CpG site hypomethylation at ETS1-binding region regulates DLK1 expression in Chinese patients with Tetralogy of Fallot

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Abstract. Tetralogy of Fallot (TOF) is the most common cyanotic congenital heart malformation accounting for ~10% of cases. Although the pathogenesis of TOF is complex and largely unknown, epigenetics plays a huge role, specifically DNA methylation. The protein δ like non-canonical Notch ligand 1 (DLK1) gene encodes a non-canonical ligand of the Notch signaling pathway, which is involved in heart development. However, the epigenetic mechanism of DLK1 in the pathogenesis of TOF is yet to be elucidated. Therefore, the present study aimed to clarify its specific mechanism. In this study, immunohistochemistry was used to detect the protein expression of DLK1 and the methylation status of the DLK1 promoter was measured via bisulfite sequencing PCR. Dual-luciferase reporter assays were performed to examine the influence of transcription factor ETS proto-oncogene 1 (ETS1) on DLK1 gene expression. The electrophoretic mobility shift assay and chromatin immunoprecipitation assay, both *in vivo* and *in vitro*, were used to verify the binding of the ETS1 transcription factor to the DLK1 promoter as well as the influence of methylation status of DLK1 promoter on this binding affinity. The expression of DLK1 in the right ventricular outflow tract was significantly lower in patients with Tetralogy of Fallot (TOF) than that in controls (P<0.001). Moreover, the methylation level of CpG site 10 and CpG site 11 in the DLK1_R region was significantly decreased in TOF cases compared with controls (P<0.01). The integral methylation levels of DLK1_R and the methylation status of the CpG site 11 were both positively associated with DLK1 protein expression in TOF cases. ETS1 was found to inhibit DLK1 transcriptional activity by binding to the CpG site 11 and this affinity could be influenced by the methylation level of the DLK1 promoter. These findings demonstrated that the hypomethylation of the DLK1 promoter could increase the binding affinity of ETS1 transcription factor, which in turn inhibited DLK1 gene transcriptional activity and contributed to the development of TOF.

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Introduction

Tetralogy of Fallot (TOF) is the most common cyanotic congenital heart disease with a prevalence of 1/3,600 live births (1). Its symptoms consist of pulmonary outflow tract obstruction, ventricular septal defects (VSDs), overriding aortic roots and right ventricular hypertrophy (2). Although advances in pharmacotherapy and surgical procedures have improved the survival rate of patients with TOF, the exact pathogenesis of TOF is yet to be elucidated. However, only 20% of total TOF cases have a known cause, which may be associated with gene mutations or chromosomal anomalies, thus the exact etiology for the remaining TOF cases remains unclear (2). In addition to genetic mechanisms, epigenetics may play an important role in the development of TOF (3).

The Notch signaling pathway is highly conserved and related to the formation of the atrioventricular ducts, valves, outflow tracts and trabecula (4,5). Mutations in the genes of the Notch signaling pathway can cause heart defects in humans or mice, proving its important role in cardiac development (6-8). δ like non-canonical Notch ligand 1 (DLK1) is a non-canonical Notch ligand that regulates the Notch signaling pathway (9).

Several studies have found that DLK1 is involved in the cell differentiation process throughout embryonic development and adulthood (9). During cardiac development, a previous study demonstrated that DLK1 is highly expressed in the heart of embryos (10). In addition, DLK1 can negatively regulate the differentiation of cardiac fibroblasts into myofibroblasts, and therefore control myocardial fibrosis (11). Furthermore, a previous study reported that mutations of zinc finger protein 57 homolog, the target gene of DLK1, can cause a variety of cardiac defects, including atrial septal defect and VSD, via the Notch signaling pathway (10). Moreover, Page *et al* (2) found that there was a very low frequency of genetic variants in the DLK1 coding region in 829 patients with TOF (2). Therefore, these previous studies indicate that epigenetic changes of the DLK1 gene may be a risk factor for the pathogenesis of TOF.

DNA methylation is the most thoroughly studied epigenetic regulatory mechanism and can alter gene expression in both development and disease (12). Numerous studies have shown that aberrant DNA methylation may be associated with cardiovascular disease (13-15). For example, abnormal DNA methylation of several candidate genes involved in cardiac development, such as transcription factor GATA-4, has been found in patients with congenital heart disease (CHD) (3). In addition, aberrant methylation levels of the homeobox protein Nkx-2.5 gene body and heart and neural crest derivatives-expressed protein 1 gene promoter region are negatively correlated with their mRNA expression (16). In the present study, the changes in DNA methylation of the DLK1 promoter region in TOF were explored and its influence on gene expression was analyzed. These findings may provide important clues in understanding the etiology of this disease.

Materials and methods

Clinical tissue samples. The Ethics Committee of the Children's Hospital of Fudan University [Shanghai, China; approval no. 2015(26)] and Soochow University (Suzhou, China) approved the collection of cardiac tissues. Written informed consent of all study participants was obtained from their parents or relatives, and the study was registered in the Chinese Clinical Trial Registry (registration no. ChiCTR2100051811). TOF was diagnosed in 25 patients recruited from the Children's Hospital of Fudan University between January 2016 and July 2018. They were diagnosed by echocardiogram and confirmed by surgery. The five control samples obtained from the Department of Forensic Medicine of Soochow University were patients who had passed away from accidents without any known heart problems. The clinical characteristics of the samples are summarized in Table I.

Table I. Demographic characteristics of TOF cases (n=25) and controls (n=5).

