

ORIGINAL ARTICLE

Dynamic Regulative Biomarker: Long Noncoding RNA (lncRNA) in Metastatic Breast Cancer

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SUMMARY

Background: Breast tumor is a common cancer in women all over the world. Long noncoding RNA (lncRNA) provides a significant and new perspective on understanding biomarkers as well as on the potential prognostic regulation of breast cancer. Its transcription, in turn, serves as a regulator in diagnosing breast cancer and preventing risk of recurrence. Here, we review the evolution of lncRNAs and discuss their regulative roles in the metastasis of breast cancer. Moreover, we aim to detect the expression level of lncRNA HOTAIR in different stages of breast cancer.

Methods: Sixty patients with breast cancer at different stages were divided into four groups based on different stages. Reverse transcription-polymerase chain reaction (RT-PCR) was used to detect the expression level of lncRNA HOTAIR in breast tumor tissue.

Results: Compared to stage I breast cancer, the expression profiles of lncRNA HOTAIR in stage II, III, IV breast cancer are significantly elevated ($p < 0.05$). The expression profiles of lncRNA HOTAIR in stage III and IV breast cancer are significantly increased compared with stage II breast cancer.

Conclusions: Consistent with microRNAs (miRNA), lncRNAs could function as underlying effective biomarkers to affect the biogenesis and gene control across all lifetime. The interaction between lncRNA and miRNA plays a crucial role in the metastasis of breast cancer and provides a potential biomarker target for breast cancer metastasis therapy. Our study has also demonstrated that the expression profiles of lncRNA HOTAIR in stage II, III, IV breast cancer are significantly elevated.

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KEY WORDS

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INTRODUCTION

Breast cancer has become the most frequent cancer and malignant disease among females all over the world and now ranks second in mortality among all cancers in women [1]. This cancer is characterized by a distinct metastatic pattern involving the regional lymph nodes, bone marrow, lung, and liver [2]. For early breast cancer, it is considered safe and should be treated with default therapy according to Baum, Vaidya³; however, when metastasis is present, it will lead to death. For pa-

tients with solid tumors, generally, ~90% of deaths were caused by metastasis [4]. Metastasis has always been portrayed as the ultimate step of the progressing breast cancers [5]. Thus, studying metastasis has become a hotspot issue for more and more scientists.

Long non-coding RNAs (lncRNAs), similar to microRNAs (miRNA), are not the template for coding protein. This somewhat arbitrary limit distinguishes lncRNAs from small regulatory RNAs such as miRNAs, short interfering RNAs (siRNAs), piwi-interacting RNAs (piRNAs), small nucleolar RNAs (snoRNAs), and other short RNAs [6]. Certain functional lncRNAs such as Air and Xist are poorly conserved, in contrast, miRNAs and snoRNAs were highly conserved [7]. While many association studies have identified lncRNAs that are aberrantly expressed in disease states, they have little understanding of their contribution within disease etiology. Expression analyses that compare tumor cells and normal cells have revealed changes in the expression of lncRNAs in several forms of cancer [8].

Recent recognition that lncRNAs function in various aspects of cell biology has focused increasing attention on their potential to contribute towards disease etiology. For example, they are currently used as biomarkers and therapeutic targets in many cancer types, based mainly on their involvement in diverse cellular regulative processes, such as signal transduction and allosteric regulation of cytoplasmic enzymatic activities via transcription or post-transcription [9]. Some literature has implicated lncRNAs in a variety of disease states and supports an involvement and co-operation in neurological disease and oncogenesis [10]. They supported that lncRNAs were related to the metastasis of breast cancer and also ways that lncRNAs could be activated by TGF- β , which was the most significantly up-regulated lncRNA in TR SKBR-3 cells. Moreover, lncRNAs could regulate Hedgehog signal transduction pathways based on a series of systematic analyses and the technology of open-ended lncRNA pulldown [9]. The inhibitors of Hedgehog pathways played a role of prevention in breast cancer treatment [11]. The discovery of new biological functions of lncRNA and novel RNA binding proteins involved in the metastasis of breast cancer suggests that lncRNAs maybe become a new prospective and evidence-based biomarker on the potential prognostic regulation of breast cancer.

