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Case Report

SNP and DNA methylation analyses of a monozygotic twins discordant for complete endocardial cushion defect: a case report

Bihui Bao[1*], Hua Hu[2*], Limei Chen[1], Shiyong Lu[1], Qifeng Tang[3], Zhiqing Liang[4]

[1]Department of Obstetrics and Gynecology, Qingbaijiang Women's and Children's Hospital (Maternal and Child Health Hospital), West China Second University Hospital, Sichuan University, Chengdu 610300, Sichuan, China; [2]Department of Obstetrics and Gynecology, The Second Affiliated Hospital (Xinqiao Hospital), Army Medical University, Chongqing 400037, China; [3]Department of Anesthesiology, Shanghai Hechuan-Rhine TCM Hospital, Shanghai 201103, China; [4]Department of Obstetrics and Gynecology, The First Affiliated Hospital (Southwest Hospital), Army Medical University, Chongqing 400038, China. [*]Equal contributors and co-first authors.

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Abstract: The exact cause of complete endocardial cushion defect (ECD) is still unknown. This report describes a unique pair of monozygotic twins (MZ twins) discordant for ECD. The chromosome karyotyping analysis revealed normal karyotype of 46, XY, 16qh+ and mat in both MZ twins. A genome-wide analysis of DNA using the Affymetrix SNP 6.0 revealed identical genotyping of single nucleotide polymorphisms (SNPs) and copy number variations (CNVs). An extensive methylation assay was carried out by NimbleGen 3 × 720 K CpG Island Plus RefSeq Promoter Arrays to analyze the potential epigenetic differences. The DNA methylation profiles of the affected twin seemed increased compared with that of the unaffected twin. However, further validation of Notch1 promoter hypermethylation and six top-ranked differentially methylated CpG sites by sodium bisulfate modification and methylation-specific PCR, failed to reveal consistent methylation differences between the twins. Other relevant factors, such as heritability and penetrance of the condition that place the MZ twins near to a threshold for ECD or variations in local epigenetic events in the twins' heart tissues, are probably responsible for the phenotypic discordance.

Keywords: Monozygotic twins, discordant phenotype, complete endocardial cushion defects, DNA polymorphism, epigenetic difference

Introduction

Complete endocardial cushion defect (ECD) is a serious congenital heart defect (CHD) that can cause stillbirth or neonatal death with a prevalence of 0.83/10,000 live birth [1, 2]. Though complete ECD can be treated surgically, 14.4% of ECD patients still fail to survive even after the second operation [3, 4]. Identification of genetic and epigenetic changes, the related environmental risk factors for the pathogenesis of CHDs, and subsequent efforts in developing new preventive and therapeutic approaches for birth defect are important research priorities.

Some mutations in gene or chromosomal regions that involve primarily in cardiovascular system constitute potential risk factors for CHD [5, 6]. Notch activity is crucial in heart with complex architecture, and Notch1 signaling is required for the proliferation and differentiation of trabecular myocardium, but the molecular mechanisms and cellular processes in the developing cardiovascular system remain only partially understood [7, 8]. A polymorphism in the VEGF-gene causing reduced VEGF-expression can increase the risk of developing congenital heart malformations [9]. Cytogenetic techniques have identified chromosomal abnormalities in 16.8% karyotyped CHD cases, and submicroscopic chromosomal variants contribute to the genetic basis of ECD [10-13]. But genomic imbalances contain many genes, and identifying loci and relevant information cannot

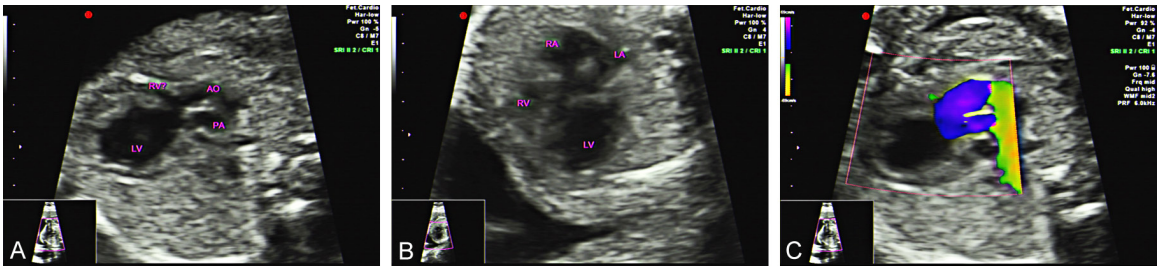

Figure 1. Imaging characteristics of color doppler echocardiography, which revealed the complete endocardial cushion defect (A), transposition of great artery (B) and blood flow (C) of the twin.

