Low expression of lncRNA CTB-104H12.5 induces resistance to adriamycin in HER2 negative breast cancer by downregulating GAS7C

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Primary research

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Abstract

Background Adriamycin-based neoadjuvant chemotherapy is widely used in HER2- breast cancer patients, however, some patients have no response or acquire resistance to these regimens. Therefore, it is important to screen patients who respond to adriamycin with biomarkers. Methods A human IncRNA microarray analysis followed by a quantitative real-time polymerase chain reaction (qRT-PCR) assay was utilized to identify the differentially expressed IncRNAs. The proliferation viability of cells transfected with or without CTB 104H12.5 was measured by CCK-8 and clone formation assay, cell apoptosis and cell cycle were evaluated by Flow cytometry. And the expression of RNA and proteins was tested by qRT-PCR and Western blot, respectively. Results Ten whole blood samples from HER2- breast cancer patients who received docetaxel combined with adriamycin regimen were enrolled, a human IncRNA microarray analysis showed that the low expression of CTB-104H12.5 was associated unfavorable prognosis. And the mRNA and protein level of CTB-104H12.5 is much lower in MCF-7/ADR cells than that of MCF-7 cells while CTB-104H12.5 knockdown in MCF cells induces cell resistance to adriamycin, while CTB-104H12.5 overexpression in MCF7/ADR cells reversed their resistance to adriamycin. Also, the mRNA and protein levels of GAS7C were associated with CTB-104H12.5. Conclusions Low expression of CTB-10412.5 predicted resistance to adriamycin in HER2 negative breast cancer, which may serve as a potential biomarker to dynamically monitor adriamycin resistance for breast cancer patients.

Background

Breast cancer is the most common cancer among women and the second-leading cause of cancer death worldwide[1]. Anthracyclines, like adriamycin (ADR), widely applied in the neoadjuvant treatment of breast cancer. However, many patients failed to benefit from them for drug resistance, which related to many factors, such as P-glycoprotein, drug efflux, DNA damage repair, target mutation, etc.[2]. Recent studies have indicated that non-coding RNAs (ncRNA) also play an important role in the drug resistance[3].

As one type of ncRNA transcripts, long non-coding RNAs (IncRNA) have been reported to function in a wide variety of cellular processes including drug resistance[4]. For example, NONHSAT101069, which serve as a ceRNA (competing endogenous RNA), promotes the epirubicin resistance of breast cancer cells by sponging miR-129-5p[5]. EPB41L4A-AS2 was identified as a potential biomarker for docetaxel sensitivity in breast cancer cells [6]. Knockdown of IncRNA-HOTAIR weakens the resistance of breast cancer cells to doxorubicin though PI3K/AKT/mTOR signaling while its overexpression contributes to tamoxifen resistance by promoting ER signaling[7, 8]. Besides HOTAIR,IncRNA H19 is also reported to promote tamoxifen resistance via autophagy[9]. And IncRNA TINCR can promote trastuzumab resistance and epithelial-mesenchymal transition (EMT) by targeting microRNA-125b[10]. Furthermore, extracellular IncRNA-SNHG14 was incorporated into exosomes and transmitted to sensitive breast cancer cells, thus inducing trastuzumab resistance[11]. However, all these IncRNAs were identified in drug-sensitive or resistant cell lines, which lower their values on predicting response to systemic treatments in breast cancer patients.
To explore the potential lncRNAs of predicting response to neoadjuvant chemotherapy (NAC) regimen consisting of docetaxel and adriamycin (TA regimen), we enrolled 10 breast cancer patients who treated with TA, divided into two groups according to prognosis. A human lncRNA microarray identified a novel lncRNA CTB-104H12.5 (Seqname: ENST00000578276, also named NONHSAG020811.2 or Lnc-MYH13-2) which associated with unfavorable prognosis of HER2- breast cancer patients, and adriamycin resistance in MCF7 and MCF7/ADR cells. In this study, we explored its contributions to the adriamycin resistance in breast cancer and its possible targeted genes.

