LncRNA DCST1-AS1 drives the malignant progression of pediatric acute myeloid leukemia *via* regulating p53 methylation

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ABSTRACT

To uncover the biological role of lncRNA DCST1-AS1 in the process of pediatric AML and its regulatory effect on p53 methylation. Serum level of DCST1-AS1 in AML children and healthy participants was detected by qRT-PCR. The role of DCST1-AS1 in mediating biological functions of AML193 and U937 cells was assessed by functional experiments. p53 methylation level was examined. Through BSP, MSP and dual-luciferase reporter assay, the regulatory effect of DCST1-AS1 on p53 methylation was explored. The correlation between DCST1-AS1 and p53 in the serum of AML children was assessed. Serum level of DCST1-AS1 was higher in AML children than in healthy subjects. Knockdown of DCST1-AS1 decreased proliferative and migratory rates in AML193 and U937 cells. DCST1-AS1 was able to induce methylation of p53 promoter. P53 was markedly upregulated by the knockdown of DCST1-AS1, presenting a negative correlation. LncRNA DCST1-AS1 drives the malignant progression of pediatric AML through inducing methylation of the p53 promoter.

Introduction

Acute leukemia frequently affects children's hematopoietic systems. Acute myeloid leukemia (AML) accounts for 20% of pediatric acute leukemia. In the past 20 years, the cure rate of pediatric AML is about 40%, which is far less than that of pediatric ALL (1,2). The pathogenesis of AML is a multi-step process. Disordered gene regulation, uncontrolled cell differentiation, proliferation and apoptosis ultimately lead to the malignant transformation of hematopoietic stem progenitor cells (3-5). It is of significance to enhance the survival of pediatric AML by exploring its pathogenesis and developing novel strategies with a low rate of side effects (6-8).

LncRNAs belong to a type of non-coding RNAs with over 200 bp long. They are able to regulate gene expressions at multiple levels of epigenetics, transcription and post-transcription (9,10). LncRNAs display very important biological functions in the occurrence and development of hematological malignancies, which can be used as indicators for stratified treatment and prognosis evaluation of leukemia (11,12). So far, there have been few comprehensive and systematic reports on the correlation between lncRNAs and AML (13,14). LncRNA DCST1-AS1 is served as a proto-oncogene in hepatocellular carcinoma and glioblastoma and upregulated DCST1-AS1 triggers carcinogenesis (15,16). In the present study, we mainly explore the mechanism of DCST1-AS1 affecting the malignant process of AML by inducing p53 methylation. Meanwhile, the potential of DCST1-AS1 as a biomarker for AML is assessed through analyzing the clinical data of recruited AML children.

Materials and Methods

Subjects and samples

Serum samples were collected from 20 AML children and age-matched healthy subjects. None of the AML patients received chemotherapy or radiotherapy. Tumor staging of AML was evaluated based on UICC criteria. Clinical data of them were completely recorded. This study was approved by the research ethics committee of our hospital and complied with the Helsinki Declaration. Informed consent was obtained from subjects and their parents.

Cell culture

Human AML cell lines (AML2, AML5, AML193, HL-60, Kasumi-1, U937) and human bone marrow cell line (HS-5) were purchased from American Type Culture Collection (ATCC) (Manassas, VA, USA). They were cultivated in dulbecco's modified eagle medium (DMEM) containing 10% fetal bovine serum (FBS) at 37°C, and 5% CO_2 (Gibco, Rockville, MD, USA).

Transfection

Transfection plasmids were synthesized by GenePharma, Shanghai, China. Cells were cultured to 40-60% density in a 6-well plate and transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). After 48-h transfection, cells were collected to verify transfection efficacy and functional experiments.

Cell counting kit-8 (CCK-8) assay

Cells were inoculated in a 96-well plate with 2×10^3 cells/well. At 24, 8, 72 and 96 h, the optical density at 450 nm of each sample was recorded using the CCK-8 kit

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(Dojindo Laboratories, Kumamoto, Japan) for plotting the viability curves.

5-Ethynyl-2'-deoxyuridine (EdU) assay

Cells were induced with 50 μM EdU (RiboBio, Guangzhou, China) for 2 h, and dyed using AdoLo and DAPI in the dark. EdU-labeled cells were captured for calculating EdU incorporation.

Transwell migration assay

200 μl of suspension (2×10^5 cells/ml) was applied on the top of a Transwell insert, which was placed in each well containing 700 μl of medium and 20% FBS. Cells were allowed to migrate for 48 h, and they were fixed, dyed and captured for counting in five random fields per sample.