The large number of studies about lncRNAs in metastatic breast cancer published in recent years aroused our interest. In this study, we reviewed the evolution of lncRNAs and discussed their regulative roles in the metastasis of breast cancer and verified the expression level of lncRNA HOTAIR in different stages of breast cancer. Our study provides an in-depth view of lncRNAs in metastatic breast cancer by reviewing the latest studies and using our own data regarding the interaction between lncRNA and miRNA that plays a crucial role in the metastasis of breast cancer and providing a potential biomarker as target for metastatic breast cancer therapy.

Classification

The size of lncRNAs are very different, ranging from two hundred nucleotides (nt) to several hundred KB. They are transcribed by RNA polymerase II or RNA polymerase III. While the abundance of lncRNAs was unanticipated, this number, nevertheless, represents a conservative lower estimate, since it omitted many singleton transcripts and non-polyadenylated transcripts (tiling array data shows more than 40% of transcripts are non-polyadenylated) [12]. According to the diversity in size, lncRNAs can be divided into three different groups, small-lncRNA (200 ~ 950 nt), medium-lncRNA (950 ~ 4,800 nt), and large-lncRNA (> 4,800 nt) [13]. In humans, small-lncRNAs are the major member, approximately 58%; however, medium-lncRNAs (approximately 78%) are the leading actor in mice. Moreover, the proportion of small-lncRNAs and large-lncRNAs in the human genome are higher than that in the mouse [14].

Similar to microRNAs, lncRNAs do not participate in the process of protein translation in the role of coding template and are located either in the nucleus or in the cytoplasm. Based on the position in the chromosome and the different features of molecular function, molecular model, and special mechanisms, lncRNAs could be divided into four distinct groups from the view of genomic location and context, long intergenic noncoding RNAs (lncRNAs), intronic lncRNAs, sense lncRNAs, and antisense lncRNAs. There are four different locations in the genome, more specifically, intergenic lncRNA means they are transcribed intergenically from both strands; intronic lncRNA means they are transcribed entirely from introns of protein-coding genes; sense lncRNA means they are transcribed from the sense strand of protein-coding genes and contain exons from protein-coding genes, overlapping with part of protein-coding genes or covering the entire sequence of a protein-coding gene through an intron; antisense lncRNA means they are transcribed from the antisense strand of protein-coding genes, overlapping with exonic or intronic regions or covering the entire protein-coding sequence through an intron [15].

A major function of lncRNAs is regulating gene expression through changes in chromatin state. Currently, more and more literature revolves around the research of lncRNAs and antisense lncRNAs, especially lncRNAs. In contrast, less is known about intronic lncRNAs and sense lncRNAs, especially sense lncRNAs. Additionally, it is more difficult to study antisense lncRNAs and sense lncRNAs as they are correlated differently with coding genes and they are trying to exert different effects on gene locus or mRNAs [14]. An individual lncRNA can downregulate a subset of Staufen 1 (STAU1)-mediated messenger RNA decay (SMD) targets which involves the degradation of translationally active the 3'-UTRs binding to STAU1 which is a protein that binds to double-stranded RNA [16].

A biomarker in metastasis of breast cancer

Emerging evidence indicates that lncRNAs play a crucial role in regulating cellular processes, such as cell growth, apoptosis, cancer progression as well as cancer metastasis. The discovery of lncRNAs and their regulatory roles challenges the initially miRNA-centered regulatory networks, which may help comprehensively understand gene regulation by lncRNAs, and accordingly, provide new insights into complex processes of gene regulation. For patients with solid tumors, generally, ~90% of deaths were caused by metastasis [4]. Metastasis has always been portrayed as the ultimate step of progressing breast cancers [5]. Recent evidence, however, indicates that about a third of women diagnosed with small asymptomatic breast tumors already harbor disseminated BC cells in their bone marrow [17].

