Chemoresistance-related long non-coding RNA expression profiles in human breast cancer cells

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Abstract. Breast cancer is the most commonly diagnosed cancer and the leading cause of cancer death in females worldwide. Chemoresistance has been a major reason for the drug therapy failure. The present study performed a microarray analysis between MCF-7 and MCF-7/adriamycin (ADR) cells, and intended to identify long non-coding (lnc)RNA expression character in drug resistant breast cancer cells. MCF-7/ADR cells were induced from MCF-7 cells via pulse-selection with doxorubicin for 4 weeks, and the resistance to doxorubicin of ADR cells was confirmed by MTT assay. Microarray analysis was performed between MCF-7 and MCF-7/ADR cells. Total RNA was extracted from the two cell lines respectively and was transcribed into cDNA. The results of the microarray were verified by reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Gene Ontology (GO) and pathways analysis were conducted to enrich the dysregulated lncRNAs presented in the microarray results. Compared to the MCF-7 cells, 8,892 lncRNAs were differentially expressed in MCF/ADR cells (absolute fold-change >2.0). A total of 32 lncRNAs were selected for RT-qPCR by fold-change filtering, standard Student’s t-test, and multiple hypothesis testing. Among the dysregulated lncRNAs, AX747207 was prominent because its associated gene RUNX3 was previously reported to be relative to malignant tumor chemoresistance. GO analysis results also indicated some biological processes and molecular functions linked to chemoresistance. The pathway enrichment results provided some potential pathways associated with chemoresistance. In the present study, the authors intended to identify lncRNA expression character in drug resistant cell line MCF-7/ADR, corresponding to the parental MCF-7 cell line. In addition, the study identified the lncRNA AX747207, and its potential targeted gene RUNX3, may be related to chemoresistance in breast cancer. These results may new insights into exploring the mechanisms of chemoresistance in breast cancer.

Introduction

Breast cancer is the most commonly diagnosed cancer and the leading cause of cancer death in females worldwide (1,2). Incidence and mortality rates vary internationally by five-fold. The highest incidence rates were reported in many European countries, while low rates were in Asia, Africa and South America (3). However, the incidence of breast cancer has increased by 3% per year in China, which has threatened the health of women, and created a great burden on the society (4). With the development of chemotherapy, hormonal therapy, immunotherapy, gene therapy and other treatment technologies, the long-term survival of breast cancer patients has become possible. Breast cancer patients continue to succumb to the disease due to tumor metastasis, drug resistance and other reasons, including hemorrhage, infection and recurrence (5).

Long non-coding (lnc)RNAs are defined as RNA transcripts which are >200 bp and lack open reading frames. Thousands of lncRNAs have been identified in mammalian cells, many with expression patterns specifically restricted by cell or tissue-type and development stage (6,7). A number of lncRNAs were initially identified through the whole-genome tiling array and the next generation sequencing of transcriptomes (8-10). These studies suggested that lncRNAs have complicated structures and intrinsic origins, and therefore, they can no longer be defined just by their length and protein-coding incapability (11).
Generally, lncRNAs are linked to diverse gene-regulatory roles, such as chromosome dosage compensation, imprinting, epigenetic regulation, cell-cycle control, nuclear and cytoplasmic trafficking, transcription, translation, splicing and cell differentiation (11,12). Most importantly, aberrant expression of lncRNAs is linked to several disease states, including cancer (13).

A few studies have associated certain lncRNAs with poor outcome and disease progression in different types of cancer: High HOTAIR expression was identified in several types of cancer, including breast and colorectal cancer (14-16), overexpression of prostate cancer associated transcript-1 in prostate cancer and overexpression of metastasis associated lung adenocarcinoma transcript-1 in several types of cancer, including early-stage non-small cell lung cancer (17,18).

Some research indicated that lncRNAs were associated with chemoresistance: MEG3 and HOTAIR were considered to contribute to the cisplatin resistance of human lung adenocarcinoma (19,20). HOTITIP promotes progression and gemcitabine resistance by regulating HOXA13 in pancreatic cancer (21). Takahashi et al (22) suggest that extracellular vesicle lncRNA is a mediator of the chemotherapeutic response, and supports targeting long intergenic non-protein coding RNA (LINC-ROR) to enhance chemosensitivity in hepatocellular carcinoma.

