

RESEARCH ARTICLE

High CpG island methylation of *p16* gene and loss of p16 protein expression associate with the development and progression of tetralogy of Fallot

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Abstract

We examined CpG island methylation in *p16* gene and its effect on p16 protein expression in tetralogy of Fallot (ToF) patients to explore its potential implications in the development and progression of ToF. The study subjects consisted of 75 healthy controls and 63 ToF patients recruited at Linyi People's Hospital between January 2012 and June 2014. The 4 mL of peripheral venous blood of each subject was obtained and saved in ethylene diamine tetraacetic acid (EDTA) tubes. Methylation-specific polymerase chain reaction (MSP) was employed to detect CpG island methylation in *p16* promoter region and Western blotting was used to detect p16 expression of all subjects. Real-time fluorescence quantitative polymerase chain reaction (FQ-PCR) was performed to test p16 mRNA expression. The results showed that p16-methylation rates in ToF group were significantly higher than the control group (ToF group, 58.73%; control group, 13.33%; $P < 0.001$). Remarkably, Western blotting and FQ-PCR results derived from RVOT revealed that p16 protein expression was significantly lower in ToF group compared to the control group (0.76 ± 0.21 versus 2.31 ± 0.35 ; $P < 0.001$), and *p16* gene expression was also markedly decreased in ToF group (1.212 ± 0.152 versus 1.346 ± 0.191 , $P < 0.001$). Additionally, our analysis suggested that CpG island methylation in *p16* promoters in ToF patients was negatively correlated with p16 protein and gene expression (both $P < 0.05$). Our study reports that high CpG island methylation of *p16* gene and loss of p16 protein expression associate with the development and progression of ToF, which may have significant therapeutic applications for ToF.

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Introduction

Tetralogy of Fallot (ToF) is the most common cyanotic heart defect and the cause of blue baby syndrome. The severe form of ToF is known as ventricular septal defect (VSD) and involves complete obstruction of the pulmonary artery, resulting in diversion of all blood from the right ventricle (RV) into the aorta (Dennis *et al.* 2014). ToF accounts for 8–10% of all congenital heart defects (Bellinger *et al.* 2015). Surgical intervention for ToF includes pulmonary valvotomy, resection of right ventricle outflow tract (RVOT) muscle bundles and ventricular septal defect closure (Weinberg and McElhinney 2014). ToF treatment with corrective surgery during infancy results in acceptable outcomes into young adulthood (Kirsch *et al.* 2014). However, a variety of disastrous postoperative consequences are observed, and pulmonary regurgitation (PR) due to pulmonary valve

excision is a common sequel after repair of ToF, leading to RV dilatation (Schwerzmann *et al.* 2007). During the next 30 years, postrepair patients may undergo progressive exercise intolerance, arrhythmia, right heart failure and sudden death (Bichell 2014). The occurrence of adverse effects after surgical repair of ToF indicates that alternative approaches, such as cell therapy or gene therapy, may be safer and more effective for treatment of ToF patients. Such a possibility is currently unavailable, because the genes and mechanisms leading to ToF are completely unknown and efforts to understand the basic biology of ToF are still in their infancy.

Human *p16* gene is a tumour suppressor gene contains three exons and two introns, with a total 8.5 kb in length, and located on chromosome 9q21 (Piepkorn 2000). The protein product, p16INK4a inhibits cyclin-dependent kinases (CDK4 and CDK6). CDK4 and CDK6 initiate the phosphorylation of retinoblastoma (RB) protein, thus p16 negatively regulates cell cycle through inhibiting RB phosphorylation, thereby promoting cell cycle arrest

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(Wang *et al.* 2014). CpG islands mark most gene promoters and majority of cytosines in CpG dinucleotides are methylated at the 5' position by cytosine methyltransferase (Wachter *et al.* 2014). CpG island methylation represses transcription, and demethylation of endogenous methylated CpG islands using DNA methyltransferase inhibitors restores gene expression (Shimoda *et al.* 2014). Deletion of the *p16INK4a* locus is a common mechanism of gene dysregulation, observed in some cancers, but CpG island hypermethylation still remains the main mechanism of *p16* inactivation (Yang *et al.* 2014). Inactivation of *p16* by methylation is detected in early carcinogenesis and results in loss of cell cycle arrest in G1-phase (Cao *et al.* 2009; Fujiwara-Igarashi *et al.* 2014). In recent studies, human foetal ventricular cardiomyocytes (HFCs) are proposed for cell therapy based on interventions in heart failure, and HFC senescence is associated with upregulation of *p16* expression (Golubnitschaja *et al.* 2003; Ball and Levine 2005). Although, the involvement of CpG island methylation in suppression of *p16* gene expression has been discussed in cancer settings, the status of *p16* gene methylation and its effects on development of ToF is completely unknown. In this study, we detected CpG island methylation in *p16* gene and its effects on p16 protein expression in ToF patients to explore its potential implications to the development and progression of ToF.