Characteristics	TOF	Control
Age, years, median (IQR)	0.59 (0.37-0.95)	0.17 (0.01-0.79)
<1, n (%)	20 (80)	4 (80)
1~2, n (%)	4 (16)	1 (20)
>2, n (%)	1 (4)	0 (0)
Sex		
Female, n (%)	10 (40)	0 (0)
Male, n (%)	15 (60)	5 (100)

TOF, Tetralogy of Fallot.

Immunohistochemistry. The right ventricular outflow tract (RVOT) tissues of patients with TOF and controls were fixed with 10% neutral buffered formalin for ~48 h at room temperature. The fixed tissues were embedded in paraffin and cut into 4 μm thick sections. The paraffin-embedded sections were baked in 56˚C for ~3 h, dewaxed in dimethylbenzene, hydrated in descending alcohol series (100%, 5 min; ~100%, 5 min; ~95%, 5 min; ~85%, 5 min; ~75%, 5 min) and boiled (100˚C) for antigen retrieval with citrate buffer (0.01 mol/l; pH, 6.0) (Beyotime Institute of Biotechnology). Next, endogenous peroxidase activity was blocked with 3% hydrogen peroxide for ~30 min at room temperature, and 5% bovine serum (0.05 g/ml; BioFroxx; neoFroxx GmbH) was used to reduce non-specific staining. The sections were incubated with a primary antibody against DLK1 (1:200; cat. no. ab210471; Abcam) overnight at 4˚C, followed by incubation with horseradish peroxidase (HRP)-conjugated anti-rabbit/anti-mouse IgG antibodies (cat. no. GK500710; Gene Tech Co., Ltd.) for 2 h at 25˚C. Lastly, DAB and hematoxylin were applied for staining. The intensity of DLK1 protein expression was detected under a light microscope (Lecia Microsystems GmbH). For each sample, three fields of view without repeating areas were selected under the light microscope (magnification, x200) and images were captured in order to analyze the expression of DLK1 in RVOT tissues using ImageJ software version 1.48 (National Institutes of Health). Segmentation was set at a level that allowed for the detection of positive immunostaining and the positive area % of each image was measured. The mean value of the total of three visual fields was calculated.

DNA extraction and bisulfite sequencing PCR (BSP). Genomic DNA from the RVOT tissues of controls and patients with TOF was extracted using the E.Z.N.A.® Tissue DNA Kit (cat. no. D3396; Omega Bio-Tek, Inc.) following the manufacturer's instructions. Bisulfite modification of genomic DNA was carried out using the EZ DNA Methylation-Gold kit (Zymo Research Corp.) and amplified by PCR with ZymoTaq PreMix (Zymo Research Corp.). The primers were designed using Methyl Primer Express™ v1.0 software (Applied Biosystem; Thermo Fisher Scientific, Inc.): DLK1-BSP-forward (F), 5'-TAGTTGGGTATGTGTGTTTGTG-3' and reverse (R), 5'-TTACCCAACCATAAACATCCT-3'. Thermocycling conditions were as follows: 95˚C for 5 min; 40 cycles of 95˚C for 30 sec, 58˚C for 30 sec and 72˚C for 40 sec; and 72˚C for 7 min.

The PCR products were purified (Axygen; Corning, Inc.), inserted into a pGEM-T easy vector (Promega Corporation), and then transformed into *E. coli* DH5α competent cells (cat. no. DL1002; Shanghai Weidi Biotechnology Co., Ltd.). A total of 10 clones were selected randomly to determine the methylation status. The sequencing results were analyzed using the QUMA website (http://quma.cdb.riken.jp).

Table II. Sequences of oligonucleotide probes used for electrophoretic mobility shift assay.

Probe	Sequences (5'→3')
Biotin-DLK1_-744/-720	F: TCTGTTTATACGTGTGTTTGCGTGT
	R: ACACGCAAACACACGTATAAACAGA
Unlabeled-DLK1_-744/-720	F: TCTGTTTATACGTGTGTTTGCGTGT
	R: ACACGCAAACACACGTATAAACAGA
MU-DLK1_-744/-720	F: TCTGTTTATACGTGTGGCGATATGT
	R: ACATATCGCCACACGTATAAACAGA
Me-DLK1_-744/-720	F: TCTGTTTATACGTGTGTTTGCGTGT
	R: ACACGCAAACACACGTATAAACAGA

DLK1, δ like non-canonical Notch ligand 1; Me, methylated; MU, mutant; F, forward; R, reverse.

Luciferase reporter plasmid construction. pGL3-DLK1_-902/-597 was constructed by amplifying the fragment containing DLK1-R (-899 to -641 bp) using the following primers: DLK1-*Kpn*I-F, 5'-ATAGGTACCAGTCAGCTGGGTATGTGTGC-3'; DLK1-*Xho*I-R, 5'-GATCTCGAGTATAGACACAGTCCTAGGTGGCAG-3'. Next, the amplified fragment was inserted into the pGL3-Promoter (Promega Corporation) vector to determine the influence of the DLK1_R region on gene transcriptional activity.

Cell culture, transfection and dual-luciferase reporter gene assay. 293 cells (The Cell Bank of Type Culture Collection of The Chinese Academy of Sciences) were plated in 96-well plates at 2-4x10^5 cells/well and grown in DMEM (Gibco; Thermo Fisher Scientific, Inc). containing 10% FBS (Gibco; Thermo Fisher Scientific, Inc.) and penicillin-streptomycin (1:100; Gibco, Thermo Fisher Scientific, Inc.) at 37˚C under 5% CO_2. Once cells reached 60% confluency, 100 ng pGL3-basic, pGL3-promoter and pGL3-DLK1_-902/-597 were separately co-transfected with 4 ng internal reference pRL-TK plasmids (Promega Corporation) using Lipofectamine® 3000 (Invitrogen; Thermo Fisher Scientific, Inc.) at 25˚C. The transfection process lasted 30-60 min.