**Materials and methods**

**Cell culture.** The human breast cancer MCF-7 cell line was obtained from the American Type Culture Collection (Manassas, VA, USA). Cells were cultured in RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.), 100 U/ml penicillin (Gibco; Thermo Fisher Scientific, Inc.) and 100 U/ml streptomycin (Gibco; Thermo Fisher Scientific, Inc.) at 37°C in a humidified atmosphere containing 5% CO₂. MCF-7 cells were pulse-selected with doxorubicin (10 pulses, once a week for 4 h, with 1 µM doxorubicin (Zhejiang Hisun Chemical Co., Ltd., Taizhou, China)) to generate MCF-7/ADR after 6 months, as described previously (23). Pulse concentrations were decided based on changes in cell morphology and clinical doxorubicin plasma concentration. MCF-7/ADR cells were cultured in the continuous presence of doxorubicin (1 µg/ml) to maintain the drug resistance phenotype and cultured in drug-free medium for >2 weeks before subsequent experiments. The experiments were independently reproduced twice, and each cell line was tested in triplicate.

**MTT assay.** Doxorubicin-resistance was demonstrated in cell lines by means of the MTT (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) dye reduction assay. MCF-7 and MCF-7/ADR cells were seeded into 96-well plates at a density of 1x10⁴ cells per well and incubated overnight in 100 µl 10% FCS medium (Gibco; Thermo Fisher Scientific, Inc.). Cells were then exposed to varied concentrations of doxorubicin and incubated at 37°C in a humidified atmosphere containing 5% CO₂ for 48 h. After this time, cells were treated with MTT solution (5 mg/ml in phosphate-buffered saline) for a further 4 h at 37°C. Following this incubation period, the medium was rapidly removed and the MTT crystals were solubilized using 150 µl DMSO (Sigma-Aldrich; Merck KGaA). The number of viable cells was determined by measuring the absorbance at 490 nm for each well using a microplate spectrophotometer. Absorbance readings were subtracted from the value of blank wells; the reduction in cell growth was calculated as a percentage of control absorbance in the absence of any drug. Data presented the mean of at least three independent experiments ± standard deviation.

**RNA extraction and quality control.** According to the manufacturer's protocol, total RNA was extracted from the cells grown in monolayer with TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.). Quantification and quality checks were performed with the Nanodrop ND-1000 and Agilent 2100 Bioanalyzer (Agilent Technologies, Inc., Santa Clara, CA, USA), respectively. RNA Integrity and gDNA contamination were assessed by standard denaturing agarose gel electrophoresis.

**Microarray analysis.** Sample preparation and microarray hybridization were performed by Kangcheng Bio-tech (Aksomics Inc., Shanghai, China). Briefly, RNA was purified from 1 µg total RNA following removal of rRNA (mRNA-ONLY Eukaryotic mRNA Isolation kit; Epicentre; Illumina, Inc., San Diego, CA, USA). Each sample was amplified and transcribed into fluorescent cRNA along the entire length of the transcripts without 39 bias utilizing a random priming method. The labeled cRNAs were hybridized onto the Human LncRNA Array (version, 2.0; 8660 K; ArrayStar, Inc., Rockville, MD, USA). Following the washing of the slides, the arrays were scanned by the Agilent Scanner G2505B (Agilent Technologies, Inc., Santa Clara, CA, USA). Agilent Feature Extraction software (version, 10.7.3.1; Agilent Technologies, Inc.) was utilized to analyze acquired array images. Quantile normalization and subsequent data processing were carried out using the GeneSpring GX software package (version, 11.5.1; Agilent Technologies, Inc.). Differentially expressed LncRNAs and mRNAs were identified through fold change filtering (fold change ≥3.0 or ≤0.5), standard student t-test (P<0.05) and multiple hypothesis testing (FDR<0.05). P-values and FDR were calculated by Microsoft Excel (Microsoft Corporation, Redmond, WA, USA) and MATLAB 8.2 (The MathWorks, Inc., Natick, MA, USA), respectively.

**Gene ontology and pathway analysis.** Pathway analysis and GO terms were used to determine the roles of these differentially expressed mRNAs in these biological pathways or GO terms. Differentially regulated mRNAs were uploaded into the Database for Annotation, Visualization and Integrated Discovery (http://david.abcc.ncifcrf.gov/) to analyze the enrichment of these coding genes. The result of pathway enrichment analysis was confirmed by the online database of Kyoto Encyclopedia of Genes and Genomes (KEGG; http://www.kegg.jp). The potential functions of these differentially expressed LncRNAs were identified by functional annotation clustering and ranked by enrichment scores.

