Original article

Microarray expression profile analysis of long non-coding RNAs in human breast cancer: A study of Chinese women

Nan Xu a, Fengliang Wang b, Mingming Lv b, Cheng Lu b, *a

a First Clinical Medicine College, Nanjing University of Chinese Medicine, Nanjing, China
b Nanjing Maternity and Child Health Care Hospital Affiliated to Nanjing Medical University, Nanjing, China

A R T I C L E   I N F O

Article history:
Received 30 October 2014
Accepted 1st December 2014

Keywords:
Breast cancer
LncRNA
Immune system
Microarray
ZBTB46

A B S T R A C T

Breast cancer (BC) is the most commonly diagnosed cancer and the second leading cause of cancer death among women. Long non-coding RNAs (lncRNAs) are key regulators of gene expression. Numerous lncRNAs have performed critical roles in cancer biology including breast cancer (BC). The expression levels of certain lncRNAs are associated with tumor development, recurrence, metastasis, and prognosis. However, the potential roles that lncRNAs regulate breast cancer tumorigenesis and tumor progression are still poorly understood. To investigate the potential roles of lncRNAs in the breast cancer, we constructed BC related lncRNA libraries by using microarray. Microarray expression profiling suggests 790 up-regulated and 637 down-regulated (log fold-change > 2.3) lncRNAs were differently expressed between BC tissues and its paired adjacent tissues. Furthermore, we found differently expressed lncRNAs associated with immune regulation. RP4-583P15.10, an up-regulated lncRNA, was found to be located downstream of the natural antisense of the ZBTB46 gene, which may regulate breast cancer through influence immune system. In conclusion, our results for the first time indicate that distinct lncRNAs expression profiles of BC, which related to the immune network, may provide information for further research on immune regulation during the BC process.

© 2014 Elsevier Masson SAS. All rights reserved.

1. Introduction

Breast cancer (BC) is the most common type of tumor in women. The incidence of breast cancer in western countries has decreased or at least been stable over the last few decades, but the incidence is increasing in China and in many developing countries [1]. The potential mechanisms that regulate breast cancer progression are still poorly understood. The development of BC is a complex multistep process associated with numerous genetic alterations [2]. Accordingly, the elucidation of the molecular mechanisms in BC has been the subject of extensive research over past decades. Clinically, delayed diagnosis, recurrence, and metastasis are still the biggest obstacles to the treatment of BC [3]. Therefore, searching for the ideal biomarkers and novel therapeutic targets of BC are essential for the diagnosis and treatment of BC.

Immune system functions as a host defensive mechanism protecting against invading pathogens and transformed cells, including cancer [4]. It has long been recognized that the immune system plays dual roles in the development of tumors [5]. Many experimental in vitro and in vivo studies have demonstrated that the immune network plays a significant role in the development and progression of BC [6,7]. However, the underlying mechanisms are still poorly understood, especially the mechanisms explaining how the immune system dysfunction in breast cancer development. Long non-coding RNA (lncRNA) are functional RNAs longer than 200 nucleotides in length. Over past few decade advances in genome-wide analyses have revealed that the human genome encodes over 10,000 lncRNAs with little coding capacity [8]. For a long time these lncRNAs have been considered as transcriptional noises, however, growing evidence suggests that lncRNAs are key regulators which governing various biological processes such as genomic imprinting, transcription activation and inhibition, chromatin modification and tissue development [9]. Dysregulation of lncRNAs is associated with many human diseases, including various types of cancers [10]. More recently, many lncRNAs have been shown to exert oncogenic or tumor suppressor properties in BC [11]. The well-studied lncRNA HOTAIR, for example, was found to be overexpressed in breast tumors, and the expression of HOTAIR in primary breast tumors was characterized as a negative prognostic factor in BC patients [12]. Although a few lncRNAs' information accumulated in cancer, the functions of majority of lncRNAs remain largely unknown.

http://dx.doi.org/10.1016/j.biopharma.2014.12.002
0753-3322/© 2014 Elsevier Masson SAS. All rights reserved.
Hence, the aim of the present study was to perform IncRNA expression profiling to identify IncRNAs that might help to better diagnose and treat breast cancer. We identified a set of IncRNAs that were differentially expressed in breast cancer. Moreover, this study indicated that the dysregulated IncRNAs may disturb the immune network and promote the development of breast cancer. These findings will aid in our understanding of IncRNA function in immune system and may provide a basis for the diagnosis and therapy of breast cancer.