The JASPAR database (version JASPAR 2020) was used to analyze the sequence of the DLK1_R region and binding sites for the protein C-ets-1 (ETS1) transcription factor containing the CpG site 11 were found (17). Therefore, 100 ng pcDNA3.1-ETS1 and 100 ng constructed plasmids were co-transfected into cells. Luciferase activity was detected using the dual-luciferase reporter assay system (Promega Corporation) 48 h after transfection. Three independent luciferase activity assays were conducted. PGL3-basic vector served as a negative control, and the pGL3-promoter vector was a positive control. PRL-TK plasmids were used to normalize the luciferase activity to avoid errors caused by differences in the number of cell and efficiency of plasmid transfection to cells. To compare the differences between the two groups, data of three times experiments were normalized relative to the pGL3-promoter group.

Electrophoretic mobility shift assay (EMSA) and Shift-western blotting. 293 cells were plated in 10 cm dishes at 2x10^6 cells/well. Once cells reached 70% confluency, 12.5 μg pcDNA3.1 and pcDNA3.1-ETS1 were separately transfected into cells using Lipofectamine 3000 at 25˚C according to the manufacturer's protocol. The transfection process lasted 30-60 min. The pcDNA3.1 plasmid (the empty vector) was used as the negative control. After 48 h, the nuclear protein was extracted by using cytoplasmic extraction reagent and nuclear extraction reagent (Thermo Fisher Scientific, Inc.), and the concentration of the nuclear protein was measured using BCA protein assay (Takara Biotechnology Co., Ltd.) according to the manufacturer's protocol. Furthermore, western blotting was performed to verify the overexpression of ETS1. Samples containing equal amounts of protein (15 μg) were separated by SDS-PAGE on 10% gels (Epizyme, Inc.), and subsequently transferred to nitrocellulose membranes (MilliporeSigma) and blocked with 5% skimmed milk (0.05 g/ml) for ~1 h at room temperature. The membranes were probed with primary antibodies against ETS1 (1:1,000; cat. no. D8O8A; Cell Signaling Technology, Inc.) and PCNA (1:5,000; cat. no. 10205-2-AP; ProteinTech Group, Inc.) at 4˚C overnight. Then, membranes were incubated with HRP-conjugated anti-rabbit secondary antibody (1:5,000; cat. no. M21002; Abmart Pharmaceutical Technology Co., Ltd.) for ~2 h at room temperature. The blots were visualized using enhanced chemiluminescence reagents (Thermo Fisher Scientific, Inc.).

Biotin-labeled oligonucleotide probes containing the binding sequence of ETS1 were synthesized and labeled with biotin at the 5' end. Unlabeled probes used for the competition were also synthesized and the methylated probes were modified at CpG sites. These probes were purchased from Generay Biotech Co., Ltd. The sequences of the DNA probes are shown in Table II. ETS1 protein (10 μg) was co-incubated with 4 pmol unlabeled probes (1:100 dilution) for ~30 min at room temperature and subsequently with 20 fmol biotin-labeled probes (1:10,000 dilution) for ~30 min at room temperature. The protein-DNA complexes were separated from free DNA probes on a 6% polyacrylamide gel in 0.5X TBE at 100 V for ~50 min and then transferred to a nylon membrane (MilliporeSigma). Subsequently, the membrane was detected with the LightShift™ EMSA kit (Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. The Shift-western blotting was performed using the same methods as EMSA, except that EMSA involved the use of nylon membranes, while Shift-western blotting required nitrocellulose membranes for

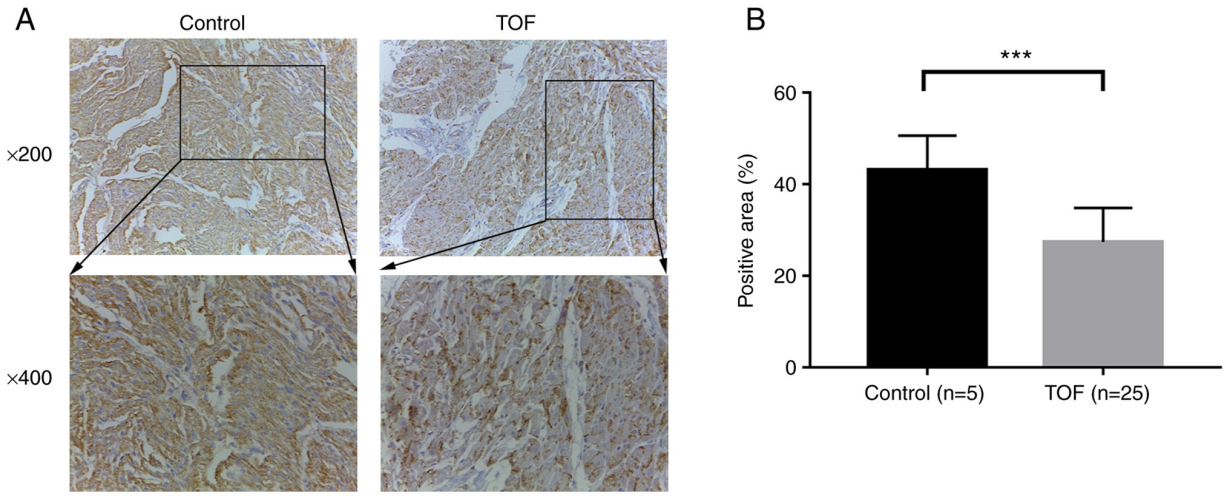

Figure 1. Expression analysis of DLK1 protein in cardiac tissues. (A) DLK1 protein expression was detected by immunohistochemistry [anti-DLK1 (1:200); magnification, x200 and 400] in cardiac tissues from 25 patients with TOF and five controls. The x400 image is the enlarged part, which is inside the rectangle in the x200 image. (B) Semi-quantitative analysis of DLK1 expression. ***P<0.001. DLK1, δ like non-canonical Notch ligand 1; TOF, Tetralogy of Fallot.