Materials and methods

Ethics statement

This study was approved by the Institutional Ethics Committee of Linyi People's Hospital. Written informed consent was obtained from guardians of the subjects, and the study confirmed to the declaration of Helsinki.

Patients

A total of 63 ToF children who were examined by cardiac catheterization and surgical operation at Linyi People's Hospital between January 2012 and June 2014 were enrolled in the present study. Among the 63 patients, there were 37 males (58.73%) and 26 females (41.27%), with age of 1 month to 15 years old (2.3 ± 1.9 years old average). All enrolled patients were tested for chest radiography and type-B ultrasonic inspection and had no chromosome karyotype abnormality or family history of congenital heart disease, as

well as the exclusion of previous history of cancers, except for cardiac vascular malformation. ToF patients were designated as 'ToF group'. The control group consisted of 75 children without congenital heart diseases, including 43 males (57.33%) and 32 females (42.67%). Their age ranges from 2 months to 18 years old (2.7 ± 2.1 years old average). The 4 mL of peripheral venous blood of each subject was obtained and saved in ethylene diamine tetraacetic acid (EDTA) tubes for following experiments. As shown in table 1, age and gender of the children in the two groups were similar ($P > 0.05$).

Bisulphite conversion

Four millilitre of peripheral venous blood was extracted from each subject, and saved in EDTA tubes. Subsequently, peripheral blood leucocyte was obtained. The DNA extraction used Genomic DNA kit (Tiangen, Beijing, China). DNA concentration and purity was measured with an ultraviolet spectrophotometer. Genomic DNA ($10 \mu\text{g}/\mu\text{L}$) was subjected to bisulphite conversion using the EpiTect® Fast DNA Bisulphite kit (50) reagent kit (Qiagen, Hilden, Germany) according to the manufacture's protocol. A total of $45 \mu\text{L}$ of DNA was denatured by sodium hydroxide (NaOH) (2 mol/L) at 37°C for 5 min. Next, $20 \mu\text{L}$ of hydroquinone (10 mol/L) and $520 \mu\text{L}$ of sodium bisulphite (pH = 5.0, 3 mol/L) were added and mixed. The mixture was centrifuged and incubated under mineral oil overnight at 55°C . Subsequently, $500 \mu\text{L}$ of samples under the oil layer were carefully extracted, and modified DNA samples were purified with UNIQ-10 Column Clinical Sample Genomic DNA purification kit (Sangon, Shanghai, China). The $8 \mu\text{L}$ of NaOH (2 mol/L) was added to DNA samples, incubated at 37°C for 5 min, followed by precipitation with sodium acetate ($5 \mu\text{L}$, pH = 5.0, 3 mol/L) and precooled absolute ethyl alcohol ($120 \mu\text{L}$) at -20°C for 2 h. DNA was resuspended in distilled water ($30 \mu\text{L}$) and stored at -20°C .

Methylation-specific polymerase chain reaction (PCR)

A schematic representation of p16 promoter with its CpG islands and the primer sites are illustrated in figure 1. Detection of *p16* methylation was performed using methylation-specific polymerase chain reaction (MSP) method. The primers for MSP were synthesized by Sangon Biotech, which were used for amplification of methylated *p16* sequencing (*p16-M*) and unmethylated *p16* (*p16-U*) sequencing,

Table 1. Detection of *p16* methylation in subjects of the ToF group and control group.

	ToF group ($n = 63$)	Control group ($n = 75$)	t/χ^2	P
Age	2.3 ± 1.9	2.7 ± 2.1	1.17	0.242
Gender				
Male	37	43	0.03	0.869
Female	26	32		
<i>p16</i> methylation				
Methylation	37	10	31.42	<0.001
Unmethylation	26	65		
<i>p16</i> methylation (%)	58.73	13.33		

ToF, tetralogy of Fallot.