2. Materials and methods

2.1. Tissues collection

Samples of breast cancer tissues consisting of tumors and adjacent sections from patients who had invasive breast cancer were collected consecutively between January 2013 and March 2014 at Nanjing Maternity and Child Health Care Hospital, Nanjing, China. This study was approved by the ethical review committee of Nanjing Maternity and Child Health Care Hospital affiliated to Nanjing Medical University. None of these patients accepts radiotherapy or chemotherapy prior to the breast tumor resection. The adjacent tissues were taken 5 cm from the edge of the tumor. Histological evaluation of adjacent tissue samples showed no indication of contamination from tumor or other abnormal cells. The patients were biologically unrelated, but all belonged to the Han Chinese ethnic group from the Jiangsu province in China. Informed consent was obtained from the subjects. Finally, 22 cases of paired tissues were selected. For further study, all the tissues were immediately frozen in liquid nitrogen and stored at −80 °C freezer until use.

2.2. RNA extraction and quality control

To isolate total-RNA from each tissue, the frozen tissues were resuspended in TRIzol reagent (Life Technologies) and were finally eluted into 50 µL of Elution Solution according to the manufacturer’s instructions. Quantification and quality check were performed with Nanodrop and Agilent 2100 Bioanalyzer (Agilent Technologies), respectively. All the RNA samples were stored at −80 °C until further processing. Complementary DNA (cDNA) was synthesized from 1000 ng of total-RNA by using a Primerscript TM RT Master Mix Kit (Applied TAKARA, DaLian, China) with random hexamer primers in a final volume of 20 µL. The reverse-transcription reaction were performed at 37 °C for 13 minutes, 85 °C for 5 seconds and 4 °C for 10 minutes.

2.3. Microarray analysis

An ArrayStar, Inc. (Rockville, MD, USA) Human IncRNA Microarray V 3.0 is designed for the global profiling of human IncRNAs and protein-coding transcripts. The IncRNAs were obtained from authoritative databases (RefSeq, Ensembl, UCSC_knowngenes et al.). The mRNAs were collected from RefSeq and GENCODE. Each transcript is represented by a specific exon or splice junction probe, which can identify individual transcript accurately. Positive probes for housekeeping genes and negative probes are also printed onto the array for hybridization quality control. The sample preparation and microarray hybridization were performed according to the Agilent One-Color Microarray-Based Gene Expression Analysis protocol (Agilent Technology). Briefly, mRNA was purified from 1 µg cleaned total-RNA (RNeasy Mini Kit; Qiagen). Then, each sample was amplified and transcribed into fluorescent cRNA along the entire length of the transcripts without 3’bias utilizing a random priming method. Agilent Quick Amp Labeling Kit was used for sample labeling. Hybridization was performed in Agilent’s SureHyb Hybridization Chambers. After washing, slides were scanned with the Agilent DNA Microarray Scanner (G2565BA). Data normalization and analysis was performed using the GeneSpring GX v11.5.1 software (Agilent Technologies). All microarray data were deposited into the Gene Expression Omnibus (GEO) at the National Center for Biotechnology Information, National Institutes of Health under Series Number GPL16956.

2.4. Quantitative real-time reverse-transcription PCR

The selected IncRNAs and the primers used for qRT-PCR were designed and synthesized by Generay Biotech (Shanghai, China). GAPDH was used as an internal control for tissue samples. qRT-PCR analysis was performed using ABI ViiA7 (Applied Biosystems) with SYBR expression assay system (TaKaRa, Dalian, China). The PCR reaction conditions were: an initial denaturation at 95 °C for 30 s, followed by 40°PCR cycles at 95 °C for 5 s and 60 °C for 34 s. Finally annealing and extension at 95 °C for 15 s, 60 °C for 60 s and 95 °C for 15 s. Each sample was assayed in triplicates. We used the 2−( ΔΔCt) method to determine the fold-change in gene expression in the cancer samples relative to the adjacent samples.