protein transfer. The membrane was blocked with 5% skimmed milk (0.05 g/ml) for ~1 h at room temperature, incubated with the primary antibody against ETS1 (1:1,000; cat. no. D8O8A; Cell Signaling Technology, Inc.) at 4˚C overnight, and then incubated with a HRP-conjugated anti-rabbit secondary antibody (1:5,000; cat. no. M21002; Abmart Pharmaceutical Technology Co., Ltd.) for ~2 h at room temperature. Finally, the blots were visualized using enhanced chemiluminescence reagents (Thermo Fisher Scientific, Inc.).

Chromatin immunoprecipitation-quantitative (ChIP-q) PCR. 293 cells were seeded in two 10 cm dishes at a density of $2x10^6$ cells/well. After 24 h, the experimental group was treated with 40 μM 5-Aza-2'-deoxycytidine (Sigma Aldrich; Merck KGaA), and the control group was treated with the same volume of DMSO. The treatment of both groups lasted for ~48 h at 37˚C under 5% CO_2. The ChIP assay was performed using EZ-Magna ChIP™ A/G Chromatin Immunoprecipitation kit (MilliporeSigma) according to the manufacturer's instructions. Briefly, cells were incubated with 225 μl 37% formaldehyde (final concentration, 1%) for ~10 min at room temperature, then 1 ml 10X glycine was added to stop crosslinking for ~5 min at room temperature and collected with cold 1X PBS. After resuspension in 500 μl SDS lysis buffer, samples were then sonicated by ultrasonic disruptor (Diagenode Bioruptor; intensity 5, 30 sec on and 30 sec off, 25 cycles) and centrifuged at 10,000 x g at 4˚C for 10 min to remove insoluble material. Sheared chromatin (50 μl) was incubated with 450 μl ChIP dilution buffer and antibody overnight at 4˚C with rotation. Antibodies used for ChIP included: Anti-ETS1 antibody Chip grade (1:50; cat. no. D8O8A; Cell Signaling Technology, Inc.) and anti-rabbit IgG Chip grade (5 μg; cat. no. 2729S; Cell Signaling Technology, Inc.). The antibody of IgG was used as the negative control. The next day, the magnetic beads were washed with low salt wash buffer, high salt wash buffer, LiCl wash buffer and TE buffer at room temperature. Each sample was incubated with 100 μl ChIP elution buffer at 62˚C for 2 h with shaking, at 95˚C for 10 min and then cooled down to room temperature. Beads were then separated using magnets, and the supernatant was purified using FastPure Gel DNA Extraction Mini Kit (cat. no. DC301; Vazyme Biotech Co., Ltd.) to acquire DNA fragments. The enriched DNA fragments were quantified via qPCR using TB Green® Premix Ex Taq™ (cat. no. RR420A; Takara Bio, Inc.). Thermocycling conditions were as follows: 95˚C for 30 sec; 40 cycles of 95˚C for 5 sec and 60˚C for 20 sec; and 95˚C for 15 sec and 60˚C for 60 sec. Three independent qPCRs were performed. The primers used for ChIP-qPCR were as follows: ChIP-qPCR-F, 5'-TTTGTGTTTCAGCGCGGCTAAG-3' and ChIP-qPCR-R, 5'-TTACCCAACCATGGGCATCCTCC-3'. The obtained results were expressed as % input (18).

Statistical analysis. All data are presented as the median (interquartile range) of three independent experiments. Data were analyzed using GraphPad Prism software (version 7.0, GraphPad Software, Inc.). The differences between DLK1 expression and the methylation status of the DLK1 gene promoter in the two groups were determined with a Mann-Whitney test. Pearson's correlation was used to test the correlation between methylation levels and protein expression. The differences in luciferase activity and the enrichment of the amplified fragments between multiple groups were tested by one-way ANOVA, followed by the LSD post hoc test. P<0.05 was considered to indicate a statistically significant difference.

Results

Expression of DLK1 protein in the RVOT tissues of patients with TOF and controls. To determine the clinical relevance of DLK1 in the development of TOF, immunohistochemistry was used to detect the expression level of DLK1 protein in the RVOT tissues of 25 patients with TOF and five controls. As shown in Fig. 1A, the intensity of positive yellow-brown immunohistochemical staining of DLK1 protein in the RVOT tissues was markedly weaker in patients with TOF than that in controls (Fig. 1A). The further semi-quantitative analysis showed that the expression of DLK1 protein in the patients with TOF was significantly lower than that in the controls (P<0.001; Fig. 1B).