Methods

Patients and blood samples collection

Ten biopsy-proven patients with invasive primary breast cancer treated with TA regimen at Chongqing University Cancer Hospital from April 2017 to October 2018 were enrolled in this study. The clinicopathological characteristics of the patients are summarized in Figure 1A. Whole blood was collected before any treatments, frozen in liquid nitrogen immediately, and preserved at -80°C until used. The clinical response was assessed according to RECIST criteria. Thus, patients with complete remission (CR) or partial remission (PR) were regarded as clinical responders, while patients with stable disease (SD) or progressive disease (PD) were regarded as non-responders for further analysis. The present study was approved by the Ethics Committee of Chongqing University Cancer Hospital, and all patients signed informed consent.

Microarray analysis

Total RNA was quantified by the NanoDrop 2000 and RNA integrity was assessed by standard denaturing agarose gel electrophoresis. The sample preparation and microarray hybridization were performed based on the manufacturer’s standard protocols. The microarray data were selected by threshold values of >2 and < 2-fold change under FDR protection (P < 0.05).

Cell culture

The breast cancer cell lines MCF-7 and MCF-7/ADR (adriamycin-resistant) were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). The cells were cultured in DMEM (Gibco, Suzhou, China) supplemented with 10% heat-inactivated fetal bovine serum (Zhejiang TianHang Biotechnology Co. LTD, Hangzhou, China) 100 units/ml penicillin and 100 μg/ml streptomycin (KeyGen Biotech, Jiangsu, China) in a humidified atmosphere at 37 °C under 5% CO2. The MCF-7/ADR cells were cultured in the above-mentioned media that were additionally supplemented with 1 mg/mL ADR (KeyGen Biotech, Nanjing, China) to maintain the drug-resistant phenotype.

Transfection

LncRNA CTB-104H12.5 small interfering RNAs (si-122, si-1365, si-2484), negative control (si-control), LncRNA CTB-104H12.5 vector (CTB-104H12.5) and control vector (Control) were purchased from RiboBio
Guangzhou, China). The cells were transfected with Lipofectamine® 2000 (Invitrogen, USA) according to the manufacturer's instructions. Cells were harvested for further evaluation at 48 h after transfection. The sequence of siRNA as followed: si-control, 5’-GAGAAACAATTAAAAATACCC-3’, si-122, 5’-CTCAAAAAAAGAAGTGGACACT-3’, si-1365, 5’-TTGGCCCCAAGCATACAGCCCCCTA-3’, si-2484, 5’-TGAGCTATGATTGTACCCT-3’

CCK8 assays

The viability ability and ADR chemosensitivity were detected by CCK8 assay (Biotool, Shanghai, China) according to the manufacturer’s instructions. Briefly, MCF-7 and MCF-7/ADR cells were plated at the density of 1×10^4 cells per well in 96-well plates and incubated with 100 µl anticancer drugs adriamycin (KeyGen Biotech, Nanjing, China) at a final concentration of (0.02, 0.1, 0.5, 1, 2.5, 5, 10) µg/ml and (1, 2.5, 5, 10, 20, 40, 60) µg/ml for 48 h, respectively. Each well was added with a 10µl CCK-8 working solution and incubated for another one hour. The absorbance was tested at a wavelength of 450nm on the synergy2 microplate reader (BioTek, NV, USA). The IC50 values were calculated with these absorbance data. Each sample was performed in triplicate and repeated three independent experiments.

Colony formation assay

A colony formation assays were conducted to evaluate cell proliferation capability. Briefly, cells (500 cells/well) were seeded into 6-well plates and transfected with siRNA or CBT 104H12.5 vectors. Then, the plates were incubated for 8 days and the medium was refreshed every two days. Cells were fixed in 10% formaldehyde for 40min and stained with a 0.1% crystal viola solution for 20min. Finally, the clone number was counted under a microscope.

Apoptosis and cell cycle analysis

Twenty-four hours after the transfection as described above, ADR was added, with the final concentration of 10 mg/mL for MCF-7/ADR and 1 mg/mL for MCF-7. After incubation for 24 hours, cells were harvested and washed twice with cooled PBS and stained with FITC Annexin V and PI. Cell apoptosis was measured by a FITC Annexin V apoptosis detection kit (KeyGen Biotech, China) while cells were stained by PI for cell cycle analysis according to the manufacturer's protocols. Analyses were conducted by FACS flow cytometer (BD Biosciences, San Jose, CA, USA).