2.5. Gene Ontology (GO) Enrichment and pathway Analysis

The Gene Ontology (GO) project provides a controlled vocabulary to describe gene and gene product attributes (http://www.geneontology.org) [13]. GO categories were considered as significantly enriched only if the Fisher’s exact probability P-value < 0.05. Pathway analysis is a functional analysis mapping genes to KEGG pathways [14]. The P-value (EASE-score, Fisher P-value or Hypergeometric P-value) denotes the significance of the pathway correlated to the conditions. The lower the P-value, the more significant considered the pathway.

2.6. Statistical analysis

The data were analyzed using SPSS 20.0 software package (SPSS, Chicago, IL, USA). Differential expression levels of IncRNAs were compared by Independent-samples t-test between two groups. Fisher’s exact test was used in GO and pathway analysis. All values are expressed as the mean ± SEM. All experiments were repeated at least three times. Statistical significance was accepted for P < 0.05.

3. Results

3.1. Profile of microarray data

ArrayStar, Inc. (Rockville, MD, USA) Human IncRNA Microarray V 3.0 is designed for the global profiling of human IncRNAs and protein-coding transcripts. According microarray expression profiling data, 33664 IncRNAs and 23516 mRNAs were detected. In addition, 1486 IncRNAs (log fold-change > 2.3) were found differently expressed between BC samples and its paired adjacent tissue samples. All these IncRNAs were obtained from authoritative databases, RefSeq, UCSC Knowngenes, Ensembl and many related literatures (Fig. 1A). Scatter-plot is used for assessing the IncRNA expression variation between the two groups of BC and adjacent (Fig. 1B). Hierarchical Cluster shows IncRNA expression patterns (Fig. 1C and D); 790 up-regulated and 637 down-regulated IncRNAs were identified in BC tissues compared with paired adjacent tissues (log fold-change > 2.3).
Fig. 1. The differently expressed profiling in BC was compared with paired adjacent samples. (A) Using second-generation lncRNA microarray, 1486 lncRNAs (log fold-changes > 2.3) were detected. Pie chart was showing the most authoritative databases. (B) Differentially expressed lncRNAs and mRNAs in BC and its paired adjacent tissues were analyzed using hierarchical clustering. Hierarchical clustering analysis arranges samples into groups by expression level, ‘red’ indicates high relative expression, and ‘green’ indicates low relative expression. The scatter plot is a visualization method used for assessing the lncRNAs (C) and mRNAs (D) expression variations between BC tissues and paired adjacent tissues. The values of the X and Y axes in the scatter plot are the averaged normalized signal values of the group (log2 scaled). The green lines are fold-change lines.

3.2. Real-time quantitative PCR confirmation

By using quantitative real-time reverse-transcription PCR (qRT-PCR), 3 up-regulated lncRNAs and 4 down-regulated lncRNAs with log fold-changes > 2.3 were randomly selected to test and verify the microarray data in different samples of BC tissues and paired adjacent tissues (Fig. 2). The qRT-PCR results and microarray data are consistent. Thus, microarray data profiling indicated a series of lncRNAs constantly differentially expressed between BC tissues and paired adjacent tissues.

3.3. Expression signatures of deregulated lncRNAs between BC tissues and paired adjacent tissues

As lncRNA expression has tissue specific. To further study the lncRNA expression pattern in BC tissues, we first investigate general signatures of deregulated lncRNAs which log fold-changes > 2.3, including lncRNA classification, length distribution, and chromosome distribution. According lncRNA position on the genome, we attributed lncRNA into 6 categories (bidirectional, exon sense-overlapping, intergenic, intronic antisense and natural antisense). Classification analysis showed that lncRNA in our microarray belong to intergenic lncRNA (Fig. 3A) and distribute 401-800 nt in length (Fig. 3B). Chromosome distribution shows that up and down lncRNAs has a different chromosomes location (Fig. 3C and D).

