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Hsa_circ_0003998 Promotes Chemoresistance via Modulation of miR-326 in Lung Adenocarcinoma Cells

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Circular RNAs (circRNAs) represent a new class of noncoding RNAs that is involved in the development of cancer. However, little is known about their role in chemoresistance. In the present study, we found that hsa_circ_0003998 expression levels in lung adenocarcinoma (LAD) tissues and docetaxel-resistant cell lines (A549/DTX and H1299/DTX) were upregulated. Knockdown of hsa_circ_0003998 decreased chemoresistance, inhibited proliferation, and enhanced apoptosis in docetaxel-resistant LAD cells. Moreover, by using bioinformatics and luciferase reporter assays, we found that miR-326 was a direct target of hsa_circ_0003998. Functional analysis revealed that miR-326 mediated the effect of hsa_circ_0003998 on chemosensitivity. Our findings provide a molecular insight on understanding drug resistance in LAD cells. Therefore, inactivation of hsa_circ_0003998 or activation of miR-326 could be a novel approach for the treatment of LAD.

Key words: Circular RNA (circRNA); hsa_circ_0003998; Chemoresistance; miR-326; Lung adenocarcinoma (LAD)

INTRODUCTION

Lung cancer is a predominant cause of cancer-related deaths worldwide[1]. Approximately 85% of lung cancer cases are non-small cell lung cancer (NSCLC), among which lung adenocarcinoma (LAD) is the most common type[2]. Currently, chemotherapy is the main strategy used to manage advanced LAD. However, successful treatment is often hampered by the development of drug resistance[3,4]. Thus, a detailed understanding of the mechanisms underlying chemoresistance is essential to improve treatment of LAD patients.

Circular RNAs (circRNAs) are another class of noncoding RNAs that are widely expressed in mammals. They have covalently linked ends of a single RNA molecular and appear highly stable compared to their linear types[5,6]. Recently, certain kinds of circRNAs have been shown to be aberrantly expressed in gastric cancer, colorectal cancer, and prostate cancer, and these deregulated circRNAs are suggested to participate in cancer development[7–9].

Hsa_circ_0003998 is one of the circRNAs with 304 nt in spliced sequence length. Its gene is located at chr20:47570092-47580435. Abnormal expression of hsa_circ_0003998 has been detected in breast cancer and renal cell carcinoma through RNA-seq and microarray[10].To date, however, the function of hsa_circ_0003998 remains unknown. In this study, we examined the expression of hsa_circ_0003998 in docetaxel-resistant LAD cells. Also, we further explored the biological function and mechanism of hsa_circ_0003998. Our results showed that hsa_circ_0003998 was upregulated in LAD tissues and promoted the acquisition of chemoresistance in docetaxel-resistant LAD by modulation of miR-326.

MATERIALS AND METHODS

Cell Culture and Transfection

Human lung adenocarcinoma A549 and H1299 cells were purchased from the Institute of Biochemistry and Cell Biology of the Chinese Academy of Sciences (Shanghai, P.R. China). Docetaxel-resistant cell lines

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A549/DTX and H1299/DTX were established in our laboratory. Cells were maintained in RPMI-1640 medium containing 10% fetal bovine serum (FBS) with 100 μg/ml penicillin/streptomycin at 37°C with 5% CO_2. Small interfering RNA (siRNA), miR-326 mimics, and inhibitors were synthesized by GenePharma Company (Shanghai, P.R. China). Transfection was performed with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. The sequence of siRNA targeting hsa_circ_0003998 is as follows: 5 -UUAAAGUUGCAGGAGGCCAGA-3 .

Tissue Samples

A total of 30 LAD tissues and pair-matched noncancerous tissues were obtained from patients diagnosed with advanced LAD at Yinzhou Hospital Affiliated to Medical School of Ningbo University. Informed consent was obtained from patients, and this study was approved by the Clinical Research Ethics Committee of Ningbo University.

RNA Extraction and qRT-PCR Analyses

Total RNA was extracted from cells or the tissue samples using TRIzol (Invitrogen). Real-time quantitative (qRT)-PCR analysis was performed with TransStart Tip Green qPCR SuperMix (TransGen Biotech, Beijing, P.R. China) according to the manufacturer's protocol, and GAPDH was utilized as an internal control. The primers used in this study were as follows: hsa_circ_0003998 forward, 5 -AAA GAG GCT CAT CAC TGT CAG G-3 , reverse, 5 -GGA CTG GGG TTT TGA CTG GAT-3 ; GAPDH forward, 5 -TCA GTG GTG GAC CTG ACC TG-3 , reverse, 5 -TGC TGT AGC CAA ATT CGT TG-3 .