RNA isolation and qRT-PCR assay

Total RNA was extracted from whole blood or cells using the Trizol reagent (TianGEN, Beijing, China) according to the manufacturer's instructions. Reverse transcription was performed with PrimeScript RT-PCR Kit (Takara Bio companies, Beijing, China) following the manufacturer's protocols. The qRT-PCR analysis was conducted with the SYBR Green PCR master mix (QIAGEN, Germany). The RNA amount of target genes was quantified by the 2^-ΔΔCT method, and GAPDH used as an internal control. Primer sequences are listed as followed. Each PCR assay was performed on Applied Biosystems 7500 for
triplicates. GAPDH: Forward 5’-TGTTCGTCATGGGTGTGAA-3’, Reverse 5’-ATGGCATGGACTGTGGTCAT-3’, RP11-53019.: Forward, 5’-CATGGTGACGGACCTTGGAC-3’, Reverse 5’-TCGGGACTACAGGAAGAGTGGAA-3’, RP11-264F23.3, Forward, 5’-CATGCAGCCATCCATTGTTG-3’, Reverse 5’-GGTCTGATAGCCAGCCAAG-3’, RP11-414K1.3, Forward 5’-GGAACGGCAAGCAGAAAGAT-3’, Reverse 5’-GGCTGGGACACTGGAAACATA-3’, CHEK1, Forward 5’-GGAAAGCATTGGTCTCCACC-3’, Reverse 5’-TAAATCAATCGCCACTCCC-3’, CTB-104H12.5, Forward 5’-CCTCCTTTTGATCCATAACCTG-3’, Reverse 5’-CGTCCCTTTGAATGTCTGTCC-3’, ARHGEF26, Forward 5’-ATGGAGATGAAGGATGGGAGAT-3’, Reverse 5’-AGCGGACAGTATGGGGAGCAGAT-3’, GAS7C, Forward 5’-CGAGCTACGTCAGTTGCT-3’, 5’-CATGTGGGCAGTCTCTGGAG-3’

**Western blot analysis.**

Cells were harvested and suspended in RIPA buffer (TianGEN, Beijing, China) and quantified with a BCA protein assay kit (Boster, Wuhan, China). After denatured by boiling for 5 minutes, total protein samples (10μg each sample) were separated with 10% SDS-PAGE and subsequently transferred into the PVDF membrane (EMD Millipore, Billerica, MA, USA). Then the membranes were blocked by 5% non-fat milk for 1 h at 37°C, incubated overnight at 4°C with following primary antibodies: anti-CDK1, anti-Cyclin D1, anti-Caspase-3, anti-Bcl-2 (Cell Signaling Technology, USA), anti-GAS7C (Invitrogen, USA) and anti-GAPDH (Santa Cruz, USA). And Horseradish peroxidase-conjugated anti-mouse, anti-rabbit antibodies were used as secondary antibodies. The bands were visualized by LICOR C-digit (Biocompare, USA) and quantified by ImageJ. Each experiment was conducted at least three times.

**Statistical analysis**

All data are displayed as mean ± standard deviation (SD) and analyzed by using SPSS 17.0 software (SPSS, Chicago, IL, USA). The Student’s t-test was used to evaluate the significance of differences in multiple comparisons. The statistical significance was defined as *P* < 0.05, **P* < 0.01 or ***P* < 0.001. All experiments were performed at least three times.

**Results**

**LncRNA CTB-104H12.5 is downregulated in ADR resistant cells and whole blood samples of breast cancer patients who have no response to the TA regimen.**

To systematically explore the lncRNA biomarkers for predicting the response to adriamycin, we enrolled 10 HER2- breast cancer patients (Figure 1A) who received TA regimen, divided into 2 groups according to prognosis. A human lncRNA microarray analysis revealed that there were 1564 up-regulated and 838 down-regulated between these 2 groups, the top 100 most significantly down-regulated lncRNAs were shown in Figure 1B.