**Validation of differentially expressed LncRNA by reverse transcription-quantitative polymerase chain reaction.** Total RNA was isolated from tissues by the TRIzol reagent...
(Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's instructions. A total of 2.5 μg RNA for each sample was reversely transcribed into cDNA by using random hexamer primer with PrimeScript™ RT MASTER MIX (Perfect Real Time kit; Takara Bio, Inc., Otsu, Japan). Primers for each lncRNA were designed according to Primer 3 (http://sourceforge.net/projects/primer3/) online and checked with Basic Local Alignment Search Tool of NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi) to ensure a unique amplification product. RT-qPCR was performed on an Applied BiosystemsViiA™ 7 Dx (Thermo Fisher Scientific, Inc.) using the SYBR Green (Invitrogen; Thermo Fisher Scientific, Inc.) method according to the manufacturer's instructions. The PCR reaction conditions were: Denaturation at 10 min at 95°C, followed by 40 cycles of 15 sec at 95°C and 30 sec at 60°C and 30 sec and then 30 sec at 72°C. Elative gene expression levels were quantified based on the cycle threshold values and normalized to the internal control gene GAPDH. The 2^(-ΔΔCq) method was used to comparatively quantify the levels of mRNA (24).

Statistical analysis. The differences of lncRNA levels were determined by using a standard Student's t-test and multiple hypothesis testing. The sensitivity and specificity were analyzed according to the standard formulas. All the P-values are two-sided and P<0.05 was considered to indicate a statistically significant difference. Calculations of the mean ± standard deviation were conducted using SPSS software (version 20.0; SPSS, Inc., Chicago, IL, USA).

Results

Cell viability of MCF-7 and MCF-7/ADR cells after doxorubicin treatment. To determine whether MCF-7/ADR cells are resistant to the chemotherapeutic agent doxorubicin, cell viability was measured via an MTT assay. MCF-7 and MCF-7/ADR cells were treated with different concentrations of doxorubicin (0, 1, 10, 100, 500 and 1,000 nM). The results indicated that the semi-effective inhibitory concentration (IC50) of MCF-7 cells was 9.007 nM doxorubicin. However, the IC50 of the MCF-7/ADR cells was 800.853 nM doxorubicin. When compared to the IC50 of MCF-7 cells, the IC50 of MCF-7/ADR cells was elevated 88.91-fold (Fig. 1), which indicated that MCF-7/ADR cells were resistant to doxorubicin.

LncRNA microarray data of MCF-7 and MCF-7/ADR cells. MCF-7 and MCF-7/ADR cells were collected to perform a standard lncRNA microarray. The boxplot presented that after normalization the distribution of expression values of each sample was consistent to each other (Fig. 2A). The outcome of microarray demonstrated that 30,575 lncRNAs and 23,682 mRNAs were detected in total. The LncRNA expression patterns of the samples are presented as a hot-spot and cluster map (Fig. 2B and C). Compared to the MCF-7, 8,892 lncRNAs differentially expressed in MCF/ADR cells absolute fold-change ≥2.0, among which 3,594 were upregulated and 5,298 were downregulated.

Annotation of differentially expressed lncRNAs in MCF-7/ADR cells. To make a further investigation into the expression pattern of these differently expressed lncRNAs, general characteristics of these RNAs were taken into consideration, including the chromosome location, length distribution and classification. Chromosome 11 possessed the most upregulated lncRNAs, while chromosome 1 contained the most downregulated ones (Fig. 3A and B). The length of these lncRNAs was between 400 and 2,400 nt (Fig. 3C). The relationship between these lncRNAs and nearby coding genes included: i) Exon sense overlapping, where the exon of lncRNA overlaps with coding transcript exon on the same genomic strand; ii) intronic, where the lncRNA overlaps with intron of a coding transcript on the same genomic strand; iii) natural antisense, where the lncRNA is transcribed from the antisense strand and overlaps with a coding transcript; iv) non-overlapping antisense, where the lncRNA is transcribed from the antisense strand without sharing overlapping exons; v) bidirectional, where the lncRNA is oriented head to head to a coding transcript within 1,000 bp; and vi) intergenic, where there are no overlapping or bidirectional coding transcripts nearby the lncRNA. Among them, natural antisense consisted >40% in total differentially expressed lncRNAs (Fig. 3D).

