Available online at www.sciencedirect.com
SCIENCE DIRECT
Biochemical and Biophysical Research Communications 342 (2006) 135–144
BBRC
www.elsevier.com/locate/ybbrc

Identification of differentially expressed genes in human heart with ventricular septal defect using suppression subtractive hybridization

Hao Zhang, Lei Zhou, Rong Yang, Yanhui Sheng, Wei Sun, Xiangqing Kong *, Kejiang Cao

Department of Cardiology, First Affiliated Hospital of Nanjing Medical University, No. 300 Guangzhou Road, Nanjing 210029, PR China

Received 2 January 2006
Available online 2 February 2006

Abstract

Ventricular septal defect (VSD) accounts for the largest number of birth congenital heart defects in human, but the genetic programs that control ventricular septation are poorly understood. To identify differentially expressed genes between ventricular septal defect and normal ventricular septum myocardium, we have undertaken suppression subtractive hybridization (SSH) and generated reciprocal cDNA collections of representative mRNAs specific to human heart with ventricular septal defect versus normal control. Following SSH, 1378 clones were sequenced and found to derive from 551 different genes. These predominately expressed genes included genes involved in energy metabolism, cell cycle and growth, cytoskeleton and cell adhesion, LIM protein, zinc finger protein, and development. It is anticipated that further study of genes identified will provide insights into their specific roles in the etiology of VSD, even in cardiac development, aging, and disease.

Keywords: Ventricular septal defect; Suppression subtractive hybridization; Gene expression; Heart development

Congenital heart defects (CHDs) account for the largest number of birth defects in human, with an incidence of 1% in live births and 5–10% in spontaneously aborted pregnancies [1,2]. As a single cardiac malformation, the ventricular septal defect (VSD) is the most frequent form of CHDs and is present in the 33% of all affected infants [3]. Endocardial cushion and myocardium play important roles in the formation of interventricular septum. The muscular portion of the interventricular septum arises from ingrowth and folding of the myocardial wall of the developing ventricle, whereas the membranous portion of the interventricular septum is derived primarily from endocardial cushion tissue [4]. The ontogeny of ventricular septation is complex, which requires cell coordinated growth, specification, differentiation, migration and apoptosis, morphogenesis and cell–cell interaction [5].

To date, a few genes, which cause CHDs, have been identified. The mutations of NKX2.5 [6], GATA4 [7], and BMP10 [8] are associated with atrial and ventricular septal defects. However, like most CHDs, VSD is thought to be multigenic disorders, and the basic mechanism for VSD in human is still incompletely defined.

There are several PCR-based approaches to analyze changes in gene expression. These include differential display polymerase chain reaction (DD-PCR) [9], RNA fingerprinting by random primed PCR [10], representational difference analysis (RDA) [11], and suppression subtractive hybridization (SSH) [12]. Using SSH has the advantage to combine normalization and subtraction in a single procedure. SSH is a highly effective method for generating subtracted cDNA libraries. It dramatically increases the probability of obtaining low-abundance differentially expressed cDNAs and simplifies the analysis of the subtracted cDNA libraries. Using SSH in the present study to identify differentially expressed genes related to VSD could elucidate the mechanism of the development of

* Corresponding author. Fax: +86 25 83672050.
E-mail address: kongxq@njmu.edu.cn (X. Kong).

0006-291X/$ - see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2006.01.113

VSD and lead to new aspect of diagnosis and primary prevention of VSD, even the mechanism in cardiac development, aging, and disease.

Materials and methods

Samples. All cardiac biopsies weighing about 50–100 mg were obtained from patients with VSD in the septum of right ventricle and near the defect during corrective cardiac surgery at Department of Thoracic-Cardiac Surgery, the First Affiliated Hospital of Nanjing Medical University, after ethical approval by the Ethics Committee at Nanjing Medical University and informed consent of the patients or parents. Tissues from normal human heart's septum of right ventricle were obtained from donor hearts without cardiac diseases that were not used for transplantation. Clinical data of VSD patients and normal human are presented in Table 1. All samples were directly snap-frozen in liquid nitrogen after excision and stored at −80 °C.

Total RNA and polyadenylated RNA isolation. According to the manufacturer's instructions, total RNA was extracted from ventricular septum myocardium with Trizol reagents (Invitrogen Life Technologies, Carlsbad, CA), and then polyadenylated RNA was isolated from total RNA using Oligotex mRNA spin columns (Qiagen, Valencia, CA). The quantity and quality of each sample were determined spectrophotometrically by A_{260} and $A_{260/280}$ ratio, and checked by electrophoresis on a 1.2% agarose/formaldehyde gel.

Table 1
Clinical data

	Control ($n = 10$)	VSD patients for SSH ($n = 10$)	VSD patients for RT-PCR ($n = 6$)	p value
Gender (female/male)	2/8	4/6	2/4	NS
Age (years)	24.1 ± 3.1	28.5 ± 2.2	26.0 ± 2.3	NS
PAP (mmHg)	15.0 ± 1.1	18.3 ± 0.8	18.7 ± 1.1	NS
LVDd (mm)	42.6 ± 0.9	45.2 ± 1.1	45.0 ± 1.1	NS
EF (%)	69.2 ± 0.6	68.0 ± 0.7	68.2 ± 0.9	NS

Data were presented as mean values ± SEM.
EF, ejection fraction; LVDd, left ventricular end diastolic dimension; NS, not significant; PAP, pulmonary arterial pressure.

Suppression subtractive hybridization. According to manufacturer's protocol, SSH (suppression subtractive hybridization) was performed using PCR-Select cDNA Subtraction Kit (Clontech, Palo Alto, CA). We used ventricular septum myocardial cDNA of VSD patients as tester and that of normal human ventricular septum myocardial cDNA as driver for forward subtraction to identify cDNA upregulated in VSD patients, and vice versa for reverse-subtraction to identify cDNA downregulated in VSD patients. Ventricular septum myocardial cDNA was pooled from 10 VSD patients and 10 normal human (clinical data are shown in Table 1), respectively. Unsubtracted control for each subtraction was prepared by the same procedures but without the subtractive hybridization step.

Analysis of subtraction efficiency. To evaluate the subtraction efficiency, the subtracted cDNA products were subjected to 18, 23, 28, or 33 cycles of PCR amplification of glyceraldehyde 3-phosphate dehydrogenase (G3PDH) with specific primers. The forward (5′-ACC ACA GTC CAT GCC ATC AC-3′) and reverse (5′-TCC ACC ACC CTG TTG CTG TA-3′) G3PDH primers were provided by the PCR-Select cDNA Subtractive Kit. Unsubtracted controls were amplified in parallel.