3.4. GO analysis and pathway analysis

GO analysis was performed to determine the gene and gene product enrichment, which covered three domains: biological processes, cellular components and molecular functions. We found that the highest GO classification targeted by under-regulated transcripts were regulation of biology process (biological process [BP]), plasma membrane (cellular component [CC]), receptor binding (molecular function [MF]). Meanwhile, highest GO classification targeted by the over-regulated transcripts were regulation of biology process (biological process [BP]), membrane part (cellular component [CC]), signal transducer activity and molecular transducer...
Fig. 2. The differential expression of lncRNAs between BC tissues and paired adjacent tissues were validated by qRT-PCR. *P < 0.05, **P < 0.01, ***P < 0.001.

Fig. 3. (A) Classification of deregulated lncRNAs. The lncRNAs are mainly intergenic. (B) Length distribution of the dysregulated lncRNAs. The lncRNAs are mainly between 400 and 800 bp in length. Chromosome distribution show the numbers of up (D) and down (E) regulated lncRNAs location in different chromosomes.
activity (molecular function [MF]) (S1, Supplementary data). To analyze the candidate biology process of IncRNAs, we enriched the biological process of both up and down-regulated IncRNAs. The interesting is most biological process of up-regulated IncRNAs are involved in immune regulation, such as immune response, immune system process, defense response, regulation of immune system process, lymphocyte activation, regulation of immune response, positive regulation of immune system process, leukocyte activation, cell activation and cellular response to interferon-gamma which shows that up-regulated IncRNA in breast tissues has a tight association with immune actions (Fig. 4A). As far as our knowledge, it is the first time to report that breast cancer related IncRNAs are associated with immune system in microarray profiling. However, we found the biological process of down-regulated IncRNA is enriched in system development, organ development and other development process, which is very similar with previous microarray profiling [15] (Fig. 4B).

We performed pathway analysis by mapping genes to KEGG pathways, Pathway analysis indicated that 44 pathways corresponded to under-regulated transcripts and 31 pathways corresponded to up-regulated transcripts. The top 10 pathway that the associated with up-regulated and down-regulated IncRNAs are both analyzed (Fig. 4C and D). Pathways that down-regulated IncRNAs enriched involved in a category “calcium signaling” has been reported involved in the development of BC [16]. Furthermore, most of the pathways that up-regulated IncRNA enriched are also related with immune system, such as systemic lupus erythematosus, Rheumatoid arthritis, Intestinal immune network for IgA production and Inflammatory bowel disease. This analysis showed that IncRNAs may play a key role in regulating immune system and may involve in tumor development process of BC.

For we found that up-regulated IncRNAs may associated with immune regulation and down expressed IncRNA may influence BC development. We selected up-regulated IncRNAs that log fold-change > 2.3 in BC and its paired adjacent tissues and their associated coding gene with a function of immune regulation, meanwhile we also selected down-regulated that log fold-change > 2.3 and their associated coding gene with a function of immune regulation. RP4-583P15.10, an up-regulated IncRNA, was found to be located downstream of the natural antisense of the ZBTB46 gene (Fig. 4E). Previous research reported that ZBTB46 as a key immune regulator and influence immune system, which was specifically expressed by pre-DCs and cDCs [17]. Meanwhile, Song et al. reported that the SNP of ZBTB46 was

---

**Fig. 4.** GO and pathway analysis. The top 10 GO terms that associated with coding gene functions of up-regulated IncRNAs (A) and down-regulated IncRNAs (B) are listed. The top 10 pathway that associated coding gene of up-regulated IncRNAs (C) and down-regulated IncRNAs (D) are listed. IncRNA RP4-583P15.10 (E) was verified up-regulated in BC tissues and shows a function in regulate immune system.
significantly associated with both glioblastoma and astrocytoma [18]. However, the importance of RP4-583P15.10 in dendritic cells needs to be determined, as does its role in promoting breast cancer. In addition, an under-regulated IncRNA RP11-445H22.4 was found to be located downstream of the intron of the WNT1-inducible signaling pathway protein 2 (WISP2). Previous research reported that WISP2 was associated with the progression of breast cancer [19].