As mentioned before, lncRNAs have critical roles in various biological processes ranging from embryonic development to human diseases, including cancer progression. The lncRNA could promote the metastasis as well as inhibit one, for example, increasing the expression level of the lncRNA HOX-antisense intergenic RNA (HOTAIR) was most likely to predict metastasis and death in primary breast tumors as HOTAIR was a powerful predictor of eventual metastasis and death [18]. Experimental evidence suggests that the expression level of lncRNA in the HOX locus, including HOTAIR, is a predictor of breast cancer metastasis [19]. In matched primary and metastatic cancers, both HOTAIR and EZH2, which is the human homolog of the *Drosophila* Protein Enhancer of Zeste and a PcG protein in the PRC2 complex [20], had increased expression in the metastatic carcinomas. Kleer et al. [21] also identified high levels of EZH2 in breast cancer metastases. Of note, Gupta et al. [18] used qRT-PCR to demonstrate that elevated levels of HOTAIR in stage I and II breast cancer, defined as 125-fold more than that detected in normal breast epithelia, is strongly associated with eventual metastasis and death. Except for breast cancer, the lncRNA HOTAIR was also a predictor of poor prognosis and promotes metastasis in non-small cell lung cancer, gastric cancer, skin cancer, etc. The misexpression of lncRNAs in cancer naturally raises the question as to how structural variations in lncRNA genes, either germline or somatic, may contribute to cancer predisposition [22].

Transforming growth factor β (TGF- β) has come to be accepted as the prototypical multifunctional peptide growth factor [23] and orchestrates an intricate signaling network to modulate carcinogenesis and progression [24]. The lncRNA activated by TGF- β (lnc-ATB) was the other pathway to promote the breast cancer metastasis and confer resistance to various therapeutics through enhancing proliferation, migration, and invasion to induce epithelial-mesenchymal transition (EMT) [25]. It was the pathway which was the most significantly up-regulated lncRNA in TR SKBR-3 cells and the tissues of TR breast cancer patients [26]. The role of TGF- β -induced EMT in trastuzumab resistance [27], which is the

leading cause of mortality in HER2-positive breast cancer and has the second-poorest prognosis among breast cancer subtypes, is well established [28].

By contrast, lncRNA-ROR, first discovered in induced pluripotent stem cells (iPSCs), was upregulated in breast tumor samples and could have a key role in the maintenance of iPSCs and embryonic stem cells (ESCs) by preventing the activation of cellular stress pathways. Ectopic overexpression of lncRNA-ROR in immortalized human mammary epithelial cells induced an EMT program [29], which may function as a key competing endogenous RNA to link the network of miRNAs and core transcription factors and is regulated by the key pluripotency factors Oct4, Sox2, and Nanog [30]. Like miRNA, lncRNA or lncRNAs have recently been identified as novel regulators of the transcriptional and epigenetic networks [31]. Overexpressed lncRNA-ROR cells presented decreased sensitivity to 5-FU and paclitaxel with decreased E-cadherin expression, increased Vimentin, N-cadherin expression, and invasion ability, thus its up-regulation is important for chemotherapy tolerance and invasion of breast cancer [32].

Targeting metastasis mechanisms of lncRNAs

lncRNAs may play an important role in the progression of melanoma. Further research on lncRNA expression profiles is needed to define the impact of lncRNAs on the progress of melanoma [33]. Tumor metastasis suppressors are promoters or inhibitors of metastasis, but their mechanisms of action are generally not understood. Antagomirs are synthetic RNA molecules that are designed to directly hybridize with miRNAs, thus potentially limiting the availability of the miRNA for Argonaute loading and 3'-UTR hybridization [34]. Other previous studies indicated that the suppressor Raf kinase inhibitory protein (RKIP) inhibits breast tumor metastasis in part via let-7 targets (HMGA2, BACH1) in turn upregulating bone metastasis genes (MMP1, OPN, CXCR4) [35]. As the mechanisms of lncRNAs promote or inhibit the metastasis of breast cancer, Gupta et al. [18] indicated that enforced expression of HOTAIR in epithelial cancer cells induced genome-wide re-targeting of polycomb repressive complex 2 (PRC2) to an occupancy pattern more resembling embryonic fibroblasts, leading to altered histone H3 lysine 27 methylation, gene expression, and increased cancer invasiveness and metastasis in a manner dependent on PRC2. Conversely, loss of HOTAIR can inhibit cancer invasiveness, particularly in cells that possess excessive PRC2 activity. Through RNA in situ hybridization of probes to three different lncRNAs (HOTAIR, ncHoxA1, and ncHoxD4), of the 283 lesions on the primary breast carcinoma tissue microarray, unequivocal scores were obtained in 221/283 (78%) HOTAIR, 118/283 (42%) ncHoxA1, and 142/283 (50%) ncHoxD4 carcinomas [19]. Moreover, lncRNA-ROR had a key role in the maintenance of iPSCs and ESCs by preventing the activation of cellular stress pathways. Hou et al. [29] showed that lncRNA-ROR enhanced breast cancer cell migration