be verified with certainty in clinical heart defects till set up a reliable animal model [10, 14-16]. Epigenetics, such as histone modifications and DNA methylation, is increasingly associated with the pathology of many disorders, so it has become the most widely investigated causative factor of somatically heritable changes of gene expression without a change in the primary DNA sequence [17]. Maternal age, stresses, smoking, alcohol use, gestational diabetes, fever, influenza and drugs may perturb the biological processes of cardiac development through various signaling pathways in a temporal and spatial specific manner, although the molecular mechanisms underlying the associations are unclear [18, 19].

Human karyotyping can detect both numerical and structural chromosomal aberrations with resolution of 5 to 15 Mega bases (Mb). High-resolution melting (HRM) in conjunction with a sequencing method can greatly benefit precise mutation screening, single Nucleotide Polymorphisms (SNPs) detection and genotyping of all possible base combinations at one position of clinical samples for many genetic disorders [20]. Methylation-sensitive HRM (MS-HRM) analysis can distinguish methylated samples by comparing two samples with the HRM profiles, then heterogeneous methylation can be investigated by sequencing-based methodologies [17].

In-depth monozygotic twin studies have been proved to be extremely valuable for crucial genetic-phenotype relationship and epidemiological information of complex diseases in human since monozygotic (MZ) twins share an identical genotype. Putative mechanisms for minor degrees of inconsistent phenotypes in MZ twins range asymmetry of the inner cell masses, post-zygotic gene mutation, epigenetic changes, the influence of the local placenta, laterality defects and complex gene-environment interactions [21-23]. SNP and copy number variations (CNVs), epigenetic analyses of identical twins can help us explore the genetical mechanisms of discordant lesions [24]. Here, we report a unique pair of MZ twins discordant for complete ECD with the results of cytogenetic analyses, as well as the DNA and epigenetic comparisons to better understand the specific pathogenesis.

Case presentation

Subjects, karyotype and zygosity tests

A detailed ultrasound scan and fetal echocardiography found that one of the fetuses in a twin pregnancy had complete ECD (**Figure 1**) while the other was developing normally. The 24-year-old pregnant woman came to our Prenatal Diagnostic and Counseling Clinic at 25 weeks of gestation, gravida 1 para 0. The parents of the twins were at the same age but not consanguineous. During early pregnancy, the mother-to-be had some cold medicine for two days, with no other risk factors. The pregnant woman had a family history of twins, with 4 pairs of twins from her grandfather.

With a signed informed consent from the patients and the approval from the ethics review board of Southwest Hospital (NO: KY-2021055), this research was performed in line with the Ethical Principles for Biomedical Research Involving Human Subjects (Ministry of Health of the People's Republic of China) and with the Declaration of Helsinki principles. Following parental written consent for etiologic research and publication of the case, the cord blood was sampled at 29 weeks of gestation by diagnostic cordocentesis with transabdominal

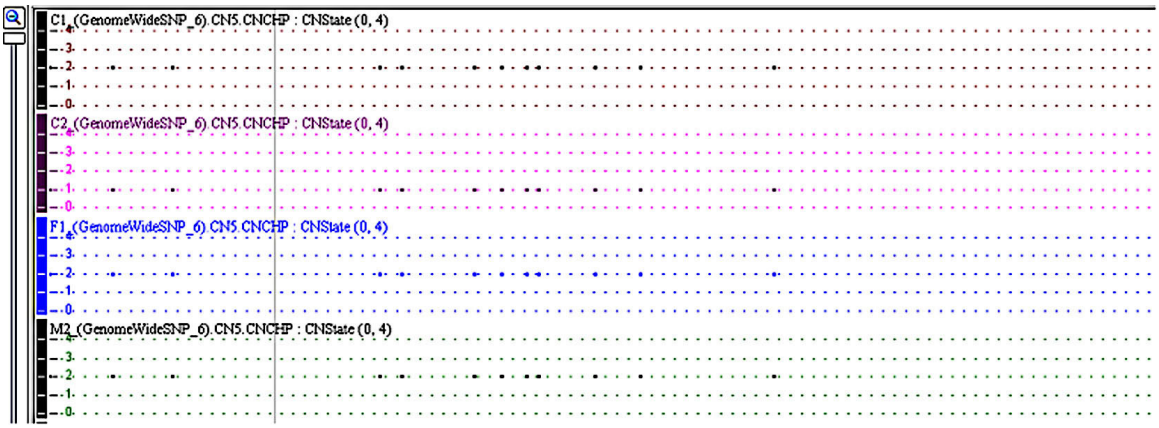

Figure 2. DNA CNV analyses roughly revealed no differences among C1, C2, F1 and M2. C1-the ECD twin; C2-the normal twin; F1-the father; M2-the mother. CNV: Copy Number Variation; ECD: Endocardial Cushion Defect.

