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

Running title: *p16* methylation and ToF risk

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Abstract

Objectives: 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 to the development and progression of ToF.**Methods:** 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.**Results:** 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 ± 0.21vs. 2.31 ± 0.35; $P< 0.001$), and *p16* gene expression was also markedly decreased in ToF group (1.212 ± 0.152 vs. 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 $P< 0.05$).**Conclusion:** 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.

Keywords: Tetralogy of Fallot;*p16* gene;p16 protein;CpG islands;Methylation;Promoter regions

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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 thirty years, post-repair 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 tumor suppressor gene containing 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 via inhibiting RB phosphorylation, thereby promoting cell cycle arrest (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

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transcription, and demethylation of endogenous methylated CpG islands using DNA methyltransferase inhibitorsrestores 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 fetal ventricular cardiomyocytes (HFCs) are proposed for cell therapy based on interventions in heart failure, and HFC senescence is associated with up-regulation of p16expression (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.

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Materials and Methods

Ethics statement

The 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 conformed to the Declaration of Helsinki.

Objects

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 into the present study.

Among the 63 patients, there are 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 by 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 were 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 ($P > 0.05$).

Bisulfite conversion

The 4 ml 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 Biotech Co., Ltd., Beijing, China). DNA concentration and purity was measured with an ultraviolet spectrophotometer. Genomic DNA (10μg/μl) was subjected to bisulfite conversion using the EpiTect® Fast DNA Bisulfite Kit (50) reagent kit (QIAGEN, Germany) according to the manufacture's protocol. A total of 45 μL of DNA was denatured by sodium hydroxide (NaOH) (2 mol/L) at 37 °C for 5 min. Next, 20 μL of hydroquinone (10 mol/L) and 520 μL of sodium bisulfite (pH = 5.0, 3mol/L) were added and mixed. The mixture was centrifuged and incubated under mineral oil overnight at 55 °C. Subsequently, 500 μL 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 Biotech Co., Ltd., Shanghai, China). The 8 μL of NaOH (2mol/L) was added to DNA samples, incubated at 37 °C for 5 min, followed by precipitation with sodium acetate (5 μL, pH=5.0, 3mol/L) and pre-cooled absolute ethyl alcohol (120 μL) at -20 °C for

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2 hours. DNA was resuspended in distilled water (30 μL) and stored at -20 °C.

Methylation-specific polymerase chain reaction

A schematic representation of p16 promoter with its CpG islands and the primer sites were illustrated in Fig. 1. Detection of *p16* methylation was performed using methylation-specific polymerase chain reaction (MSP) method. The primers for MSP were synthesized by Sangon Biotech Co., Ltd., which were used for amplification of methylated *p16* sequencing (*p16*-M) and unmethylated *p16* (*p16*-U) sequencing, respectively. The sequences of *p16*-M primer were as follows: 5'-TTATTAGAGGGTGGGGCGGATCGC-3' (sense) and 5'-AAAAAAAACGCAATGGCTTCACGTGC-3' (antisense), and the amplified products are 150 bp. The sequences of *p16*-U primers were 5'-TTATTAGAGGGTGGGGTGGATTGT-3' (sense) and 5'-CCACCTAAATCAACCTCCAACCA-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 μL) were loaded onto 2% agarose gel for electrophoresis, and visualized under a gel imaging system (BTS-20M; Unvitec, England).

Real-time fluorescence quantitative polymerase chain reaction (FQ-PCR)

Peripheral blood (4 ml) was collected from the case and control groups and anticoagulated by EDTA. Following, total RNA in blood samples was extracted with TRIzol® reagent (Invitrogen, Carlsbad, CA, 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

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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 μl) was performed in 12.5 μl of SYBR Premix Ex Taq^{TM} (2×), 1 μl of each primer, 2 μl 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 10min). 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 analyzed with 3 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}-$CT_{\beta\text{-actin}}$) experimental group – (CT_{mRNA}－$CT_{\beta\text{-actin}}$) control group).

