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Original Article

Differential Expression of *lincRNA-ROR* Spliced Transcript Variants in Breast Cancer

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Abstract

Background: We investigated the expression pattern of a human stem cell-specific, large intergenic *noncoding* RNA *(lincRNA)* regulator of reprogramming (*lincRNA-ROR*) and its spliced transcript variants in breast tumors. Breast cancer is the leading cause of cancer mortality in women; therefore, finding a reliable diagnostic tumor marker, based on the molecular profile of tumor cells, is warranted.

Methods: qRT-PCR was used to investigate the expression alteration of a specific stem cell-related *lincRNA* and its spliced transcript variants in breast tumors which provided by the Iran National Tumor Bank (2014-2016). Suitability of *lincRNA-ROR* and expression alterations of its spliced transcript variants as breast tumor biomarkers were examined by ROC curve analysis.

Results: Expression was significantly upregulated in *lincRNA-ROR* variants 1 (NR-048536) and 4 (AB844432) and downregulated in variant 3 (AB844431), with expression levels failing to distinguish between breast tumor types, grades, and malignancy stages. Whereas ROC curve analysis gave good scores to the expressions of variants 1 (AUC=0.7675, *P*=0.003) and 3 (AUC=0.9383, *P*=0.00173), suggesting their suitability as potential breast tumor biomarkers, it gave an AUC score of 0.6033 for *lincRNA-ROR* spliced variant 4 (*P*=0.4118), denoting its unsuitability as a breast cancer biomarker.

Conclusion: Aberrant expressions of *lincRNA-ROR* spliced transcript variants could serve as reliable biomarkers with potential usefulness in breast cancer diagnosis. However, further research should elucidate the role and tissue expression of *lincRNA-ROR* spliced transcript variants in various cancers.

Keywords: Spliced variants; Breast tumor tissues

Introduction

A complicated heterogeneous disorder and the most frequent malignancy, breast cancer is the leading cause of global female mortality (1). The etiology and pathological symptoms of breast cancer vary from person to person (2-4). Despite the advances in the diagnosis and treatment of

breast cancer, the prognosis remains very poor, hence the need for an extensive understanding of the molecular mechanisms of the progression of this malignancy (5).

Long noncoding RNAs (lncRNAs) comprise a group of noncoding RNAs with a length exceeding 200 base pairs (bp) without protein-coding potential. LncRNAs play critical roles in tumor initiation, progression, and metastasis and are, thus, deemed potential novel biomarkers and therapeutic targets for the diagnosis and prognosis of cancer (5-8). Some lncRNAs such as HOX transcript antisense intergenic RNA (*lncRNA-HOTAIR*), metastasis-associated-lung-adenocarcinoma-transcript-1 (*MALAT-1*), and large intergenic noncoding RNA regulator of reprogramming (*lincRNA-ROR*) have regulatory roles in breast cancer (9-11).

Among studied lncRNAs, *lincRNA-ROR* is 2.6 kb lncRNA situated in chromosome 18. Introduced (12), *lincRNA-ROR* was initially reported as a regulator of the reprogramming of human-induced pluripotent stem cells by sequestering microRNA-145 (miR-145) (13). With its crucial role in pluripotency and reprogramming, *lincRNA-ROR* has been demonstrated to participate in oncogenesis. Since the discovery of *lincRNA-ROR*, mounting evidence has demonstrated the deregulation of *lincRNA-ROR* expression in various tumor malignancies, including breast (9, 14, 15), hepatic (16), gastric (17) colorectal (18), ovarian (19), pancreatic (20), and esophageal (21) cancers (14, 22).

Remarkably, some studies have reported the dramatic upregulation of *lincRNA-ROR* in many breast cancer cell lines and tissues and its contribution to the malignancy and treatment resistance of advanced breast cancer (9, 14, 22-24). Recently, our group introduced 13 novel spliced transcript variants of *lincRNA-ROR* featuring a unique array of exons (25).

We aimed to investigate the expression pattern of the spliced transcript variants of *lincRNA-ROR* in breast tumor tissues and normal tissues and to assess the potential of *lincRNA-ROR* spliced transcript variants as reliable cancer biomarkers with high sensitivity and specificity.

Materials and Methods

Tissues

Thirty-six fresh frozen tissues (biopsies) of breast tumor and marginal non-tumor tissues were provided by the Iran National Tumor Bank, founded by the Cancer Institute of Tehran University of Medical Sciences, for Cancer Research (2014-2016). Histopathological parameters were evaluated according to the grading and TNM system for stage classification by the WHO.

