Screening for differential methylation status in fetal myocardial tissue samples with ventricular septal defects by promoter methylation microarrays

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Abstract. To identify and provide a global assessment of DNA methylation in fetal ventricular septal defect (VSD), genomic DNA extracted from fetal myocardial tissue samples with VSD (n=21) and from normal fetal myocardial tissue samples (n=15) was analyzed for gene methylation using array-based technology. Furthermore, the KIAA0310, RAB43, SIVA1 and NDRG2 genes were randomly selected for validation analysis using methylation-specific PCR. Our results revealed that 70 and 85 genes were regulated by hypermethylation and hypomethylation, respectively, in VSD. Different clusters of genes were associated with functions including embryo development, signal transduction, cell apoptosis and cell proliferation. In conclusion, this study identified a set of candidate genes whose expression is regulated by DNA methylation in fetal VSD.

Introduction

Congenital heart disease (CHD) is the most common type of developmental defect, occurring in almost 1% of all neonates (1). Ventricular septal defect (VSD) is the most commonly recognized CHD (2). VSD may exist alone or as an integral part of complex CHD (3). VSD is a multifactorial complex disease, in which genetic and environmental factors play important roles. Despite the availability of several surgical techniques to treat VSD, the exact molecular mechanism of this type of CHD remains unclear.

DNA methylation has been widely recognized as a potent mechanism for silencing gene expression and maintaining genome stability (4). DNA methylation is the predominant epigenetic alteration occurring in mammalian genomes, and plays a critical functional role in development, differentiation and disease (5). Previous studies have revealed that during embryonic development, the mammalian genome undergoes profound reprogramming of DNA methylation patterns in the germ and early pre-implantation embryos (6). Furthermore, the different prototypes of genes in each cell, tissue and organ are thought to be regulated by DNA methylation even during early development (7).

The recent advent of array-based techniques offers the opportunity for more comprehensive DNA methylation profiling (8). Comparing the DNA methylation profiles of myocardial tissue samples from VSD and normal fetuses, we provide novel information for identifying gene methylation that may be implicated in the pathological consequences of VSD.

Materials and methods

Tissue samples. Fetal myocardial tissue samples were obtained from Nanjing Maternal and Child Health Hospital. Myocardial tissue samples from 21 VSD and 15 normal fetuses at 26 weeks of gestation were obtained during surgery for pregnancy termination owing to trauma of the pregnant women. All samples were collected with the approval of the appropriate institute ethics committee, and written consent was provided by each pregnant woman and her family. The speciments were immediately snap frozen in liquid nitrogen and then stored at -80˚C until analysis.

DNA methylation profiling by methylated DNA immunoprecipitation. The methylation profiling by methylated DNA immunoprecipitation (MeDIP) assay was performed using 3 mg of sonicated genomic DNA (300-1,000 bp) and 10 mg

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Table I. Primer sequences for amplifying the methylated (M) and unmethylated (U) genes

Primers	Sequences (5'-3')	Product size (bp)	Annealing temperature (˚C)
KIAA0310	MF-GTTGATGTCGTAAGTCGGATAC MR-ACCACCGCGATCCAACCTAAACAAC UF-GTTGATGTTGTAAGTTGGATAT UR-ACCACCACAATCCAACCTAAACAAC	198	55
RAB43	MF-GTTTTTGATCGGCGGTTTGGGAGGT MR-CGACTCTACCTTCAAACCCACCTCA UF-GTTTTTGATTGGTGGTTTGGGAGGT UR-CAACTCTACCTTCAAACCCACCTCA	409	54
SIVA1	MF-AAATTAGATTCGTTTCGACGTC MR-TCGATATACTAAACTCGACGCC UF-TTTAAATTAGATTTGTTTTGATGTT UR-TCAATATACTAAACTCAACACCACA	321	55
NDRG2	MF-AGAGGTATTAGGATTTTGGGTACG MR-GCTAAAAAAACGAAAATCTCGC UF-AGAGGTATTAGGATTTTGGGTATGA UR-CCACTAAAAAAACAAAAATCTCACC	125	55

of antibody against 5-methylcytidine (BI-MECY-1000; Eurogentec) as previously described (9). For PCR, 20 ng of sonicated genomic input DNA and 1/40 of an MeDIP reaction were used. In each array, seven unamplified MeDIP reactions were pooled and hybridized together with sonicated genomic input DNA. Final promoter methylation $\log_2$ ratios of bound over input signals represent the average of three independent experiments, including one dye swap.