GO and pathway analysis of differentially expressed lncRNAs. To research the potential functions of those dysregulated lncRNAs in MCF-7/ADR cells preliminarily, the associated target genes of those lncRNAs were predicted based on the principles of chromosome location of nearby coding genes and base-pairing. Subsequently, GO analysis was performed for the lncRNAs and the target genes. The GO project (http://www.geneontology.org) mainly covers biological process, molecular function and cellular component, as well as providing controlled annotations to describe gene and gene product attributed in any organism. In terms of the upregulated lncRNAs, the GO analysis results inferred that the relative gene products were primary involved in the biological process of biological adhesion, cell adhesion, nervous system development and system development (Fig. 4A); they were
mainly relative to the molecular functions such as binding, ion channel binding and protein binding (Fig. 4B). Cell junction, cell-cell junction, plasma membrane part were the main cell components those lncRNAs associated with (Fig. 4C). Among the downregulated lncRNAs, regulation of metabolic process, cell cycle and negative regulation of cellular process ranked the top three of ‘biological process’; protein binding, bitter taste receptor activity and binding were the top three of ‘molecular functions’. The majority of the cell components in which those lncRNAs involved in were intracellular and in the nucleus (Fig. 5A-C). In addition, the pathway results suggested that the upregulated lncRNAs were part of several signaling pathways, including systemic lupus erythematosus (hsa05322), alcoholism (hsa05034), calcium signaling pathway (hsa04020) and the Hippo signaling pathway (hsa04390). However, the downregulated lncRNAs participated in the following pathways: p53 signaling pathway (hsa04115), circadian rhythm (hsa04710), taste transduction (hsa04742), pathways in cancer (hsa05200) and cell cycle (hsa04110) (Fig. 6A and B). The P-value (EASE-score, Fisher P-value or hypergeometric P-value) indicates the significance of the GO term and pathway correlated to the conditions. The lower the P-value, more significant is the GO term and pathway (P<0.05 is recommended).

Validation of the dysregulated lncRNAs. In order to verify the expression levels of the dysregulated lncRNAs in the microarray pattern, 32 lncRNAs were selected for (RT-qPCR) by fold-change filtering (absolute fold-change >2.0), standard Student’s t-test (P<0.05), multiple hypothesis testing (FDR<0.05). Among the 32 lncRNAs, 17 were proven to be upregulated and 15 were downregulated. According to the RT-qPCR results, LOC100506178, AC007271.3, AC093673.5, RP11-113K21.5 and RP3-512B11.3 were upregulated in MCF-7/ADR cells when compared with MCF-7 cells, while AX747207, HOXC-AS5, RP11-152P17.2, RP11-1136G11.7, BC005081, RP11-143E21.7 and AC091878.1 were downregulated. Basically, the results of the RT-qPCR presented a considerable consistence with that of the microarray (Fig. 7A and B).

Discussion

Breast cancer has become a major cancer in females worldwide. According to international statistics, on a global scale for women in 2013, breast cancer caused the highest incidence and disability-adjusted life-years, and was regarded as the second largest cause of cancer death (25). Depending on the tumor grade/stage and the molecular characteristics of the malignancy, treatments vary, ranging from surgery, chemotherapy, radiation, hormone treatment and targeted therapy. Chemotherapy is the most commonly used treatment where it is often used with other therapies, even following surgery (26). However, chemoresistance has become a major reason for treatment failure (27,28).

Using lncRNA has been a novel field since the year 2000. Some research has been focused on the association between lncRNA and chemoresistance. LncRNA MEG3 and HOTAIR were demonstrated to contribute to cisplatin resistance of lung adenocarcinoma (19,20). LncRNA HOTTIP was reported to
promote gemcitabine resistance by regulating HOXA13 in pancreatic cancer (21). LncRNA linc-ROR was confirmed to contribute to the effects of transforming growth factor-β (TGF-β) on chemoresistance in hepatocellular carcinoma (22). LncRNA UCA1 was proven to increase the cisplatin resistance of bladder cancer cells by enhancing the expression of Wnt6 (29). HOTAIR may regulate breast cancer proliferation and chemoresistance using oncogenic lncRNA (30-33).

In the present study, the gene microarray results were verified by RT-qPCR. Totally, 32 lncRNAs were screened for
Among the dysregulated lncRNAs, some were associated with those genes that may be linked with chemoresistance of tumors. AX747207 is a sequence of 2,874 bp located on chromosome 1, which is relative to the gene RUNX3. The RUNX3 gene serves a critical role in the regulation of cell proliferation, apoptosis and angiogenesis, as well as cell adhesion and invasion (34,35). Additionally, RUNX3 functions as a tumor suppressor involved in the TGF-β signaling pathway.
Previously, some research indicated that RUNX3 was associated with the chemoresistance of tumors. Barghout et al. (37) reported that overexpression of RUNX3 rendered epithelial ovarian cancer cells more resistant to carboplatin, whereas inhibition of RUNX3 increased the sensitivity of epithelial ovarian cancer cells to carboplatin. In addition, Guo et al. (38) indicated that overexpression of Runx3 in gastric cancer cells sensitized the cells to chemotherapeutic drugs, while blocking Runx3 expression in immortalized gastric epithelial cells or gastric cancer cells conferred the cells multidrug resistance. Zhang et al. (39) demonstrated that, compared with human gastric adenocarcinoma cell line SGC7901, RUNX3 was significantly decreased in two multidrug resistance variants, SGC7901/ADR and SGC7901/VCR cells. Although the function of RUNX3 in breast cancer still remains unknown, this pathway in breast, colon, gastric and ovarian cancers (36).
gene deserves further research to reveal its association with chemoresistance in malignant tumors.

