Original Paper

Knockdown of Long Non-Coding RNA XIST Inhibited Doxorubicin Resistance in Colorectal Cancer by Upregulation of miR-124 and Downregulation of SGK1

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Key Words

LncRNA • XIST • miR-124 • SGK1 • Doxorubicin • Colorectal cancer

Abstract

Background/Aims: Doxorubicin (DOX) is a widely used chemotherapeutic agent for colorectal cancer (CRC). However, the acquisition of DOX resistance limits its clinical application for cancer therapy. Mounting evidence has suggested that aberrantly expressed lncRNAs contribute to drug resistance of various tumors. Our study aimed to explore the role and molecular mechanisms of lncRNA X-inactive specific transcript (XIST) in chemoresistance of CRC to DOX. **Methods:** The expressions of XIST, miR-124, serum and glucocorticoid-inducible kinase 1 (SGK1) mRNA in DOX-resistant CRC tissues and cells were detected by qRT-PCR or western blot analysis. DOX sensitivity was assessed by detecting IC₅₀ value of DOX, the protein levels of P-glycoprotein (P-gp) and glutathione S-transferase- π (GST- π) and apoptosis. The interactions between XIST, miR-124 and SGK1 were confirmed by luciferase reporter assay, qRT-PCR and western blot. Xenograft tumor assay was used to verify the role of XIST in DOX resistance in CRC *in vivo*. **Results:** XIST expression was upregulated and miR-124 expression was downregulated in DOX-resistant CRC tissues and cells. Knockdown of XIST inhibited DOX resistance of CRC cells, as evidenced by the reduced IC₅₀ value of DOX, decreased P-gp and GST- π levels and enhanced apoptosis in XIST-silenced DOX-resistant CRC cells. Additionally, XIST positively regulated SGK1 expression by interacting with miR-124 in DOX-resistant CRC cells. miR-124 suppression strikingly reversed XIST-knockdown-mediated repression on DOX resistance in DOX-resistant CRC cells. Moreover, SGK1-depletion-elicited decrease of DOX resistance was greatly restored by XIST overexpression or miR-124 inhibition in DOX-resistant

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CRC cells. Furthermore, XIST knockdown enhanced the anti-tumor effect of DOX in CRC *in vivo*. **Conclusion:** XIST exerted regulatory function in resistance of DOX possibly through miR-124/SGK1 axis, shedding new light on developing promising therapeutic strategy to overcome chemoresistance in CRC patients.

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Introduction

Colorectal cancer (CRC) is one of the most frequently diagnosed cancers worldwide and the third leading cause of cancer-associated mortality in the United States in men and females worldwide [1]. In recent years, the incidence and fatality rates of CRC present an increasing trend in developed countries due to genetic and environmental factors [2]. Chemotherapy is one of the important adjuvant therapy approaches for CRC patients, particularly for those with advanced disease. Doxorubicin (DOX), an anthracycline drug, is widely used as a chemotherapeutic agent to fight with a broad range of malignancies including CRC [3]. However, the development of drug resistance has become a major challenge for the clinical application of DOX in CRC treatment [4]. Hence, it is imperatively needed to clarify the molecular mechanism of DOX resistance in CRC in order to identify more effective intervention strategies for CRC.

Long noncoding RNAs (lncRNAs) are defined as evolutionarily conserved non-protein coding transcripts longer than 200 nucleotides [5]. It has been proposed that lncRNAs play important roles in a series of physiological and pathological processes in tumorigenesis through affecting mRNA stability, RNA splicing, protein structure, and miRNA-mediated gene regulation [6]. Moreover, ample evidence has suggested that aberrantly expressed lncRNAs also contribute to drug resistance in various tumors via different regulation mechanisms and signaling pathways [7]. LncRNA X-inactive specific transcript (XIST) is a product of XIST gene and a master regulator for X-chromosome inactivation in female mammals [8]. Dysregulation of XIST has been reported in the genesis and development of multiple neoplasms, highlighting its potential and clinicopathological value in cancers [9]. Recent studies have documented that XIST expression was significantly increased in CRC tissues and cells, and functioned as an oncogene in human CRC through facilitating cell proliferation, migration, invasion and EMT [10, 11]. Moreover, it was also reported that XIST was significantly upregulated in 5-fluorouracil (5FU)-resistant CRC cells, and enhanced XIST restrained 5FU-induced cytotoxicity in CRC cells by promoting the expression of thymidylate synthase [12]. However, whether XIST participated in DOX resistance in CRC cells remains to be further elucidated.

