Methylation status of CpG sites in the \textit{NOTCH4} promoter region regulates \textit{NOTCH4} expression in patients with tetralogy of Fallot

Yanjie Zhu$^{1,2}$*, Ming Ye$^{3}$, Hongfei Xu$^{4}$, Ruoyi Gu$^{3}$, Xiaojing Ma$^{3}$, Mingwu Chen$^{2}$, Xiaodi Li$^{1,2}$, Wei Sheng$^{1,2}$ and Guoying Huang$^{1,3}$

$^{1}$Institute of Paediatrics; $^{2}$Shanghai Key Laboratory of Birth Defects; $^{3}$Cardiovascular Centre, Children's Hospital of Fudan University, Shanghai 201102; $^{4}$Department of Forensic Medicine, Soochow University, Suzhou, Jiangsu 215006; $^{5}$Division of Life Sciences and Medicine, The First Affiliated Hospital of The University of Science and Technology of China, Hefei, Anhui 230036, P.R. China

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\textbf{Abstract.} Tetralogy of Fallot (TOF) is the most common form of cyanotic congenital heart disease (CHD). Although a lower methylation level of whole genome has been demonstrated in TOF patients, little is known regarding the DNA methylation changes in specific gene and its associations with TOF development. \textit{NOTCH4} is a mediator of the Notch signaling pathway that plays an important role in normal cardiac development. However, the role of epigenetic regulation of the \textit{NOTCH4} gene in the pathogenesis of TOF remains unclear. Considering the \textit{NOTCH4} low mutation frequency and reduced expression in the TOF patients, we hypothesized that abnormal DNA methylation change of \textit{NOTCH4} gene may influence its expression and responsible for TOF development. In this study, we measured the promoter methylation status of \textit{NOTCH4} and was measured and its regulation mechanism was explored, which may be related to TOF disease. Additionally, the promoter methylation status of \textit{NOTCH4} was measured in order to further understand epigenetic mechanisms that may serve a role in the development of TOF. Immunohistochemical analysis was used to examine \textit{NOTCH4} expression in right ventricular outflow tract myocardial tissues in patients with TOF. Compared with healthy controls, patients with TOF displayed significantly reduced in \textit{NOTCH4} expression (P=0.0055). Moreover, bisulphite sequencing suggested that the methylation levels of CpG site 2 in the \textit{NOTCH4} promoter was significantly higher in the patients than in the controls (P=0.0459). \textit{NOTCH4} expression was negatively associated with CpG site 2 methylation levels (r=-0.51; P=0.01). ETS1 transcription factor can serve as transcriptional activators by binding to specific DNA sequences of target genes, such as \textit{DLL4} and \textit{NOTCH4}, which serves an important role in normal heart development. Dual-luciferase reporter and electrophoretic mobility shift assays indicated that the ETS1 transcription factor could bind to the \textit{NOTCH4} promoter region. However, binding of ETS1 to the \textit{NOTCH4} promoter was abrogated by methylation at the putative ETS1 binding sites. These findings suggested that decreased \textit{NOTCH4} expression in patients with TOF may be associated with hypermethylation of CpG site 2 in the \textit{NOTCH4} promoter region, due to impaired binding of ETS1.

\textbf{Introduction}

Congenital heart defects (CHDs), are the most common type of birth defect, affecting ~ eight in 1,000 livebirths (1,2). Among these congenital conditions, tetralogy of Fallot (TOF) is the most common cyanotic heart malformation and accounts for approximately 10\% of all CHD cases (3). TOF results in anatomic defects, such as obstruction of the right ventricular outflow tract (RVOT), ventricular septal defect, aortic dextroposition and right ventricular hypertrophy (4). The pathogenesis of TOF is not completely understood. Previous studies have demonstrated that cardiac tissue-specific transcription factors, genes and associated signalling pathways, such as the Notch signalling pathway, play important roles in normal cardiac development, and that abnormal expression of such genes can contribute to TOF (5-7). In addition to DNA sequence variants, environmental factors, such as opioid exposure during early pregnancy and maternal pre-pregnancy obesity (8), may also be associated with the aetiology of TOF, as indicated by epidemiological data (9).