Cloning and sequencing. Products from the secondary PCR were inserted into pGEM-T Easy Vector (Promega Corp., Madison, WI) and transformed into *Escherichia coli* strain XL2-Blue MRF' Ultracompetent Cells (Stratagene, San Diego, CA). Plasmid DNA of each clone was purified and the presence of insert was tested by digestion with *Eco*RI. Successfully cloned cDNA fragments were sequenced with M13 forward primers in an ABI PRISM 3730 automatic sequencing machine (Applied Biosystems, Foster City, CA) using the ABI Prism dRhodamine Terminator Cycle Sequencing Kit.

Data analysis. Obtaining sequences were compared against all sequences in the nonredundant database at the National Center for Biotechnology Information (NCBI) using the Basic Local Alignment Search Tool (BLAST; www.ncbi.nlm.nih.gov/BLAST).

Validation of differential expression of genes by RT-PCR. The differential expression of randomly selected genes was further validated by RT-PCR. Each selected gene was performed RT-PCR using the templates from the ventricular septum of six different VSD patients and six different normal human (clinical data are shown in Table 1), respectively. GAPDH was used as an internal control. GAPDH and eight pairs of gene-specific primers are listed in Table 2. For RT-PCR, chromosomal DNA was removed from total RNA by use of DNase I. Total RNA was reverse-transcribed by MMLV reverse transcriptase (Promega Corp., Madison, WI) at 42 °C for 1 h and subsequently heated at 95 °C for 5 min. After the RT reaction, PCR was carried out in a total volume of 20 μl of a mixture containing 1 μl of RT samples under the following conditions: 95 °C for 5 min (initial denaturation), then 94 °C for 30 s (denaturation), 54–58 °C

Table 2
Sequences of primer pairs for RT-PCR

Gene name	Ref. sequence	Sequence of primer (5′-3′)	Annealing temperature (°C)	Length (bp)
GAPDH	NM_002046	ACCACAGTCCATGCCATCAC TCCACCACCCTGTTGCTGTA	58	452
ANKRD1	NM_014391	AGACTCCTTCAGCCAACATG AGAGCAGCCTTCAGAAACGT	58	403
BVES	BC040502	CCAAGATGAAACCGATAGAA AACAAGGCATAGTCAGAAGG	54	325
CORIN	NM_006587	TGCTTGGCATCAACAATCTA CAAATAATGTCCACCGTCCT	54	435
DMD	NM_004007	TGGAAACTCCCGTTACTCTG TTATGTTCGTGCTGCTGCTT	54	385
EVI1	BX647613	TCCCTCCATTCTACATCCCA CCTTCAGCCCACCAAGTTTT	58	387
GATA4	NM_002052	CTGGGACTTGGAGGATAGCA CCAGGTACATGGCAAACAGA	58	387
LMO7	AF330045	CCACATCAAACCGTGCCTAC AGCAGCAACCACCTCTTCTT	58	453
TTN	NM_133437	TTTACCAGACTCACCGAAGG ATGGGTTTGGAAGGACGACT	58	395

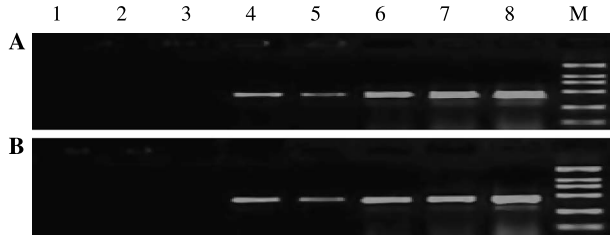

Fig. 1. Evaluated subtraction efficiency of subtracted cDNAs. Subtracted cDNA products were subjected to PCR amplification (for 18, 23, 28, or 33 cycles as indicated) of glyceraldehyde 3-phosphate dehydrogenase (G3PDH) with specific primers. Unsubtracted controls were amplified in parallel. (A) Forward subtracted cDNAs, ventricular myocardium cDNA of VSD patients as tester and normal human ventricular myocardium cDNA as driver; (B) reverse-subtracted cDNAs, normal human ventricular myocardium cDNA as tester, and ventricular myocardium cDNA of VSD patients as driver. Lanes 1 and 5, 18 cycles; lanes 2 and 6, 23 cycles; lanes 3 and 7, 28 cycles; lanes 4 and 8, 33 cycles. Lane M, marker (2000 bp).

for 40 s (annealing), 72 °C for 30 s (extension), 25–33 cycles total, and a final extension step was at 72 °C for 7 min. Aliquots (5 μl) of the PCR products were analyzed on 2% agarose gel.

Statistics. Differences in clinical data between VSD patients and normal control were analyzed using student's t test of SPSS 10.0 statistical package. Two-sided p value < 0.05 was considered statistically significant. All values were expressed as means ± SEM.

Results

Fig. 1 shows that obvious bands were seen after 23 cycles in unsubtracted cDNA and after 33 cycles in subtracted cDNA. The amount of G3PDH was significantly decreased after subtraction, indicating that the subtracted cDNA library had high subtraction efficiency.

About 1378 positive clones were randomly selected and sequenced. We stopped sequencing when only five new clones were obtained upon sequencing the last batch of 100 clones. The results suggested that the screening of the subtracted cDNA library was all but complete and that few new sequences were probably derived by further screening.

After comparing these sequences to the sequences in the GenBank nucleotide database, 1378 clones showed more than 95% identity to 551 known human genes, and 87 clones did not show significant homology to any sequence in the GenBank database and 73 clones showed homology only to genomic DNA sequence (summary of ESTs are shown in Table 3). Of 551 unique genes, 299 genes were upregulated and 252 genes are downregulated in VSD patient's heart. After bioinformatics analysis, these 551 known genes were associated with much biological process, such as energy metabolism, cell cycle and growth, cytoskeleton, and cell adhesion, LIM protein, zinc finger protein, development, and others. These genes were listed in Table 4.

To further verify and validate the results obtained by SSH, we also performed RT-PCR analysis of 21 randomly chosen differentially expressed genes which were either upregulated or downregulated in VSD patients. GAPDH was used as an internal control. The RT-PCR results of eight genes are shown in Figs. 2 and 3. There were only 3 false positive genes among the 21 randomly selected genes for RT-PCR. Overall, the expression patterns obtained by RT-PCR reflected the results obtained by SSH, demonstrating the low false positive rate associated with SSH in this experiment.

Discussion

Suppression subtractive hybridization is a powerful approach to identify differentially expressed genes. This technique permits screening of differentially expressed genes for possible involvement in pathological processes. SSH is successfully used to identify differential myocardial upregulation of genes between idiopathic dilated cardiomyopathy and normal control [13]. Gene expression differences between VSD and normal myocardium have not been studied before. Using this approach, we identified several differentially expressed genes which would give us some clues to partly elucidate the development of VSD. These genes are involved in energy metabolism, cell cycle and growth, cytoskeleton and cell adhesion, LIM protein, zinc finger protein, and development.