Western blotting

Total protein was extracted from all blood samples using a BCA Protein Assay Kit (Wuhan Boster Biological Engineering Co., Ltd.), adding 300 μl of tissue lysates (Beijing Biosynthesis Biotechnology Co., Ltd.). Samples were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) system, and transferred topolyvinylidene difluoride (PVDF) membrane with 5% blocking buffer at 4 °C for 3 h. Adding primary antibody of rabbit monoclonal anti-p16 monoclonal antibody (1: 1000; Abcam Inc., Cambridge, MA, USA), the member was incubated overnight at 4 °C, washing 3 times 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 Co., Ltd.) was added, incubating at 37 °C for 1 h and washing 3 times for 15 min with TBST. Positive bands were evaluated using ultrasensitive ECL aptamer sensor (Beijing Berson Biotechnology Co., Ltd.). Quantity One analysis software (Bio-Rad,

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Hercules, CA) was conducted to analyze optical densitometry data.

Statistical analysis

SPSS17.0 software was applied for the data analysis. The comparison of positive expression rates between groups was performed with Chi-square test or Fisher's exact test of the fourfold table. The correlation between *p16* methylation and p16 protein expression was analyzed with 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 bp and151 bp products, *p16* gene was partially methylated. The methylated data include methylated *p16* and partially methylated *p16* (Fig. 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 the 16 kD bands represent p16 positive expression, while the 43 kD bands represent β-actin expression (Fig. 3A). In the ToF group, p16 positive expression was 0.76 ± 0.21, and p16 protein expression in the control group was 2.31 ± 0.35. This result suggested that the

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positive expression rate of p16 protein in TOF group was significantly lower than the control group, which was statistically different ($P< 0.001$) (Fig. 3B).

Results of p16 mRNA expression

As illustrated in Fig. 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 (Fig. 4A). Furthermore, there were no detectable signs of non-specific dissolution peak or miscellaneous peak in the dissolution curves, which indicated a high specificity and confirmed the absence of non-specific amplification products (Fig. 4B). The *p16* mRNA expression in the ToF group and the control group was 1.212 ± 0.152 and 1.346 ± 0.191, respectively (Fig. 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 ($r = -0.793$, $P< 0.001$). This results indicated that the CpG island methylation in *p16* promoters can reduce p16 protein expression by silencing of gene expression ($r = -0.853$, $P < 0.001$) (Table 2).

Discussion

Methylation of CpG islands in*p16* gene is observed in several tumors, including non-small cell lung cancer, colon cancer, bladder cancer and prostate cancer (Majid et al., 2008; 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 leads to uncontrolled cell growth and proliferation, and loss of p16 activity can occur due to

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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 tumor suppressor gene, previous reports suggested that a conditional *p16* knockout mice model creation might develop heart defects, hematopoietic abnormalities, increased matrix deposition or myofibroblast differentiation etc. (Wolstein et al., 2010; Shao et al., 2011; An S, 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 tumor suppressors can alter their expression, thus hypermethylation of tumor suppressor genes is a common mechanism of controlling their expression, as evident in many tumors 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

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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 Y et al. 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 (Feng Y, 2013). Recent study conducted by Xu M and his colleague 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 pediatric congenital heart disease might be partially attributed to the mutations and methylation of *CITED2* gene (Xu M, 2014). Importantly, genetic changes included mutations and abnormal methylation in genes, which may strengthen the 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 (De Luca A, 2011; Goodship JA, 2012; Palomino Doza J, 2013). Besides, abnormal methylation in *LINE-1*, *VANGL2* and *DNMT3A/3B* was suggested to provide potential important clues for the development of ToF (Sheng W, 2012; Yuan Y, 2014; Sheng W, 2013). In this context, a previous study showed that down-regulation of p16 can stimulate cell cycle in cardiomyocytes, and up-regulation 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. Aging and cardiomyocyte senescence is strongly correlated with p16 activation, leading to impaired ventricular function (Gonzalez et al., 2008). Nozato Toshihiro 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 (Nozato et al., 2001). Our findings that p16 has a role in heart development is consistent with previous studies. Further study of p16 mechanisms involved in ToF may provide a