Notch is a highly conserved signalling pathway that regulates cell specification, differentiation, and organ formation and morphogenesis involved in development (10). Moreover, tissue-specific endocardial Notch signalling regulates cardiac morphogenesis through interactions with multiple myocardial,
epicardial and neural crest-derived signals (11). Mutations in Notch signalling elements result in CHD in humans and mice, demonstrating its important role in normal cardiac development (12-14). NOTCH4 serves as a membrane-bound receptor that regulates cell fate (15). Notch1-deficient embryos display severe vascular developmental defects, which are exacerbated in Notch1/Notch4 double-mutant embryos (16). Constitutive activation of Notch4 in the embryonic vasculature also leads to defects in vascular remodelling (17,18). In addition, a previous study have demonstrated that Notch4 activation in endothelial cells causes trans-differentiation to a mesenchymal phenotype, suggesting that implicates Jagged1-Notch interactions promote epithelial-mesenchymal transition, which is required for normal endocardial cushion differentiation and vascular smooth muscle cell development (19). Furthermore, a previous study has demonstrated that the NOTCH4 gene was down-regulated and had low frequency of genetic variants in the NOTCH4 coding region in patients with TOF (20).

DNA methylation is an epigenetic modification that is vital for embryonic development, and changes in methylation may be associated with the development of cardiovascular diseases (21). The interactions between DNA and transcription factors could be influenced directly or indirectly by DNA methylation, due to recruitment of methyl-CpG-binding proteins (22). Sheng et al (23,24) suggested that aberrant methylation levels at the promoter CpG island shore of the ZFPM2 gene and HAND1 genes may be responsible for gene transcription regulation in patients with TOF. Gong et al (25) demonstrated that demethylation in theTXB20 promoter region may be associated with overexpression in the cardiac tissue of patients with TOF. However, a recent study on severe anxiety has reported that a single CpG site located in the promoter of the Asb1 gene may be responsible for a methylation increase of 48.5% (26). Methylation at specific loci in genes, such as tissue factor F3, interleukin-6 and toll-like receptor 2, is influenced by exposure to air pollution, thus leading to several adverse health effects (27-29). Therefore it may be hypothesised that changes in DNA methylation contribute to downregulation of NOTCH4 in patients with TOF.

NOTCH4 has been demonstrated to be a ETS factor-regulated gene, and ETS may activate the expression of Notch signalling components to initiate Notch signalling in the early artery (30). The ETS1 oncogene belongs to a large family within the ETS domain family of transcription factors (31). It is widely expressed in the developing embryo, and could be detectable in the day-15 embryos of murine (32). Previous studies have demonstrated that ETS1 plays an important role in human heart development and was associated with the development of CHD (33,34). ETS proteins can function as transcriptional activators by binding to specific DNA sequences of target genes, thereby increasing (such as DLL4, NOTCH4 and NKp46) or decreasing (such as MMP1 and BCL2) gene transcription in response to various stimuli, including changes of genetics, histone modification or DNA methylation (30,35-37).

The aim of the present study was to examine the epigenetic mechanisms that regulate the NOTCH4 gene and their effect on NOTCH4 protein expression in patients with TOF. The findings of this study may provide insight into the aetiology of TOF.

Materials and methods

Clinical samples. The present study was approved by The Ethics Committee of The Children's Hospital of Fudan University (approval no. 2015) (26). Written informed consent was obtained from the parents or relatives of all study participants. Patients with TOF were recruited from the Children's Hospital of Fudan University between January 2016 to July 2018. TOF was diagnosed using an echocardiogram and confirmed by surgery. The control samples from autopsy specimens were provided by the Department of Forensic Medicine of Soochow University. In total, 24 patients with TOF were enrolled, including 14 (58.3%) males and 10 (41.7%) females. Patient age ranged from 1 month to 14 years (mean ± SD, 2.54 ± 0.86 years). Control samples were obtained from five male subjects, aged 1 day to 7 months (mean ± SD, 0.35 ± 0.19 years). All samples were taken from RVOT myocardial tissue and immediately stored at –80°C in RNAlater solution (Ambion; Thermo Fisher Scientific, Inc.). Patient characteristics are summarized in Table I.