The muscle tissue is by far one of the most energy demanding organs in the body. Although infants with VSD may match the energy intake of healthy infants, they are unable to meet their increased energy demands, resulting in growth retardation [14]. In this study, we found that several genes inducing fatty acid metabolism were upregulated in VSD patients, such as ACADVL, ACSL1, HSD17B4, and PECI. And genes involved in carbohydrate metabolism were also increased. GPD1L, HK1, ENO3, GPD1L, MAN2A2, MGAT4B, and PDHA1 were upregulated in VSD patients. These genes were upregulated and enhanced energy support, to meet the increased energy demands. However, whether these genes contribute to the etiology of VSD is still in further study.

The transformation of the endocardial cushion into septa is a crucial step in cardiac morphogenesis as it

Table 3
Summary of EST data from SSH (Data are numbers of ESTs, with percentages in parentheses)

	Upregulated in VSD patient's heart	Downregulated in VSD patient's heart	Total
Match with known genes	547 (84.4%)	593 (81.2%)	1140 (82.7%)
Only match with ESTs	37 (5.7%)	41 (5.6%)	78 (5.7%)
Only match with genomic DNA sequence	29 (4.5%)	44 (6.1%)	73 (5.3%)
No database match	35 (5.4%)	52 (7.1%)	87 (6.3%)
Total	648 (100%)	730 (100%)	1378 (100%)

Table 4
Part of the differential expressed genes identified in VSD and normal human ventricular septum myocardium using SSH

Biological process	Gene symbol	Gene name	Accession No.	Chromosomal localization	Expression
Genes related to energy metabolism	ACADVL	*acyl-Coenzyme A dehydrogenase, very long chain*	NM_000018	17p13-p11	U[a]
	ACSL1	*acyl-CoA synthetase long-chain family member 1*	NM_001995	4q34-q35	U
	ACO2	*aconitase 2, mitochondrial*	NM_001098	22q13.2-q13.31	U
	ADCY5	*adenylate cyclase 5*	AF497517	3q13.2-q21	U
	AFG3L2	*AFG3 ATPase family gene 3-like 2 (yeast)*	BC065016	18p11	U
	AKR1B1	*aldo-keto reductase family 1, member B1 (aldose reductase)*	NM_001628	7q35	U
	ALDH2	*aldehyde dehydrogenase 2 family (mitochondrial)*	NM_000690	12q24.2	U
	ALDOA	*aldolase A, fructose-bisphosphate*	BC000367	16q22-q24	U
	ASAH1	*N-acylsphingosine amidohydrolase (acid ceramidase) 1*	NM_177924	8p22-p21.3	U
	CTSB	*cathepsin B*	NM_147783	8p22	U
	ENO3	*enolase 3 (beta, muscle)*	NM_053013	17pter-p11	U
	GNPAT	*glyceronephosphate O-acyltransferase*	NM_014236	1q42.11-42.3	U
	GPD1L	*glycerol-3-phosphate dehydrogenase 1-like*	NM_015141	3p24.1	U
	GYS1	*glycogen synthase 1 (muscle)*	NM_002103	19q13.3	U
	HK1	*hexokinase 1*	BC008730	10q22	U
	HSD17B4	*hydroxysteroid (17-beta) dehydrogenase 4*	NM_000414	5q21	U
	MAN2A2	*mannosidase, alpha, class 2A, member 2*	NM_006122	15q26.1	U
	MGAT4B	*Mannosyl(alpha-1,3-)-glycoprotein beta-1,4-N-acetylglucosaminyltransferase, isoenzyme B*	BC009464	5q35	U
	OSBPL9	*oxysterol binding protein-like 9*	NM_148909	1p32.3	U
	OXA1L	*oxidase (cytochrome c) assembly 1-like*	NM_005015	14q11.2	U
	OXCT1	*3-oxoacid CoA transferase 1*	NM_000436	5p13.1	U
	PECI	*peroxisomal D3,D2-enoyl-CoA isomerase*	NM_206836	6p24.3	U
	PDHA1	*pyruvate dehydrogenase (lipoamide) alpha 1*	NM_000284	Xp22.2-p22.1	U
	PLCL2	*phospholipase C-like 2*	AB029015	3p24.3	U
	PYGB	*phosphorylase, glycogen; brain*	NM_002862	20p11.2-p11.1	U
	RNF167	*ring finger protein 167*	BC010139	17p13.2	U
Genes related to cell cycle and growth	APRIN	*androgen-induced proliferation inhibitor*	NM_015032	13q12.3	U
	ARHGEF6	*Rac/Cdc42 guanine nucleotide exchange factor (GEF)6*	NM_004840	Xq26	U
	CAV1	*caveolin 1, caveolae protein, 22kDa*	NM_001753	7q31.1	U
	CCND1	*cyclin D1 (PRAD1: parathyroid adenomatosis 1)*	NM_053056	11q13	U
	CCNL2	*cyclin L2*	AF087903	1p36.33	U
	CD81	*CD81 antigen (target of antiproliferative antibody 1)*	NM_004356	11p15.5	U
	CD9	*CD9 antigen (p24)*	NM_001769	12p13.3	U
	CORIN	*corin, serine protease*	NM_006587	4p13-p12	U
	CYR61	*cysteine-rich, angiogenic inducer, 61*	NM_001554	1p31-p22	D[b]
	DEDD	*death effector domain containing*	NM_032998	1q23.3	U
	DNAJA2	*DnaJ (Hsp40) homolog, subfamily A, member 2*	NM_005880	16q11.1-q11.2	D
	DST	*Dystonin*	NM_183380	6p12-p11	U
	DUSP1	*dual specificity phosphatase 1*	NM_004417	5q34	D
	FABP3	*fatty acid binding protein 3, muscle and heart (mammary-derived growth inhibitor)*	BC007021	1p33-p32	U
	FABP4	*fatty acid binding protein 4, adipocyte*	NM_001442	8q21	U
	FAT	*FAT tumor suppressor homolog 1 (Drosophila)*	NM_005245	4q34-q35	U
	FTH1	*ferritin, heavy polypeptide 1*	NM_002032	11q13	D
	FTL	*ferritin, light polypeptide*	BC067772	19q13.3-q13.4	D
	FTS	*fused toes homolog (mouse)*	NM_022476	16q12.2	D
	GPNMB	*glycoprotein (transmembrane) nmb*	AY359124	7p15	D
	IGFBP3	*insulin-like growth factor binding protein 3*	AK127537	7p13-p12	U
	NDRG2	*NDRG family member 2*	AF304051	14q11.2	U
	NDRG4	*NDRG family member 4*	NM_020465	16q21-q22.1	U
	NME1	*nonmetastatic cells 1, protein (NM23A) expressed in*	AF487339	17q21.3	D
	PEA15	*phosphoprotein enriched in astrocytes 15*	BC010469	1q21.1	D
	PERP	*PERP, TP53 apoptosis effector*	AJ251830	6q24	D
	PHB	*Prohibitin*	NM_002634	17q21	U
	PMP22	*peripheral myelin protein 22*	NM_153322	17p12-p11.2	U
	PPIF	*peptidylprolyl isomerase F (cyclophilin F)*	NM_005729	10q22-q23	U
	PTPRA	*protein tyrosine phosphatase, receptor type, A*	NM_080841	20p13	D
	RAF1	*v-raf-1 murine leukemia viral oncogene homolog 1*	NM_002880	3p25	U
	SERPINF1	*serine (or cysteine) proteinase inhibitor, clade F (alpha-2 antiplasmin, pigment epithelium derived factor), member 1*	BT007222	17p13.1	D