Table 1. The expression profiles of lncRNA HOTAIR in patients with breast cancer at different stages.

Groups	lncRNA HOTAIR
Stage I	1
Stage II	3.62 ± 0.48
Stage III	7.23 ± 0.73
Stage IV	8.12 ± 0.85

and invasion, which was accompanied by generation of stem cell properties. Silencing it would repress breast tumor growth and lung metastasis *in vivo*. Mechanistically, lncRNA-ROR was associated with miRNPs and functioned as a competing endogenous RNA to mi-205. Specifically, lncRNA-ROR prevented the degradation of miR-205 target genes, including the EMT inducer ZEB2. On the other hand, lncRNA-ROR promotes invasion, metastasis, and tumor growth in pancreatic cancer by activating ZEB1 pathway. As master gene regulators, on the other hand, lncRNAs are generally capable of forming lncRNA-protein (ribonucleoprotein) complexes to regulate a large number of genes. For example, lncRNA-RoR suppresses p53 in response to DNA damage, which means lncRNA-RoR is a p53 repressor by interaction with heterogeneous nuclear ribonucleoprotein I (hnRNP I) [36]. The present study indicated hnRNP I can also form a functional ribonucleoprotein complex with lncRNA urothelial carcinoma-associated 1 (UCA1) and increase the UCA1 stability.

lnc-ATB has been reported to be activated by TGF- β , promote trastuzumab resistance and invasion-metastasis cascade in breast cancer by competitively binding miR-200c, upregulating ZEB1 and ZNF-217, and then inducing EMT, and also found that miR-200c was down-regulated in the tissues of trastuzumab resistant breast cancer patients, and inversely correlated with lnc-ATB expression [26]. Interestingly, increased lncRNA-ROR, a stress-responsive lncRNA, was highly expressed in hepatocellular cancers (HCC), which are highly resistant to chemotherapy, and could reduce chemotherapy-induced cell death [37]. The result implicates extracellular vesicle lncRNAs as mediators of the chemotherapeutic response and supports targeting lnc-ROR to enhance chemosensitivity. There were generally three functional mechanisms of lncRNAs, transcriptional regulation, post-transcriptional regulation, and other functional mechanisms. Cis-lncRNA and trans-lncRNA also function through transcriptional interference, whereas trans-lncRNA also functions through chromatin modification. Although the most efficient signaling pathway of lncRNA associated with breast cancer was unknown, the biomarker of lncRNA will be a new perspective to prevent metastasis of breast cancer. In triple-negative breast cancer, lncRNA for kinase activation (LINK-A) mediates HB-EGF-triggered, EGFR: GPNMB heterodi-

mer-dependent HIF1 phosphorylation at Tyr 565 and Ser 797 by BRK and LRRK2, respectively [33], which cause HIF1-p300 interaction, HIF1 stabilization, and activation of HIF1 transcriptional programs under normoxic conditions.