Quantitative real-time polymerase chain reaction (qRT-PCR)

Serum samples were processed for isolating RNAs. After purification of cellular RNAs, they were reversely transcribed to cDNAs and subjected to qRT-PCR using SYBR®Premix Ex Taq™ (TaKaRa, Tokyo, Japan). Relative levels of PCR products were calculated by $2^{-\Delta\Delta Ct}$ and normalized to that of GAPDH. DCST1-AS1: Forward: 5'-CCACACAGTTAGGAAGCCCA-3', Reverse: 5'-CAGATCGATGACCGCAGGAA-3'; p53: Forward: 5'-ACAAGGGCACTGATTCCGAG-3', Reverse: 5'-TGGGTTCCGTGGTCAATTCC-3'; GAPDH: Forward: 5'-ATGGAGAAGGCTGGGGCTC-3', Reverse: 5'-AAGTTGTCATGGATGACCTTG-3'.

Western blot

Radioimmunoprecipitation assay (RIPA) was used to lyse cells on ice, and the lysate was subjected to 14000×g centrifugation at 4°C. Fifteen minutes later, the precipitant was resuspended for measuring protein concentration. Prepared protein samples were loaded on sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and polyvinylidene fluoride (PVDF) membranes (0.22 μm) (Millipore, Billerica, MA, USA), which were blocked for non-specific antigens in 5% skim milk for 2 h. Later, membranes were immunoblotted with primary and secondary antibodies for grey value analysis.

Methylation-specific PCR (MSP)

Genomic DNA was extracted from cells and tissues using a genomic DNA purification kit (Qiagen company, Hilden, Germany), followed by DNA modification with sodium bisulfite using Intergen CpGenome DNA modification kit (Intergen, New York, NY, USA). Unmethylated cytosines were converted to thymine, while the others remained. MSP was conducted using p53 methylation primers. DNA treated with M.SssI methylase was used as a positive control, while untreated DNA was used as a non-methylated negative control. To eliminate false positive results, ddH$_2$O was used as a control instead of the DNA template. PCR products were loaded on TBE and analyzed.

Dual-luciferase reporter assay

HEK293T were seeded in a 24-well plate, and co-transfected with sh-DCST1-AS1/sh-NC and p53-MUT/p53-WT vector, respectively. After 48 h, cells were lysed to measure luciferase activity.

Statistical analysis

Statistic Package for Social Science (SPSS) 22.0 (IBM, Armonk, NY, USA) was used for statistical analyses and data were expressed as mean ± standard deviation. Differences between groups were compared by the *t*-test. The correlation between DCST1-AS1 and p53 levels was assessed by the Pearson correlation test. $P < 0.05$ was considered as statistically significant.

Results

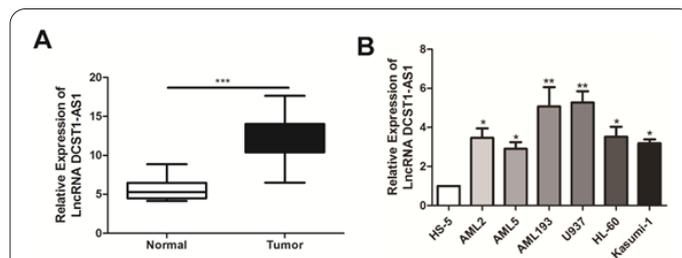### DCST1-AS1 was highly expressed in the serum of AML children and AML cell lines

Serum level of DCST1-AS1 was detected in AML children and healthy subjects by qRT-PCR, which was higher in the former group (Figure 1A). In addition, DCST1-AS1 was upregulated in AML cell lines than that of bone marrow cell lines (Figure 1B). Among six tested AML cell lines, the highest level of DCST1-AS1 was detected in AML193 and U937 cells, and they were used for the following experiments.

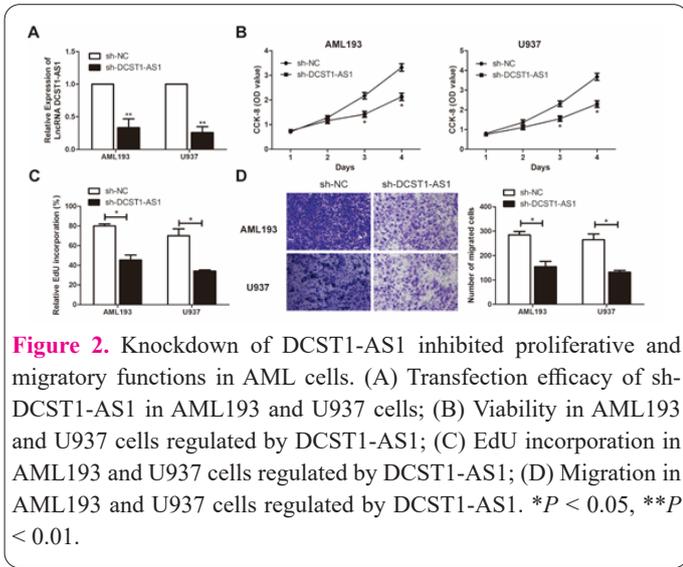### Knockdown of DCST1-AS1 inhibited proliferative and migratory functions in AML cells