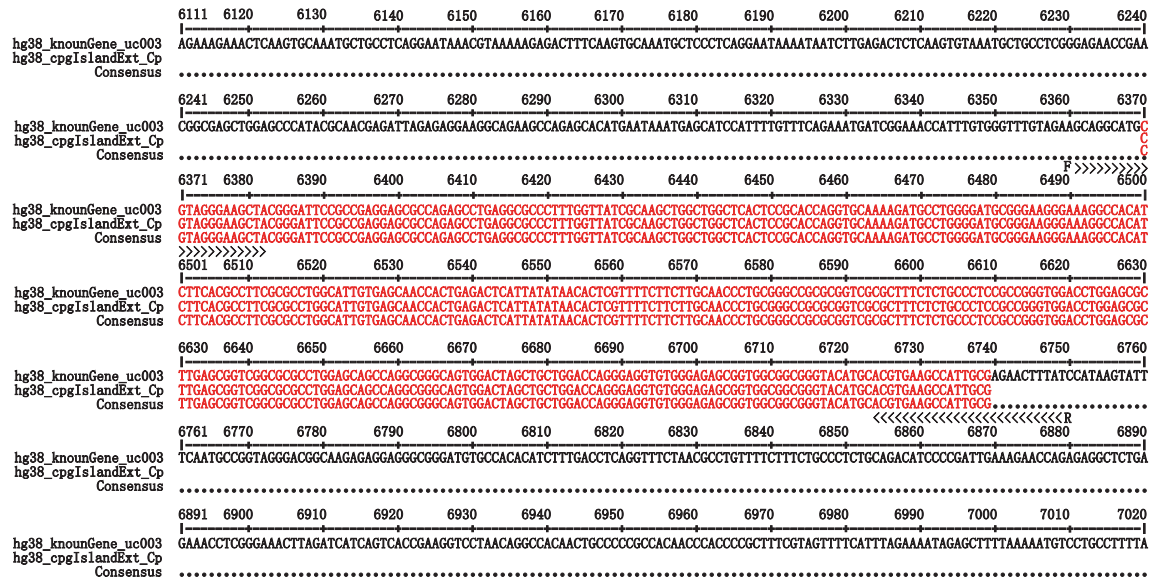


Figure 1. Methylation status of CpG islands in the promoter region of *p16* gene. Red area, CpG islands; F/R, the sequences of primers.

respectively. The sequences of *p16*-M primer were as follows: 5'-TTATTAGAGGGTGGGGCGGATCGC-3' (sense) and 5'-AAAAAAACGCAATGGCTTACGTGC-3' (antisense), and the amplified products are 150 bp. The sequences of *p16*-U primers were 5'-TTATTAGAGGGTGGGGTGGATTGT-3' (sense) and 5'-CCACCTAAATCAACCTCCAA CCA-3' (antisense), and the amplified products are 151 bp. If PCR products were amplified from either *p16*-M primer or *p16*-U primer, partial methylation of CpG islands in *p16* gene was observed. The PCR amplification cycles were as follows: initial denaturation at 95°C for 3 min, followed by 35 cycles of denaturation at 95°C for 45 s, annealing at 65°C for 45 s, and extension at 72°C for 45 s. Final extension step was performed at 72°C for 7 min. PCR products (5 μ L) were loaded onto 2% agarose gel for electrophoresis, and visualized under a gel imaging system (BTS-20M; Unvitec, England).

Real-time fluorescence quantitative PCR (FQ-PCR)

Peripheral blood (4 mL) was collected from the case and control groups, and anticoagulated by EDTA. Following this, total RNA in blood samples was extracted with TRIzol[®] reagent (Invitrogen, Carlsbad, USA). SYBR[®] PrimeScript[™] RT-PCR kit (Takara, Tokyo, Japan) was used for reverse transcription (RT) procedure and FQ-PCR reactions. Primers were synthesized by Takara. The sequences for p16 are: forward sequence: 5'-TGG CAC CCA GCA CAA TGA A-3', reverse sequence: 5'-CTA AGT CAT AGT CCG CCT AGA AGC A-3'. The β -actin is used as internal control and the primer sequences are: forward sequence: 5'-TGG CAC CCA GCA CAA TGA A-3', reverse sequence: 5'-CTA AGT CAT AGT CCG CCT AGA AGC A-3'. The PCR system (25 μ L) was performed in 12.5 μ L of SYBR Premix Ex Taq[™] (2 \times),

1 μ L of each primer, 2 μ L of DNA template and distilled water. The PCR procedures were under the following conditions: an initial denaturation step (95°C for 30 s) and 40 cycles of denaturation (95°C for 5 s), annealing step (60°C for 30 s) and extension step (72°C for 10 min). Finally, dissociation curve analysis was employed at rates of 15 s at 95°C, 30 s at 60°C and 15 s at 95°C. β -actin was used as an internal reference, the average value of each sample was analysed using three parallel tubes. The expression of mRNA in the ToF group and the control group was detected by using relative quantitative method. The relative value of mRNA was expressed using $2^{-\Delta\Delta CT}$ ($\Delta\Delta CT = (CT_{mRNA} - CT_{\beta-actin})_{experimental\ group} - (CT_{mRNA} - CT_{\beta-actin})_{control\ group}$).