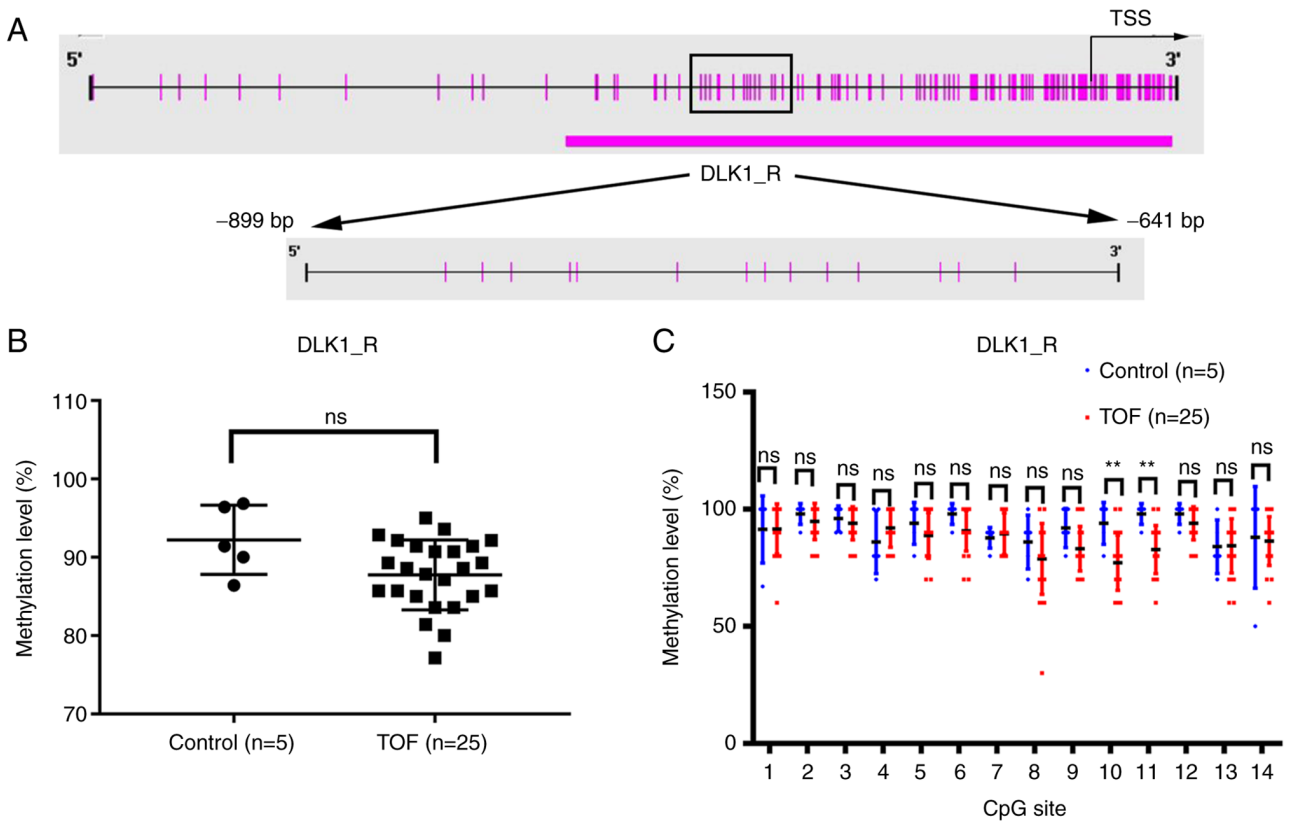

Figure 2. Methylation status of the DLK1 promoter region in cardiac tissues. (A) The presentation of DLK1_R (-899 bp/-641 bp) in the DLK1 promoter region. (B) The integral methylation status of DLK1_R (-899 bp/-641 bp) in the 25 patients with TOF and five controls. (C) The methylation level of each CpG site in the DLK1_R region in patients with TOF (n=25) and healthy controls (n=5). **P<0.01. DLK1, δ like non-canonical Notch ligand 1; TOF, Tetralogy of Fallot; ns, no significance; TSS, transcription start site.

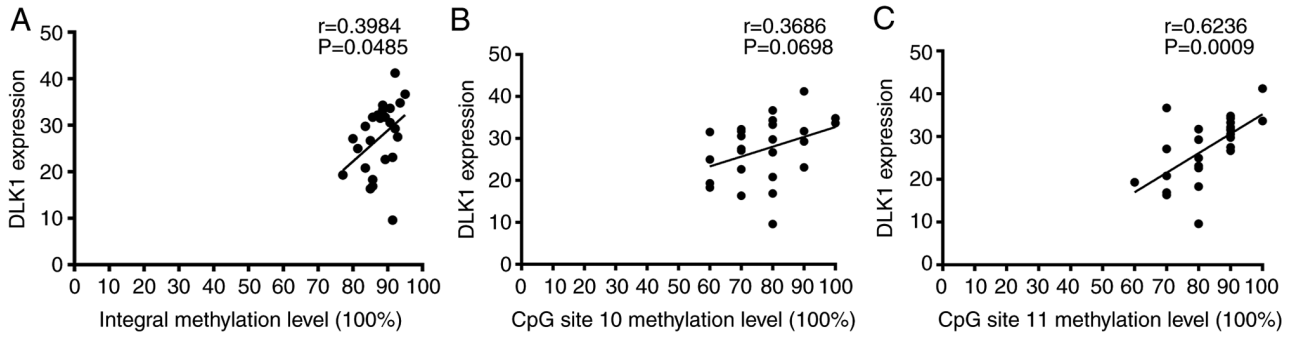

Figure 3. Correlation between methylation level and DLK1 expression in 25 patients with TOF. (A) Correlation between integral methylation level of the DLK1_R region and DLK1 expression in 25 patients with TOF. (B) Correlation between the CpG site 10 methylation level and DLK1 expression in 25 patients with TOF. (C) Correlation between the CpG site 11 methylation level and DLK1 expression in 25 patients with TOF. DLK1, δ like non-canonical Notch ligand 1; TOF, Tetralogy of Fallot.

Methylation status analysis for DLK1 gene in patients with TOF and controls. BSP was performed to measure the methylation level of the DLK1 gene promoter. The amplicon (DLK1_R, -899 to -641 bp) from the DLK1 gene promoter was analyzed in 25 patients with TOF and five age-matched controls (Fig. 2A). The results underwent quality control to remove unreliable methylation data.