Then the expression of the top six lncRNAs was tested in MCF/ADR and MCF7 cells by qRT-PCR. Among them, CTB-104H12.5 was downregulated mostly in MCF7/ADR cells (Fig 2B). Furthermore, CTB-104H12.5 expression was examined by qRT-PCR in breast cancer whole blood of clinical responders (n = 5) and
non-responders (n = 5). As shown in Fig 1C, CTB-104H12.5 expression was considerably downregulated in non-responders compared with that in the responders. The above data indicated that CTB-104H12.5 involved in adriamycin resistance in breast cancer.

**Knockdown of CTB-104H12.5 induces adriamycin resistance in MCF-7 cells**

To uncover the function of CTB-104H12.5 in adriamycin resistance, we constructed three siRNAs to knockdown CTB-104H12.5 and si-1365 was used in the following assays for its highest interferent efficiency (Figure 2A). As shown in Figure 2B, the IC50 value of MCF7 cells that transfected with si-1365 was about 2.5-fold as much as control cells transfected with empty vectors (si-control). Additionally, the cell proliferation capacity of MCF-7 cells transfected si-1365 increased in MCF-7 cells (Figure 2C).

Moreover, ADR-induced apoptosis remarkably decreased in si-1365-transfected MCF-7 cells, and the percentage of cells in the G1 phase also decreased while that of S and G2 increased (Figure 2D and 2E). Data shown in Figure 2F implied that knockdown of CTB-104H12.5 could upregulate the protein level of CDK1 and cyclin D1, which promote cell cycle transit from G1 phase to S phase[12], and downregulate the level of Caspase 3 and Bcl2, which serve as markers of apoptosis[13]. All these data supported that knockdown of CTB-104H12.5 can induce MCF7 cells resistant to adriamycin.

**Overexpression of CTB-104H12.5 reverses adriamycin resistance in MCF7/ADR cells.**

To further confirm the CTB-104H12.5 function, MCF7/ADR cells were transfected with CTB-104H12.5. As data are shown in Figure 3A, the IC50 of the MCF7/ADR cells transfected with CTB-104H12.5 was about half of that of control cells (10.35±1.84μg/ml vs 20.40±3.45μg/ml), and their proliferation ability was also weaker than MCF7/ADR cells transfected with control vector (Figure 3B). Moreover, the ADR-induced apoptosis remarkably increased in CTB-104H12.5-transfected MCF7/ADR cells (Figure 3C). Similarly, CTB-104H12.5 overexpression prevented MCF-7/ADR cells from transiting to S phase, which caused the percentage of cells at the G1 phase to increase (Figure 3D). Western blot data showed that CTB-104H12.5 overexpression in MCF-7/ADR cells displayed opposite results compared to CTB-104H12.5 knockdown in MCF7 cells (Figure 2F and Figure 3E), the protein level of CDK1 and Cyclin D1 decreased while that of Caspase 3 and Bcl2 increased. Taken all those data together, we concluded that the low level of CTB-104H12.5 may predict resistance to adriamycin in breast cancer.

**GAS7C is associated with CTB-104H12.5, may serve as one of its targets.**

Bioinformatics analysis suggested that GAS7C introns partially overlapped with CTB-104H12.5, which gave us a hint that GAS7C may serve one of its targets. To explore the relationship between CTB-104H12.5 and GAS7C, we tested the mRNA and protein level of CTB-104H12.5 and GAS7C in MCF-7 transfected with or without si-1365 and MCF-7/ADR cells with or without CTB-104H12.5. Data showed that the mRNA and protein levels of GAS7C declined in MCF-7 cells transfected with si-1365 while raised in MCF-7/ADR cells transfected with CTB-104H12.5 (Figure 4A and 4B). Meanwhile, the mRNA and protein levels of GAS7C in MCF7 cells were higher than those in MCF7/ADR cells, which was consistent
with the level of CTB-104H12.5 (Figure 4C). These results suggested that GAS7C was associated with
CTB-104H12.5, which may be one of its target genes. However, more data are needed to clear GAS7C
roles in adriamycin resistance.