Methylation-specific PCR (MSP). Four differentially regulated genes identified with promoter methylation microarray analysis were randomly selected for validation analysis by MSP. DNA methylation patterns in the CpG islands of the *KIAA0310, RAB43, SIVA1* and *NDRG2* genes were determined by chemical treatment with sodium bisulfite and subsequent MSP, according to a previously described method (10). Primer sequences of these genes are described in Table I. Primers were purchased from Invitrogen (USA). Myocardial tissue DNA samples, either original or methylated *in vitro* by excess CpG (Sss.I) methyltransferase (NEB, USA), were used as positive controls for unmethylated and methylated DNA, respectively. Distilled water was used as a negative control.

Statistical analysis. Statistical analysis was performed using the χ^2 test or Fisher's exact test and the Student's t-test if the data followed a normal distribution. A P-value of <0.05 (two-sided) was regarded as statistically significant. All data were analyzed with SPSS 13.0 for Windows.

Results

Methylation profiles of fetal ventricular tissue. A promoter methylation microarray was used to evaluate 21 ventricular tissues samples from VSD fetuses and 15 samples from normal healthy controls. The array identified 70 and 85 candidate genes regulated by hypermethylation and hypomethylation, respectively, in VSD (Table II).

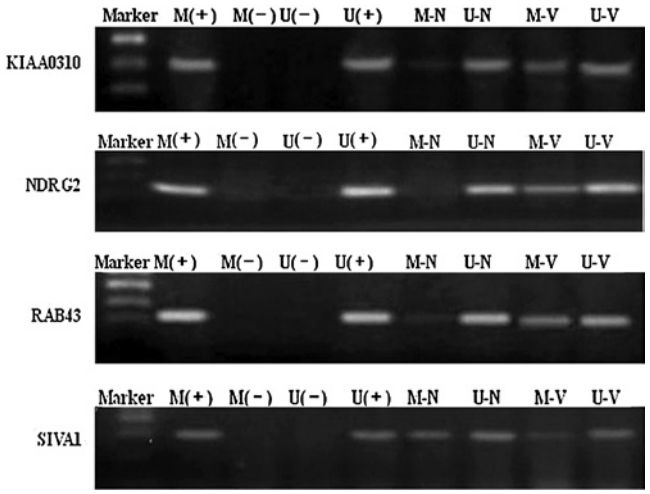

Figure 1. Representative results of methylation-specific PCR analyses of myocardial DNA samples from normal and VSD fetuses. The PCR products in lanes M and U reveal the presence of methylated and unmethylated templates, respectively, of the NOX5 gene. Lanes N and V contain the control and VSD templates, respectively. Marker, 100-bp DNA ladder; M(+), methylated positive control; U(+), unmethylated positive control; M(-) and U(-), negative controls.

Validation of the microarray results by MSP. To further evaluate and validate the results obtained by the microarrays, MSP analysis was performed in four randomly selected differentially expressed genes. Both hypermethylation and hypomethylation genes were selected in the ventricular tissues of the VSD fetuses for subsequent MSP analysis. Representative gel images of these four genes are shown in Fig. 1. Overall, the hypermethylation rate of *RAB43, NDRG2*

Table II. Promoter methylation in VSD genome.