The integral methylation level of DLK1_R was not significantly different in 25 TOF patients with a median of 88.57% (IQR, 85-91.43%), compared with the five controls with a median of 91.43% (IQR, 88.21-96.64%) (P=0.0716; Fig. 2B). Of note, the subsequent analysis of the methylation status of each CpG site in the DLK1_R region revealed that the methylation level of CpG site 10 and CpG site 11 was significantly lower in the TOF group than in the control group (P<0.01; Fig. 2C).

Pearson's correlation analysis was used to determine whether DLK1_R methylation level was related to the expression level of DLK1 protein. There was a moderate positive correlation between the integral methylation status of DLK1_R and DLK1 expression in 25 patients with TOF (r=0.3984, P=0.0485; Fig. 3A). DLK1 protein expression was not significantly correlated with the methylation level of CpG site 10 (r=0.3686, P=0.0698; Fig. 3B).

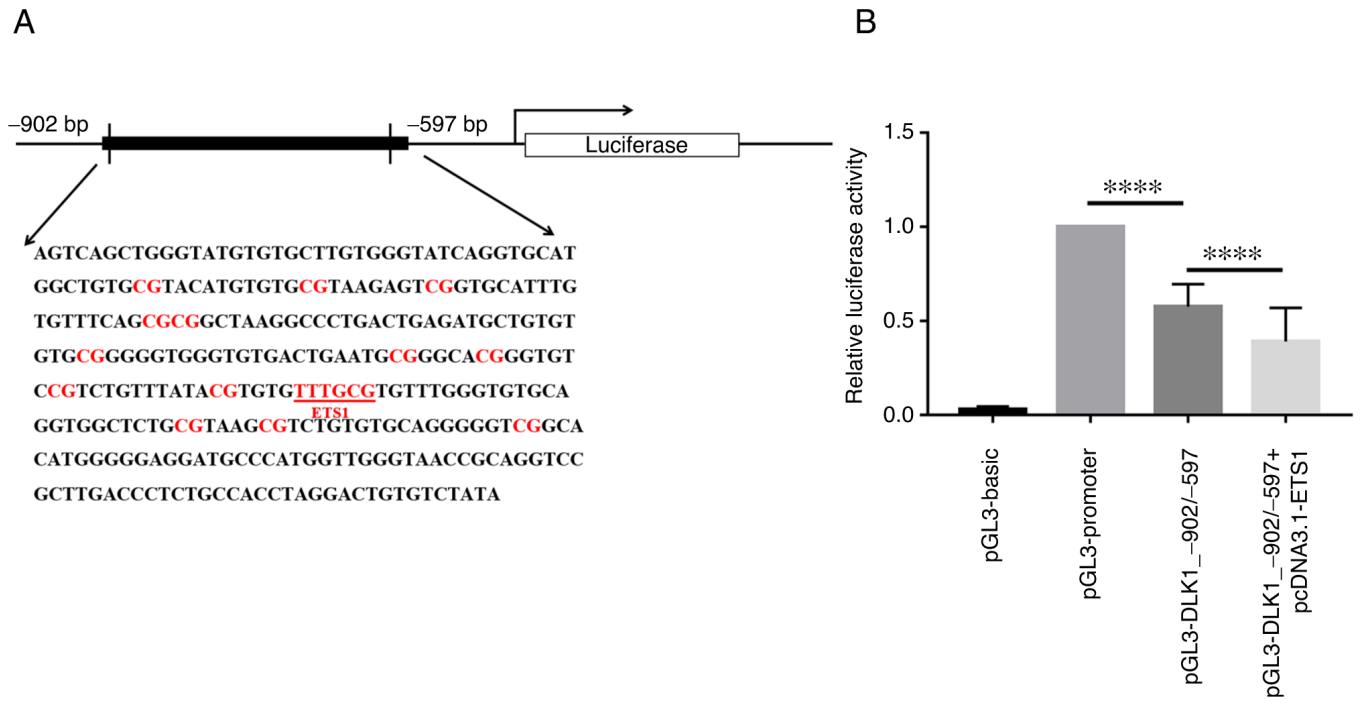

Figure 4. Regulation of ETS1 on the DLK1_-902/-597 region. (A) The sequence of the DLK1_-902/-597 region and the binding sites of ETS1. (B) Luciferase activity of pGL3-DLK1_-902/-597 co-transfected with ETS1 in the 293 cell line. ****P<0.0001. DLK1, δ like non-canonical Notch ligand 1; ETS1, ETS proto-oncogene 1.

However, a moderate positive correlation was also observed between DLK1 expression and the methylation status of CpG site 11 in patients with TOF (r=0.6236, P=0.0009; Fig. 3C).

ETS1 transcription factor binds to the promoter region of DLK1 and inhibits gene transcription activity. To explore the influence of the DLK1_R region on gene transcription activity, a dual-luciferase assay was performed using 293 cells. The pGL3-DLK1_-902/-597 plasmid was constructed by placing the DLK1_-902/-597 fragment containing the DLK1_R region (-899 bp to -641 bp) under pGL3-promoter (Fig. 4A). As shown in Fig. 4B, the luciferase activity in the case of the pGL3-DLK1_-902/-597 vector was significantly lower than in the case of the plasmid harboring the pGL3-promoter and lacking the DLK1_-902/-597 fragment, which indicated that the examined DLK1_-902/-597 region can inhibit gene transcription activity (P<0.0001).