Table 4 (*continued*)

Biological process	Gene symbol	Gene name	Accession No.	Chromosomal localization	Expression
	SERPINH1	*serine (or cysteine) proteinase inhibitor, clade H (heat shock protein 47), member 1, (collagen binding protein 1)*	BT007094	11q13.5	D
	SIVA	*CD27-binding (Siva) protein*	NM_021709	14q32.33	D
	SUMO2	*SMT3 suppressor of mif two 3 homolog 2 (yeast)*	NM_001005849	17q25.1	U
	TENS1	*tensin-like SH2 domain containing 1*	NM_022748	7p13-p12.3	U
	TM4SF1	*transmembrane 4 superfamily member 1*	BC034145	3q21-q25	D
	TRIB1	*tribbles homolog 1 (Drosophila)*	NM_025195	8q24.13	U
Genes related to cytoskeleton and cell adhesion	ACTA1	*actin, alpha 1, skeletal muscle*	NM_001100	1q42.13-q42.2	D
	ACTC	*actin, alpha, cardiac muscle*	NM_005159	15q11-q14	D
	ACTR1B	*ARP1 actin-related protein 1 homolog B, centractin beta (yeast)*	NM_005735	2q11.1-q11.2	D
	ACTR3	*ARP3 actin-related protein 3 homolog (yeast)*	NM_005721	2q14.1	D
	ADD3	*adducin 3 (gamma)*	NM_019903	10q24.2-q24.3	U
	AHNAK	*AHNAK nucleoprotein (desmoyokin)*	BC017483	11q12.2	D
	ANKHD1	*ankyrin repeat and KH domain containing 1*	NM_017747	5q31.2	U
	ANKRD1	*ankyrin repeat domain 1 (cardiac muscle)*	NM_014391	10q23.31	U
	ASB8	*ankyrin repeat and SOCS box-containing 8*	NM_024095	12q13.11	D
	BVES	*blood vessel epicardial substance*	BC040502	6q21	U
	CAPZB	*capping protein (actin filament) muscle Z-line, beta*	BC012305	1p36.1	D
	CAST	*Calpastatin*	NM_173060	5q15-q21	U
	CLASP1	*cytoplasmic linker associated protein 1*	BC032563	2q14.2-q14.3	D
	CLASP2	*cytoplasmic linker associated protein 2*	NM_015097	3p23	D
	CORO6	*coronin 6*	BC028205	17q11.2	U
	CTNNA1	*catenin (cadherin-associated protein), alpha 1, 102kDa*	NM_001903	5q31	D
	DDR2	*discoidin domain receptor family, member 2*	BC052998	1q12-q23	D
	DLC1	*deleted in liver cancer 1*	NM_182643	8p22	D
	DMD	*dystrophin (muscular dystrophy, Duchenne and Becker types)*	NM_004007	Xp21.2	D
	EPLIN	*epithelial protein lost in neoplasm beta*	NM_016357	12q13	D
	FN1	*fibronectin 1*	BC005858	2q34	U
	ILK	*integrin-linked kinase*	BC001554	11p15.5-p15.4	D
	IMMT	*inner membrane protein, mitochondrial (mitofilin)*	NM_006839	2p11.2; 2	D
	ITGB1	*integrin, beta 1*	NM_133376	10p11.2	D
	ITM2B	*integral membrane protein 2B*	NM_021999	13q14.3	D
	LAMP1	*lysosomal-associated membrane protein 1*	BC007845	13q34	D
	LAMP2	*lysosomal-associated membrane protein 2*	NM_013995	Xq24	D
	LSAMP	*limbic system-associated membrane protein*	NM_002338	3q13.2-q21	D
	MB	*Myoglobin*	NM_005368	22q13.1	D
	MSN	*Moesin*	NM_002444	Xq11.2-q12	D
	NEXN	*nexilin (F actin binding protein)*	AF114264	1p31.1	U
	PDE4DIP	*phosphodiesterase 4D interacting protein (myomegalin)*	NM_001002810	1q12	U
	PNN	*pinin, desmosome associated protein*	NM_002687	14q21.1	U
	QKI	*quaking homolog, KH domain RNA binding (mouse)*	NM_006775	6q26-27	U
	RHOB	*ras homolog gene family, member B*	NM_004040	2p24	D
	SVIL	*Supervillin*	NM_021738	10p11.2	D
	TFIP11	*tuftelin interacting protein 11*	NM_012143	22q12.1	D

(*continued on next page*)

Table 4 (*continued*)