Immunohistochemistry. RVOT myocardial tissues were fixed in 10% neutral buffered formalin at room temperature for 48 h and embedded in paraffin. The sections were then cut into 4-μm sections and dried overnight at 56°C. After deparaffination, hydration and antigen retrieval with citric acid buffer (0.01 mol/l; pH 6.0), endogenous peroxidase activity was blocked by incubating the sections with 3% hydrogen peroxide (H2O2) at room temperature for 20 min and blocking with 5% bovine serum (cat. no. 143183; Bio Forxx) for 1 h at room temperature. The slides were incubated with a rabbit anti-human monoclonal antibody against NOTCH4 (dilution 1:200, cat. no. ab184742; Abcam) overnight at 4°C. After primary antibody incubation, the slides were incubated with goat anti-rabbit/mouse IgG (cat. no. GK500710; Gene Tech Co., Ltd.) for 30 min at room temperature, then washed in TBST three times for 5 min each time. Finally, the slides were stained with 3,3-diaminobenzidine (DAB) for 40 sec and counterstained with haematoxylin for 1 min. After dehydration, the sections were mounted with mounting medium. For each sample, three visual fields were randomly chosen and examined under a light microscope at x200 magnification. Images of cardiomyocytes were quantified using ImageJ (version 1.48; National Institutes of Health). To quantify the intensity of NOTCH4 protein expression, images were captured with a light microscope. Three randomly selected fields (x200 magnification) per tissue section were scanned and analyzed using ImageJ software (version 1.48). The integrated optical density (IOD) sum of each image was measured after the optical density was adjusted with the segmentation set at a level to allow for detection of positive immunostaining. For statistical analysis, the mean value of the total three counted fields was calculated.

DNA extraction and bisulphite sequencing PCR (BSP). Genomic DNA was extracted from RVOT myocardial tissue samples from patients with TOF and control subjects using a QIAamp DNA Mini kit (Qiagen GmbH) according to the manufacturer's instructions. For each sample, bisulphite treatment of genomic DNA from RVOT myocardial tissues was
performed using the EZ DNA Methylation-Gold kit (Zymo Research Corp.) according to the manufacturer's protocol. The bisulphite-treated DNA samples were then used as templates for BSP. The primers used to amplify the *NOTCH4* _r_ region (from position -240 to +113 bp, containing five CpG sites) were designed using Methyl Primer Express v1.0 software (Applied Biosystem; Thermo Fisher Scientific, Inc.). Primer sequences were as follows: *NOTCH4*-BSP-F, 5'-aTa GGT acc aGa TTc cTT cTc ccc GGG aTT GTT TaG G-3' and *NOTCH4*-BSP-R, 5'-ACAAAA ACCACCTCCTCTAACTCC-3'. For sequencing, the PCR products were purified using the AxyPrep DNA Gel Extraction kit (Axygen; Corning, Inc.) according to the manufacturer's protocol. The purified PCR products were then cloned into the pGEM-T easy vector system (Promega Corporation) and transformed into DH5α competent cells (Tiangen Biotech Co., Ltd.). After a 12-h incubation at 37°C, blue/white and ampicillin screening was carried out. For each p-GeM-T vector, 10 clones were determined the methylation status using Sanger sequencing. The fidelity of the plasmids was verified by Sanger sequencing. This construct is referred to as pGL3-*NOTCH4*-179/-53 plasmid thereafter.

In addition, The Mut-pGL3-*NOTCH4*-179/-53 plasmid was constructed by mutating a C base in the CpG site 2 to a T base to simulate its hypermethylation status which might abrogate binding of the target to *NOTCH4* _r_. The Mut-pGL3-*NOTCH4*-179/-53 plasmid was constructed using the KOD-plus-mutagenesis kit (cat. no. SMK-101; Toyobo Life Science) according to the manufacturer's protocol. The primers used for mutagenesis were synthesized by Generay Biotech Co., Ltd., as follows: Mut-*NOTCH4*-F, 5'-TGCTCCTACTCC CCTCATTCCCCCA-3' and Mut-*NOTCH4*-R, 5'-GTGTGTC CTGGAGGCCAGTGATAGG-3'.

Lastly, the Me-pGL3-*NOTCH4*-179/-53 plasmid was generated by incubating the pGL3-*NOTCH4*-179/-53 plasmid with the M.SssI CpG methyltransferase (cat. no. M0226V; New England BioLabs) for 4 h at 37°C. The methylation status was confirmed by BSP, as aforementioned.