Discussion

Adriamycin is one of the most choices in breast cancer neoadjuvant chemotherapy, but some patients are
not sensitive to those regimens based on adriamycin. So, it is necessary to screen patients who can
benefit from regimens containing adriamycin. Several biomarkers have been reported to be able to predict
the efficacy of anthracycline-based chemotherapy in breast cancer, for example, the expression levels of
CREB3L1 in TNBC responsive to doxorubicin-based chemotherapy were significantly higher than that in
resistant cancers[14], and loss of FKBP12 function in invasive breast cancers predicts poor prognosis
and chemo resistance to doxorubicin[15] while HOXD3 overexpression enhances breast cancer cell drug
resistance via integrin β3-mediated Wnt/β-catenin signaling[16]. Recently, increasing evidence has
supported that ncRNAs also play an important role in drug resistance of breast cancer[17, 18]. Among
these ncRNAs, IncRNAs, as the promising molecular markers for forecasting drug resistance of breast
cancer, are becoming new targets for treating breast cancer in the future. For example, LOC645166,
IncRNA GAS5, and IncRNA H19 regulate adriamycin resistance in breast cancer cells[4, 19, 20], and
IncRNA UCA1 and IncRNA NONHSAT141924 modulates paclitaxel resistance[21, 22], while IncRNA
HOTAIRM1 and BLACAT1 can induce tamoxifen resistance in ER+ breast cancer cells[23, 24]. However, all
these IncRNAs were identified from breast cancer tissue or cell lines, which limits their utilities in the clinic
as a dynamic monitoring biomarker.

To explore the liquid IncRNA biomarkers that can predict patients’ response to standard anthracycline-
based chemotherapy, we analyzed the differential expression of IncRNAs in whole blood samples from
breast cancer patients who treated with TA regimen. According to the analysis results of RNA-seq and
treatment response to TA regimen, we found that the expression level of CTB-104H12.5, a novel IncRNA,
was associated with adriamycin resistance, which had been confirmed in MCF-7 and MCF-7/ADR cells
(Fig. 1). And the expression difference was also verified in the clinical blood sample by qRT-PCR.

Then the role of CTB-104H12.5 in adriamycin resistance was confirmed in breast cancer cells by
regulating CTB-104H12.5 expression level. Transfection with CTB-104H12.5 small interference RNA
induced adriamycin resistance in MCF-7 cells while CTB-104H12.5 overexpression reversed adriamycin
resistance in MCF-7/ADR cells (Figs. 2 and 3). Moreover, the bioinformatics analysis indicated that the
location of CTB-104H12.5 has partially overlap with that of gene GAS7, which involves gefitinib
resistance[25]. So, we speculated that GAS7 may play a role in CTB-104H12.5-mediated adriamycin
resistance. Western blot and qRT-PCR data suggested that the expression of GAS7C, one isoform of GAS7
which correlates with poor prognosis and promotes metastasis in lung cancer[26], showed similar trends
as CTB-104H12.5 in MCF7 and MCF7/ADR cells (Fig. 4C). And the mRNA and protein level of GAS7C
increased in CTB-104H12.5 overexpressed MCF7/ADR cells while reduced in CTB-104H12.5 interfered
MCF7 cells (Fig. 4A and 4C).
GAS7 is expressed primarily in terminally differentiated brain cells and plays a putative role in neuronal development[27]. Recent researches supported that GAS7 involves cancer development and metastasis[28, 29]. Moreover, GAS7 was also reported to associate with drug resistance. For example, GAS7B knockdown can desensitize neuroblastoma cells to cisplatin[30]. Downregulation of GAS7 expression could antagonize gefitinib re-sensitivity in gefitinib resistance non-small cell lung cancer cells[25]. Consistently, our data that GAS7C expression in MCF-7/ADR was lower than that in MCF-7 cells, which suggested GAS7C may contribute to CTB-104H12.5 associated adriamycin drug resistance in breast cancer cells, but the underlying mechanism that how CTB-104H12.5 regulates GAS7 expression remains to be further elucidated.

**Conclusions**

Our study identified a novel IncRNAs CTB-104H12.5 that contributes to adriamycin drug resistance in breast cancer by regulating GAS7. Our current data provide a novel perspective on understanding the underlying mechanism of adriamycin resistance. Especially, CTB-104H12.5 was identified in patients’ whole blood samples, not tissue or cell lines, which makes it to be a potential liquid biomarker for predicting the efficiency of adriamycin-based neoadjuvant chemotherapy in breast cancer.