Biological process	Gene symbol	Gene name	Accession No.	Chromosomal localization	Expression
	TTN	*Titin*	NM_133437	2q31	U
	VCL	*Vinculin*	BC039174	10q22.1-q23	U
Genes related to LIM protein	CSRP3	*cysteine and glycine-rich protein 3 (cardiac LIM protein)*	NM_003476	11p15.1	U
	FHL1	*four and a half LIM domains 1*	BC088369	Xq26	U
	FHL2	*four and a half LIM domains 2*	NM_201557	2q12-q14	U
	LDB3	*LIM domain binding 3*	NM_007078	10q22.3-q23	U
	LMO7	*LIM domain 7*	AF330045	13q22.2	U
	NRAP	*nebulin-related anchoring protein*	NM_198060	10q24-q26	U
	PDLIM1	*PDZ and LIM domain 1 (elfin)*	NM_020992	10q22-q26.3	U
Genes related to zinc finger protein	EVI1	*ecotropic viral integration site 1*	BX647613	3q24-q28	U
	DDEF1	*Development and differentiation enhancing factor 1*	NM_018482	8q24.1-q24.2	U
	GATA4	*GATA binding protein 4*	NM_002052	8p23.1-p22	D
	KLF9	*Kruppel-like factor 9*	NM_001206	9q13	U
	ZA20D2	*zinc finger, A20 domain containing 2*	NM_006007	9q13-q21	U
	ZNF313	*zinc finger protein 313*	NM_018683	20q13.13	U
	ZNF207	*zinc finger protein 207*	BC000962	17q11.2	D
	ZNF346	*zinc finger protein 346*	NM_012279	5q35.2	D
Genes related to development	ANKH	*ankylosis, progressive homolog (mouse)*	NM_054027	5p15.1	U
	CASQ2	*calsequestrin 2 (cardiac muscle)*	NM_001232	1p13.3-p11	U
	CRYM	*crystallin, mu*	NM_001888	16p13.11-p12.3	U
	DDX1	*DEAD (Asp-Glu-Ala-Asp) box polypeptide 1*	NM_004939	2p24	U
	DDX3X	*DEAD (Asp-Glu-Ala-Asp) box polypeptide 3, X-linked*	NM_001356	Xp11.3-p11.23	U
	EPAS1	*endothelial PAS domain protein 1*	NM_001430	2p21-p16	U
	EZH1	*enhancer of zeste homolog 1 (Drosophila)*	NM_001991	17q21.1-q21.3	U
	FLII	*flightless I homolog (Drosophila)*	NM_002018	17p11.2	U
	MEF2A	*MADS box transcription enhancer factor 2, polypeptide A (myocyte enhancer factor 2A)*	BC053871	15q26	D
	NRD1	*nardilysin (N-arginine dibasic convertase)*	BC008775	1p32.2-p32.1	U
	PCOLCE	*procollagen C-endopeptidase enhancer*	NM_002593	7q22	U
	QKI	*quaking homolog, KH domain RNA binding (mouse)*	NM_006775	6q26-27	U
	WWP1	*WW domain containing E3 ubiquitin protein ligase 1*	NM_007013	8q21	U
Other genes	ANXA2	*annexin A2*	NM_004039	15q21-q22	D
	ANXA5	*annexin A5*	BC001429	4q26-q28; 4q28-q32	D
	ARF1	*ADP-ribosylation factor 1*	AK130737	1q42	D
	ARL1	*ADP-ribosylation factor-like 1*	NM_001177	12q23.3	D
	ATF7IP	*activating transcription factor 7 interacting protein*	AY337596	12p13.1	U
	BTBD15	*BTB (POZ) domain containing 15*	BC071729	11q24.3	U
	BTNL9	*Butyrophilin-like 9*	NM_152547	5q35.3	U
	CALU	*calumenin*	NM_001219	7q32	U
	CANX	*calnexin*	BC042843	5q35	U
	CBWD1	*COBW domain containing 1*	BC013432	9p24.3	U
	CHD4	*chromodomain helicase DNA binding protein 4*	NM_001273	12p13	U
	FBXO40	*F-box protein 40*	AB033021	3q13.33	U
	FBXO9	*F-box protein 9*	NM_012347	6p12.3-p11.2	U
	HNRPDL	*heterogeneous nuclear ribonucleoprotein D-like*	BC007392	4q13-q21	D
	MYBPC3	*myosin binding protein C, cardiac*	NM_000256	11p11.2	U
	NIF3L1BP1	*Ngg1 interacting factor 3 like 1 binding protein 1*	BC020599	3p14.1	U
	NOMO1	*NODAL modulator 1*	NM_014287	16p13.11	D
	NPPA	*natriuretic peptide precursor A*	NM_006172	1p36.21	D
	PLN	*phospholamban*	NM_002667	6q22.1	D
	RBX1	*ring-box 1*	BC017370	22q13.2	U

Table 4 (*continued*)

Biological process	Gene symbol	Gene name	Accession No.	Chromosomal localization	Expression
	SOD1	*superoxide dismutase 1, soluble (amyotrophic lateral sclerosis 1 (adult))*	BC001034	21q22.1; 21q22.11	D
	SORBS1	*sorbin and SH3 domain containing 1*	NM_015385	10q23.3-q24.1	U
	SSB	*Sjogren syndrome antigen B (autoantigen La)*	NM_003142	2q31.1	D
	TNKS	*tankyrase, TRF1-interacting ankyrin-related ADP-ribose polymerase*	AF082557	8p23.1	U
	VPS35	*vacuolar protein sorting 35 (yeast)*	NM_018206	16q12	U
	WBSCR1	*Williams-Beuren syndrome chromosome region 1*	BC010021	7q11.23	U
	WDR1	*WD repeat domain 1*	NM_005112	4p16.1	U
	WDR6	*WD repeat domain 6*	NM_018031	3p21.31	U
	WSB1	*WD repeat and SOCS box-containing 1*	NM_015626	17q11.1	U

[a] Genes upregulated in VSD patient's heart.

[b] Genes downregulated in VSD patient's heart.

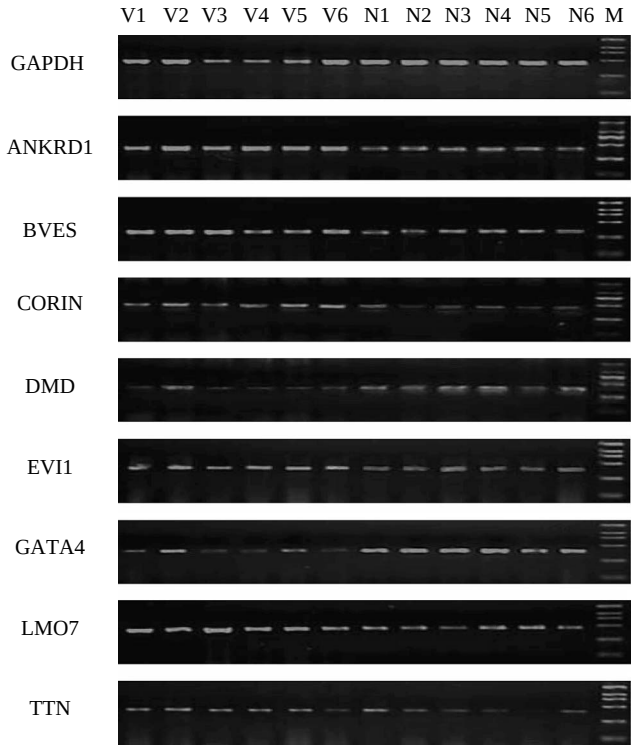

Fig. 2. RT-PCR for randomly validation of the SSH subtracted library. V1–V6, VSD ventricular myocardium; N1–N6, normal human ventricular myocardium. Lane M, marker (2000 bp).

initiates the development of the four-chambered heart. This transformation results from a region-specific balance between cellular proliferation, apoptosis, and differentiation [15]. The genes inhibiting cellular proliferation, such as APRIN, FABP3, FABP4, FAT, PHB, and PMP22, were all upregulated in VSD patients, at the same time, DEDD, RAF1, ARHGEF6, and CCNL2 which have the effect of inducing apoptosis were up-expressed and anti-apoptosis gene PEA15 was downregulated in VSD patients. The excessive apoptosis and inhibitive proliferation of the cell may lead to the disorder of the septa.