**Transfections and dual luciferase reporter assays.** HeLa and HL-1 cells were purchased from the Cell Bank of the Type Culture Collection of the Chinese Academy of Sciences, which were cultured in Dulbecco’s modified Eagle's medium (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% foetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.) at 37°C with 5% CO2. For transfection, the cells were plated in 96-well plates 12 h before transfection at a density of 1-4x10^4 cells/well, then separately transfected with 100 ng of pGL3-basic (negative control), pGL3-promoter (empty vector), pGL3-*NOTCH4*-179/53 or Me-pGL3-*NOTCH4*-179/53 vector using Lipofectamine® 3000 (Invitrogen; Thermo Fisher Scientific, Inc.).

In order to study the mechanism underlying the effect of the *NOTCH4* _r_ region on gene transcription activity, the *NOTCH4* _r_ region sequence was analysed by a TF search (http://www.cbrc.jp/research/db/TFSEARCH.html) and the JASPAR database (http://jaspar.genereg.net/cgi-bin/jaspar_db.pl?-rmbrowse&db=core&tax_group=vertebrates), and the results demonstrated the

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### Table I. Clinical characteristics of patients with TOF and healthy controls.

| Clinical variable | Patients with TOF | Controls | P-valuea |
|-------------------|-------------------|----------|----------|
| Age, years, mean ± SD | 2.54±0.86 | 0.35±0.19 |          |
| Age category, years, n (%) |        |          |          |
| <1 | 15 (62.5) | 4 (80.0) | 0.796a |
| 1-2 | 4 (16.7) | 1 (20.0) |          |
| >2 | 5 (20.8) | 0 (0) |          |
| Sex, n (%) |        |          |          |
| Male | 14 (58.3) | 5 (100) | 0.134a |
| Female | 10 (41.7) | 0 (0) |          |

*a*P-value for patients with TOF (n=24) vs. healthy controls (n=5); Fisher's exact test. TOF, tetralogy of Fallot.
potential ETS1 transcription factor binding sites, and the CpG site 2 was within the ETS1 binding site. For dual luciferase reporter assays, 100 ng of pGL3-NOTCH4-179/-53 (unmethylated), Mut-pGL3-NOTCH4-179/-53 (mutated) and Me-pGL3-NOTCH4-179/-53 (methylated) were co-transfected with 100 ng of ETS1 transcription factor expression vector which was constructed by cloning the entire human ETS1 cDNA (accession no. NM_00143820) into a pcDNA3.1 (+) expression vector (cat. no. G105592; YouBio). The pGL3-basic and pGL3-promoter vectors were also used as controls. The pRL-TK plasmid (Promega Corporation) was co-transfected with 2.5 µg pcDNA3.1-eTS1 expression plasmid, and Me-pGL3- promotive factor construct was used as competitor-M. All luciferase activities were measured using a LightShift™ EMSA kit (GE Healthcare Life Sciences). GAPDH was used as a loading control.

Biotin-labelled oligonucleotide probes (Biotin-probe) specific for the ETS1-binding site of the NOTCH4 gene (5'-CGTCTT-3' position 138 to -133) were synthesized by General Biotech Co., Ltd. and the oligonucleotide probes were annealed into double strands by heating it to 95°C, then cooling to room temperature. The mutant biotin-labelled oligonucleotide probes (Biotin-Mut-probe) were used to confirm the ETS1 binding specificity. An unlabelled oligonucleotide probe (Competitor-WT), a mutant unlabelled oligonucleotide probe (Competitor-Mut) and an methylated oligonucleotide probe (Competitor-Met) were used as competitors. The sequences of these oligonucleotide probes are listed in Table II.