MTT and Colony Formation Assays

The MTT assay was used to assess the 50% inhibition concentration (IC_{50}) value for the drugs as described previously[11]. For colony-forming assays, after 48 h of transfection, the cells were reseeded in six-well plates at 500 or 1,000 cells per well, and the medium was replaced every 3 days. After incubation at 37°C for 2 weeks, the cells were fixed with methanol and stained with 0.5% crystal violet. The number of colonies was counted under a microscope.

Flow Cytometric Analysis

Cells were harvested after transfection for 48 h via ethylene diamine tetraacetic acid-free trypsinization. Apoptosis rate was detected via flow cytometric analysis using Annexin V-FITC Apoptosis Detection Kit (Transgen Biotech) according to the manufacturer's instructions.

Luciferase Reporter Assay

The fragment of hsa_circ_0003998 was cloned to the downstream of the *Renilla* pGL3 vector. The reporter plasmid was cotransfected with a control *Renilla* luciferase vector into A549 cells in the presence of either miR-326 or negative control (NC). After 48 h, cells were harvested, and the luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA).

Statistical Analysis

All data from three independent experiments were expressed as mean ± SD. Differences were assessed by two-tailed Student's *t*-test. The association between miR-326 and hsa_circ_0003998 expression was explored by Pearson correlation. SPSS version 17.0 software (SPSS Inc., Chicago, IL, USA) was employed to analyze the data. A value of $p < 0.05$ was considered statistically significant.

RESULTS

Hsa_circ_0003998 Is Upregulated in LAD Tissues and Docetaxel-Resistant Cell Lines

To examine whether hsa_circ_0003998 is involved in the molecular etiology of chemoresistance in LAD, we detected hsa_circ_0003998 expression in 30 paired LAD tissues and adjacent normal tissues using qRT-PCR. As shown in Figure 1A, hsa_circ_0003998 expression was increased in cancerous tissues compared with normal counterparts. We also evaluated hsa_circ_0003998 expression in docetaxel-resistant cells. The results showed that hsa_circ_0003998 was upregulated in docetaxel-resistant A549/DTX and H1299/DTX cells compared with parental cells (Fig. 1B). These results imply that hsa_circ_0003998 may play important roles in docetaxel-resistant of LAD.

Knockdown of hsa_circ_0003998 Reverses Docetaxel Resistance in LAD Cells

To confirm the functional role of hsa_circ_0003998 in docetaxel resistance, siRNA for hsa_circ_0003998 was transfected into A549/DTX and H1299/DTX cells, respectively. Satisfactory transfection efficiency was obtained at 48 h posttransfection (Fig. 2A). MTT assay revealed that suppression of the hsa_circ_0003998 level in docetaxel-resistant cells resulted in an enhanced sensitivity to docetaxel as indicated by substantially decreased IC_{50} values (Fig. 2B). In addition, colony formation assays demonstrated that growth of A549/DTX and H1299/DTX cells transfected with siRNA was attenuated compared with control cells (Fig. 2C). Furthermore, flow cytometry analysis revealed that knockdown of hsa_circ_0003998 expression induced apoptosis in docetaxel-resistant cells (Fig. 2D).

Identification of miR-326 as a Target of hsa_circ_0003998

Because circRNAs function mainly as miRNA sponges to bind functional miRNAs and then regulate gene expression, we next examined the potential miRNAs