An ETS1-binding sequence (-728 bp to -723 bp) containing the CpG site 11 (-724 bp) was predicted to be located in the DLK1_R region of the DLK1 promoter using the JASPAR database (Fig. 4A). To verify this prediction, pcDNA3.1-ETS1 was co-transfected with pGL3-DLK1_-902/-597 into 293 cells and the luciferase activity assay was performed. The luciferase activity in the case of pGL3-DLK1_-902/-597 + pcDNA3.1-ETS1 was significantly reduced compared with the pGL3-DLK1_-902/-597 transfection alone (P<0.0001; Fig. 4B).

These findings suggested that the ETS1-transcription factor could bind to the promoter region of DLK1 and inhibit the transcriptional activity of DLK1.

Validation of binding of ETS1 to the DLK1 promoter in vitro. To determine whether the transcription factor ETS1 could directly bind to the CpG site 11 and whether the methylation of DLK1_R could affect ETS1 binding affinity, EMSAs were performed using the overexpressed ETS1 protein (Fig. 5A) and oligonucleotide probes that contained ETS1 binding sequences. As shown in Fig. 5B (lane 2), a DNA-protein complex was formed when the biotin-labeled probes were incubated with ETS1. When unlabeled probes were added for competition, the ETS1 binding was inhibited, which resulted in a lighter band (lane 3). However, the bands in lane 4 and lane 5, where the mutation probes and methylated probes were added, were even lighter than that in lane 3. The subsequent Shift-western blotting showed that the delayed band detected in EMSA was the result of ETS1 protein binding (Fig. 5B). These findings demonstrated that the ETS1 transcription factor could bind to the promoter of DLK1 and that the methylation status of DLK1_R was able to influence ETS1 binding affinity.

Validation of binding of ETS1 to the DLK1 promoter in vivo. ChIP DNA samples and input DNA samples were quantified via qPCR. The fragments containing the predicted ETS1 transcription factor binding sequences were significantly enriched in the anti-ETS1 group, when compared with the anti-IgG group (P<0.001; Fig. 5C). When 293 cells were treated with DNA-demethylating agent 5-AZA-dC, the enrichment of the fragment was higher than that in the control group without 5-AZA-dC (P<0.01; Fig. 5C).

Discussion

The Notch pathway is a highly conserved cell signaling pathway required for cell fate specification and tissue patterning in the embryo (19). A consistent set of data in animal models and

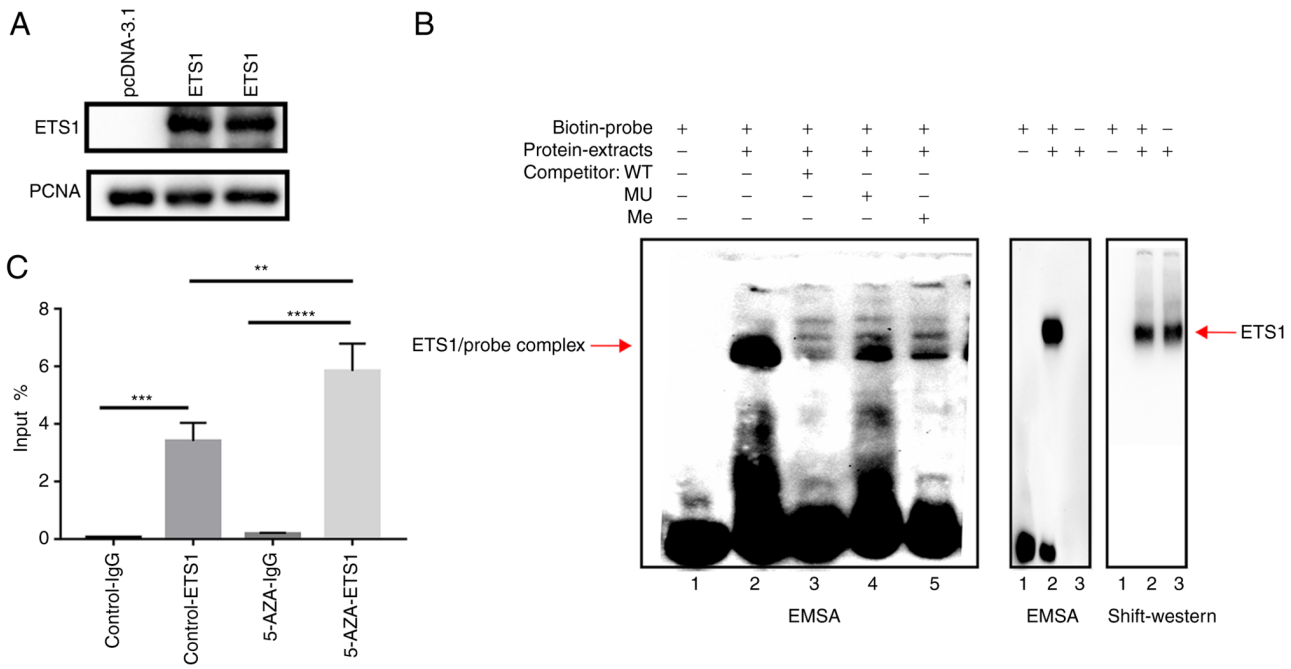

Figure 5. Binding of ETS1 to the DLK1 promoter. (A) Overexpression of ETS1 was verified via western blotting. (B) EMSA: i) Lane 1, biotin-labeled probes alone; ii) lane 2, biotin-labeled probes were incubated with ETS1 nuclear protein; iii) lane 3, unlabeled probes as competitors were added based on lane 2; iv) lane 4, mutation probes as competitors were added based on lane 2; and v) lane 5, methylated probes as competitors were added based on lane 2. Shift-western blotting confirmed ETS1 binding. (C) Chromatin immunoprecipitation assay was performed with anti-ETS1 using 293 cells treated with or without 5-aza. Quantitative PCR was used to verify the enrichment of DLK1_R. **P<0.01, ***P<0.001, ****P<0.0001. DLK1, δ like non-canonical Notch ligand 1; ETS1, ETS proto-oncogene 1; EMSA, electrophoretic mobility shift assay; 5-AZA, 5-azacytidine; WT, wild-type; MU, mutant; Me, methylated; PCNA, proliferating cell nuclear antigen.