The DNA-binding ability of ETS1 to the NOTCH4 gene was detected by EMSA using a LightShift™ EMSA kit (cat. no. 20418; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. Specifically, 10 µg nuclear extract were incubated with 20 fmol biotin-labelled probes in binding buffer at room temperature for 30 min, and a 200-fold excess of unlabelled/methylated probes was added to the reaction as competitors. The protein-DNA complexes were separated from the free probes by a 6% polyacrylamide gel at 100 V for 50 min, then transferred onto a nylon membrane at 380 mA for 30 min. Subsequently, the membrane was analysed using a Fujifilm Las3000 Luminescence Image Analyzer (FUJIFILM Wako Pure Chemical Corporation). Shift-western blotting was performed by transferring the protein-DNA complexes from the polyacrylamide gel to a PVDF membrane in 0.5X Tris-borate-EDTA (TBE) buffer for 30 min. For protein detection, the membrane was blocked with blocking buffer (PBS with 1% Tween-20 and 5% BSA) for 2 h at room temperature, then incubated with a primary antibody against ETS1 (1:1,000; cat. no. ab220361; Abcam) and GAPDH (1:3,000; cat. no. ab9482; Abcam) at 4°C overnight, then incubated with horseradish peroxidase (HRP)-conjugated anti-rabbit secondary antibody (1:3,000; cat. no. M21002; Abmart) and HRP-conjugated anti-mouse secondary antibody (1:3,000; cat. no. M21001; Abmart). The blots were detected by enhanced chemiluminescence (Thermo Fisher Scientific, Inc.), and were visualized using GE ImageQuant LAS4000 mini (GE Healthcare Life Sciences). GAPDH was used as a loading control.

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

| Probe                  | Sequence (5'-3')                        |
|------------------------|----------------------------------------|
| Biotin-probe-F         | GCCCTCCAGGCACACCGTCTACTTCCC            |
| Biotin-probe-R         | GGAAGATGAGGCGTGTGCTGGAGGGGC            |
| Biotin-Mut-probe-F     | GCCCTCCAGGCACACATAAACACTAGCC           |
| Biotin-Mut-probe-R     | GGGCTAGTGTATGTGTCGTGGAGGGGC            |
| Competitor-WT-F        | GCCCTCCAGGCACACCTCTACTTCCC             |
| Competitor-WT-R        | GGAAGATGACGCGTGTGCTGGAGGGGC            |
| Competitor-Mut-F       | GCCCTCCAGGCACACATACCTAGCC              |
| Competitor-Mut-R       | GGCGTATGTTATGTGTGGCTGGAGGGGC           |
| Competitor-Mut-F       | GCCCTCCAGGCAACCCGTCTACTTCCC            |
| Competitor-Met-F       | GGAAGATGAGGCGTGTGCTGGAGGGGC            |

Mut, mutant; me, methylated; F, forward; R, reverse.
and were visualized using GE ImageQuant LAS4000 mini (GE Healthcare Life Sciences).

**Statistical analysis.** All data are presented as the mean ± SD of three independent experiments. Statistical analysis was performed using SPSS software v20.0 (IBM Corp.). Differences between two groups were analysed by the Mann-Whitney test. Differences of luciferase activity assays between multiple groups were analysed using one-way ANOVA, followed by the Least Significant Difference post hoc test. Pearson's correlation analysis was performed to analyse the relationship between the immunohistochemistry data and bisulphite sequencing data. P<0.05 was considered to indicate a statistically significant difference.

**Results**

**Expression of the NOTCH4 protein in patients with TOF and controls.** Immunohistochemistry was carried out to detect NOTCH4 protein expression in RVOT myocardial tissue from 24 patients with TOF and five controls. NOTCH4 protein was detectable by yellow or brown staining in the nucleus or cytoplasm. The staining intensity of cardiomyocytes from patients with TOF was weaker than that in the control subjects (Fig. 1A). Statistical analysis confirmed that NOTCH4 expression was significantly lower in the patients, compared with the controls (3.0 ± 0.3 vs. 6.3 ± 1.2; P=0.0055; Fig. 1B).

**NOTCH4 promoter methylation status is associated with protein expression.** BSP was carried out in order to determine whether reduced NOTCH4 expression was caused by changes in its epigenetic regulation, BSP was performed on the NOTCH4 promoter using tissue samples collected from patients with TOF and controls. Considering that there is no CpG island in the promoter region (≤2,000 to 200 bp), only the region that was targeted for sequencing (NOTCH4_R: -240 to +113 bp, containing 5 CpG sites; Fig. 2A) was studied.

In an initial screen, the methylation levels in NOTCH4_R were analysed in five patients with TOF and five control subjects (Fig. 2B). The overall methylation levels of NOTCH4_R (CpG sites 1-5) in patients with TOF did not significantly differ from the controls (42.4 ± 5.1 vs. 42.8 ± 5.8; Fig. 2C). Interestingly, only one CpG site (CpG site 2) in NOTCH4_R exhibited significantly higher methylation levels, compared with the controls (54.0 ± 6.8 vs. 20.0 ± 10.5; P=0.0476).