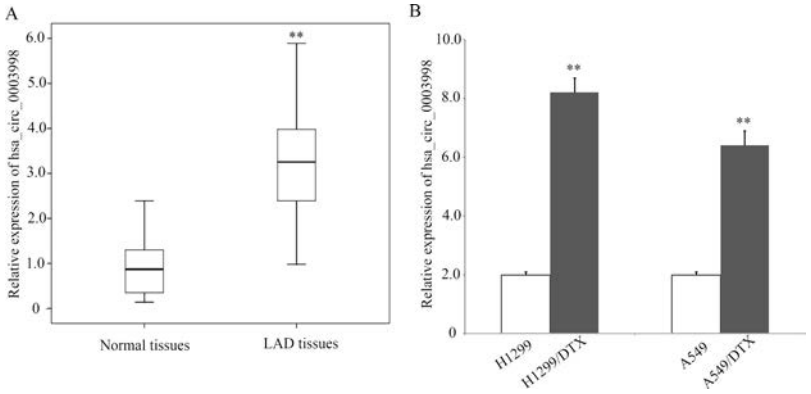

Figure 1. Hsa_circ_0003998 expression was upregulated in lung adenocarcinoma (LAD) tissues and docetaxel-resistant cell lines. (A) Real-time quantitative (qRT)-PCR analysis of hsa_circ_0003998 expression in 30 paired LAD and their corresponding normal samples. (B) Expression level of hsa_circ_0003998 in parental and docetaxel-resistant LAD cell lines. The level of hsa_circ_0003998 expression was normalized to GAPDH. $^{**}p < 0.01$.

associated with hsa_circ_0003998. By using bioinformatics program (Circular RNA Interactome, https://circinteractome.nia.nih.gov/index.html), we selected miR-326 as a possible target of hsa_circ_0003998 (Fig. 3A). As illustrated in Figure 3B, the expression levels of miR-326 in A549/DTX and H1299/DTX were lower than those in the corresponding parental cells. After knockdown of hsa_circ_0003998, miR-326 expression was increased compared with control (Fig. 3C). To further validate the regulatory relationship between hsa_circ_0003998 and miR-326, we performed dual-luciferase reporter assay. As shown in Figure 3D, miR-326 mimics reduced the luciferase activity of wild-type hsa_circ_0003998 reporter vector but not that of mutant reporter vector. These data confirmed the direct binding between hsa_circ_0003998 and miR-326.

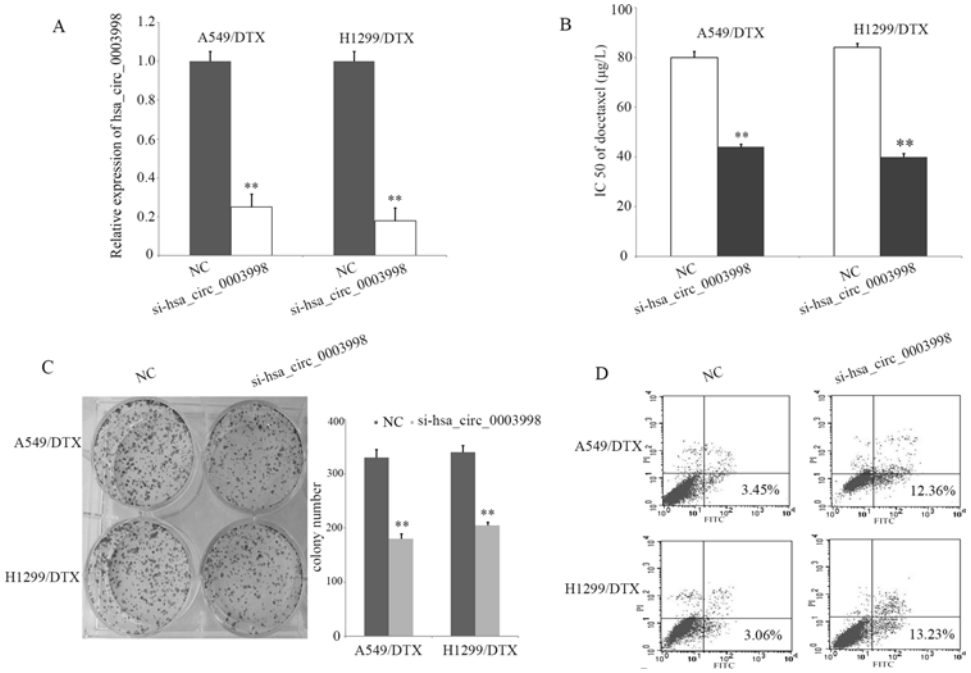

Figure 2. Knockdown of hsa_circ_0003998 reverses chemoresistance in docetaxel-resistant LAD cells. (A) qRT-PCR analyses of hsa_circ_0003998 expression level following treatment of A549/DTX and H1299/DTX cells with small interfering RNAs (siRNAs) targeting hsa_circ_0003998. (B) IC_{50} values for docetaxel in A549/DTX and H1299/DTX cells transfected with si-hsa_circ_0003998. Colony formation (C) and flow cytometry (D) assays on A549/DTX and H1299/DTX cells transfected with si-hsa_circ_0003998. $^{**}p < 0.01$.