humans has demonstrated that the Notch signaling pathway plays an important role in cardiogenesis (20-22). DLK1, one of the Notch ligands, is commonly expressed during fetal development and highly expressed in cardiac development (9). In the current study, by using immunohistochemistry, it was found that DLK1 protein expression in RVOT tissues of patients with TOF was significantly lower than that in controls. Considering the very low frequency of DLK1 in patients with TOF and the importance of epigenetics on the pathogenesis of TOF, it was deduced that the epigenetic changes may be closely associated with the abnormal expression of the DLK1 gene.

Several studies have demonstrated that epigenetic mechanisms play an important role in cardiac development by altering the expression of cardiac-related genes (23,24). One of the epigenetic modifications regulating gene transcription is DNA methylation status (25). Aberrant methylation of promoter CpG islands is known to contribute to the phenotypic expression of CHDs, including TOF and VSD (3). Our previous studies identified that the altered expression of some genes involved in cardiac development, such as Notch4 and COUP transcription factor 2 in TOF, may be associated with the abnormal methylation of their promoters (26,27). In the current study, no significant difference was observed in the integral methylation level of DLK1 promoter between the patients with TOF and controls. However, previous studies have demonstrated that the methylation of certain CpG sites can affect gene expression (26,28,29). Therefore, the methylation status of each CpG site in the DLK1_R region was analyzed in the present study. CpG site 10 and CpG site 11 had lower methylation levels in patients with TOF compared with controls. Moreover, a significant positive correlation between the DLK1 methylation level and DLK1 expression in patients with TOF could be observed in the CpG site 11. It was deduced that the hypomethylation level of the CpG site 11 may be associated with lower expression of DLK1 in patients with TOF compared with controls.

The present findings differ from previous studies that have reported a repressive role of hypermethylation in gene expression, which acts by preventing transcription factors from binding to CGI promoters (30,31). However, several publications have reported similar findings that promoter hypermethylation can facilitate gene transcription, mainly in tumor occurrence, development and metastasis (32-34). The most commonly hypothesized mechanism is that the increased methylation of the promoter can prevent the binding of a repressive transcription factor, which facilitates active gene transcription (35). To verify this hypothesis, the JASPAR database was used in the current study and it was found that ETS1 is a potential transcription factor that can bind to the DLK1_R region containing the CpG site 11. ETS1, as a member of the ETS family required for normal vascular development, can mediate early cardiomyocyte development in the embryo (36,37). The current research determined that ETS1 could bind to the DLK1 promoter and inhibit gene activity. This finding was consistent with the aforementioned previous studies.

To further investigate whether the methylation status of the DLK1_R region could affect the binding of ETS1, EMSA Shift-western blotting and ChIP-qPCR were performed. EMSA demonstrated that ETS1 could directly bind to the promoter of DLK1, and its binding could be blocked by

methylation. Moreover, Shift-western blotting confirmed that the band detected as a result of EMSA was indeed the ETS1 protein. Furthermore, ChIP-qPCR was performed and it was determined that DNA-demethylating agent 5-AZA-dC increased the enrichment of the DLK1_R fragment in ETS1. Taken together, these findings suggested that ETS1 could bind to the DLK1 promoter, with its affinity affected by the methylation status of the DLK1 promoter. This dependence proved to influence DLK1 protein expression. Although these results clarified that ETS1 could regulate gene expression by directly binding to the DLK1 promoter, the specific mechanism of demethylation and repression needs further exploration.

There are several limitations of the present study. The first limitation of this study was the insufficient number of samples due to the difficulty in obtaining cardiac tissues, especially from healthy controls. In addition, it could not be determined whether TOF caused abnormal methylation of DLK1 or whether this abnormality contributed to TOF. Therefore, the aforementioned issues need to be further explored in animal models or a prospective cohort. The second limitation was that 293 cells were used rather than cardiomyocytes, 293 cells were used in order to improve transfection efficiency as the transfection efficiency using cardiomyocytes was very low and not suitable for experiments.

In summary, this study demonstrated that the hypomethylation of specific sites in the DLK1_R region may influence DLK1 gene expression in patients with TOF. ETS1 proved to be a repressive transcription factor binding to the DLK1 promoter. Therefore, hypomethylation of CpG site 10 and CpG site 11 may increase the binding affinity of ETS1 and reduce DLK1 expression levels in patients with TOF. Given the aforementioned information, this study has the potential to provide novel epigenetic insights into the pathogenesis of TOF and contribute to further research on this subject.

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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

WS and GH made major contributions to the design of this study. GT, LH and RG performed the experiments and wrote the manuscript. JS, WC and YQ collected the samples and patient information, and analyzed the clinical data. ZZ, XM and WY participated in analyzing the experimental data. ZX and MS prepared the tables and figures, and made contributions to the interpretation of data. GT and WS confirm the authenticity of all the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

The Ethics Committee of the Children's Hospital of Fudan University [Shanghai, China; approval no. 2015(26)] and Soochow University (Suzhou, China) approved the collection of cardiac tissues. Written informed consent of all study participants was obtained from their parents or relatives.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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