MicroRNAs (miRNAs) are a class of small, single-stranded ncRNAs of 20-22 nucleotides in length and are crucial regulators of gene expression through binding to complementary sequences in the 3' untranslated region (3'UTR) of mRNA [13]. Abundant studies have shown that miRNAs, acting as potential oncogenes or tumor suppressors, are involved in the regulation of drug resistance in a variety of cancers [14], including CRC [15]. Recently, miR-124, an enriched miRNA, has been a focus of interest for its tumor-suppressive effect in the development of many malignant tumors including CRC [16]. However, the function of miR-124 on chemoresistance in CRC has been scarcely understood till date. Recent studies suggest that lncRNAs can suppress the expressions and biological functions of miRNAs by functioning as a molecular sponge or a competing endogenous RNA (ceRNA), hence leading to derepression of its target mRNA [17]. Interestingly, XIST was reported to directly interact with miR-124 to modulate bladder cancer progression [18]. Therefore, this study was designed to investigate whether XIST could interact with miR-124 to participate in the regulation of DOX resistance in CRC.

In the present study, we demonstrated the upregulation of XIST in DOX-resistant CRC cells. Functional analysis proved that XIST silencing reversed DOX resistance by facilitating

DOX-induced apoptosis in CRC cells. Mechanistic analyses revealed that XIST functioned as a molecular sponge to inhibit the expression and activities of miR-124, thus leading to increased expression of serum and glucocorticoid-inducible kinase 1 (SGK1) and enhanced resistance of DOX in CRC cells.

Materials and Methods

Patients and tissues

The study was approved by the Ethics Committee of Liaoning Cancer Hospital & Institute, and written informed consent was obtained from each patient prior to surgery. A total of 31 advanced CRC patients who received 2 cycles of DOX-based adjuvant chemotherapy between 2014 and 2016 at Liaoning Cancer Hospital & Institute were enrolled in this study. After completion of the chemotherapy, patients were divided into chemosensitive group (n=13) and chemoresistant group (n=18) according to RECIST Response Evaluation Criteria. All tumor specimens were obtained by surgical resection prior to undergoing chemotherapy.

Cell lines and cell culture

Human CRC cell lines (HCT116 and LoVo) were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). All cells were maintained in RPMI-1640 medium (Gibco, Gaithersburg, MD, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Gibco, Logan, UT, USA), 100 µg/mL streptomycin (Invitrogen, Carlsbad, CA, USA), and 100 units/mL penicillin (Invitrogen) in a humidified incubator with a air flow of 5% CO₂ and 95% O₂ at 37°C.

The DOX-resistant CRC cells (HCT116/DOX and LoVo/DOX) were generated from the parental HCT116 and LoVo cells through repeated exposure to increasing concentrations of DOX (0.01, 0.02, 0.04, 0.08, 0.16, 0.32, and 0.50 µg/mL) (Sigma, St. Louis, MO, USA) over a 6-week period. To maintain the DOX-resistant phenotype, HCT116/DOX and LoVo/DOX cells were incubated with the culture medium containing 0.50 µg/mL DOX.

Cell transfection

siRNA targeting XIST (si-XIST), control siRNA (si-Control), pcDNA empty control (pcDNA-Control), XIST-overexpressing plasmid (pcDNA-XIST), miR-124 mimic (miR-124), control miRNA (miR-con), miR-124 inhibitor (anti-miR-124), and miRNA inhibitor control (miR-Control) were purchased from GenePharma (Shanghai, China). Transient transfection with these above plasmids or oligonucleotides into HCT116/DOX and LoVo/DOX cells was performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA).

Lentivirus production and infection

Short hairpin (sh)RNA targeting XIST (sh-XIST) were designed and synthesized by Genewiz, Inc. (Suzhou, China) and inserted into the pFH-L plasmid to generate lentiviral based shRNA expressing vectors. After confirmed by DNA sequencing, lentivirus vector carrying sh-XIST and packaging vectors pCMV-Δ8.92 and pMD2G (VΔR8.92) were cotransfected into HEK293T cells. The supernatant was collected at 48 h post-transfection by centrifugation (4000 g, 4°C, 10 min) and filtered through 0.45 µm cellulose acetate filters. A lentiviral vector that contained an irrelevant sequence was used as a negative control (lenti-control). For lentivirus infection, LoVo/DOX cells were seeded into 6-well plate at a density of 5 × 10⁴ cells/well and infected with lenti-sh-XIST or lenti-sh-Control at a multiplicity of infection (MOI) of 50 in the presence of puromycin (2 µg/mL) and incubated for 72 h prior to performing the subsequent analyses. The infection efficiency was determined through counting the number of infected green fluorescent protein (GFP)-expressing cells at 100× magnification under a fluorescence microscope (Olympus Corporation, Tokyo, Japan).