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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 was examined owning to the limitation of biopsies. Accordingly, studies on the association of *p16* methylation and ToF incidence are future 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, p16may 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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Acknowledgements

We are grateful to our instructors for their valuable advice. We also thank the reviewers for their precious comments on this paper.

Competing interests

The authors have no financial and non-financial conflicts of interest to declare with regard to the study.

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Received 20 November 2015, in final revised form 11 March 2016; accepted 17 March 2016

Unedited version published online: 22 March 2016

TABLE 1.DETECTION OF *P16* METHYLATION IN SUBJECTS OF THE TOF GROUP AND THE CONTROL GROUP

		TOF group (n=63)	Control group (n=75)	$t/\chi 2$	*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		
P16 methylation	Methylation	37	10	31.42	<0.001
	Unmethylation	26	65		
P16 methylation (%）		58.73	13.33		

Note: ToF, Tetralogy of Fallot.

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TABLE 2. COMPARISON OF *P16* METHYLATION STATUS WITH P16 PROTEIN EXPRESSION IN THE TOF GROUP

	p16 methylation	
	r	*P*
p16 protein expression	-0.793	<0.001
p16mRNA expression	-0.853	<0.001

Note: ToF, Tetralogy of Fallot.

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

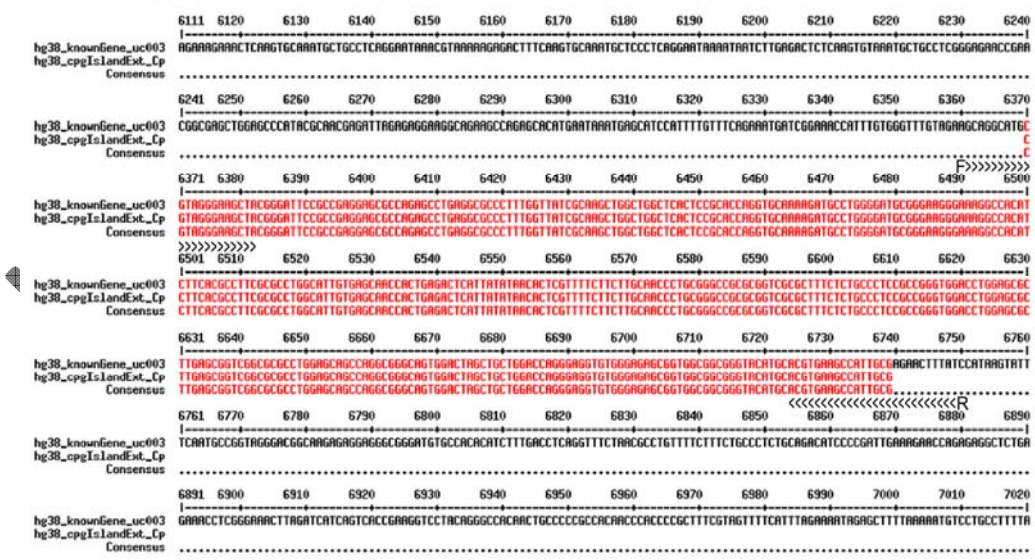


Figure 2. Methylation-specific polymerase chain reaction testing *p16* methylation in subjects of the ToF group and the control group. Mo: DNA marker; 1, 3: the ToF group; 2, 4: the control group; M: *p16* methylated PCR product; U: *p16* unmethylated PCR product.