The biopsies were obtained from patients before they consumed any special medication or underwent chemotherapy. The clinical and pathological characteristics of the breast tissues are presented in Supplementary Table 1.

RNA extraction and cDNA synthesis

Total RNA was isolated from the frozen breast tumor and marginal non-tumor tissues by using TRIzol reagent (Invitrogen, USA) according to the manufacturer's instructions (26). The quality of RNA was evaluated by gel electrophoresis, and the concentration of RNA was measured with the NanoDrop 2000 Spectrophotometer (Thermo, USA). RNase Free DNase I (Takara, Japan) treatment was employed to eliminate any possible traces of DNA contamination. Reverse transcription was done by using the PrimeScript TM Reagent Kit (Takara, Japan), primed with an oligo (dT) primer and a random hexamer.

Quantitative real-time polymerase chain reaction (qRT-PCR)

Specific primers of each variant were designed to examine the expression levels of *lincRNA-ROR* variants (Supplementary Table 2). The primers designed for different spliced transcript variants are shown in Fig. 1.

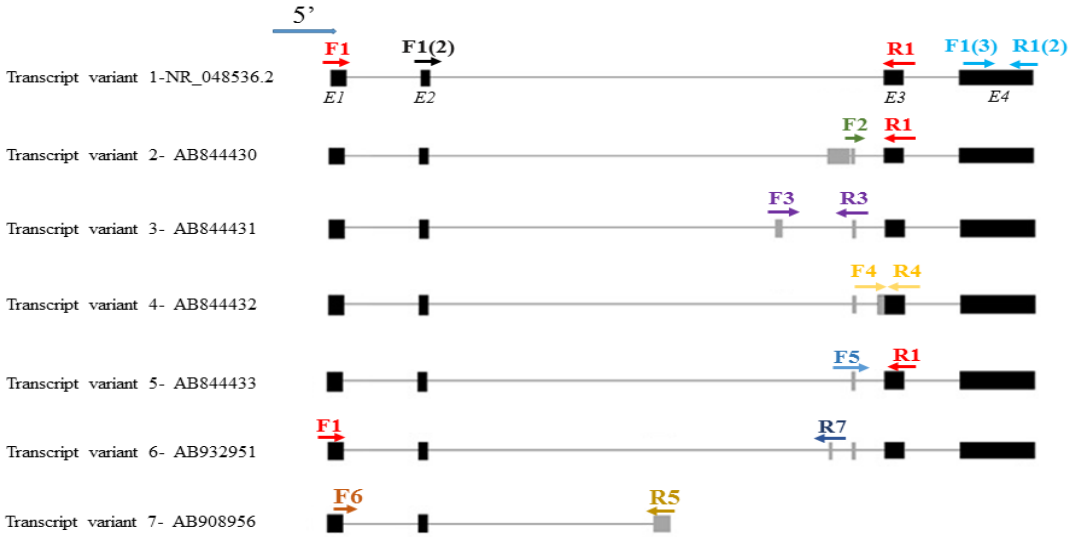

Fig. 1: The image depicts a schematic view of the location of the primers applied to amplify different *lincRNA-ROR* spliced transcript variants.
E: exon

The qRT-PCR test was carried out with 2 µL of synthesized cDNA, 10 µL of SYBR Green Ready Mix (TAKARA, Japan), 0.1 µL of ROX reference dye, and 0.5 µM of each specific primer. The *GAPDH* gene was utilized as an internal control so that the expression levels of other genes could be normalized to its expression level. The cycling condition was as follows: initiation at 95 ℃ for 15 min, amplification for 40 cycles, denaturation at 95 ℃ for 15 sec, annealing at 62 ℃ for 30 sec, and extension at 72 ℃ for 30 sec.

Statistical Analysis

For fold-change expression analysis, $2^{-\Delta\Delta Ct}$ was used. The statistical analyses were performed with GraphPad Prism 8 software, and *P*-values less than 0.05 were considered statistically significant. The Student paired t-test and receiver operating characteristic (ROC) curve analysis were applied to determine whether the spliced transcript variants had enough sensitivity and specificity to distinguish tumor from non-tumor breast tissue samples.