RNA extraction and quantitative real-time (qRT-PCR) assay

Total RNA was extracted from the collected tissues and cultured cells using TRIZOL reagent (Invitrogen). 1 µg of total RNA was reversely transcribed to complementary DNA (cDNA) in a final volume of 10 µl using random primers and PrimeScript RT Reagent Kit (Invitrogen). Then the relative expression levels of XIST and miR-124 were performed using SYBR Premix Ex Taq (TaKaRa, Dalian, China) and TaqMan MicroRNA

Assay Kit (Applied Biosystems, Foster City, CA, USA) on an ABI 7500 Fast Real-Time PCR system (Applied Biosystems), respectively. The qPCR reaction was set at 95°C for 10 min, 40 cycles at 95°C for 15 s, 55°C for 30 s, and 72°C for 30 s. The primer sequences were displayed as below: XIST, 5'-ACGCTGCATGTGCTTAG-3' (forward) and 5'-GAGCCTCTTATAGCTGTTTG-3' (reverse); miR-124, 5'-GCTTAAGGCACGCGG-3' (forward) and 5'-GTGCAGGGTCCGAGG-3' (reverse); GAPDH, 5'-CATGAGAAGTATGACAACAGCCT-3' (forward) and 5'-AGTCCTTCCACGATACCAAAAGT-3' (reverse); U6, 5'-CTCGCTTCGGCAGCAC-3' (forward) and 5'-AACGCTTCACGAATTTGCGT-3' (reverse). Expression levels of XIST and miR-124 were normalized to that of GAPDH and U6 small nuclear RNA (snRNA) using $2^{-\Delta\Delta Ct}$ method.

Western blot

The cellular proteins were extracted from resected tissues or treated cells using the RIPA lysis buffer (Beyotime, Beijing, China) supplemented with protease inhibitor cocktail (Sigma). Equal amount of protein extractions (50 µg) were subjected to 10% sodium dodecyl sulfate-polyacrylamide (SDS-PAGE) and transferred to nitrocellulose membranes (Millipore, Billerica, MA, USA). After blocked with bovine serum albumin for 2 h at room temperature, the membranes were then incubated at 4°C overnight with primary antibodies against P-glycoprotein (P-gp, Santa Cruz Biotechnology, Santa Cruz, CA, USA), glutathione S-transferase-π (GST-π) (Abcam, Cambridge, UK), SGK1 (Santa Cruz Biotechnology), and β-actin (Sigma). After washed, followed by probed with horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology) for 1 h. The protein signals were detected by enhanced chemiluminescence system (ECL, Pierce and Warriner, Chester, UK). One analysis software (Bio-Rad, San Francisco, California, USA).

Drug resistance assay

3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay was performed to evaluate DOX sensitivity. Transfected HCT116/DOX and LoVo/DOX cells were seeded into 96-well plates (3000 cells/well) and incubated overnight. Cells were then treated with DOX at various concentrations (0.05, 0.1, 0.5, 1, 5, and 10 µg/mL) for 24 h. Subsequently, 20 µL of MTT solution (5 mg/mL; Sigma) was added to each well and incubated for 4 h, followed by removal of culture medium and addition of 150 µL dimethyl sulfoxide (DMSO; Sigma). The optical density (OD) was measured at 490 nm using an ELISA microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The half maximal inhibitory concentration (IC₅₀) of DOX, at which drug produced 50% inhibition of growth, was calculated according to the charted dose-response curve at different concentrations.

Flow cytometry analysis

Transfected HCT116/DOX and LoVo/DOX cells were inoculated into 96-well plates and treated with 2 µg/mL DOX for 72 h. Cells were then subjected to annexin V/PI staining using an annexin V-FITC Apoptosis Detection Kit (KeyGEN Biotech, Jiangsu, China). The apoptotic rate was measured by flow cytometry using a FACS flow cytometer (Becton Dickinson, San Jose, CA, USA).

Luciferase reporter assay

The wild-type or mutated XIST containing the predicted miR-124 binding sites were amplified and inserted into the luciferase reporter vector pMIR-Report (Promega, Madison, WI, USA), namely XIST-WT (XIST-WT1 or XIST-WT2) and XIST-MUT (XIST-MUT1 or XIST-MUT2). Similarly, the predicted or mutated miR-124 binding sites in 3'UTR of SGK1 were amplified and cloned into pMIR-Report vector to generate (SGK1-WT) and (SGK1-MUT). The mutant miR-124 target sites for XIST (XIST-MUT1 or XIST-MUT2) or SGK1-3'UTR (SGK1-MUT) were produced by using the QuikChange II Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA). HCT116/DOX and LoVo/DOX cells were cotransfected with luciferase reporter constructs, as well as internal control pRL-TK (Promega) and miR-124 or miR-Control using Lipofectamine 2000 (Invitrogen). At 48 h posttransfection, cells were harvested and luciferase activity was measured using a Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA). The relative luciferase activity was normalized to Renilla luciferase activity.

