	13	0.546
Negative	12	7		8	10	
Her-2 status						
Positive	9	10	0.768	10	9	0.765
Negative	15	14		13	14	
ER status						
Positive	21	23	0.296	22	21	0.550
Negative	3	1		1	2	
PR status						
Positive	18	16	0.525	19	15	0.179
Negative	6	8		4	8	

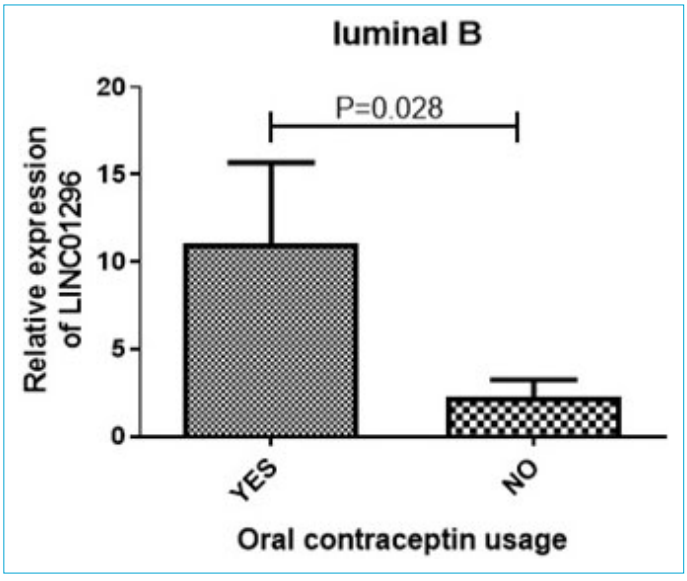

Figure 2. Comparison of the LINC01296 expression levels in patients with luminal B type of BC who had consumed OCD and those who did not consume OCD.

pression levels of LncRNAs and other risk factors including menopause status, age at onset of menopause, abortion history, and use of hormone replacement therapy. Further analysis using Spearman's rank correlation coefficient showed no significant correlation between the expression levels of either LINC01296 or LINC00152 and quantitative variables including BMI, age at onset of menopause, age at first delivery, and breastfeeding.

Discussion

Research on the deregulation of an increasing number of ncRNAs in diverse human cancers and their association with disease progression has recently gained momentum. For example, it has been shown that modulating the androgen receptor signal pathway prostate cancer antigen 3, which is highly expressed in prostate cancer, can promote cancer cell proliferation.[23] Metastasis associated in lung adenocarcinoma transcript 1, also referred to as nuclear-enriched abundant transcript 2, which is a highly conversed lncRNA and overexpressed in many kinds of cancers especially in nonsmall cell lung carcinoma (NSCLC), can promote cancer cell migration by regulating the expression of targeted genes.[24] Not all lncRNAs affect tumors by pro-oncogenic capability. For instance, maternally expressed gene 3 and growth arrest-specific transcript (GAS5) have low expression and play tumor-suppressive roles.[25] Such lncRNAs not only can act as novel molecular biomarkers for diagnosis and prognosis but also are useful for developing therapeutic strategies in treatments of various types of cancers. The first finding in this study was the increased expression of LINC01296 and LINC00152 in the cancerous tissues compared with their adjacent normal tissue. Additionally, we demonstrated that the increased expression of LINC00152 was positively correlated with the advanced TNM stage. A 2019 study by Shen et al. identified that LINC00152 expression was dramatically elevated in triple negative breast cancer (TNBC) cells. Inhibition or overexpression of LINC00152 obviously increased or suppressed PTEN protein expression, a tumor suppressor involved in the regulation of the cell cycle and division. Furthermore, analysis of clinical samples suggested that high LINC00152 expression was correlated with ER or PR negative expression, late TNM stage and lymphatic invasion, as well as shorter OS time in patients.[26] A similar study by Wu et al. in 2018 found that LINC00152 was highly expressed in all basal-like cell lines and in the majority of TNBC tissues. LINC00152 suppression by shRNA significantly inhibited invasion and colony growth and triggered apoptosis in vitro. It was revealed that LINC00152 partially enhanced breast cancer tumorigenesis by inactivation of the BRCA1/PTEN through DNA methyltransferase.[27] Regarding LINC01296, an investigation by Jiang et al. in 2018 showed that upregulation of this lncRNA is correlated with larger tumor size, positive LNM, advanced TNM stage, and unfavorable prognosis of patients with breast cancer. Moreover, silencing of LINC01296 inhibited breast cancer cell growth, cell migration, and invasion in vitro and in vivo and enhanced cell apoptosis via Caspase 3 and 9 proteins.[28] A 2019 meta-analysis by Feng et al., elucidated the correlation between LINC01296 with clinicopathological features and survival outcomes in tumors including breast cancer. In total, nine studies comprising 720 participants were enrolled in this analysis. The pooled results showed that increased LINC01296 expression could predict unfavorable OS which was correlated with the clinical stage. The results of this meta-analysis indicated that LINC01296 was a novel biomarker for prognosis in cancer patients.[29]

In addition, our findings showed a significant association between the expression levels of LINC1296 in breast cancer patients with luminal B type and the use of oral contraceptives. This result is comparable to reports of the association between oral contraceptives and increased risk of breast cancer.[30] Although the link between the risk of breast cancer and hormone-dependent factors, including reproductive history and obesity, has long been confirmed by epidemiological and experimental studies, the molecular mechanisms underlying this association are not well understood. Previous studies have shown that various lncRNAs are linked with hormone-dependent markers of breast cancer. A 2018 study by Mansoori et al. evaluated the association of obesity and reproductive history with

expression levels of two breast cancer-related lncRNAs, namely ZFAS1 and SRA1, in cancer-free breast tissues of 145 healthy women.[31] It was found that women with (BMI) ≥25 kg/m^2 showed a lower expression of ZFAS1 compared with those with BMI <25 kg/m^2. Interestingly, this analysis demonstrated a negative correlation between low expression of the ZFAS1 and high BMI in women with menarche age less than 14. Another study by Mansoori et al. analyzed the expression levels of two breast cancer-related lncRNAs, GAS5 and LSINCT5, in 145 normal breast tissues obtained from cosmetic surgery and found lower levels in the overweight-obese (BMI ≥25) subgroup than in the normal BMI (<25) subgroup.[32] Moreover, the expression level of GAS5 was negatively correlated with BMI. The expression level of GAS5 was higher in women with late menarche (>13 years) than those with early menarche (≤13 years). Our findings suggested that oral contraceptives might be role players in increased expression of LINC01296. Further research is needed to investigate the association of circulating sex hormones such as estrogen and progesterone and LINC01296 in breast cancer patients.

In summary, the present study demonstrated that LINC00152 and LINC01296 expressions were upregulated in cancerous breast tissues in comparison with normal breast tissues. Moreover, there was a significant relationship between the expression pattern of LINC01296 and the use of oral contraceptives. Our results may help better understand the role of lncRNAs in the development of breast cancer.

Disclosures

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Ethics Committee Approval: The study was approved by the Local Ethics Committee.

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Conflict of Interest: None declared.

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