Western blot

Total protein was extracted from all blood samples using a BCA Protein Assay kit (Wuhan Boster Biological Engineering, Wuhan, China), with addition of 300 μ L of tissue lysates (Beijing Biosynthesis Biotechnology). Samples were separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) system, and transferred to polyvinylidene difluoride (PVDF) membrane with 5% blocking buffer at 4°C for 3 h. Adding primary antibody of rabbit monoclonal anti-p16 antibody (1 : 1000; Abcam, Cambridge, USA), the membrane was incubated overnight at 4°C, washing thrice for 15 min with Tris buffered saline Tween-20 (TBST) buffer. Thereafter, horseradish peroxidase-conjugated goat anti-rabbit IgG (secondary antibody, 1 : 1000; Wuhan Boster Biological Engineering) was added, incubating at 37°C for 1 h and washed thrice for 15 min with TBST. Positive bands were evaluated using ultrasensitive ECL aptamer sensor (Beijing Berson Biotechnology). Quantity One analysis software (Biorad, Hercules, USA) was conducted to analyse optical densitometry data.

Statistical analysis

SPSS 17.0 software was applied for the data analysis. The comparison of positive expression rates between groups was performed using χ^2 test or Fisher's exact test of the four-fold table. The correlation between *p16* methylation and *p16* protein expression was analysed using Spearman's correlation analysis. A *P* value below 0.05 was considered significant.

Results

Association between *p16* methylation and ToF

For each sample, both *p16*-M (for methylated *p16* gene) and *p16*-U (for unmethylated *p16* gene) primers were used in MSP. The methylated *p16* gene amplification resulted in a 150 bp product, while the unmethylated *p16* gene amplification resulted in a 151 bp product. If both PCR products were amplified, yielding both 150 and 151 bp products, then *p16* gene was partially methylated. The methylated data include methylated *p16* and partially methylated *p16* (figure 2). As seen in table 1, there were 37 patients with methylated *p16* gene (58.73%) among the 63 patients in the ToF group, while in the control group, *p16* methylation was detected in only 10 of the 75 subjects in the control group (13.33%). This result showed that *p16*-methylation rates in ToF group are significantly higher than the control group, which showed statistical significance ($\chi^2 = 31.42$, $P < 0.001$).

Results of *p16* protein expression

Further results indicated that 16 kD bands represent p16 positive expression, while the 43 kD bands represent β -actin expression (figure 3a). In the ToF group, p16 positive expression was 0.76 ± 0.21 , and p16 protein expression in the

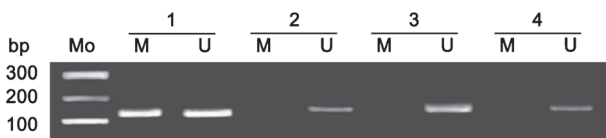


Figure 2. MSP testing *p16* methylation in subjects of the ToF group and control group. Mo, DNA marker; 1, 3: the ToF group; 2, 4: the control group; M, *p16* methylated PCR product; U, *p16* unmethylated PCR product.

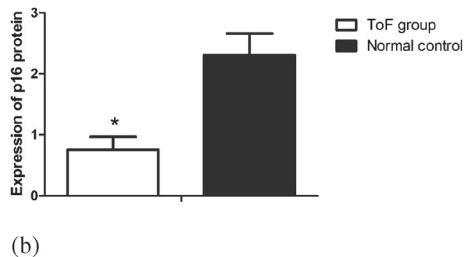
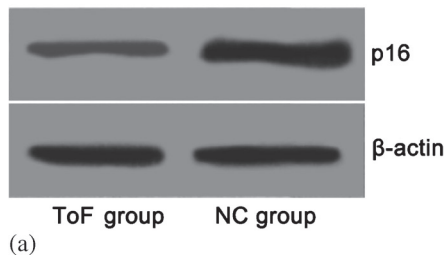


Figure 3. Western blot analysis testing p16 protein expression in subjects of the ToF group and control group. (a) Electrophoresis diagram of Western blot; (b) p16 protein expression was significantly lower in subjects of the ToF group when compared to those of control group.

control group was 2.31 ± 0.35 . This result suggested that the positive expression rate of p16 protein in ToF group was significantly lower than the control group, which was statistically different ($P < 0.001$) (figure 3b).