GO analysis and KEGG pathway analysis were both conducted among the differentially expressed lncRNAs. These lncRNAs are involved in regulating several biological processes (Figs. 4A and 5A), and the top three included regulation of metabolic process, cell cycle, and negative regulation of cellular process for downregulated lncRNAs and biological adhesion, cell adhesion, and nervous system development for upregulated lncRNAs, which are closely related to the malignancy of the tumor. In addition, the authors classified the potential functions into 10 categories through analyzing the target gene pool (Figs. 4B and 5B), and the top five for upregulated and downregulated lncRNAs respectively involved binding, ion channel binding, protein binding, metal ion binding, transmembrane receptor protein tyrosine phosphatase activity, protein binding, bitter taste receptor activity, binding, nucleic acid binding, and helicase activity.

Moreover, pathway analysis results indicated that these dysregulated lncRNAs primarily participated in the signaling pathways in Fig. 6A and B, and some were demonstrated to participate in chemoresistance of the tumor. The phosphatidylinositol 3-kinase (PI3K)-protein kinase B (Akt) signaling pathway (hsa04151) was identified to have a close connection with chemoresistance, not only in breast cancer, but also in pancreatic cancer, glioma, colon cancer, ovarian cancer, gastric cancer and hepatocellular carcinoma (40-48).

The Hippo signaling pathway (hsa04390) serves critical roles not only in mammary gland development but also in breast cancer. Besides, the Yes-associated proteins (YAP, YAP1), an effector of the Hippo signaling pathway, was reported to serve an important role in the chemoresistance of malignant tumors, including hepatocellular carcinoma and gastric cancer (49-53). Whether the Hippo-YAP signaling pathway participates in the chemoresistance of breast cancer requires further studies.
The p53 signaling pathway (hsa04115) was considered to take part in the chemoresistance process by interacting with other pathways. The role that p53 pathway plays in chemoresistance has been validated in breast cancer, renal cancer, glioma, lung cancer and colorectal cancer (54-58).

The ErbB signaling pathway (hsa04012) was also involved in the pathway analysis results. In this pathway, ErbB2 has been prominent in research as ~20% of human breast cancers overexpress the human epidermal growth factor receptor 2 (HER2) (59). HER2 belongs to the type I receptor tyrosine kinase family that includes four members: EGFR, HER2 (ErbB2/neu), HER3 and HER4 (60). HER2-positivity confers aggressive tumor growth, early metastases, worse prognosis and variable response to conventional chemotherapy (61,62). Zhang et al. (63) demonstrated that HER2 overexpression led to an increased resistance of MCF7 cells to multiple antitumor drugs, such as paclitaxel, cisplatin, etoposide, doxorubicin, mitoxantrone and 5-fluorouracil (5-FU), which was consistent to previous research (64-67). However, there are some discrepancies regarding chemoresistance by overexpression of HER2 in both laboratory and clinical studies. Coley's (68) study presented no link between HER2-overexpression or HER2 amplification and resistance to cytoxan/methotrexate/fluorouracil or to fluorouracil/epirubicin/cytoxan (68). Furthermore, further study is required to resolve the apparent contradictions between ErbB2 and chemotherapy resistance.

In the present study, microarray analysis was performed on MCF-7 and MCF-7/ADR cells to screen differentially expressed lncRNAs. The results indicated a potential chemoresistance-related lncRNA, AX747207, together with its associated target gene, RUNX3. In addition, the pathway analysis results provided some pathways that may potentially participate in the chemoresistance network, such as PI3K-Akt, p53, Hippo and ErbB signaling pathways. More future studies are required to confirm the roles that lncRNA AX747207 and...
the target gene RUNX3 serve and the relative pathways in chemoresistance of breast cancer.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

LH, LZ, JC participated in data acquisition, analysis and interpretation of data, and the writing and editing of the manuscript. ZF, HX and SW participated in the conception and design of the study. PX, ML, JX, JW, WL, LW and XW assisted with the analysis and interpretation of the data and the writing and editing of the manuscript.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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