Following this initial screen, the methylation status of NOTCH4_R was determined in another 19 TOF patients and the data for the combined cohort was analysed (n=24; Fig. 2D). The methylation levels of CpG site 2 were significantly higher in 24 patients with TOF, compared with the controls (43.8 ± 4.5 vs. 20.0 ± 10.5; P=0.0459; Fig. 2D).

In addition, the methylation levels of all five CpG sites, as well as overall NOTCH4_R promoter methylation, were compared between male (n=14) and female (n=10) patients with TOF. None of the five CpG sites significantly differed between male and female patients with respect to methylation levels (Fig. 2E). Furthermore, the methylation levels of the five CpG sites were also compared between male patients with and normal controls. Consistent with the aforementioned results, only CpG site 2 in NOTCH4_R displayed significantly higher methylation levels in male patients with TOF, compared with controls (45.7 ± 4.7 vs. 20.0 ± 10.5; P=0.0263; Fig. 2F). In addition, the methylation levels of the five CpG sites were also compared between female patients with and normal controls. No significant difference was observed between female and normal controls (male) with respect to methylation levels (45.0 ± 9.2 vs. 20.0 ± 10.5; P=0.1758; Fig. 2G). After excluding two extreme values, CpG site 2 in NOTCH4_R showed higher methylation levels in female patients with TOF, compared with controls (56.2 ± 6.8 vs. 20.0 ± 10.5; P=0.0111). Thus, it can be
concluded that no sex differences exist in methylation levels in the TOF group and the normal control group.

Pearson's correlation was carried out to determine whether NOTCH4 protein expression was associated with NOTCH4 methylation status. There was no correlation between NOTCH4 protein expression and the overall methylation levels of NOTCH4 (r=-0.2966; P=0.1593; n=24; Fig. 3a) in patients with TOF. However, a significant negative association was observed between NOTCH4 expression and the methylation status of the CpG site 2 (r= -0.5063; P=0.0116; n=24; Fig. 3B) in patients with TOF.

Effect of NOTCH4 CpG site 2 methylation on gene transcription in vitro. A dual-luciferase assay was carried out in HeLa and HL-1 cells to determine whether single CpG site 2 methylation could influence NOTCH4 gene transcription activity. The pGL3-NOTCH4-179/-53 plasmid was constructed by cloning the NOTCH4 promoter region (-179 to -53 bp, containing only the CpG site 2) into the pGL3-promoter vector. In addition, a methylated plasmid was generated using M.SsSI treatment. BSP was used to determine the methylation status of the unmethylated and methylated CpG sites (Fig. 4A, B). As indicated in Fig. 4C, the luciferase activity of pGL3-NOTCH4-179/-53 was significantly higher than that of pGL3-promoter (HeLa: P=0.0022; HL-1: P=0.0022). Moreover, the luciferase activity of me-pGL3-NOTCH4-179/-53 decreased nearly six-fold, compared with pGL3-NOTCH4-179/-53. These results indicated that methylation in the NOTCH4_R region regulated gene transcription and that a methylation of a single CpG site could inhibit transcription of the NOTCH4 gene.

NOTCH4 expression is regulated by the ETS1 transcription factor binding to its promoter region. To clarify the mechanism through which NOTCH4 methylation affects gene transcription, the NOTCH4_R sequence was analysed for potential binding sites for the ETS1 transcription factor using an online TF search and the JASPAR database (38,39) (Fig. 4D). To evaluate the effect of ETS1 on NOTCH4 transcriptional activity, we co-transfected ETS1-overexpressing plasmids with pGL3-NOTCH4-179/-53, Mut-pGL3-NOTCH4-179/-53 and Me-pGL3-NOTCH4-179/-53, respectively, into HeLa and HL-1 cells (Fig. 4E). Following co-transfection with pGL3-NOTCH4-179/-53, luciferase activity was significantly increased, compared with pGL3-basic. In addition, a significant increase in luciferase activity following co-transfection with pGL3-NOTCH4-179/-53 and ETS1 transcription factor was observed. However, the luciferase gene activity driven by Mut-pGL3-NOTCH4-179/-53 was significantly reduced in the presence of ETS1, compared with pGL3-NOTCH4-179/-53 (HeLa: P=0.0022; HL-1: P=0.0159). Moreover, me-pGL3-NOTCH4-179/-53 resulted in significantly reduced luciferase activity when co-transfected with the ETS1-overexpression plasmid (HeLa: P=0.0022; HL-1: P=0.0095).
Altogether, these findings demonstrated that the ETS1 transcription factor could bind to the NOTCH4 promoter region and promote gene expression. This interaction was inhibited by methylation changes in the ETS1 binding site.