Xenograft assay

The protocol was approved by the Animal Care and Ethics Committee of Liaoning Cancer Hospital & Institute. All experimental procedures were performed in accordance with the National Institutes of Health

Animal Use Guidelines. Female BALB/c nude mice (4-6 weeks old, $n = 5/\text{group}$) were obtained from the Chinese Academy of Medical Sciences (Beijing, China) and housed under pathogen-free conditions. LoVo/DOX cells stably transfected with lenti-sh-XIST or lenti-sh-Control were harvested and resuspended at a concentration of 1×10^8 cells/mL, and then 100 μL of cell suspension was subcutaneously injected in a single side of the posterior flank of each mouse. Six days after cell injection, DOX was intraperitoneally administered at a dose of 5 mg/kg every 3 days. Tumor growth was examined every 3 days, and tumor volumes were calculated using the equation $\text{Volume} = 0.5 \times \text{Width}^2 \times \text{Length}$. Then mice were euthanized by cervical dislocation at 21 days after treatment. Tumor masses were dissected, photographed and subjected to weight measurement, qRT-PCR analysis and western blot assay.

Statistical analysis

All data are presented as the means $\pm$ standard deviation (SD). Statistical difference was analyzed using one-way analysis of variance (ANOVA) or Student's t test with SPSS version 18 software (Chicago, IL, USA). Differences were considered significant when $P < 0.05$.

Results

XIST was upregulated and miR-124 was downregulated in DOX-resistant CRC tissues and cells

To investigate the involvement of XIST and miR-124 in the DOX resistance of CRC, we initially checked the expression profiles of XIST and miR-124 in 31 CRC tumor tissues that were divided into chemosensitive group ($n = 13$) and chemoresistant group ($n = 18$) by qRT-PCR. The results showed that XIST expression was robustly upregulated (Fig. 1A) and miR-124 expression was distinctly downregulated (Fig. 1B) in tumor tissues from chemoresistant patients compared with that from chemosensitive patients. Then, the expressions of XIST and miR-124 in the DOX-resistant CRC cell lines (HCT116/DOX and LoVo/DOX) and corresponding parental CRC cell lines (HCT116 and LoVo) were further evaluated by qRT-PCR. As expected, XIST expression was apparently higher (Fig. 1C and 1D) and miR-124 expression was greatly lower (Fig. 1E and 1F) in DOX-resistant CRC cell lines than that in their parental CRC cell lines. These results suggested that abnormal expressions of XIST and miR-124 may be associated with DOX resistance in CRC.

Fig. 1. XIST expression was increased and miR-124 expression was decreased in DOX-resistant CRC tissues and cells. qRT-PCR analyses of XIST (A) and miR-124 (B) expressions in 31 cases of CRC tumor tissues, among which there were 13 chemosensitive cases and 18 chemoresistant cases. qRT-PCR analyses of XIST (C and D) and miR-124 (E and F) in DOX-resistant CRC cell lines (HCT116/DOX and LoVo/DOX) and corresponding parental CRC cell lines (HCT116 and LoVo). * $P < 0.05$.

Fig. 2. XIST downregulation impaired DOX resistance in DOX-resistant CRC cells. (A and B) The viability was measured by MTT assay after LoVo, HCT116, LoVo/DOX, and HCT116/DOX cells were exposed to DOX at various concentrations (0.05, 0.1, 0.5, 1, 5, and 10 μg/mL) for 24 h. Data were presented as a percentage normalized to the viability of cells with no DOX treatment. The abscissa was the logarithm of DOX concentration. IC50 values were calculated according to the corresponding dose-response curve. (C and D) The relative expression of XIST was detected by qRT-PCR in LoVo/DOX and HCT116/DOX cells after transfection with si-XIST or si-Control. (E and F) The protein levels of P-gp and GST-π in LoVo/DOX and HCT116/DOX cells transfected with si-XIST or si-Control were estimated by western blot. (G and H) The cell viability and IC50 values were detected by MTT assay after LoVo/DOX and HCT116/DOX cells transfected with si-XIST or si-Control were treated with the indicated concentrations (0.05, 0.1, 0.5, 1, 5, and 10 μg/mL) of DOX for 24 h. Flow cytometry analysis was performed to examine apoptosis of LoVo/DOX (I) and HCT116/DOX (J) cells introduced with si-XIST or si-Control after treatment with 2 μg/mL DOX for 24 h. *P<0.05.