Interestingly, in this study, there were several genes, TTN, ANKHD1, ANKRD1, VCL, DMD, which encode the proteins belong to cytoskeleton or membrane-associated protein, differentially expressed. TTN, encoding an integeal cytoskeletal protein, was upregulated in VSD patients. TTN is essential in the control of the assembly of the highly ordered sarcomeres and mutations in this gene cause autosomal dominant familial dilated cardiomyopathy [16]. It was reported that TTN overexpressed in Tetrology of Fallot (TOF) compared with normal heart [17]. At the same time, ANKHD1, ANKRD1, and VCL were upregulated in VSD patients. ANKRD1, the cardiac ankyrin repeat protein (CARP), is markedly increased cardiac hypertrophy and plays as a genetic marker of cardiac hypertrophy [18]. The Carp–Lbh transgenic mice have the malformations in pulmonary outflow tract valvulogenesis, cardiac septation, inflow tract morphogenesis, as well as abnormalities in ventricular cardiomyocyte growth [19]. VCL is necessary for normal neural and cardiac development [20]. However, another membrane-associated protein, DMD, was downregulated in this study. DMD missense mutation may cause X-linked dilated cardiomyopathy [21]. And cleavage of dystrophin by an enteroviral protease 2A results in cytoskeletal disruption and post-myocarditis cardiomyopathy [22]. Membrane-associated proteins have also been considered as belonging to the cytoskeleton. These proteins play a role in link with the extracellular matrix [23]. The extracellular matrix that constitutes the cardiac jelly within the cushions has long been thought to play an important role in early septal morphogenesis [24]. Abnormalities of extracellular matrix cause atrioventricular canal defects [25]. The differential expression of these genes in the heart of VSD patients may cause extracellular matrix disorder and involve in the development of VSD.

A number of LIM proteins, FHL1, FHL2, LDB3, LMO7, and PDLIM1, were found upregulated in VSD patients. Many known LIM proteins have proven roles in differentiation, function, and the maintenance of phenotype of portions of the cardiovascular system [26]. FHL2, a known transcriptional coactivator and corepressor expressed in the developing cardiovascular system, appears to have the most restricted expression pattern during development. FHL2 is the highly expressed in cardiac septa and

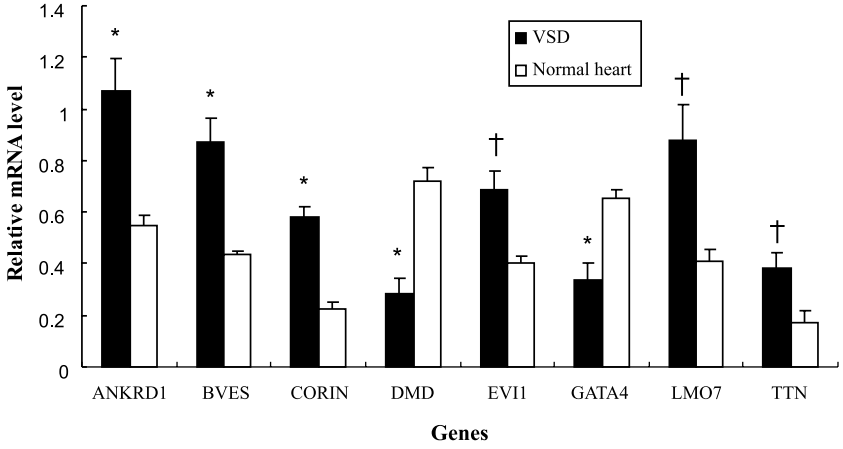

Fig. 3. Relative mRNA levels of randomly selected genes for RT-PCR verification. The eight genes were randomly selected for RT-PCR verification of the SSH subtracted library. Band intensities for eight genes were normalized for bands of GAPDH. Results are expressed as the mean value ± SEM. $^{*}p < 0.01$ and $^{\dagger}p < 0.05$ between groups, respectively.

in the region adjacent to the atrio-ventricular ring, suggesting a potential role in septation or conduction system development. In the heart, FHL1 expression was observed strongly in developing outflow tract, and to a lesser extent in myocardium. Cardiac ventricular expression of FHL1, but not FHL2, was upregulated in two mouse models of cardiac hypertrophic and dilated cardiomyopathy [27]. Scholl et al. [28] found that transcription of the FHL2 gene can be stimulated by p53, and then induces apoptosis. However, FHL2-deficient mice are viable and maintain normal cardiac function both before and after acute mechanical stress induced by aortic constriction [29]. Mutations of LDB3 gene can cause dilated cardiomyopathy [30,31]. Further study of these members of this important LIM gene family is likely to provide significant insights into the etiology of VSD and development of heart.

It was also found that several zinc finger proteins were differentially expressed in this study. GATA4, a zinc finger protein, cardiac key developmental gene, was downregulated in VSD patients. Missense mutations in GATA4 that impair its interaction with TBX5 have been shown to cause ventricular septal defects in humans [32], and GATA4-null embryos display heart defects characterized by disrupted looping morphogenesis, septation, and a hypoplastic ventricular myocardium [33]. However, some other zinc finger proteins, EVI1, DDEF1, KLF9, ZA20D2, ZNF313, were upregulated in VSD patients. EVI1 has important roles in general cell proliferation, vascularization, and cell-specific developmental signaling, at midgestation. Besides other abnormalities, there were defects in the hearts of mutant embryos [34]. Through overexpression of EVI1 in murine embryonal carcinoma P19 cells, Kazama et al. [35] suggest that EVI1 might be an important transcription factor for regulation of early neuroectodermal differentiation. DDEF1 overexpressed in high-grade uveal melanomas and may act as an oncogene in this cancer [36]. The Kruppel-like transcription factor (KLFs) family is made up of 15 C2H2 zinc-finger proteins involved in vertebrate development and able to control cell proliferation, growth, and differentiation [37]. Overexpression of ZA20D2 has redundant and distinct roles in regulating NFκB activation and inducing apoptosis [38].