ultrasonic guidance. Karyotyping was performed for the twins and the parents by GTG banding at a resolution of approximately 400 bands. The karyotypes of the twins were both 46, XY, 16qh+, with the mother 46, XX, 16qh+ and the father 46, XY, without an early postzygotic mitotic error such as chromosomal rearrangements, inversion or translocation, et al. Genomic DNA samples were extracted from cord blood, and parental peripheral blood samples were acquired using the DNeasy Blood Kit (Qiagen).

SNP and methylation analyses

To compare the genetic differences between the twins, the blood samples of the parents and the twins were analyzed using Affymetrix Genome-Wide Human SNP Array 6.0 platform at CapitalBio Corporation (Beijing, China). The raw data passed quality control were further analyzed by using the Genotyping Console Software (Affymetrix, Santa Clara, CA, US). To explore the potential influence of epigenetic factors, DNA methylation analyses were carried out using a Roche NimbleGen Human DNA Methylation 3 × 720 K CpG Island Plus RefSeq Promoter Array Kit at CapitalBio Corporation (Beijing, China). The MeDIP assay was performed following the NimbleGen's standard protocol, and analyses of microarray data were performed using NimbleScan 2.6 software. Log2-ratio and region features of significant positive enrichment in ChIP-based methylation data were identified using a modified ACME algorithm for peak identification [25].

No significant genomic CNVs were found between the twins by Affymetrix SNP 6.0 Array (**Figure 2**). But 1,191 polymorphic markers were at different sites, while 905,409 (99.86%) SNPs were at the same sites. Except for different sites shared by one of the parents (**Figure 3**), 14 different sites were chosen for validation by HRM (LightCycler 480 System, Roche) and sequencing, which suggested the twins share the same genetic information (as Supplementary Material). The genomic analyses suggested that the complete ECD was not likely caused by genome alterations since the twins cannot be discriminated with almost same chromosomal markers.

Eighty-six genes were observed to be hypermethylated by a log2-ratio of more than 1.5 on the array in the affected twin when compared with those in the other twin. We selected preliminary findings to test the overlaps with cardiovascular-related annotations or datasets. But no gene was strongly associated with heart development among the 86 hypermethlyated genes, except for Notch1 gene with log2-ratio more than 1.09 plays important roles in congenital heart diseases [7]. When the log2-ratio on array was more than 1.55, no significant differences arose (**Figure 4**). The differentially methylated CpG sites on the promoter regions of 6 top-ranked genes were chosen to validate by sodium bisulfite modification and methylation-specific PCR followed by DNA sequencing (promoter region: SNRNP25|POLR3K; promoter region: INO80E|HIRIP3; promoter region: PCDHAC1; promoter region: CYB5D1|LSMD1|TMEM88; promoter region:

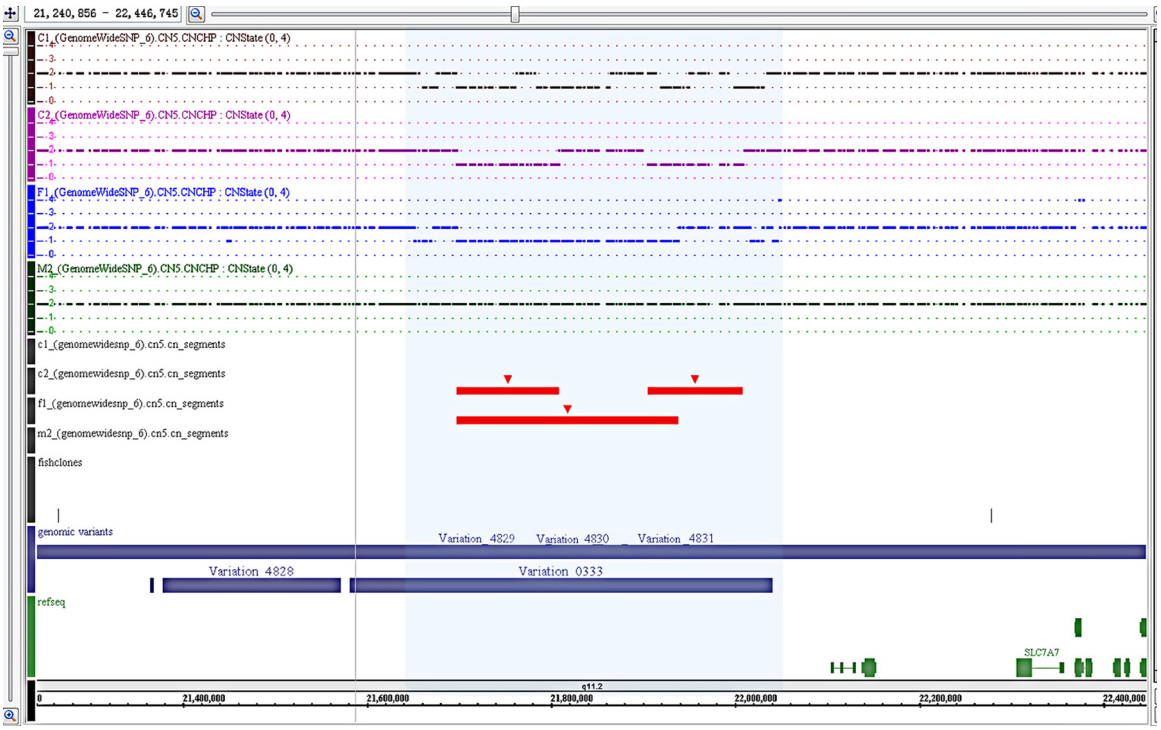

Figure 3. The twins' (C1, C2) DNA copy number deletion inherit from the father (F1), which means structural features of DNA polymorphism.

SUPT16H; promoter region: SHROOM1). However, sodium bisulfite modification, methylation-specific PCR and DNA sequencing failed to reveal significant differences in the methylation of promoter and within the Notch1 gene between the twins (**Figure 5**).

Discussion

MZ twins originate from one single zygote, but post-zygotic factors may generate genetic differences [26]. Mutations or genetic differences between MZ twins are certainly helpful to study the cause of complex diseases since SNP arrays (Affymetrix and Illumina) have shown twins with discordant genotypes and genomically concordant [27-29]. CNVs, typically a large insertion/deletion of a sequence length from 1 kb to 3 Mb, may alter the gene expression dosage of contiguous genes and result in developmental disorders during cell division [18]. To clarify possible ECD-associated alteration of regulatory gene, we investigated genome-wide genetic and epigenetic differences between the discordant MZ twins. The results indicated that not only the SNPs but also CNVs were not detected to be different. We hypothesize that other genetic or environmental insults may act stochastically. Epigenetic differences in MZ twins have been reported previously which involved in the developmental regulation of gene expression and contributed to interindividual phenotypic variation and disease susceptibility [30, 31]. Methylation has been offered as an epigenetic explanation for the discordance of MZ twins for developmental disorders that drove us to focus on interindividual DNA methylation differences [31]. Differential DNA methylation in the promoter regions of Notch 1 may affect the gene-expression of target genes and contribute to the phenotypic discordance in MZ twins [32]. Notch-signaling is involved in cardiac cushion epithelial-to-mesenchymal transition (EMT) that forms the cardiac cushion, proper outflow tract and cardiac valve development [32]. Notch1 haploinsufficiency as a consequence of targeted deletion of Notch1 results in inefficiency of EMT in endothelial cells, collapsed endocardium, absent cushions and defective outflow tract [6-8]. However, few epigenetic differences between the genetically identical twins were validated by subsequent experiments using blood lymphocyte DNA, as would be expected the possibility of minor variations in local epigenetic events during cardiogenesis between the MZ twins.