Chromosome	Gene	Description
Hypermethylation		
1	GBP2	Guanylate binding protein 2, interferon-inducible
	GJB4	Gap junction protein, β 4 (connexin 30.3)
	POGZ	Pogo transposable element with ZNF domain
	OPN3	Opsin 3
	RHOC	Ras homolog gene family, member C
2	SUSD4	Sushi domain containing 4
	CYBRD1	Cytochrome b reductase 1
	GPR148	G protein-coupled receptor 148
	RPE	Ribulose-5-phosphate-3-epimerase
3	RAB43	Member RAS oncogene family
	MFN1	Mitofusin 1
	KPNA1	Karyopherin α 1 (importin α 5)
4	DRD5	Dopamine receptor D5
	ANKRD17	Ankyrin repeat domain 17
5	KIAA1191	Kiaa1191
6	MLLT4	Myeloid/lymphoid or mixed-lineage leukemia (trithorax homolog, Drosophila) %3B translocated to, 4
	SSR1	Signal sequence receptor, α
	TMEM14C	Transmembrane protein 14C
7	HIST1H3F	Histone 1, H3f
	RNF32	Ring finger protein 32
	BCDIN3	Bin3, bicoid-interacting 3, homolog (Drosophila)
	RELN	Reelin
	ZYX	Zyxin
	C7orf20	Chromosome 7 open reading frame 20
	FASTK	Fas-activated serine/threonine kinase
8	ANGPT2	Angiopoietin 2
	FNTA	Farnesyltransferase, CAAX box, α
	PTDSS1	Phosphatidylserine synthase 1
9	KIAA0310	Kiaa0310
	STOML2	Stomatin (EPB72)-like 2
10	DYDC1	DPY30 domain containing 1
	LOC653471	Similar to Ribosome biogenesis protein BMS1 homolog
	FGF8	Fibroblast growth factor 8 (androgen-induced)
	ABI1	Abl-interactor 1
11	CHID1	Chitinase domain containing 1
	EEF1G	Eukaryotic translation elongation factor 1γ
	MUCDHL	Mucin and cadherin-like
	DKK3	Dickkopf homolog 3 (Xenopus laevis)
	LDHB	Lactate dehydrogenase B
12	CLEC4C	C-type lectin domain family 4, member C
13	THSD1	Thrombospondin, type I, domain containing 1
	EBPL	Emopamil binding protein-like
14	WDR20	WD repeat domain 20
	TRAPPC6B	Trafficking protein particle complex 6B
	NDRG2	NDRG family member 2
15	NOX5	NADPH oxidase, EF-hand calcium binding domain 5
	SNRPN	Small nuclear ribonucleoprotein polypeptide N
	CLUAP1	Clusterin associated protein 1
16	CMTM1	CKLF-like MARVEL transmembrane domain containing 1
	STUB1	STIP1 homology and U-box containing protein 1

Table II. Continued.

Chromosome	Gene	Description
17	USP6	Ubiquitin specific peptidase 6 (Tre-2 oncogene)
	SHMT1	Serine hydroxymethyltransferase 1 (soluble)
	NT5C	5', 3'-nucleotidase, cytosolic
18	MBD1	Methyl-CpG binding domain protein 1
	CXXC1	CXXC finger 1 (PHD domain)
	PPP4R1	Protein phosphatase 4, regulatory subunit 1
	RPL17	Ribosomal protein L17
19	PRKACA	Protein kinase, cAMP-dependent, catalytic, α
	NFIX	Nuclear factor I/X (CCAAT-binding transcription factor)
20	RAE1	RNA export 1 homolog (S. pombe)
	GGTLA4	γ-glutamyltransferase-like activity 4
21	LOC284821	Similar to ribosomal protein L13a
	CCT8	Chaperonin containing TCP1, subunit 8 (θ)
	U2AF1	U2 small nuclear RNA auxiliary factor 1
	TRPM2	Transient receptor potential cation channel, Subfamily M, member 2
22	ZNF74	Zinc finger protein 74 (Cos52)
	PES1	Pescadillo homolog 1, containing BRCT domain (zebrafish)
X	SOX3	SRY (sex determining region Y)-box 3
	ARSD	Arylsulfatase D
	FAM39A	Family with sequence similarity 39, member A
Hypomethylation		
1	CDC20	Cell division cycle 20 homolog (S. cerevisiae)
	DAP3	Death associated protein 3
	EXOSC10	Exosome component 10
	AGL	Amylo-1
2	FLJ13305	Hypothetical protein FLJ13305
	EEF1B2	Eukaryotic translation elongation factor 1 β 2
3	TP73L	Tumor protein p73-like
	ECT2	Epithelial cell transforming sequence 2 oncogene
	RARB	Retinoic acid receptor, β
	EIF4A2	Eukaryotic translation initiation factor 4A, isoform 2
	NR1I2	Nuclear receptor subfamily 1, group I, member 2
	MME	Membrane metallo-endopeptidase
	ZNF9	Zinc finger protein 9
	TATDN2	TatD DNase domain containing 2
	HYAL1	Hyaluronoglucosaminidase 1
	PPARG	Peroxisome proliferative activated receptor, γ
4	PIGG	Phosphatidylinositol glycan, class G
	PDLIM5	PDZ and LIM domain 5
5	RAD17	RAD17 homolog (S. pombe)
	TAF9	TAF9 RNA polymerase II, TATA box binding protein (TBP)-associated factor, 32 kDa
	BNIP1	BCL2/adenovirus E1B 19 kDa interacting protein 1
	RAD1	RAD1 homolog (S. pombe)
	CCNG1	Cyclin G1
	PART1	Prostate androgen-regulated transcript 1
6	PECI	Peroxisomal D3, D2-enoyl-CoA isomerase
	MYB	V-myb myeloblastosis viral oncogene homolog (avian)
7	CDKN1A	Cyclin-dependent kinase inhibitor 1A (p21, Cip1)
	TSC22D4	TSC22 domain family, member 4

Table II. Continued.