Ethics approval

The ethics committees of Tarbiat Modares University, Imam Khomeini Hospital, and Rajaie Cardiovascular Medical and Research center approved the research procedure (IR.RHC.REC.1397.016).
All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Results

Differential expression levels of LincRNA-ROR spliced transcript variants in the breast tumor and marginal non-tumor tissues

The expression patterns of different spliced transcript variants of *lincRNA-ROR* in the breast tumor and marginal non-tumor samples were determined by using different sets of oligonucleotides and investigating the expression patterns of spliced transcript variants 1 to 7 of *lincRNA-ROR*, corresponding to accession numbers NR_048536 (HQ315778), AB844430, AB844431, AB844432, AB844433, AB908956, and AB932951, respectively. Among the 7 variants, 5 spliced transcript variants were successfully am-

plified: variants 1, 3, 4, 5, and 6. Spliced transcript variants 2 and 7 were omitted from further analysis due to the detection of additional bands in the qRT-PCR test. Additionally, the qRT-PCR test detected only spliced transcript variants 1, 3, and 4 of *lincRNA-ROR* in the breast tumor and marginal non-tumor samples (data not shown).

The expression levels of spliced transcript variants 1 (NR_048536 (HQ315778), 3 (AB844431), and 4 (AB844432) of *lincRNA-ROR* were further quantified in the breast tumor and marginal non-tumor tissue samples. *LincRNA-ROR* variants 1 and 4 were upregulated significantly in the breast tumor samples by comparison with the marginal non-tumor tissues (fold change =2.6, *P*=0.0039 vs fold change=10.44, *P*=0.048, respectively), whereas the expression of variant 3 was significantly downregulated in the tumor samples compared with the marginal non-tumor tissues (fold change =0.22, *P*=0.0020) (Fig. 2A, B, & C).

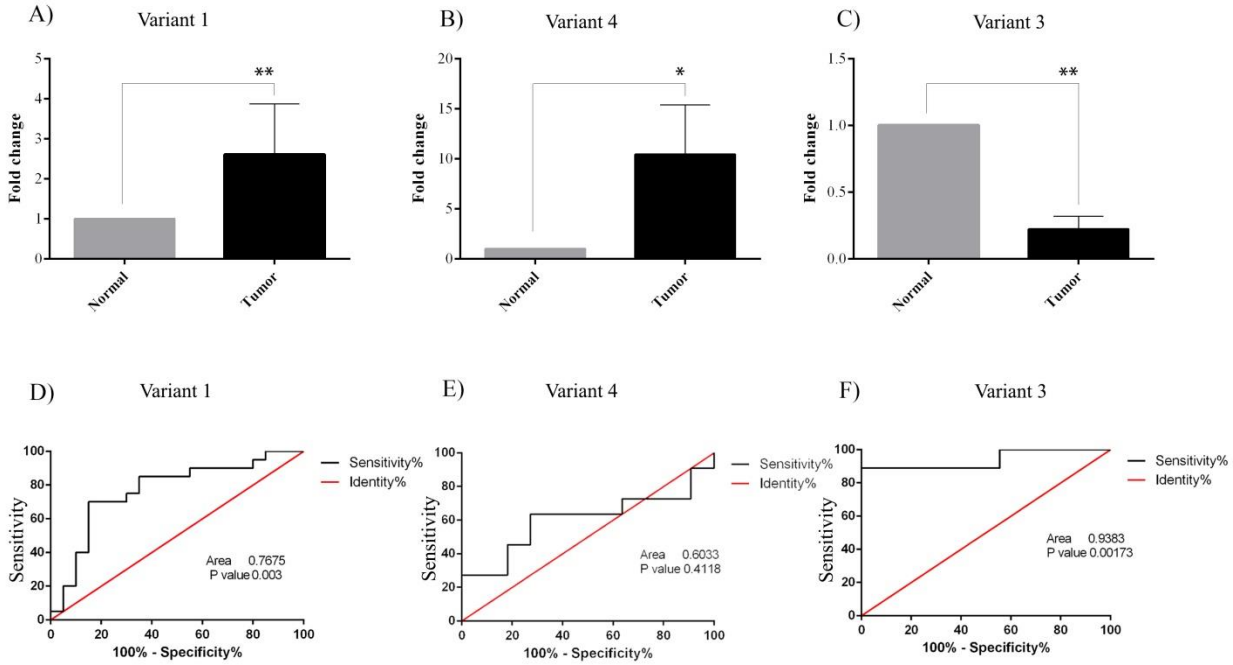

Fig. 2: The image illustrates the expression analysis of *lincRNA-ROR* spliced transcript variants in breast tumor and marginal non-tumor samples. (A) Variant 1 is significantly overexpressed in the tumor samples by comparison with the marginal non-tumor samples (P=0.003, P<0.05). (B) The expression of variant 4 is also upregulated in the breast tumor tissues compared with the marginal non-tumor tissues (P=0.048, P<0.05). (C) Conversely, the expression of variant 3 is significantly downregulated in the breast tumor tissues (P=0.002, P<0.05). (D, E, and F) The images present the ROC curve analysis of variants 1, 4, and 3 of *lincRNA-ROR*

Further, the data analysis showed no association between the expression levels of different *lincRNA-ROR* spliced transcript variants and the malignancy grade of the tumors (grading and staging).