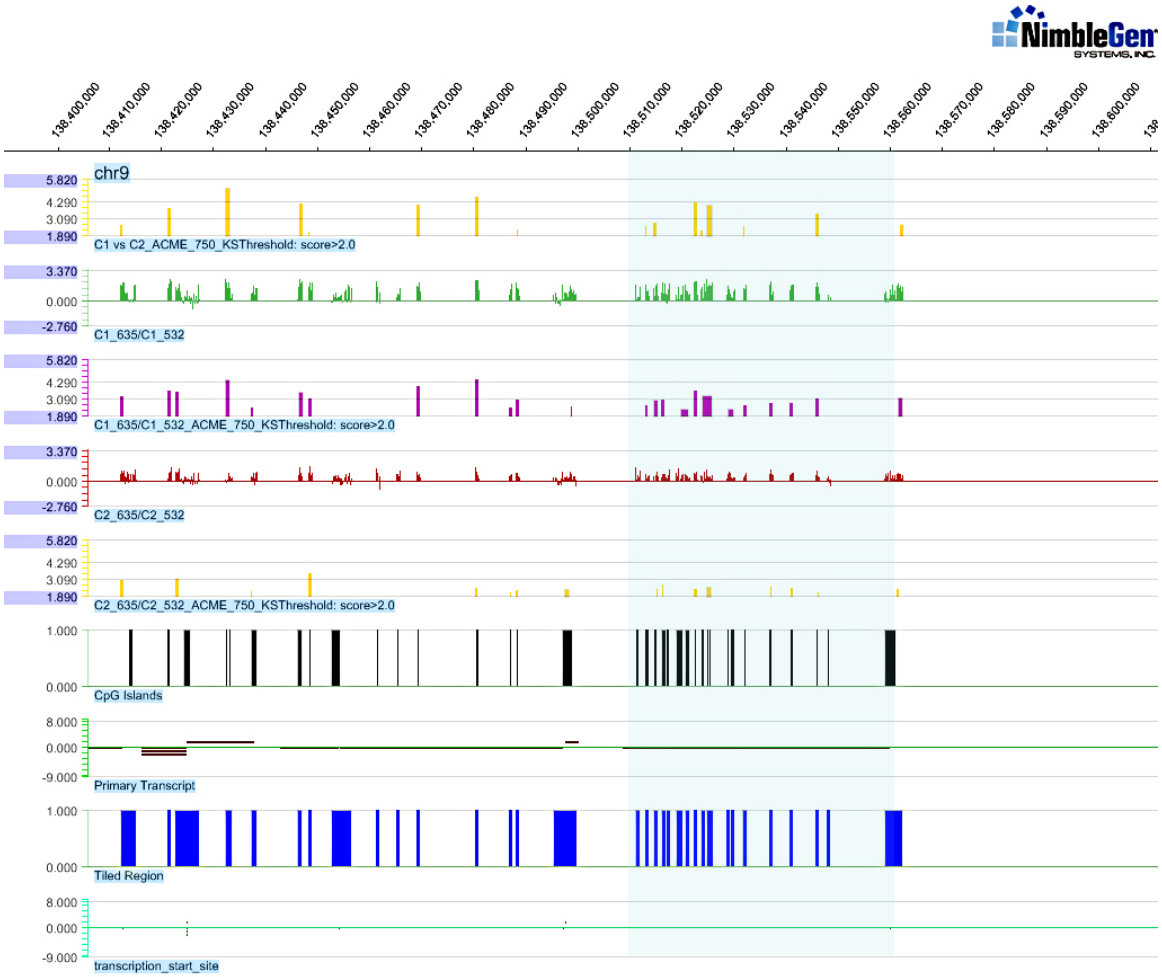

Figure 4. SignalMap software graph of differential DNA methylation in CpG islands of Notch1 gene using a Human Meth 3 × 720 K microarray. C1-the ECD twin, C2-the normal twin. The hypermethylated region in chromosome 9 occurred in C1 (the second row green strips for the Log2-ratio) when compared with C2 (the fourth row red strips). The first yellow bars were the outline of Log2-ratio and region features of the Notch 1 gene between C1 and C2. The third purple strips and the fifth yellow strips demonstrate the methylation peak score respectively corresponding C1 and C2 that showed significant methylation differences. The black bars, brown lines, blue bars and the point line below indicate CpG islands, primary transcripts, tiled regions and transcription start sites, respectively. The light blue box highlights the differentially methylated region between the cases and the control. ECD: Endocardial Cushion Defect.