Chromosome	Gene	Description
8	ALKBH4	AlkB, alkylation repair homolog 4
	TM2D2	TM2 domain containing 2
	PDLIM2	PDZ and LIM domain 2 (mystique)
	PABPC1	Poly(A) binding protein, cytoplasmic 1
	ADAM32	ADAM metallopeptidase domain 32
	YWHAZ	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, ζ polypeptide
9	MAF1	MAF1 homolog (S. cerevisiae)
	PPP2R4	Protein phosphatase 2A, regulatory subunit B' (PR 53)
	CRAT	Carnitine acetyltransferase
	ROD1	Regulator of differentiation 1 (S. pombe)
10	PITRM1	Pitrilysin metallopeptidase 1
	MAPK8	Mitogen-activated protein kinase 8
	ITGB1	Integrin, β 1
	CDC2	Cell division cycle 2, G1 to S and G2 to M
	FUT11	Fucosyltransferase 11
	HELLS	Helicase, lymphoid-specific
11	RPS6KB2	Ribosomal protein S6 kinase, 70 kDa, polypeptide 2
	WT1	Wilms tumor 1
	CD44	CD44 molecule (Indian blood group)
	KIAA0652	Kiaa0652
12	PA2G4	Proliferation-associated 2G4, 38 kDa
	BCL2L14	BCL2-like 14 (apoptosis facilitator)
13	HMGB1	High-mobility group box 1
	CDK8	Cyclin-dependent kinase 8
14	SIVA1	CD27-binding(Siva) Protein
	ATP6V1D	ATPase, H^+ transporting, lysosomal 34 kDa, V1 subunit D
	WDR20	WD repeat domain 20
15	BMF	Bcl2 modifying factor
	NUSAP1	Nucleolar and spindle associated protein 1
16	CDK10	Cyclin-dependent kinase (CDC2-like) 10
	ALDOA	Aldolase A, fructose-bisphosphate
17	UBB	Ubiquitin B
	BIRC5	Baculoviral IAP repeat-containing 5 (survivin)
	CDK5RAP3	CDK5 regulatory subunit associated protein 3
	MAPK7	Mitogen-activated protein kinase 7
	PRKAR1A	Protein kinase, cAMP-dependent, regulatory, type I, α (tissue specific extinguisher 1)
18	NFATC1	Nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 1
	CDH7	Cadherin 7, type 2
19	CAPNS1	Calpain, small subunit 1
20	TFAP2C	Transcription factor AP-2 γ (activating enhancer binding protein 2 γ)
	SNRPB2	Small nuclear ribonucleoprotein polypeptide B"
	WFDC2	WAP four-disulfide core domain 2
	GNAS	GNAS complex locus
	PRKCBP1	Protein kinase C binding protein 1
21	ATP5J	ATP synthase, H^+ transporting, mitochondrial F0 complex, subunit F6
	DSCR3	Down syndrome critical region gene 3
	SON	SON DNA binding protein

Table II. Continued.

Chr	Gene	Description
22	GNAZ	Guanine nucleotide binding protein (G protein), α z polypeptide
	PICK1	Protein interacting with PRKCA 1
	SLC2A11	Solute carrier family 2 (facilitated glucose transporter), Member 11
	EIF3S7	Eukaryotic translation initiation factor 3, Subunit 7 ζ, 66/67 kDa
	SULT4A1	Sulfotransferase family 4A, member 1
	PDGFB	Platelet-derived growth factor beta polypeptide [simian sarcoma viral (v-sis) oncogene homolog]
X	RPL10	Ribosomal protein L10
	FTSJ1	FtsJ homolog 1 (*E.coli*)
	NKAP	NF-κB activating protein
	RPL10	Ribosomal protein L10

and *KIAA0310* in VSD (85.7%, 18/21; 76.2%, 16/21 and 71.4%, 15/21; respectively) (P<0.05) was higher than that of the normal tissues. The hypomethylation rate of *SIVA1* in VSD (65.3%, 13/21) (P<0.05) was higher than that of the normal tissues.