4. Discussions

Breast cancer is the most common form of cancer in women and the second leading cause of death in females after lung cancer [20]. The development of breast cancer is a complex multistep process associated with numerous genetic factors, tumor suppressor genes, oncogenes and tumor cells hematogenous dissemination [21]. Accordingly, the elucidation of the molecular mechanisms in breast cancer has been the subject of extensive research over the past decades. Several large-scale analyses have provided evidence that many non-coding transcripts are highly regulated and functional [22]. Meanwhile, numerous studies have demonstrated the importance of non-protein part of human genome in carcinogenesis and metastasis. Among various non-proteins coding RNAs, long non-coding RNAs (lncRNAs) have been found to play a key role in cancer biology. Emerging evidence indicates that the deregulation of lncRNAs might play a crucial role in breast carcinogenesis and associated with BC recurrence, metastasis and prognosis [23]. A 2.2-kb transcript IncRNA HOTAIR, for example, is transcribed from HOX C locus and was first identified in 2007 by Rinn et al. [24] was found overexpressed in BC and the high expression level of HOTAIR was associated with metastasis, recurrence, and poor prognosis of BC [12,25].

Although the research of IncRNAs is increased rapidly, only a few of them have been well characterized. The aforementioned BC related IncRNA is only the tip of the iceberg and the IncRNA function roles with BC are not fully understood. Thus, there is need to extensive ongoing searching for the BC related IncRNAs. In current study, we investigated the IncRNA expression profiles of BC by using IncRNA microarray. We found that the IncRNA expression levels were altered compared to adjacent BC tissues; 790 up-regulated and 637 down-regulated IncRNA were identified in BC tissues compared with its paired adjacent tissues with set a filter of log fold-change > 2.3. Further, we verified microarray profiling by using quantitative real-time reverse-transcription PCR (qRT-PCR). The qRT-PCR results and microarray data are consistent. Then, we utilized Gene Ontology (GO) analysis and pathway analysis to study preliminarily the biological functions of these IncRNA. Gene Ontology provides a controlled vocabulary to note gene and gene product attributes in any organism. While Pathway analysis is considered as a method for gaining insight into the underlying biology of differentially expressed gene and proteins. According to the Gene Ontology (GO) analysis we found the biological processes of up-regulated gene profiling were present a closely association with immune network, such as immune response, immune system process, defense response, regulation of immune system process, lymphocyte activation, regulation of immune response, positive regulation of immune system process, leukocyte activation, cell activation and cellular response to interferon-gamma. Meanwhile, Pathway analysis profiling shown that mainly up-regulated gene profiling is also associated with immune system such as systemic lupus erythematosus, rheumatoid arthritis and Intestinal immune network for IGA production. In addition, one of the up-regulated IncRNA RP4-583P15.10, we randomly choose to verify our microarray data which near to the ZBTB46 gene was found to be a key regulator of immune network and could influence immune microenvironment of tumor. From the last two decades, several labs have unequivocally documented that immune can facilitate cellular transformation, prevent or control tumor outgrowth [26]. Earlier work which studies interactions between the immune system and cancer cells found that person is protected from cancer growth and the development of tumor immunogenicity by their immune system [27]. Accordingly, immune system activity cannot be ignored during the cancer progression Recently, genome-wide analysis of IncRNAs expression have indicated that thousands of IncRNAs are highly conserved among mammals and have putative roles in regulating innate immunity [28]. However, the information about the relationship between BC and IncRNA in immune system and its potential roles during BC progression is few. As far as our knowledge, the exits of our survey are for the first time indicates that IncRNAs are closely associated with immune system in BC and may influence BC progression.

In conclusion, cancer related IncRNAs information accumulated and update and it is identified large portion of IncRNAs have putative roles in regulating innate immunity. However, the 'immune' related functions of IncRNAs in BC remains largely unknown. In this study, we reported the profile of differentially expressed IncRNA between BC tissues and its paired adjacent tissues are associated with immune activity. Understanding the functions of these IncRNAs in regulating immune process in BC could help to find new immunodiagnosis and immunotherapy targets. Hence, the aim of the further study was to perform expression profiling of BC related IncRNA and to analyze its potential immune function. In addition, the candidate molecular mechanisms of the immune associated deregulated IncRNA involved in BC also need to explain.

Disclosure of interest

The authors declare that they have no conflicts of interest concerning this article.

Acknowledgments

We thank Miss Xun Lu (Jinling High School, Nanjing, China) for her help in collecting patient information. This work was supported by a Grant from the National Natural Science Foundation of China (No. 811172501).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biopharm.2014.12.002.

References