Both environmental and genetic components or stochastic factors may account for human CHD. Without chromosomal birth defects and confirmed molecular genetic evidence, phenotypic discordance for complete ECD in the monochorionic MZ twins usually indicates external influences on the intrauterine environment. Maternal fever and influenza are import factors of specific CHDs, namely right-sided obstructive defects and atrioventricular septal defects in infants [19]. The pregnant woman had a cold in early pregnancy that may possibly increase disease susceptibility to environmental insults with epigenetic processes and differential gene expression regarding phenotype differences [6]. But only one of the twins had complete ECD and had not been found to have a genetical or epigenetical basis by current technologies. So, we speculated different responses of endothelial cell lineage to minor variations in the spatial and temporal expression of local signaling molecules passing between cells during development and progression [33]. We just studied ECD and transposition of great artery (TGA) of the MZ twins by genomic and epigenomic approaches for unrecognized genomic differences and potential environmental factors on a local tissue. The occurrence may due to factors other than certain genetic variations, polymorphisms and

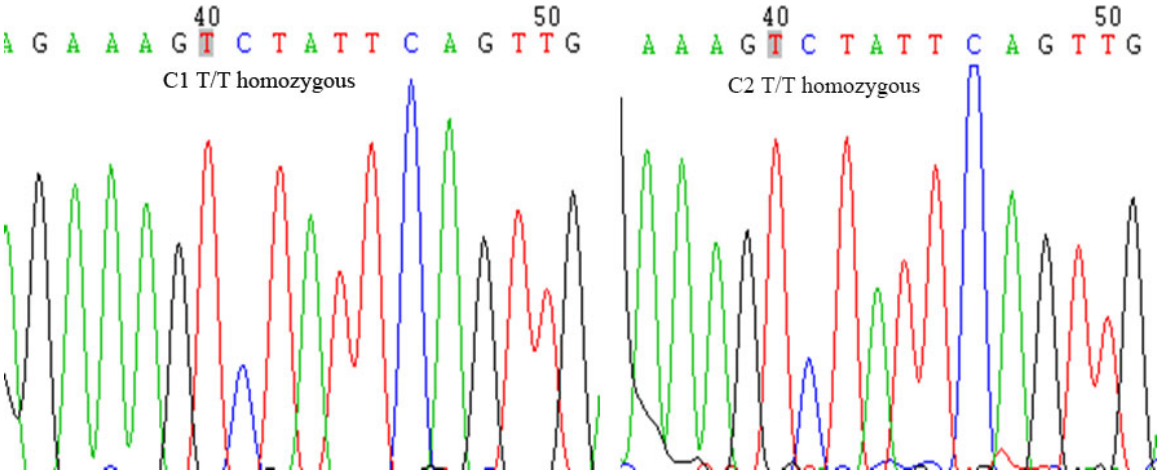

Figure 5. DNA sequencing in one of the differentially methylated CpG sites on the promoter regions of Notch1 gene between the twins after methylation-specific PCR. Promoter methylation of Notch1 in C1-the ECD twin was not significantly changed than that in C2-the normal twin with the MZ twins phenotype discordance. ECD: Endocardial Cushion Defect.

methylation in multiple genes, such as minor "disturbances" in epigenetic events at the local level during EMT, which can lead to quite major differences in the endocardium [33]. Further studies are needed to investigate whether pathological mutations, adverse effects on decidual and placental development, or unmeasured environmental factors are involved in the birth anomaly discordant.

In conclusion, no apparent molecular lesion on DNA polymorphisms and epigenome profiling was successfully identified between the disease-discordant MZ twins for complete ECD and TGA. Our ultimate goal is to discover how genetic, environmental and stochastic factors impact upon human ECD to facilitate future genetic counseling, and understand the pathophysiology and risk factors of discordant MC twins. Though recurring CNV, SNP and DNA methylation have no clinical relevance to the twins' phenotypic heterogeneity, diverse molecular mechanisms, including CHD-specific germline mutations, some miRNAs involved in cardiomyocyte signaling and communication in a variable fashion, different partial duplication or copy loss on chromosome leading to differential disruption of gene function or amount and hemodynamic perturbations, and not necessarily identical growth environment of cord or placenta, might contribute to the discordant cardiac pathology in monozygotic twins [6, 34-36]. Phenotype-based forward genetic study will significantly advance our understanding of the occurrence of congenital cardiac defects in one of the twins but not the other.

Disclosure of conflict of interest

None.

Address correspondence to: Zhiqing Liang, Department of Obstetrics and Gynecology, The First Affiliated Hospital (Southwest Hospital), Army Medical University, No. 30 Gaotanyan Street, Shapingba District, Chongqing 400038, China. Tel: +86-13320332902; E-mail: zhiqingliang0903@126.com; Qifeng Tang, Department of Anesthesiology, Shanghai Hechuan-Rhine TCM Hospital, No. 3071 Hechuan Road, Minhang District, Shanghai 201103, China. Tel: +86-15862511382; E-mail: ably.tang@qq.com

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