Results of *p16* mRNA expression

As illustrated in figure 4, PCR results clearly showed the fluorescence background signal (baseline phase), exponential amplification of fluorescence (logarithmic growth) and stable amplification of fluorescence (stationary phase) for the amplification curves of mRNA and β -actin (figure 4a). Further, there were no detectable signs of nonspecific dissolution peak or miscellaneous peaks in the dissolution curves, which indicated a high specificity and confirmed the absence of nonspecific amplification products (figure 4b). The *p16* mRNA expressions in the ToF group and control group were 1.212 ± 0.152 and 1.346 ± 0.191 , respectively (figure 4c). The result revealed that *p16* gene expression was markedly lower in the ToF group than the control group ($P < 0.001$).

Association between *p16* methylation and *p16* protein expression

The CpG island in promoters was negatively correlated with p16 protein expression and *p16* methylation ($r = -0.793$, $P < 0.001$). These results indicated that the CpG island methylation in *p16* promoters can reduce p16 protein expression by silencing the gene expression ($r = -0.853$, $P < 0.001$) (table 2).

Discussion

Methylation of CpG islands in *p16* gene is observed in several tumours, including nonsmall cell lung cancer, colon cancer, bladder cancer and prostate cancer (Majid *et al.* 2008;

Table 2. Comparison of *p16* methylation status with p16 protein expression in ToF group.

	<i>p16</i> methylation	
	<i>r</i>	<i>P</i>
p16 protein expression	-0.793	<0.001
p16 mRNA expression	-0.853	<0.001

ToF, tetralogy of Fallot.

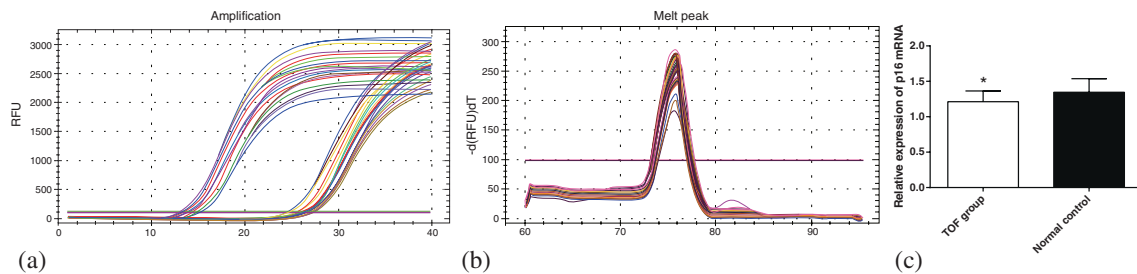


Figure 4. FQ-PCR testing *p16* mRNA expression in subjects of the ToF group and control group. (a) amplification curve. The fluorescence background signal (baseline phase), exponential amplification of fluorescence (logarithmic growth) and stable amplification of fluorescence (stationary phase) for the amplification curves of mRNA and β -actin; (b) dissolution curve. There were no detectable signs of nonspecific dissolution peak or miscellaneous peak in the dissolution curves, which indicated a high specificity and confirmed the absence of nonspecific amplification products; (c) *p16* mRNA expression was significantly lower in subjects of the ToF group when compared to those of control group.

Goto *et al.* 2009; Jablonowski *et al.* 2011; Zhang *et al.* 2011). The biological function of p16 involves cell cycle regulation to promote cell growth arrest and senescence, and loss of expression and function of p16 lead to uncontrolled cell growth and proliferation, and loss of p16 activity can occur due to mutation, deletion or promoter methylation (Liu and Sharpless 2009). And, either deletion or mutation/methylation of certain genes may contribute to gene silencing, as a tumour suppressor gene. Previous reports suggested that a conditional *p16* knockout mice model creation might develop heart defects, haematopoietic abnormalities, increased matrix deposition or myofibroblast differentiation, etc. (Wolstein *et al.* 2010; Shao *et al.* 2011; An *et al.* 2015). Previous reports have discussed the effect of *p16* promoter methylation on gene silencing and loss of p16 protein expression in various cancers (Samowitz *et al.* 2005; Nosho *et al.* 2008). In addition to the clinical significance of *p16* promoter methylation in the prediction of cancers progression, the role of *p16* promoter methylation in patients with other diseases except the previous history of cancers has also been explored and proved (Olaru *et al.* 2012; Shin *et al.* 2012; Bodoor *et al.* 2014). Our present study demonstrated that CpG island methylation of *p16* gene is negatively associated with p16 protein expression. A previous study showed that methylation of *p16* gene in promoter region was accompanied with reduced p16 protein expression in lung cancer (Kondo *et al.* 2006). In this context, p16 promoter methylation is considered as prognostic factor in several cancers, and may provide new therapeutic strategies for treating cancers (Fujiwara *et al.* 2008; Celebiler Cavusoglu *et al.* 2010).