CRYM, expressed in neural tissue, muscle, and kidney, was upregulated in VSD patients. Abe et al. [39] demonstrated that mutations in the CRYM gene can be responsible for nonsyndromic deafness. DDX1, DDX3X, upregulated in VSD patients, encode DEAD box proteins. DEAD box proteins are believed to be involved in embryogenesis, spermatogenesis, and cellular growth, and division [40]. EZH1, a human homolog of the *Drosophila* gene Enhancer of zeste, E(z), was also upregulated in VSD patients. *Drosophila* E(z) acts as a negative regulator of the segment identity genes of the Antennapedia and Bithorax complexes. The amino acids of EZH1 encoding display 70% similarity with *Drosophila* E(z). The strong sequence conservation suggested potential roles for EZH1 in human development [41]. Further studies of these genes are in progress.

In conclusion, our study suggests that the differentially expression of genes in energy metabolism, cell cycle and growth, cytoskeleton and cell adhesion, LIM protein, zinc finger protein, and development play roles in the development of VSD. It is anticipated that further study of these genes identified will provide insights into their specific roles in the etiology of VSD, even in cardiac development, aging, and disease.

Acknowledgments

This work was supported by a grant from health department of Jiangsu Province (No. RC2002050), National Natural Science Foundation of China (No. 30470709), and a grant for the graduate student of Jangsu Province. We gratefully acknowledge the technical assistance of the First Affiliated Hospital of Nanjing Medical University in the obtaining of ventricle samples of hearts.

References

[1] J.I. Hoffman, Incidence of congenital heart disease: I. Postnatal incidence, Pediatr. Cardiol. 16 (1995) 103–113.

[2] J.I. Hoffman, Incidence of congenital heart disease: II. Prenatal incidence, Pediatr. Cardiol. 16 (1995) 155–165.

[3] A. Correa-Villasenor, C. Ferencz, C. Loffredo, C. Magee, Paternal exposures and cardiovascular malformations. The Baltimore-Washington Infant Study Group, J. Expo. Anal. Environ. Epidemiol. 3 (1993) 173–185.

[4] Y. Sakata, C.N. Kamei, H. Nakagami, R. Bronson, J.K. Liao, M.T. Chin, Ventricular septal defect and cardiomyopathy in mice lacking the transcription factor /Hey2, Proc. Natl. Acad. Sci. USA 99 (2002) 16197–16202.

[5] B. Kaynak, A. von Heydebreck, S. Mebus, D. Seelow, S. Hennig, J. Vogel, H.P. Sperling, R. Pregla, V. Alexi-Meskishvili, R. Hetzer, P.E. Lange, M. Vingron, H. Lehrach, S. Sperling, Genome-wide array analysis of normal and malformed human hearts, Circulation 107 (2003) 2467–2474.

[6] J.J. Schott, D.W. Benson, C.T. Basson, W. Pease, G.M. Silberbach, J.P. Moak, B.J. Maron, C.E. Seidman, J.G. Seidman, Congenital heart disease caused by mutations in the transcription factor NKX2-5, Science 281 (1998) 108–111.

[7] A. Okubo, O. Miyoshi, K. Baba, M. Takagi, K. Tsukamoto, A. Kinoshita, K. Yoshiura, T. Kishino, T. Ohta, N. Niikawa, N. Matsumoto, A novel GATA4 mutation completely segregated with atrial septal defect in a large Japanese family, J. Med. Genet. 41 (2004) e97.

[8] H. Chen, S. Shi, L. Acosta, W. Li, J. Lu, S. Bao, Z. Chen, Z. Yang, M.D. Schneider, K.R. Chien, S.J. Conway, M.C. Yoder, L.S. Haneline, D. Franco, W. Shou, BMP10 is essential for maintaining cardiac growth during murine cardiogenesis, Development 131 (2004) 2219–2231.

[9] P. Liang, A.B. Pardee, Differential display of eukaryotic messenger RNA by means of the polymerase chain reaction, Science 257 (1992) 967–971.

[10] M. Perucho, J. Welsh, M.A. Peinado, Y. Ionov, M. McClelland, Fingerprinting of DNA and RNA by arbitrarily primed polymerase chain reaction: applications in cancer research, Methods Enzymol. 254 (1995) 275–290.

[11] N. Lisitsyn, N. Lisitsyn, M. Wigler, Cloning the differences between two complex genomes, Science 259 (1993) 946–951.

[12] L. Diatchenko, Y.F. Lau, A.P. Campbell, A. Chenchik, F. Moqadam, B. Huang, S. Lukyanov, K. Lukyanov, N. Gurskaya, E.D. Sverdlov, P.D. Siebert, Suppression subtractive hybridization: a method for generating differentially regulated or tissue-specific cDNA probes and libraries, Proc. Natl. Acad. Sci. USA 93 (1996) 6025–6030.

[13] D. Haase, M.H. Lehmann, M.M. Korner, R. Korfer, H.H. Sigusch, H.R. Figulla, Identification and validation of selective upregulation of ventricular myosin light chain type 2 mRNA in idiopathic dilated cardiomyopathy, Eur. J. Heart Fail. 4 (2002) 23–31.

[14] I.L. Ackerman, C.A. Karn, S.C. Denne, G.J. Ensing, C.A. Leitch, Total but not resting energy expenditure is increased in infants with ventricular septal defects, Pediatrics 102 (1998) 1172–1177.

[15] E. Abdelwahid, L.J. Pelliniemi, E. Jokinen, Cell death and differentiation in the development of the endocardial cushion of the embryonic heart, Microsc. Res. Tech. 58 (2002) 395–403.

[16] B. Gerull, M. Gramlich, J. Atherton, M. McNabb, K. Trombitas, S. Sasse-Klaassen, J.G. Seidman, C. Seidman, H. Granzier, S. Labeit, M. Frenneaux, L. Thierfelder, Mutations of TTN, encoding the giant muscle filament titin, cause familial dilated cardiomyopathy, Nat. Genet. 30 (2002) 201–204.

[17] G. Sun, S. Yuen Chan, Y. Yuan, K. Wang Chan, G. Qiu, K. Sun, M. Ping Leung, Isolation of differentially expressed genes in human heart tissues, Biochim. Biophys. Acta 1588 (2002) 241–246.

[18] Y. Aihara, M. Kurabayashi, Y. Saito, Y. Ohyama, T. Tanaka, S. Takeda, K. Tomaru, K. Sekiguchi, M. Arai, T. Nakamura, R. Nagai, Cardiac ankyrin repeat protein is a novel marker of cardiac hypertrophy: role of M-CAT element within the promoter, Hypertension 36 (2000) 48–53.

[19] K.J. Briegel, H.S. Baldwin, J.A. Epstein, A.L. Joyner, Congenital heart disease reminiscent of partial trisomy 2p syndrome in mice transgenic for the transcription factor Lbh, Development 132 (2005) 3305–3316.