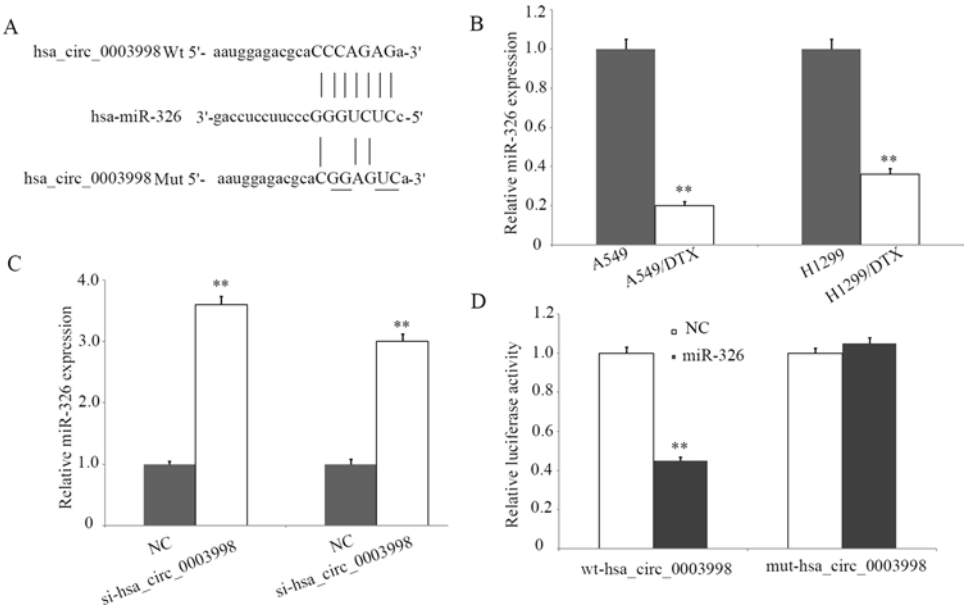

Figure 3. miR-326 is a direct target of hsa_circ_0003998. (A) The predicted miR-326/hsa_circ_0003998 binding site and its mutated version by site mutagenesis are as shown. (B) qRT-PCR analysis of miR-326 in parental and docetaxel-resistant LAD cells. (C) Expression of miR-326 following knockdown of hsa_circ_0003998. (D) Luciferase assays were performed to determine the interacting activity between miR-326 and hsa_circ_0003998. $^{**}p < 0.01$.

miR-326 Mediated the Effect of hsa_circ_0003998 on Chemosensitivity

To confirm the functional role of miR-326 in docetaxel resistance, we detected miR-326 expression in 30 paired LAD tissues and adjacent normal tissues. The results showed that miR-326 was dramatically downregulated in LAD tissues (Fig. 4A), and a significant negative correlation was also found between hsa_circ_0003998 and miR-326 expression (Fig. 4B). Besides, colony formation assays indicated that the proliferation ability of A549/DTX cells cotransfected with hsa_circ_0003998 siRNA and miR-326 inhibitors was improved compared with A549/DTX cells transfected with hsa_circ_0003998 siRNA (Fig. 4C). Moreover, the increased sensitivity to docetaxel caused by hsa_circ_0003998 siRNA was significantly reversed by miR-326 inhibitors (Fig. 4D). These data indicate that hsa_circ_0003998 inducing docetaxel resistance on LAD cells may partly improve the proliferation ability of A549/DTX cells through repressing miR-326 expression.

DISCUSSION

In this study, we found the expression level of hsa_circ_0003998 was significantly higher in LAD tissues and docetaxel-resistant cell lines. Knockdown of hsa_circ_0003998 increased sensitivity to docetaxel, inhibited proliferation, and enhanced apoptosis. These results implicated that hsa_circ_0003998 may play an important role in docetaxel-resistant LAD.

The functions of circRNAs in cancer progression have not been elucidated very clearly. The most reported function pattern for circRNAs is acting as miRNA sponge. The "sponge" characteristic was similar to long noncoding RNAs (lncRNAs), suggesting its potential regulation via binding miRNAs[12–14]. For example, CDR1 antisense RNA (Cdr1as, also known as CiRS-7), one of representative circRNAs, was shown to harbor 76 miR-7 binding sites[15]. circRNA MTO1 suppresses HCC progression by acting as the sponge of oncogenic miR-9 to promote p21 expression[16]. In lung adenocarcinoma, hsa_circ_0013958 was identified as a sponge of miR-134, and hsa_circ_0013958 levels were associated with TNM stage and lymphatic metastasis[17]. Here we used bioinformatics analysis and provide evidence that hsa_circ_0003998 acts as a potential chemoresistance promoter via modulation of miR-326.