In our study, CpG island methylation of *p16* gene is closely associated with ToF incidence, likely due to silencing of the gene and loss of p16 protein expression. CpG island methylation in promoter regions of tumour suppressors can alter their expression, thus hypermethylation of tumour suppressor genes is a common mechanism of controlling their expression, as evident in many tumours and in normal development (Dansranjavin *et al.* 2009). By comparing p16 expression with its methylation status, a study found that the methylation status and p16 expression are

correlated with cell proliferation and differentiation (Azad *et al.* 2013). In light of our novel results, it is important to understand the functions of p16 in cardiomyocytes to obtain a definitive link between the observed *p16* methylation in myocardial tissue and ToF incidence. Cardiac function is closely associated with heart health, and cardiomyocytes constitute myocardium and play pivotal roles in heart diseases, and in regulating cardiac morphology and function (Naeem *et al.* 2013). Further, existing reports have shown the close relationship between complex cardiac malformations and differences in methylation of genes. For example, Feng *et al.* (2013) found that high methylation was present in the CpG loci of *GATA-4* gene with a low expression of *GATA-4* mRNA which might be one of key mechanisms to heart defects in vitamin A-deficient offspring. Recent study conducted by Xu *et al.* (2014) pointed out that *CITED2* gene deletion or mutation was associated with the development of cardiac malformations, and in their experiment, they suggested that the development of paediatric congenital heart disease might be partially attributed to the mutations and methylation of *CITED2* gene. Importantly, genetic changes included mutations and abnormal methylation in genes, which may strengthen the opposite effect of this gene. Existing reports about ToF and genetic variants promoted that common variants in the *PTPN11* gene, *ZFPM2/FOG2* gene, *ROCK1* gene, etc., might all contribute to the changed risk of ToF (Luca *et al.* 2011; Goodship *et al.* 2012; Doza *et al.* 2013). Besides, abnormal methylation in *LINE-1*, *VANGL2* and *DNMT3A/3B* was suggested to provide potential important clues for the development of ToF (Sheng *et al.* 2012, 2013; Yuan *et al.* 2014). In this context, a previous study showed that downregulation of p16 can stimulate cell cycle in cardiomyocytes, and upregulation of p16 induces cardiomyocyte differentiation (Torella *et al.* 2004; Baker *et al.* 2011). In this case, we speculate that loss of p16 expression due to *p16* promoter methylation mediates cardiomyocyte hyperplasia, which can lead to ToF development. Ageing and cardiomyocyte senescence is strongly correlated with p16 activation, leading to impaired ventricular function (Gonzalez *et al.* 2008). Nozato *et al.* (2001) showed that p16

controls G1 stage in cell cycle and is linked to cardiomyocyte hypertrophy, offering a novel strategy for the gene therapy for cardiac hypertrophy patients. Our findings show that p16 has a role in heart development, which is consistent with previous studies. Further study of p16 mechanisms involved in ToF may provide a way for the development of novel therapeutic strategies for ToF patients.

However, some limitations should be noted in this study. First, retrospective analysis was conducted in our study, and retrospective studies are less reliable than prospective studies. Second, a case-control study cannot determine whether methylation of CpG islands in *p16* gene is able to increase risks of ToF. Another shortcoming of our study is that only *p16* methylation status and p16 protein expression were examined owing to the limitation of biopsies. Accordingly, future studies on the association of *p16* methylation and ToF incidence are required.

In conclusion, we detected a novel loss of p16 expression in ToF patients and showed that p16 loss correlates with CpG island methylation of *p16* gene. For the first time, our study provides strong evidence that CpG island methylation of *p16* gene correlates with ToF incidence. Thus, p16 may have high clinical value as a novel therapeutic intervention strategy for the treatment of ToF. However, in-depth design of animal experiments with knocking down p16 is warranted to address in future to demonstrate that p16 methylation is involved in ToF morphology.

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