To explore the biological function of DCST1-AS1 in AML, we constructed sh-DCST1-AS1 and examined its transfection efficacy in AML193 and U937 cells by qRT-PCR (Figure 2A). Transfection of sh-DCST1-AS1 not only reduced viability but also EdU incorporation in AML193 and U937 cells (Figure 2B, C). Moreover, migratory cell numbers declined after the knockdown of DCST1-AS1 in AML cells (Figure 2D).

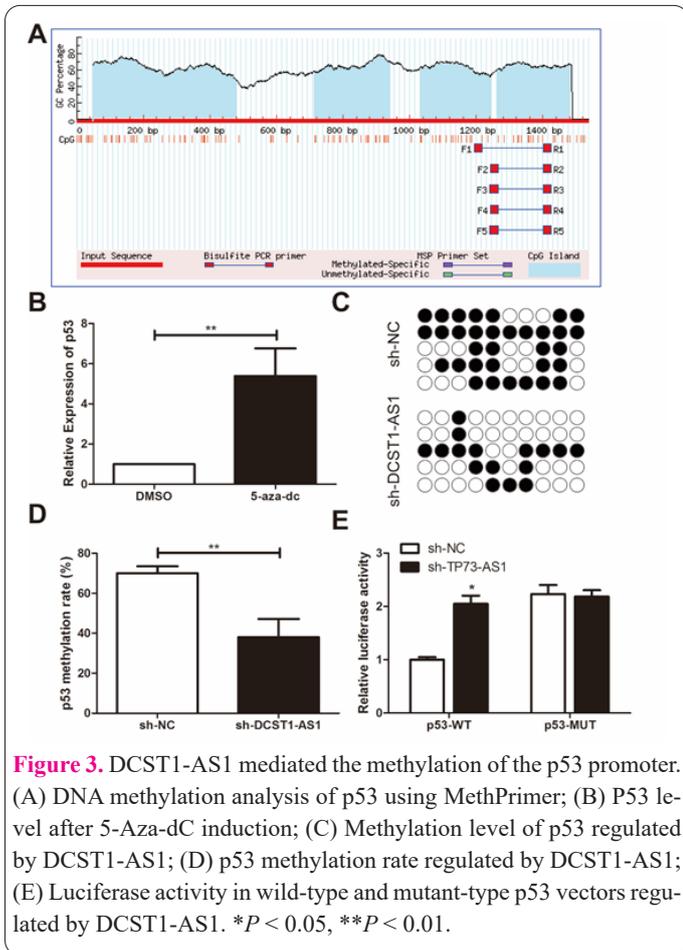### DCST1-AS1 mediated the methylation of the p53 promoter

P53 was searched as the downstream target of DCST1-AS1. The presence of the p53 promoter region was discovered using MethPrimer (http://www.urogene.org/cgi-bin/methprimer/methprimer.cgi) (Figure 3A). Compared with DMSO-induced cells, p53 was markedly upregulated in those induced with the methyltransferase inhibitor 5-Aza-dC (Figure 3B). In addition, the methylation level of the p53 promoter was detected using BSP method (Figure 3C). MSP results showed that the hypermethylation of the CpG island in the p53 promoter was inhibited by the knockdown of DCST1-AS1 (Figure 3D). Knockdown of DCST1-AS1 in HEK293T cells promoted luciferase activity in the wild-type p53 vector, while it had no influence

Figure 1. DCST1-AS1 was highly expressed in the serum of AML children and AML cell lines. (A) Serum level of DCST1-AS1 in AML children and healthy subjects; (B) DCST1-AS1 level in AML cell lines. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

Figure 2. Knockdown of DCST1-AS1 inhibited proliferative and migratory functions in AML cells. (A) Transfection efficacy of sh-DCST1-AS1 in AML193 and U937 cells; (B) Viability in AML193 and U937 cells regulated by DCST1-AS1; (C) EdU incorporation in AML193 and U937 cells regulated by DCST1-AS1; (D) Migration in AML193 and U937 cells regulated by DCST1-AS1. *$P < 0.05$, **$P < 0.01$.