Moreover, the tumor samples were divided into 2 groups based on the age of the patients: young (20-45 yr old) and older (45-80 yr old), and the expression levels of the variants were compared between the 2 groups. The results demonstrated no significant difference in the expression of variants between the 2 groups, nor was there any statistically significant difference in the expression levels of *lincRNA-ROR* spliced transcript variants between human epidermal growth factor receptor 2 (HER2)-positive and HER2-negative tumor samples. The expression of the variants in 3 grades (Grade I, II, and III) and 4 stages (T1,

T2, T3, and T4) was also examined, and the findings revealed no statistically significant difference vis-à-vis expression alterations (data not shown).

The specificity and sensitivity of the expression levels of lincRNA-ROR spliced transcript variants 1 and 3 in discriminating tumor from marginal non-tumor breast tissue samples

ROC curve analysis was employed to investigate the potential suitability of the expression levels of *lincRNA-ROR* spliced transcript variants 1, 4, and 3 to discriminate between tumor and marginal non-tumor breast tissue samples. The analysis measured an AUC of 0.7675 for variant 1 (P=0.003; 95% confidence interval (CI), 0.7421 to 1.026), and AUC of 0.9383 for variant 3 (P=0.00173; 95% CI, 0.3743 to 0.6701), and an AUC of 0.6033 for variant 4 (P=0.4118; 95% CI, 0.3519 to 0.8548) (Fig. 2D, E, & F).

Therefore, based on the ROC curve data, both *lincRNA-ROR* spliced transcript variants 1 and 3 received scores above the cutoff (0.7) needed for an appropriate biomarker. *LincRNA-ROR* spliced transcript variant 3 is a suitable biomarker to classify tumor and marginal non-tumor samples of breast tissue, whereas variant 4 lacks such suitability.

Discussion

Mounting evidence over the recent years indicates that lncRNAs play fundamental roles in gene regulation on the strength of their extensive expression. The subcellular localization and expression pattern of lncRNAs are allied to such diverse biological and physiopathological conditions as neuronal disorders (27), immune responses (28), and cancer. Accordingly, visible alterations in their expression could be used as valuable biomarkers for the diagnosis, prognosis, and treatment of cancer (8, 15, 29).

Re-expression of *lincRNA-ROR* in various breast cancer cells and tissues, as well as various other tumors (30-34), has been previously reported, promoting the hypotheses of the involvement of cancer stem cells and the potential contribution of these factors to tumorigenesis and metastasis (35-38). The expression profile of the 13 novel spliced variants of *linc-ROR* harboring unique array of exons *in* the pluripotent and non-pluripotent cells depicted all transcripts were predominantly expressed in pluripotent stem cells (25). Additionally, *linc-ROR* was a crucial factor for embryonic stem cell maintenance and take parts as an oncogenic factor in breast cancer cells via interfering on TGF-β signaling pathway, resulting in poor prognostic outcomes (39). Herein, we revealed that *lincRNA-ROR* and its spliced transcript variants were differentially expressed in tumor tissues versus non-tumor ones. Our results are in line with the result of previous studies and supported the involving of the stem cell markers in breast tumorigenesis. In addition, we determined the suitability of using the expression levels of various *lincRNA-ROR* spliced transcript variants as breast cancer biomarkers.

Several studies have reported the dramatic upregulation of *lincRNA-ROR* in breast cancer, possibly contributing to its malignancy and treatment resistance (9, 14, 22, 23). Some polymorphisms in the sequence of *linc-ROR* may regulate the *linc-ROR* expression at RNA level (40). The association of four selected SNPs (rs6420545, rs4801078, rs1942348 and rs9636089) in *linc-ROR* in the breast cancer patients' plasma revealed that TT genotype of rs4801078 in *linc-ROR* had a significant association with the higher risk of BC and the expression of *linc-ROR RNA* was closely related with the alleles of rs4801078 (40).

All 4 introduced *lincRNA-ROR* SNPs, rs6420545, rs1942348, rs4801078 and rs9636089 (Fig. 1) are harbored in the common introns (2 and 3) of the examined transcript spliced variants in the present study and the association of these SNPs with the expression pattern of *linc-ROR* spliced transcript variants require to be investigated.