XIST silencing weakened DOX resistance in DOX-resistant CRC cells

To confirm whether HCT116/DOX and LoVo/DOX cells were resistant to DOX, MTT assay was performed to test IC50 of DOX. As shown in Fig. 2A and 2B, IC50 values of DOX in LoVo/DOX and HCT116/DOX cells were significantly higher than that of their parental LoVo and HCT116 cells. To address the effect of XIST on DOX resistance of CRC cells,

Figure 2. XIST repressed miR-124 expression in DOX-resistant CRC cells by direct binding. (A) Two miR-124 binding sites were predicted on XIST by miRcode. Luciferase reporter assay was performed in LoVo/DOX (B) and HCT116/DOX (C) cells after cotransfection with miR-124 or miR-Control and XIST-WT (XIST-WT1 or XIST-WT2) or XIST-MUT (XIST-MUT1 or XIST-MUT2). The expression of miR-124 was detected by qRT-PCR in LoVo/DOX (D) and HCT116/DOX (E) cells transfected with pcDNA-XIST, si-XIST, or respective controls. * $P < 0.05$.

siRNA-mediated knockdown of XIST was performed in LoVo/DOX and HCT116/DOX cells by transfecting with si-XIST. As expected, XIST expression was greatly decreased in LoVo/DOX (Fig. 2C) and HCT116/DOX (Fig. 2D) cells following transfection with si-XIST. It is well-known that various transporter proteins including P-gp and GST- π have been shown to be involved in chemotherapeutic resistance in cancers [19]. Western blot analysis revealed that XIST depletion led to a marked reduction of P-gp and GST- π expressions in LoVo/DOX (Fig. 2E) and HCT116/DOX (Fig. 2F) cells. Consistently, MTT assay also implicated that si-

Fig. 4. XIST depletion reduced chemoresistance of DOX-resistant CRC cells to DOX via regulating miR-124. LoVo/DOX and HCT116/DOX cells were transfected with miR-Control, miR-124, si-Control, si-XIST, si-XIST + anti-miR-Control, or si-XIST + anti-miR-124. Western blot was performed to detect the protein levels of P-gp and GST- π in transfected LoVo/DOX (A) and HCT116/DOX (B) cells. MTT assay was employed to measure IC₅₀ value of DOX in transfected LoVo/DOX (C) and HCT116/DOX (D) cells after treated with different concentrations (0.05, 0.1, 0.5, 1, 5, and 10 μ g/ml) of DOX for 24 h. Flow cytometry was employed to evaluate the apoptosis of LoVo/DOX (E) and HCT116/DOX (F) cells following treated with 2 μ g/ml DOX for 24 h. *P<0.05.

XIST transfection in LoVo/DOX (Fig. 2G) and HCT116/DOX (Fig. 2H) cells rendered both cell lines more sensitive to DOX-mediated cytotoxicity compared with si-Control group, as demonstrated by the decreased IC₅₀ value of DOX following XIST downregulation. Additionally, we further evaluated whether the effect of XIST on DOX resistance of CRC cells was associated with apoptosis. LoVo/DOX and HCT116/DOX cells transfected with si-Control or si-XIST were treated with 2 µg/mL DOX for 24 h. Flow cytometry analysis results demonstrated that XIST knockdown enhanced DOX-induced apoptosis in LoVo/DOX (Fig. 2I) and HCT116/DOX (Fig. 2J) cells with respect to si-Control group. Collectively, these results indicated that XIST silencing enhanced DOX-induced cytotoxicity in DOX-resistant CRC cells.

XIST regulated miR-124 expression in DOX-resistant CRC cells by direct interaction

To elucidate the potential mechanism of XIST function in DOX-resistant CRC cells, two bioinformatic databases (starBase and TargetScanS) were employed to search for the potential miRNAs that can be regulated by XIST. Bioinformatics tools analyzed and predicted two potential binding sequences of miR-124 on XIST gene (Fig. 3A). To confirm the interaction between XIST and miR-124, luciferase reporter constructs containing the predicted wild-type or mutated miR-124 binding sites on XIST were generated. Dual-luciferase assays manifested that cotransfection with miR-124 mimic and XIST-WT1 or XIST-WT2 prominently reduced the luciferase activity, while cotransfection with miR-124 and XIST-MUT (XIST-MUT1 or XIST-MUT2) had no obvious effect on luciferase activity in LoVo/DOX (Fig. 3B) and HCT116/DOX (Fig. 3C) cells. Next, we further monitored the expression of miR-124 in response to the change XIST expression in DOX-resistant CRC cells. qRT-PCR results implied that miR-124 expression was specifically upregulated after overexpression of XIST and evidently promoted following knockdown of XIST in LoVo/DOX (Fig. 3D) and HCT116/DOX (Fig. 3E) cells. Therefore, we concluded that XIST could directly bind to miR-124 to suppress its expression in DOX-resistant CRC cells.