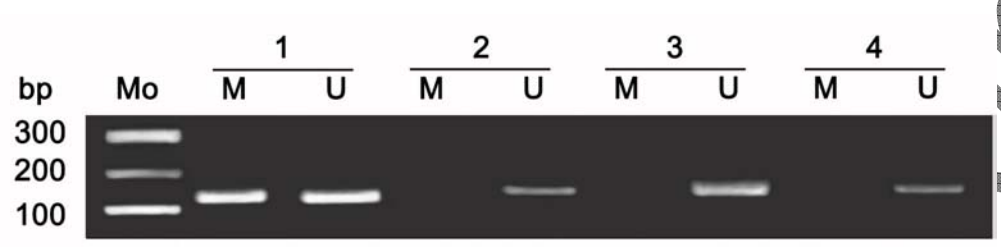


Figure 3. Western blotting analysis testing p16 protein expression in subjects of the ToF group and the control group, A, electrophoresis diagram of Western blotting; B, p16 protein expression was significantly lower in subjects of the ToF group when compared to those of the control group.

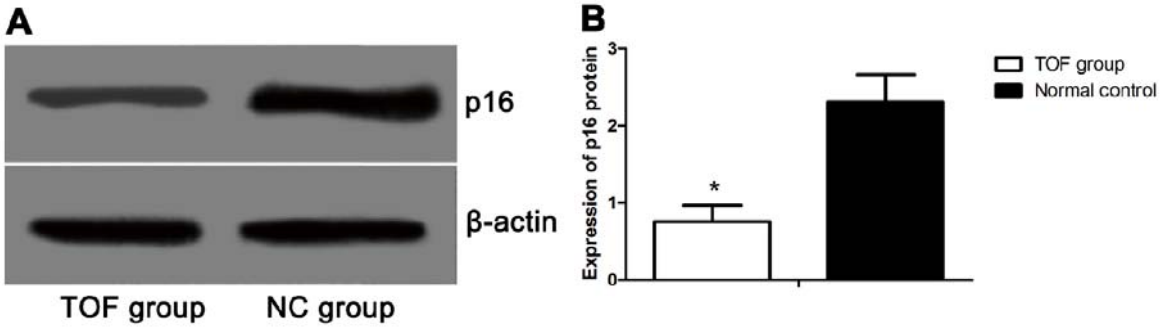
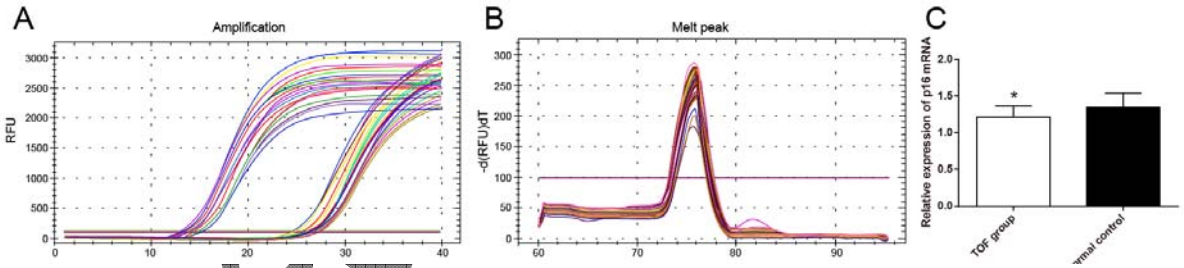


Figure 4. Real-time fluorescence quantitative polymerase chain reaction testing *p16* mRNA expression in subjects of the ToF group and the control group A, amplification curve. The fluorescence background signal (baseline phase), exponential amplification of fluorescence (logarithmic growth) and stable amplification of fluorescence (stationary phase) for the amplification curves of mRNA and β-actin; B, dissolution curve. There were no detectable signs of non-specific dissolution peak or miscellaneous peak in the dissolution curves, which indicated a high specificity and confirmed the absence of non-specific amplification products; C, p16 mRNA expression was significantly lower in subjects of the ToF group when compared to those of the control group.



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