**Declarations**

**Ethics approval and consent to participate**

The present study was approved by the Ethics Committee of Chongqing University Cancer Hospital (NO.: CZLS2020156-A), and all patients signed informed consent.

**Consent for publication**

Not applicable

**Availability of data and material**

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

**Competing interests**

The authors declare that they have no competing interests.

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Authors' contributions

Weiqi Nian: Conceptualization, Methodology. Yongpeng He: Conceptualization, Data curation, Writing Original draft preparation. Weiwei Xue: Validation. Wanyan Tang: Visualization, Investigation. Zheng Li: Visualization, Formal analysis, Software. Jing Ran: Investigation. Changhai Lin: Methodology, Validation. Enwen Wang: Resources, Writing- Reviewing and Editing Haiwei Zhang: Supervision.

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**Figures**
Figure 1

The expression of CTB-104H12.5 in breast cancer whole blood and cells. (A) Clinical characteristics of breast cancer patients. TNM, tumor, node, metastasis; ER, estrogen receptor; PR, progesterone receptor; HER-2, human epidermal growth factor receptor-2; CR, complete remission; PD, progressive disease; PR, partial remission; SD, stable disease, TA, docetaxol combined with adriamycin. (B) Heatmap of differentially expressed lncRNAs in whole blood samples from breast cancer patients (n = 5). (C) Top six downregulated lncRNAs were detected in MCF-7 and MCF-7/ADR cells by qRT-PCR. (D) The expression of CTB-104H12.5 was tested in whole bloods from responders and non-responders by qRT-PCR (n = 5), and the difference was tested by paired t-test, **P<0.01.
Figure 2

Knockdown of CTB-104H12.5 in MCF-7 cells induced adriamycin resistance. (A) The interference efficiency of CTB-104H12.5 verified by qRT-PCR. (B) MCF-7 cells transfected with negative control and CTB-104H12.5 siRNA-1365 were exposed to a series dose of ADR (0.02, 0.1, 0.5, 2.5, 5, and 10 μg/mL) for 48 hours, cell viability was detected by CCK-8 and IC50 of ADR was calculated with Excel. (C) The proliferation capacity of MCF-7 cells was assessed by colony formation assays. (D) The percentage of apoptosis was analyzed by flow cytometry, experiment repeated three times. the value was represented as mean ±S.D. (E) Cells at various cell stage were counted by flow cytometry, experiments repeated three times. (F) The protein levels of CDK1, cyclin D1, Caspase 3 and Bcl2 were evaluated by Western blot, and quantified by Image J. and the difference was tested by paired t-test, **P<0.01, ***P<0.001.
Figure 3

Overexpression of CTB-104H12.5 in MCF-7/ADR cells reversed adriamycin resistance. (A) MCF-7/ADR cells transfected with vector control and CTB-104H12.5 were exposed to a series dose of ADR (1, 2.5, 5, 10, 20, 40 and 60 μg/mL) for 48 hours, cell viability was detected by CCK-8 assay and adriamycin IC50 was calculated with Excel. (C) The proliferative capacity was assessed by colony formation assays. (D) The apoptosis percentage was evaluated by flow cytometry, experiments repeated three times. the value was represented as mean ±S.D. (E) The percentage of cell at different stage was evaluated by flow cytometry, experiment repeated three times. (F) The expression levels of CDK1, cyclin D1, Caspase 3 and Bcl2 were evaluated by Western blot, and quantified by Image J. and the difference was tested by paired t-test, *P<0.01, **P<0.01, ***P<0.001.
The expression level of GAS7C associated with that of CTB-104H12.5. (A) The protein level of GAS7C was analyzed by Western blot in MCF7 cells, experiments were replicated three times and quantified by Image J. and the difference was tested by paired t-test. (B) The level of GAS7C was analyzed by Western blot in MCF7/ADR cells, experiments were replicated three times and quantified by Image J. and the difference was tested by paired t-test. (C) The mRNA levels of GAS7C and CTB-104H12.5 were evaluated by qRT-PCR, assays were repeated three times, and the difference was tested by paired t-test. *P<0.01, **P<0.01, ***P<0.001.