Our data chime in with previous investigations that revealed the significant upregulation of *lincRNA-ROR* spliced transcript variant 1. We also found that variant 4 was significantly upregulated, while variant 3 was meaningfully downregulated in tumor versus marginal non-tumor breast tis-

sues. Spliced transcript variants 5 and 6 of *lincRNA-ROR* were undetectable in breast cancer cell lines and tissues. Our group previously published its data on the significant upregulation of variants 1, 2, and 4 of *lincRNA-ROR* in squamous cell carcinoma (21). Hence, different tumoral tissues may have differential expression patterns of *lincRNA-ROR* spliced transcript variants. Additionally, some lncRNAs such as *MALAT-1* and *Psoriasis Susceptibility 1 Candidate 3* (*PSORS1C3*) perform various regulatory roles at transcriptional and post-transcriptional levels by producing different spliced transcript variants (35, 41-43). Despite the growing knowledge on *lincRNA-ROR* expression and function, the precise profiles of the expression and activity of its spliced transcript variants need elucidation.

For all the available data on the biomarker role of lncRNAs in cancer, a consensus has yet to emerge on a reliable clinical molecular biomarker. The misregulation of mRNA splicing is considered a trigger of cancer signaling pathways and a contributor to nearly all cancer hallmarks (44). In addition, the alternative splicing of lncRNAs might impact various cellular processes and, thus, confer new potentials for biomarker discovery (45). LncRNA *miR31HG* and its spliced transcript variants, namely *MIR31HG⊿E1* and *MIR31HG⊿E3*, were highly differentially expressed in basal subtype cells and tissues of muscle-invasive bladder cancer and associated with the survival of different subgroups of patients (45). Another investigation reported that lncRNA *anti-differentiation noncoding RNA*, *ANCR,* was expressed differentially in different types of brain tumors (26). Moreover, lncRNA *PVT1* and its spliced transcript variants are greatly expressed in clear cell renal cell carcinoma and act as oncogenic transcripts (46). The different spliced transcript variants of *PSORC1C3* lncRNA are differentially expressed, and they function differently in various pluripotent and non-pluripotent cell lines (35, 47).

An ideal biomarker should have high sensitivity and specificity (48), our ROC curve analysis demonstrated that variants 1 (the main variant) and 3 of *lincRNA-ROR* were suitable biomarkers for breast cancer. The upregulation of variant 1 and the downregulation of variant 3 of *lincRNA-ROR* could be promising biomarkers to discriminate breast tumors from non-tumor samples.

Although *lincRNA-ROR* spliced transcript variant 4 was considerably upregulated in breast tumor samples, our ROC curve analysis did not recognize it as a good biomarker for breast tumor tissues. This finding is in contrast with that posited variant 4 was a moderate biomarker for squamous cell carcinoma (21).

Limitations

The noticeable limitation of the present study is its sample size. Expression pattern analyses of *lincRNA-ROR* spliced transcript variants in larger samples of breast tumor subtypes may confirm the value of alterations of *lincRNA-ROR* spliced transcript variants in expression levels as diagnosis biomarkers of different subtypes of breast tumors. Additionally, budget constraints limited our investigation to the screening of *lincRNA-ROR* spliced transcript variants expression only at the RNA level in tissue samples of the breast tumors. The expression of *lincRNA-ROR* spliced transcript variants at RNA level in body fluid such as serum should be considered in future investigations. Likewise, more prosperous information could be achieved via carrying out the functional studies of different spliced variants in in vitro and in vivo studies.

Conclusion

These results, together with those from previous studies, provide new insights into the molecular classification of different tumors based on the different spliced transcript variants of *lincRNA-ROR* and promising biomarkers in certain cancers.

The aberrant expression levels of different *lincRNA-ROR* spliced transcript variants demonstrated their different correlations with breast tumors. However, more detailed investigations are required to elucidate the exact role and tissue expression of different *lincRNA-ROR* spliced

transcript variants in breast tumors as well as other tumors.

Journalism Ethics considerations

Ethical issues (Including plagiarism, informed consent, misconduct, data fabrication and/or falsification, double publication and/or submission, redundancy, etc.) have been completely observed by the authors.

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Conflict of interest

The authors have no conflicts of interest to declare.

Data availability

The supplementary table 1 (clinical and pathological characteristics of breast tumor tissues) and supplementary table 2 (sequences of the primers utilized in the study) are accessible through request to corresponding author.

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