XIST knockdown inhibited DOX resistance by upregulating miR-124 in DOX-resistant CRC cells

To gain insight into whether XIST affected DOX resistance of DOX-resistant CRC cells via modulation of miR-124, LoVo/DOX and HCT116/DOX cells were transfected with miR-124 mimic, si-XIST or si-XIST+anti-miR-124. Western blot analysis demonstrated that ectopic expression of miR-124 or XIST knockdown distinctly reduced the protein levels of P-gp and GST-π in LoVo/DOX (Fig. 4A) and HCT116/DOX (Fig. 4B) cells, while miR-124 antagomir markedly reversed the reduction of P-gp and GST-π levels mediated by XIST silencing. MTT assay indicated that forced expression of miR-124 or downregulation of XIST greatly diminished the IC₅₀ value of DOX in LoVo/DOX (Fig. 4C) and HCT116/DOX (Fig. 4D) cells. Therefore, XIST-induced decrease of IC₅₀ was partially restored by miR-124 inhibition. Furthermore, flow cytometry analysis indicated that miR-124 overexpression or XIST knockdown dramatically aggravated DOX-induced apoptosis of LoVo/DOX (Fig. 4E) and HCT116/DOX (Fig. 4F) cells, however, si-XIST-triggered apoptosis was attenuated after cotransfection with miR-124 inhibitor. Together, these data hinted that inhibition of miR-124 expression counteracted XIST-knockdown-mediated repression on DOX resistance in DOX-resistant CRC cells.

XIST positively regulated SGK1 expression by interacting with miR-124 in DOX-resistant CRC cells

To address the underlying mechanism by which miR-124 exerted its effects on DOX resistance of CRC cells, bioinformatics tools (microRNA.org and miRBase) were used to search for the potential targets of miR-124. As displayed in Fig. 5A, binding sites of miR-124 were identified in the 3'UTR of SGK1 mRNA. SGK1 has been reported to participate in cell survival, de-differentiation, cell cycle control, apoptosis, as well as chemo- and radio-resistance in various malignancies [20]. Moreover, a recent document points out that SGK1 is essential and rate limiting in the control of precursor miRNA nuclear export

Fig. 5. XIST facilitated SGK1 expression via sponging miR-124 in DOX-resistant CRC cells. (A) The constructed luciferase reporter plasmids containing the wild-type or mutated miR-124 binding sites in the 3'UTR of SGK1. Luciferase reporter assay was performed to measure luciferase activity in LoVo/DOX (B) and HCT116/DOX (C) cells cotransfected with miR-124 or miR-Control and SGK1-WT or SGK1-MUT. Western blot was performed to examine the protein level of SGK1 in LoVo/DOX (D) and HCT116/DOX (E) cells introduced with si-Control, si-XIST, miR-Control, miR-124, miR-124 + pcDNA-Control, or miR-124 + pcDNA-XIST. (F) qRT-PCR analysis of SGK1 mRNA expression in CRC tumor tissues (chemosensitive cases, n=13; chemoresistant cases, n=18). (G and H) qRT-PCR analysis of SGK1 mRNA in DOX-resistant CRC cell lines (LoVo/DOX and HCT116/DOX) and their corresponding parental cell lines (LoVo and HCT116). *P<0.05.

Subsequent luciferase reporter assay revealed that miR-124 overexpression could effectively suppress the luciferase activity of the reporter containing the wild-type miR-124 binding sites in the 3'UTR of SGK1 in LoVo/DOX (Fig. 5B) and HCT116/DOX (Fig. 5C) cells, however, these effects were disappeared when the binding sites were mutated. Furthermore, we found that XIST knockdown or miR-124 overexpression triggered a substantial decline of SGK1 protein level in LoVo/DOX (Fig. 5D) and HCT116/DOX (Fig. 5E) cells. Moreover, miR-124-mediated decrease of SGK1 protein expression was significantly recuperated following XIST upregulation (Fig. 5D and 5E). All of these data made us draw a conclusion that XIST positively regulated SGK1 expression by interacting with miR-124 in DOX-resistant CRC cells. Additionally, as we might expect, SGK1 expression was increased in DOX-resistant CRC tumor tissues (Fig. 5F) and cells (Fig. 5G and 5H) when compared with respective controls.