Impact of single CpG site 2 methylation on ETS1 binding affinity. The ETS1 transcription factor was overexpressed in the 293T cell line (Fig. 5A), and an EMSA was carried out to further confirm the effect of single CpG site 2 methylation on ETS1 binding affinity to the NOTCH4 promoter region. The biotinylated probe (Biotin-probe) could bind to the ETS1 transcription factor in the nuclear protein extract from transfected cells, forming a visible DNA/protein complex (Fig. 5B, lane 2). However, when the unlabelled probe (Competitor-WT)
was added as a competitor, the band corresponding to the DNA/protein complex was not observed (Fig. 5B, lane 3). However, with the mutant unlabelled competition probe (Competitor-Mut), the binding of ETS1 to the biotinylated probe was not affected (Fig. 5B, lane 4). Moreover, with the addition of an unlabelled methylated probe (Competitor-Met), the band of the DNA/protein complex in lane 5 was lighter than that in lane 2, but heavier than that in lane 3 (Fig. 5B, lane 5). In addition, the complex was not detected when the mutated biotinylated probe (Biotin-Mut-probe) was added (Fig. 5B, lane 6). A super-shift western blot was conducted to confirm that the DNA/protein banding was caused by the presence of ETS1 protein. The specific DNA/protein complexes are indicated by red arrows. In addition, the results also demonstrated that the biotinylated probe could bind to the ETS1 transcription factor, and the DNA/protein complex was induced by ETS1 antibody, indicating that the complex consists of ETS1 (Fig. 5C).

Collectively, the present findings indicated that the ETS1 transcription factor could directly bind to the NOTCH4_R region and was affected by methylation of the CpG 2 site.

Discussion

The Notch signalling pathway is evolutionarily conserved and plays a critical role in the growth and development of diverse organisms, including in normal cardiac development (16-18). In vertebrates, Notch4 is distributed in the aorta, the endocardium and the endothelial cells of arteries, including pulmonary and cardiac vessels (40,41). In the present study, immunohistochemical staining demonstrated that NOTCH4 expression in RVOT myocardial tissues was significantly decreased in patients with TOF, compared with controls, suggesting that the developmental defects leading to TOF are associated with distinct changes in NOTCH4 expression. Considering the low frequency of genetic variants in the NOTCH4 coding region in patients with TOF (20), it was hypothesized that epigenetic changes might be at play in the abnormal expression of this gene.

Several studies have demonstrated that both genetic and epigenetic mechanisms control the expression of cardiac genes in a spatiotemporal manner during cardiac development (42,43). DNA methylation is one of the major epigenetic mechanisms controlling gene expression, which can increase or decrease the levels of gene transcription based on the methylation status of the target gene (44,45). Abnormal methylation can change the normal expression of genes and lead to different diseases. Abnormal DNA methylation has been associated with the pathogenesis of tetralogy of Fallot and can exacerbate defects, such as RVOT and ventricular septal defect, leading to pathogenic cardiac remodelling (46,47). Our previous study demonstrated that altered expression of NK2 homeobox 5, heart and neural crest derivatives expressed 1 and long interspersed nuclear element-1 may be associated with epigenetic regulation and involved in the development of TOF (22,48). Moreover, a single CpG site-based methylation change was
also demonstrated to be responsible for changes in gene expression in different diseases (49,50). In the current study, the methylation status of the NOTCH4 promoter region and its association with gene expression was assessed. Considering that there is no CpG island in the NOTCH4 promoter, an important regulatory region near the TSS containing five CpG sites was selected for methylation analysis. Overall, no significant difference was observed in the overall methylation level between the patients with TOF and controls. However, when the individual methylation status of each CpG site was analysed separately, CpG site 2 exhibited significantly higher methylation levels in the cardiac tissue of patients with TOF, compared with the controls. Interestingly, in the patients, a significant negative correlation was also observed between NOTCH4 expression and the methylation levels at the CpG site 2. These findings indicated that CpG site 2 methylation changes may affect NOTCH4 expression.