Discussion

Currently, pre-natal diagnosis of VSD is relatively difficult and surgical treatment of VSDs carries both mortality and morbidity risks, even though it is the most common open heart procedure performed in pediatric cardiac surgery (11,12). The heart is the first organ to form during embryogenesis, and its development is controlled by a series of important genes (13). Epigenetic processes, including DNA methylation, are thought to control gene expression during the differentiation and development of cardiac tissues (14,15). The relationship between DNA methylation and heart development is currently the focus of CHD studies.

In the present study, we utilized promoter methylation microarray technology to obtain an overall profile of the gene methylation of myocardial tissue in fetal VSD. Our analysis identified 70 and 85 genes regulated by hypermethylation and hypomethylation, respectively, in VSD. These genes are involved in embryo development, signal transduction, cell apoptosis and cell proliferation.

SSR1 and *NDRG2* were found to be hypermethylation genes. SSR1 is a subunit of the translocon-associated protein (TRAP) complex (16). Human and mouse studies have revealed that SSR1 is maternally supplied until the eight-cell stage, and is then constitutively expressed during embryogenesis (17). Previous studies have suggested that SSR1 plays a crucial role in mammalian heart development and may be involved in the translocation of factors necessary for the maturation of endocardial cushions. For example, homozygous *SSR1* mutant pups die at birth, possibly as a result of severe cardiac defects (18). *NDRG2* is one of the four members of the new NDRG (N-myc downstream-regulated gene) family (19-21). Previously, the expression of NDRG2 mRNA was examined in mouse embryos and in adult human hearts (22,23). During mouse development, NDRG2 protein expression was observed in the heart as early as E9.5, and was present at higher levels in the heart atria between E14.5 and E17.5 (24). In this study, we found that the proportion SSR1 and NDRG2 promoter hypermethylation was significantly higher in the VSD cases than in the controls. We speculate that the *SSR1* and *NDRG2* genes may display silencing by aberrant methylation during cardiac morphogenesis and maturation in fetuses with VSD.

The endoplasmic reticulum (ER) is a multifunction organelle involved in the synthesis and packaging of proteins (25). ER functions are disturbed by various stress conditions (ER stress), including the inhibition of protein glycosylation, the reduction of disulfide bond formation, calcium depletion from the ER lumen and the impairment of protein transport from the ER to the Golgi (26). Several lines of evidence indicate that ER stress may be involved in the development of the embryonic heart. Mao *et al* (27) revealed that, during early heart organogenesis, Grp78 is activated through cooperation between cell type-specific transcription factors and endoplasmic reticulum stress response element (ERSE)-binding factors. In this study, we screened and identified the promoter hypermethylation of the *RAB43* and *KIAA0310* genes in VSD tissues. Previous studies have found that overexpression and, to a lesser extent, small interfering RNA depletion of KIAA0310 inhibit ER-to-Golgi transport (28). RAB43 has also been observed to play a role in anterograde trafficking of cargo from the ER to the Golgi (29). Therefore, we propose that the hypermethylation of *RAB43* and *KIAA0310* leads to ER-to-Golgi transport dysfunction, which further leads to the occurrence of ER stress. ER stress is thought to contribute to the development of the abnormal embryonic heart, including VSD.

DNA hypomethylation is linked to genetic instability characterized by chromosomal aberration and elevated mutation rates (30), and may lead to the loss or silencing of gene function (31,32). In this study, we evaluated the

promoter hypomethylation of 85 genes in VSD. Among the genes examined, the apoptosis-related genes *SIVA1* and *MDM2* showed hypomethylation in the VSD, but not in the control, samples. Hypomethylation is a crucial step in the transformation of the endocardial cushion to ventricular septum in the development of the embryonic four-chambered heart. The development of the form and structure of the endocardial cushion is accompanied by precise patterns of abundant cell death, which has the morphological features of programmed cell death (apoptosis) (33). Hypomethylation of *SIVA1* and *MDM2* may lead to the the loss of mitotic function, which regulates mitotic balance in tissue renewal.

In conclusion, we used methylation profiling to identify a set of candidate genes whose expression is regulated by DNA methylation in VSD. The methylation profiling also identified additional candidate genes for future investigation.

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