Fig. 6. SGK1-knockdown-mediated decline of DOX resistance was regulated by XIST or miR-124 in DOX-resistant CRC cells. LoVo/DOX and HCT116/DOX cells were transfected with si-SGK1, or combined with pcDNA-XIST, or anti-miR-124. Western blot was performed to detect the protein level of SGK1 in the transfected LoVo/DOX (A) and HCT116/DOX (B) cells. MTT assay was used to measure the IC₅₀ value of DOX in the transfected LoVo/DOX (C) and HCT116/DOX (D) cells after exposed to different concentrations (0.05, 0.1, 0.5, 1, 5, and 10 µg/mL) of DOX for 24 h. Flow cytometry analysis was conducted to evaluate apoptosis of the transfected LoVo/DOX (E and G) and HCT116/DOX (F and H) cells after administrated with 2 µg/mL DOX for 24 h. *P<0.05.

XIST overexpression and miR-124 inhibition reversed SGK1-knockdown-mediated inhibition of DOX resistance in DOX-resistant CRC cells

To determine the effects of XIST and miR-124 on SGK1 expression and functions in DOX-resistant CRC cells, LoVo/DOX and HCT116/DOX cells were transfected with si-SGK1, or combined with pcDNA-XIST or anti-miR-124. Western blot analysis demonstrated that SGK1 silencing dramatically reduced SGK1 level in LoVo/DOX (Fig. 6A) and HCT116/DOX (Fig. 6B) cells, while XIST overexpression or miR-124 suppression remarkably overturned si-SGK1-induced decrease of SGK1 level. MTT assay indicated that interfering of SGK1 in LoVo/DOX (Fig. 6C) and HCT116/DOX (Fig. 6D) cells led to a substantial decline of IC50 value of DOX, but this effect was partially abated by enhanced XIST expression and reduced miR-124 expression. Flow cytometry analysis confirmed that the apoptotic rate was significantly increased following transfection with si-SGK1 in LoVo/DOX (Fig. 6E) and HCT116/DOX (Fig. 6F) cells, but this effect was distinctively counteracted by cotransfection with pcDNA-XIST or anti-miR-124. Taken together, these results revealed that SGK1 knockdown increased DOX resistance in DOX-resistant CRC cells, which was mediated by expression alteration of XIST or miR-124.

Fig. 7. XIST knockdown enhanced the anti-tumor effect of DOX in CRC in vivo. LoVo/DOX cells stably infected lenti-sh-XIST or lenti-sh-Control were subcutaneously injected into each mouse, followed by administration with DOX (5 mg/kg) every 3 days from the 6th day after cell inoculation. (A) Tumor volumes were examined every 3 days for 21 days. (B) Mice were euthanized by cervical dislocation on day 21 after implantation, and then tumors were dissected and weighed. The expressions of XIST (C) and miR-124 (D), as well as SGK1 mRNA (E) and protein (F) levels in excised tumor masses were detected by qRT-PCR and western blot. *P<0.05.

XIST knockdown enhanced the anti-tumor effect of DOX in CRC in vivo

To confirm the effects of XIST on the chemosensitivity of CRC cells to DOX *in vivo*, LoVo/DOX cells stably infected with lenti-sh-XIST or lenti-sh-Control were subcutaneously injected into each mouse, followed by administration with DOX. As shown in Fig. 7A and 7B, XIST depletion significantly reinforced DOX-induced suppression on the tumor growth, presented as decreased tumor volume and lowered tumor weight. qRT-PCR analysis clarified that tumor xenografts derived from sh-XIST-transfected LoVo/DOX cells showed a marked reduction of XIST expression (Fig. 7C) and a remarkable increase of miR-124 expression (Fig. 7D) with or without DOX treatment. Moreover, the mRNA expression of SGK1 was coincided well with the protein levels, which illustrated a dramatic decline in the sh-XIST treatment group in the presence or absence of DOX (Fig. 7E and 7F). Collectively, these results implicated that XIST knockdown displayed a synergic effect with DOX in suppressing CRC cell growth *in vivo*.