The interaction between lncRNAs and miRNAs in breast cancer

As aforementioned, these non-coding RNAs, miRNAs, and lncRNAs are crucial regulatory molecules in breast cancer. miRNA-lncRNA interactions are supported by experimental data for both human and mouse species. They can also be used as agents for delivering antitumor drugs [38]. Liz et al. have demonstrated the roles of numerous lncRNA transcripts are reducing miRNAs' stability for binding during the process of regulation, therefore alleviating the negative effect of miRNAs on their respective mRNA targets through the miRNA Response Elements (MREs) [39]. Most of the available lncRNA resources include relevant high-throughput HITS-CLIP and PAR-CLIP experimental data as well as state-of-the-art *in silico* target predictions. For example, lncRNA no. LOC554202 could encode miR-31, and its methylation promoter could decrease the level of miR-31, which contributes to the invasion and metastasis of breast cancer [5]. MiR-31 maps to the intronic sequence of a novel lncRNA, LOC554202 and the regulation of its transcriptional activity is under control of LOC554202. Loss of miR-31 expression in triple-negative breast cancer cell lines is attributed to hypermethylation of its promoter associated CpG island. Together the results provide the initial evidence for a mechanism by which miR-31, an important determinant of the invasion metastasis cascade, is regulated in breast cancer [5].

Several lncRNAs can control gene expression by directly recruiting histone-modifying enzymes to chromatin. Further, Hou et al. [29] indicated that as a miR-205 sponge, lnc-RoR prevented ZEB2 (a target host gene of miR-205) to express down regulation. Expression profiles of 6 lncRNAs that have been found to be associated with cancer or metastasis were examined by qRT-PCR in 3 pairs of primary melanoma and matched lymph node metastatic tissues [40]. Among them, HOTAIR was significantly overexpressed in metastatic lymph nodes compared to matched primary melanoma. Furthermore, lncRNA HOTAIR inhibits miR-7 and SETDB1 indirectly to reverse the EMT by downregulating the STAT3 pathway in breast cancer stem cells [41]. Chisholm et al. [19] illuminated that neither HOTAIR nor EZH2 expression correlated with the clinicopathologic feature of metastases, both HOTAIR (31%) and EZH2 (52%) expressions more often had equivalent or increased expression (52% and 40%, respectively) in the metastasis compared to the primary carcinoma. Thus, the interaction between lncRNA and miRNA play a crucial role in the metastasis of breast cancer and would be a potential biomarker in targets for breast cancer metastasis therapy.