To determine the effect of CpG site 2 methylation changes in the NOTCH4 promoter on gene expression and the underlying molecular mechanism, a dual-luciferase assay combined with an in vitro methylation assay was carried out. Following in vitro methylation, decreased transcriptional activity of pGL3-NOTCH4-179/-53 was observed, suggesting that increased methylation of the CpG site 2 had a negative impact on transcriptional regulatory activity. Several studies have suggested that CpG site-specific regulation of DNA methylation could be mediated by transcription factors binding to specific gene promoter regions (51,52). Therefore, using online prediction software was used to identify a potential transcriptional factor that could represent a target for the NOTCH4 promoter region. A potential binding site for the ETS1 transcription factor was identified in the NOTCH4 promoter that contained CpG site 2. The ETS1 transcription factor plays an important role in normal cardiac development (32), and can regulate transcriptional activity of target genes (such as DLL4, NOTCH4 and MMP1) by binding to specific sites (30,34-36).

In the present study, in vitro experiments demonstrated that NOTCH4 expression in patients with TOF was regulated by binding of ETS1 to NOTCH4_R. The present findings suggested that, although ETS1 could bind to the NOTCH4 promoter region and promote gene expression, binding affinity could be influenced when a single CpG site 2 was methylated, which might lead to a marked reduction of gene expression. However, CpG site 2 hypermethylation could result in reduced gene expression in vivo was not evaluated in this study and remains unclear. Thus, the direct function of single CpG site 2 methylation and its role in the regulation of NOTCH4 expression require further study.

Previous studies have demonstrated that DNA methylation occurs not only on CpG islands, but also on CpG island shores. Abnormal methylation in these regions may affect gene expression by altering the chromosomal structure (53,54). However, there is increasing evidence for the importance of the methylation status of individual CpG sites in the regulation of gene expression. For example, the methylation levels of a single CpG site was inversely correlated with oestrogen receptor α positivity in breast cancer specimens in a previous study (49). CpG site-specific methylation was demonstrated to alter the binding affinities of specific transcription factors that can activate or repress transcription (55,56). In the present study, CpG site 2 was differentially methylated in patients with TOF and controls, and the influence of sex and age was excluded through statistical analysis. In addition, abnormal methylation of CpG site 2 affected the binding affinity of the ETS1 transcription factor to the NOTCH4 gene and downregulates NOTCH4 expression. Furthermore, the causes of TOF development are complex, and involve many other factors, such as environmental, genetic and maternal factors such as age of the mother, radiation and drugs used by mother. Therefore, other potential interfering factors in the development of TOF cannot be excluded and should be evaluated in future studies.

A limitation of this study was the restricted sample size, due to difficulties in obtaining sufficient matched cardiac tissue from patients with TOF and controls. Further studies with a larger number of samples are required to confirm the present findings. In addition, it is uncertain whether the methylation patterns observed in the samples were effects or causes of TOF, since the onset of TOF preceded the measurement of methylation. The exact mechanism underlying NOTCH4 promoter methylation in the onset of TOF should also be explored in a large and prospective cohort, as well as animal models.

In conclusion, the present study suggested that single CpG site 2 in the NOTCH4 promoter region was hypermethylated in RVOT myocardial tissue from patients with TOF, which may lead to decreased NOTCH4 expression. Specifically, NOTCH4 expression may be regulated by an epigenetic mechanism, in which single CpG site methylation at the binding site of the ETS1 transcription factor in NOTCH4_R decreases ETS1 binding affinity and downregulates NOTCH4 expression. This, in turn, could contribute to the development of TOF.

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Availability of the data and materials

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

Authors' contributions

WS and GH made major contributions to the conception and design of this study. MY and HX collected samples and communicated with the patients' families. YZ performed the experiments and wrote the manuscript. RG, MC, XL, and XM helped collect and analyse the data. WS and GH supervised the study and edited the manuscript. All authors read and approved the final manuscript.
Ethics approval and consent to participate

The present study was approved by the Ethics Committee of The Children’s Hospital of Fudan University [approval no. 2015 (26)]. Written informed consent was obtained from the parents or relatives of all study participants.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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