Figure 3. DCST1-AS1 mediated the methylation of the p53 promoter. (A) DNA methylation analysis of p53 using MethPrimer; (B) P53 level after 5-Aza-dC induction; (C) Methylation level of p53 regulated by DCST1-AS1; (D) p53 methylation rate regulated by DCST1-AS1; (E) Luciferase activity in wild-type and mutant-type p53 vectors regulated by DCST1-AS1. *$P < 0.05$, **$P < 0.01$.

Figure 4. Knockdown of DCST1-AS1 upregulated p53 in AML cells. (A) Protein level of p53 in AML193 and U937 cells regulated by DCST1-AS1; (B) Serum level of p53 in AML children and healthy subjects; (C) Correlation between DCST1-AS1 and p53 levels. **$P < 0.01$.
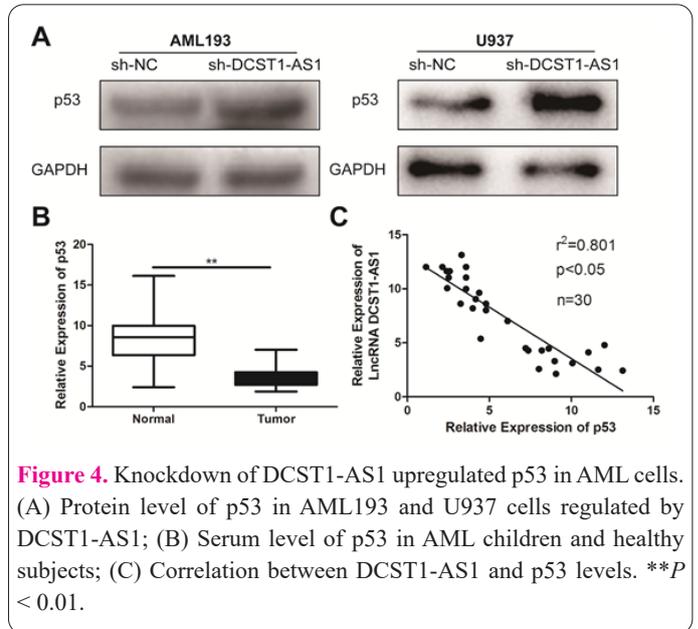
on that of the mutant-type one (Figure 3E).

Knockdown of DCST1-AS1 upregulated p53 in AML cells

The protein level of p53 was upregulated in AML193 and U937 cells transfected with sh-DCST1-AS1 (Figure 4A). In comparison to healthy subjects, the serum level of p53 was downregulated in AML children (Figure 4B). In addition, the serum level of p53 was negatively correlated to that of DCST1-AS1 in AML children (Figure 4C).

Discussion

Acute leukemia is a clonal origin, hematopoietic mali-gnant tumor caused by mutations in pluripotent stem cells or very early progenitor cells (myeloid or lymphoid) (1-3). At present, the specific etiology and pathogenesis of leukemia are still unclear, which are considered to be multifactorial involving radiological, chemical, viral, and genetic factors (4-8). Seeking for abnormally expressed lncRNAs in AML cases and analyzing their biological functions contribute to elevating diagnostic rate and therapeutic efficacy (9-12). At present, only a small number of functional lncRNAs have emerged in pediatric AML (13,14).

LncRNA DCST1-AS1 is a cancer-associated lncRNA as previously reported (15,16). Here, we detected the serum level of DCST1-AS1 in AML children and healthy participants, and its level was much higher in the former group. *In vitro, the* knockdown of DCST1-AS1 remarkably weakened the proliferative and migratory functions of AML cells. Therefore, we believed that DCST1-AS1 promoted the malignant process of pediatric AML.

We searched the potential target of DCST1-AS1 aiming to further explore its molecular mechanism in aggravating pediatric AML. Using online bioinformatic tools, p53 was predicted to have an interaction with DCST1-AS1. As a negative regulator in the cell growth cycle, p53 is related to DNA repair, cell differentiation, apoptosis, aging, angiogenesis, etc. It is regarded as a gene guardian (17,18). The mutation or deletion rate of p53 in hematological tumors is low, and whether there are other inactivation mechanisms of p53 in hematological tumors is worth exploring (19,20). We detected 16 CpG dinucleotides in the promoter region of the p53. After induction of the methyltransferase inhibitor 5-Aza-dC, p53 was markedly upregulated. In addition, DCST1-AS1 was able to inhibit p53 transcription by mediating p53 promoter methylation. As lowly expressed in the serum level of AML children, the p53 level was negatively correlated to that of DCST1-AS1. Taken together, DCST1-AS1 stimulated proliferative and migratory functions of AML as an oncogene by regulating p53 promoter methylation.

Conclusion

LncRNA DCST1-AS1 drives the malignant progression of pediatric AML through inducing methylation of the p53 promoter.

Conflict of Interests

The authors declared no conflict of interest.

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