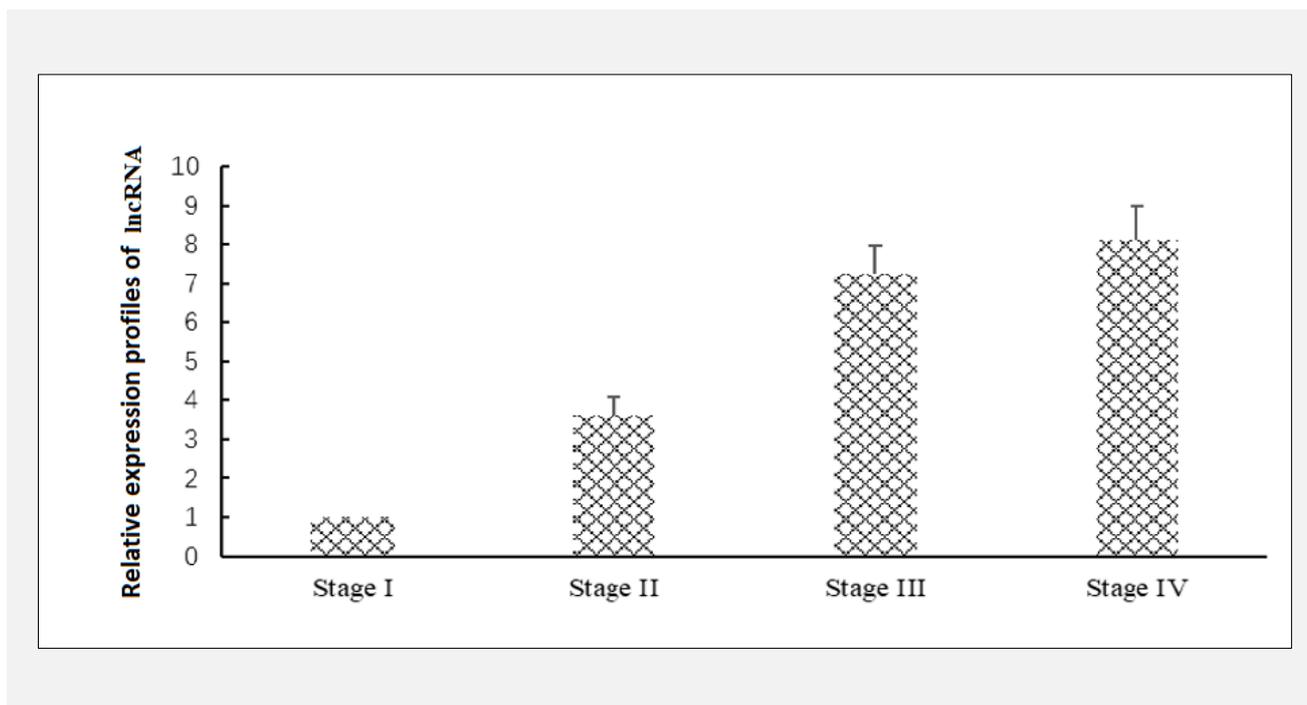


Figure 1. The expression profiles of lncRNA HOTAIR in patients with breast cancer at different stages.

Perspective

lncRNA provides a significant perspective on understanding biomarkers as well as on the potential prognostic regulation of breast cancer. Its transcription in turn serves as regulator in diagnosing breast cancer and preventing risk of recurrence. In this review, we focus on the classification, metastasis targeting mechanisms and the interplay between lncRNAs and miRNAs in metastasis of breast cancer. lncRNAs have critical roles in various biological processes ranging from embryonic development to breast cancer. Future experimental analyses will be required to investigate the exact function of lncRNAs and miRNAs in invasion and metastasis of breast cancer.

Methods and results of our study

This study was carried out with the approval of ethics committee of HwaMei Hospital, University of Chinese Academy of Science. The informed consent was obtained from all patients. Between June 2016 and June 2017, sixty patients of breast cancer with different stages who were treated by surgery in our institution of HwaMei Hospital, University of Chinese Academy of Science were included to investigate the expression profiles of lincRNA HOTAIR. The breast cancer tissues were stored at -80°C for study. They were divided into TNM stages I (14 cases), II (21 cases), III (15 cases), and IV (10 cases) according to the National Comprehensive Cancer Network (NCCN) Clinical Practice Guidelines in Oncology: Breast Cancer (version 3.2014). The me-

dian age of all patients was 52 years (range 36 ~ 65 years).

Reverse transcription-polymerase chain reaction (RT-PCR) was performed with Recover AllTM Total Nucleic Acid Isolation kit (Thermo Fisher, USA), RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher, USA), and PowerUp SYBR Green Master Mix Kit (Thermo Fisher, USA) according to manufacturer's instructions. All tests were performed in duplicate for each sample. Data was analyzed using $2^{-\Delta\Delta\text{CT}}$. The prime sequence of lncRNA HOTAIR and control gene U6 were as follows:

Forward 5'-3':

AAGGCTGAAATGGAGGACCG;

Reverse 5'-3':

TACCGATGTTGGGGACCTCT.

Furthermore, the statistical analysis was performed with analysis of variance using SPSS 19.0 (ANOVA). The value of 0.05 was confirmed as statistically significant. The results of RT-PCR showed that the expression profiles of lncRNA HOTAIR in stage II, III, IV breast cancer were significantly elevated ($p < 0.05$) compared with stage I breast cancer. Moreover, the expression profiles of lncRNA HOTAIR in stage III and IV breast cancer have increased significantly compared with stage II breast cancer ($p < 0.05$). However, the comparison of the expression patterns of lncRNA HOTAIR between stage III and IV breast cancer showed no significance ($p \geq 0.05$). These results were shown in Figure 1 and Table 1. The results of the expression profiles of lncRNA

HOTAIR in stage II, III, and IV breast cancer were consistent with the above review of lncRNA HOTAIR in metastasis breast cancer.

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Declaration of Interest:

All five authors declared that there are no conflicts of interest.

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