[20] W. Xu, H. Baribault, E.D. Adamson, Vinculin knockout results in heart and brain defects during embryonic development, Development 125 (1998) 327–337.

[21] R. Ortiz-Lopez, H. Li, J. Su, V. Goytia, J.A. Towbin, Evidence for a dystrophin missense mutation as a cause of X-linked dilated cardiomyopathy, Circulation 95 (1997) 2434–2440.

[22] C. Badorff, G.H. Lee, B.J. Lamphear, M.E. Martone, K.P. Campbell, R.E. Rhoads, K.U. Knowlton, Enteroviral protease2A cleaves dystrophin: evidence of cytoskeletal disruption in an acquired cardiomyopathy, Nat. Med. 5 (1999) 320–326.

[23] S. Hein, S. Kostin, A. Heling, Y. Maeno, J. Schaper, The role of the cytoskeleton in heart failure, Cardiovasc. Res. 45 (2000) 273–278.

[24] C.L. Maslen, Molecular genetics of atrioventricular septal defects, Curr. Opin. Cardiol. 19 (2004) 205–210.

[25] B. Marino, M.C. Digilio, Congenital heart disease and genetic syndromes: specific correlation between cardiac phenotype and genotype, Cardiovasc. Pathol. 9 (2000) 303–315.

[26] R. Tabibiazar, R.A. Wagner, A. Liao, T. Quertermous, Transcriptional profiling of the heart reveals chamber-specific gene expression patterns, Circ. Res. 93 (2003) 1193–1201.

[27] P.H. Chu, P. Ruiz-Lozano, Q. Zhou, C. Cai, J. Chen, Expression patterns of FHL/SLIM family members suggest important functional roles in skeletal muscle and cardiovascular system, Mech. Dev. 95 (2000) 259–265.

[28] F.A. Scholl, P. McLoughlin, E. Ehler, C. de Giovanni, B.W. Schafer, DRAL is a p53-responsive gene whose four and a half LIM domain protein product induces apoptosis, J. Cell Biol. 151 (2000) 495–506.

[29] P.H. Chu, W.M. Bardwell, Y. Gu, J. Ross Jr., J. Chen, FHL2 (SLIM3) is not essential for cardiac development and function, Mol. Cell. Biol. 20 (2000) 7460–7462.

[30] M. Vatta, B. Mohapatra, S. Jimenez, X. Sanchez, G. Faulkner, Z. Perles, G. Sinagra, J.H. Lin, T.M. Vu, Q. Zhou, K.R. Bowles, A. Di Lenarda, L. Schimmenti, M. Fox, M.A. Chrisco, R.T. Murphy, W. McKenna, P. Elliott, N.E. Bowles, J. Chen, G. Valle, J.A. Towbin, Mutations in Cypher/ZASP in patients with dilated cardiomyopathy and left ventricular non-compaction, J. Am. Coll. Cardiol. 42 (2003) 2014–2027.

[31] T. Arimura, T. Hayashi, H. Terada, S.Y. Lee, Q. Zhou, M. Takahashi, K. Ueda, T. Nouchi, S. Hohda, M. Shibutani, M. Hirose, J. Chen, J.E. Park, M. Yasunami, H. Hayashi, A. Kimura, A Cypher/ZASP mutation associated with dilated cardiomyopathy alters the binding affinity to protein kinase C, J. Biol. Chem. 279 (2004) 6746–6752.

[32] V. Garg, I.S. Kathiriya, R. Barnes, M.K. Schluterman, I.N. King, C.A. Butler, C.R. Rothrock, R.S. Eapen, K. Hirayama-Yamada, K. Joo, R. Matsuoka, J.C. Cohen, D. Srivastava, GATA4 mutations cause human congenital heart defects and reveal an interaction with TBX5, Nature 424 (2003) 443–447.

[33] A.J. Watt, M.A. Battle, J. Li, S.A. Duncan, GATA4 is essential for formation of the proepicardium and regulates cardiogenesis, Proc. Natl. Acad. Sci. USA 101 (2004) 12573–12578.

[34] P.R. Hoyt, C. Bartholomew, A.J. Davis, K. Yutzey, L.W. Gamer, S.S. Potter, J.N. Ihle, M.L. Mucenski, The Evi1 proto-oncogene is required at midgestation for neural, heart, and paraxial mesenchyme development, Mech. Dev. 65 (1997) 55–70.

[35] H. Kazama, T. Kodera, S. Shimizu, H. Mizoguchi, K. Morishita, Ecotropic viral integration site-1 is activated during, and is sufficient for, neuroectodermal P19 cell differentiation, Cell Growth Differ. 10 (1999) 565–573.

[36] J.P. Ehlers, L. Worley, M.D. Onken, J.W. Harbour, DDEF1 is located in an amplified region of chromosome 8q and is overexpressed in uveal melanoma, Clin. Cancer Res. 11 (2005) 3609–3613.
[37] F. Chiambaretta, F. De Graeve, G. Turet, G. Marceau, P. Gain, B. Dastugue, D. Rigal, V. Sapin, Cell and tissue specific expression of human Kruppel-like transcription factors in human ocular surface, Mol. Vis. 10 (2004) 901–909.
[38] J. Huang, L. Teng, L. Li, T. Liu, L. Li, D. Chen, L.G. Xu, Z. Zhai, H.B. Shu, ZNF216 Is an A20-like and IkappaB kinase gamma-interacting inhibitor of NFkappaB activation, J. Biol. Chem. 279 (2004) 16847–16853.
[39] S. Abe, T. Katagiri, A. Saito-Hisaminato, S. Usami, Y. Inoue, T. Tsunoda, Y. Nakamura, Identification of CRYM as a candidate responsible for nonsyndromic deafness, through cDNA microarray analysis of human cochlear and vestibular tissues, Am. J. Hum. Genet. 72 (2003) 73–82.
[40] R. Godbout, J. Squire, Amplification of a DEAD box protein gene in retinoblastoma cell lines, Proc. Natl. Acad. Sci. USA 90 (1993) 7578–7582.
[41] K.J. Abel, L.C. Brody, J.M. Valdes, M.R. Erdos, D.R. McKinley, L.H. Castilla, S.D. Merajver, F.J. Couch, L.S. Friedman, E.A. Ostermeyer, E.D. Lynch, M.C. King, P.L. Welcsh, S. Osborne-Lawrence, M. Spillman, A.M. Bowcock, F.S. Collins, B.L. Weber, Characterization of EZH1, a human homolog of Drosophila Enhancer of zeste near BRCA1, Genomics 37 (1996) 161–171.