miR-326, a recognized tumor-suppressing miRNA, has been shown to be downregulated in a variety of diseases including cancers, such as pulmonary fibrosis[18], colorectal cancer[19], lung cancer[20], and glioma[21]. Liang et al. reported that miR-326 is involved in chemotherapy resistance of breast cancer through modulating expression of multidrug resistance-associated protein 1[22]. Our previous studies have revealed that miR-326 can restore chemosensitivity in the human lung adenocarcinoma cells by targeting specificity protein 1 (SP1)[23]. In the present study, we demonstrated that miR-326 is negatively associated with hsa_circ_0003998 expression in LAD

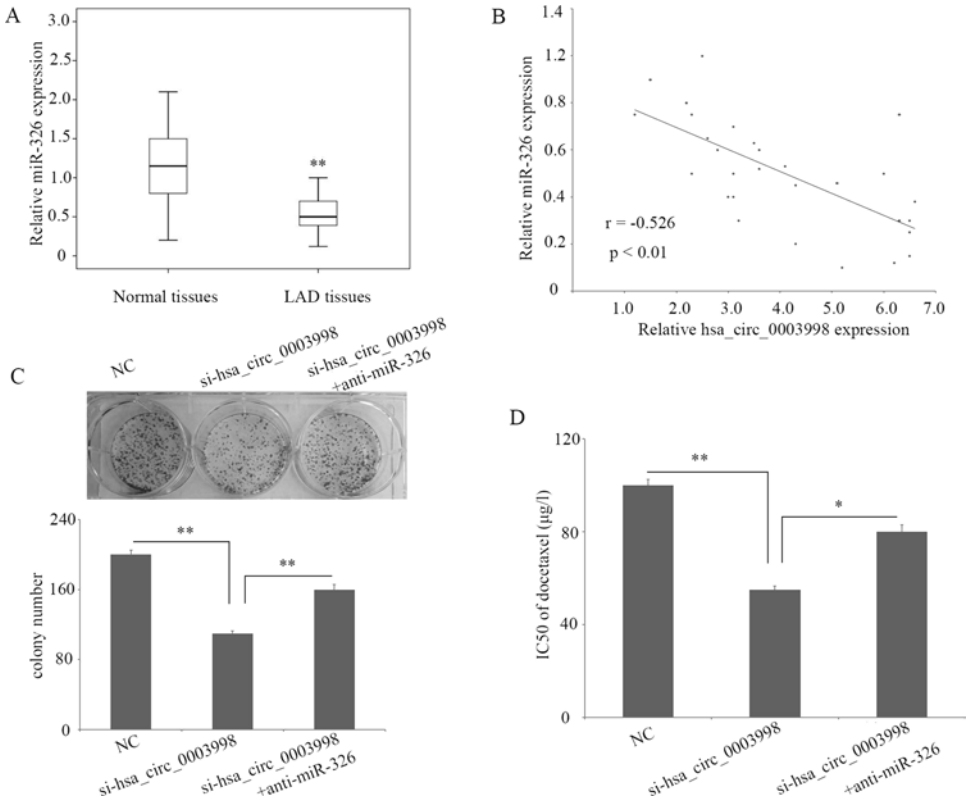

Figure 4. miR-326 mediated the effect of hsa_circ_0003998 on chemosensitivity. (A) Relative expression levels of miR-326 were detected in LAD tissues via qRT-PCR. (B) Expression levels of miR-326 are negative correlation with hsa_circ_0003998 among LAD samples ($n = 30$) as indicated by two tailed Pearson's correlation analysis ($r = -0.526$, $p < 0.01$). Colony formation (C) and MTT (D) assays were used to determine the cell proliferation and IC_{50} of docetaxel for A549/DTX cells transfected with NC and si-hsa_circ_0003998 and cotransfected with si-hsa_circ_0003998 and anti-miR-326. *$p < 0.05$, **$p < 0.01$.

tissues. Particularly, miR-326 mediated the effect of hsa_circ_0003998 on chemosensitivity. These results indicate that the hsa_circ_0003998-miR-326-SP1 pathway might be an important pathway regulating chemoresistance of LAD cells.

In conclusion, our study revealed that the function of hsa_circ_0003998 in docetaxel-resistant LAD is partially exerted via sponging of miR-326. Silencing hsa_circ_0003998 might represent a promising therapeutic strategy for the treatment of chemoresistance in LAD.

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