Discussion

Drug resistance is a frequent cause of chemotherapy failure in CRC treatment [22]. It has been proposed that numerous lncRNAs play vital functional roles in modulating drug resistance of CRC cells. For instance, knockdown of prostate cancer-associated ncRNA transcript 1 (PCAT-1) inhibited c-Myc-dependent aggressive and sensitized cells to 5FU in CRC cells [23]. Overexpression of maternally expressed gene 3 (MEG3), a prognostic factor for CRC, promoted chemosensitivity by enhancing oxaliplatin-induced cell apoptosis [24]. H19 conferred methotrexate resistance through activating Wnt/ β -catenin pathway [25]. Increasing studies revealed the oncogenic function of lncRNAs in several tumors, such as gastric cancer [26], nasopharyngeal carcinoma [27] and hepatocellular carcinoma [28], through inducing cell proliferation, invasion and metastasis. There have been documents reporting the carcinogenicity of XIST in CRC [11, 12]. Moreover, a previous study demonstrated that XIST was significantly upregulated in DOX-resistant CRC cells, and enforced expression of XIST inhibited 5FU-induced cytotoxicity in CRC cells [12]. However, it is still unclear whether XIST could also affect DOX resistance in CRC cells. To address this question, we initially detected the expression of XIST in DOX-resistant CRC tissues and cells, and found that XIST expression was aberrantly upregulated in DOX-resistant CRC tissues and cells. Moreover, functional analyses demonstrated that knockdown of XIST inhibited DOX resistance of CRC cells, as evidenced by the reduced IC₅₀ value of DOX, decreased P-gp and GST- π protein levels, and enhanced apoptosis in XIST-silenced DOX-resistant CRC cells.

One of the most functional mechanisms is the ceRNA-miRNA-mRNA regulatory circuitry, which maintains the overall activity and functional balance of gene network in a cell. Any disturbance of this system may trigger a series of pathological processes in different human diseases, including cancers [29]. XIST inhibited cell proliferation and mobility in gastric cancer by interacting with miR-21-5p and upregulating programmed cell death 4 (PDCD4) expression [30]. Knockdown of XIST exerted tumor-suppressive effects in gastric cancer by acting as a molecular sponge of miR-101 to modulate enhancer of zeste homolog 2 (EZH2) expression [31]. XIST overexpression enhanced cell growth in nasopharyngeal carcinoma through upregulating E2F3 in part through sponging miR-34a-5p [27]. In our study, luciferase reporter assay and qRT-PCR analysis revealed that XIST suppressed miR-124 expression by functioning as a molecular sponge in DOX-resistant CRC cells. miR-124 is a well-recognized tumor suppressor and is epigenetically silenced in various types of cancer including CRC [16, 32]. Additionally, it was reported that restoration of miR-124 repressed Frizzled 5 to overcome P-gp-mediated multidrug resistance in renal cell carcinoma [33]. Moreover, miR-124 was found to be downregulated in gefitinib-resistant non-small cell lung cancer (NSCLC) cells, and overexpression of miR-124 sensitized gefitinib-resistant cells to gefitinib [34]. Also, miR-124 was found to be a potential therapeutic agent to improve the efficacy of chemotherapy with DNA-damaging agents in breast cancer [35]. Our study demonstrated that miR-124 expression was downregulated in DOX-resistant CRC tissues

and cells. Ectopic expression of miR-124 enhanced DOX cytotoxicity in DOX-resistant CRC cells. Moreover, we found that suppression of miR-124 strikingly reversed XIST knockdown-mediated repression on DOX resistance in DOX-resistant CRC cells.

SGK1, belonging to the AGC family of serine/threonine protein kinases, has been suggested to be overexpressed and associated with cell proliferation, apoptosis, invasiveness, motility, epithelial to mesenchymal transition, adhesiveness and chemoresistance in a variety of epithelial tumors [20, 36]. Amato *et al.* firstly revealed the association of SGK1 and doxorubicin-induced apoptosis in kidney cancer cells [37]. Moreover, a previous report elucidated that SGK1 was highly expressed in CRC tumor tissues, and SGK1 downregulation impeded cell proliferation, migration and increased apoptosis induced by fluorouracil (5-FU) [38]. In this study, SGK1 was identified as a target of miR-124 in DOX-resistant CRC cells. Moreover, XIST positively regulated SGK1 expression by interacting with miR-124 in DOX-resistant CRC cells. SGK1 expression was increased in DOX-resistant CRC tissues and cells. Furthermore, SGK1 knockdown alleviated DOX resistance and promoted DOX-induced apoptosis in DOX-resistant CRC cells, while these effects were attenuated following XIST overexpression or miR-124 inhibition. *In vivo* experiments also confirmed that XIST knockdown enhanced the anti-tumor effect of DOX on CRC *in vivo* possibly via modulating miR-124 and SGK1 expressions. Together, all these results suggest that knockdown of XIST inhibited DOX resistance in CRC by upregulation of miR-124 and downregulation of SGK1.

Conclusion

In the present study, we demonstrated that XIST expression was upregulated and miR-124 expression was downregulated in DOX-resistant tissues and cells. In addition, we provided the evidence that knockdown of XIST reduced resistance to DOX by working as a molecular sponge of miR-124 and subsequently reducing SGK1 protein level in DOX-resistant CRC cells. Therefore, XIST might be a potential therapeutic target for improving the efficacy of DOX-based chemotherapy in CRC patients.

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Disclosure Statement

The authors declare no conflict of interests.

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