Original Article on Current Management Aspects in Adult Congenital Heart Disease (ACHD): Part IV

Molecular signaling pathways in right ventricular impairment of adult patients after tetralogy of Fallot repair

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Background: Right ventricular impairment (RVI) secondary to altered hemodynamics contributes to morbidity and mortality in adult patients after tetralogy of Fallot (TOF) repair. The goal of this study was to describe signaling pathways contributing to right ventricular (RV) remodeling by analyzing over lifetime alterations of RV gene expression in affected patients.

Methods: RV tissue was collected at the time of cardiac surgery in 13 patients with a diagnosis of TOF. RNA was isolated and whole transcriptome sequencing was performed. Gene profiles were compared between a group of 6 adults with signs of RVI undergoing right ventricle to pulmonary artery conduit surgery and a group of 7 infants, undergoing TOF correction. Definition of RVI in adult patients was based on clinical symptoms, evidence of RV hypertrophy, dilation, dysfunction or elevated pressure on echocardiographic, cardiovascular magnetic resonance, or catheterization evaluation.

Results: Median age was 34 years in RVI patients and 5 months in infants. Based on P adjusted value <0.01, RNA sequencing of RV specimens identified a total of 3,010 differentially expressed genes in adult patients with TOF and RVI as compared to infant patients with TOF. Gene Ontology and Kyoto Encyclopedia of Genes databases highlighted pathways involved in cellular metabolism, cell-cell communication, cell cycling and cellular contractility to be dysregulated in adults with corrected TOF and chronic RVI.

Conclusions: RV transcriptome profiling in adult patients with RVI after TOF repair allows identification of signaling pathways, contributing to pathologic RV remodeling and helps in the discovery of biomarkers for disease progression and of new therapeutic targets.

Keywords: Right ventricular impairment (RVI); congenital heart disease; transcriptome profiling; molecular signaling pathways

Submitted Oct 29, 2020. Accepted for publication Feb 26, 2021.
doi: 10.21037/cdt-20-894
View this article at: http://dx.doi.org/10.21037/cdt-20-894

Introduction

Due to improvements in diagnostic techniques, interventional procedures and surgical treatment more children with complex congenital heart disease (CCHD) survive into adulthood (1,2). The right ventricle is often stressed by the underlying anatomical abnormalities, surgical lesions, or pulmonary valve defects leading to pressure and/or volume overload (3). Right ventricular impairment (RVI) subsequently occurs in about 26% of CCHD patients (4) and might lead to sudden cardiac death and life-threatening long-term complications. Secondary to altered hemodynamics and myocardial stress, right ventricular (RV) remodeling, such as hypertrophy, fibrosis, arrhythmias, inflammation and dysfunction, occurs (3,5). Activation of fibroblasts, upregulation of inflammatory pathways, increased release of neurohormones and differences in gene regulation of cardiac metabolism, energy production and mitochondrial function occur in the stressed right ventricle (3,6). Additionally, the right ventricle has been suggested to suffer earlier than the left ventricle from oxidative stress in response to hemodynamic stress, which might contribute to the development of pathologic conditions (3). Preventing the transition from compensated- to non-compensated right heart failure (HF) in affected patients by timely interventions, operations, or targeted medical therapy would decrease morbidity and mortality in affected CCHD patients. However, at this point, the severity of pathologic RV remodeling can only inaccurately be predicted by clinical examination and imaging techniques (7). Consequently, the identification of biomarkers to optimize patient treatment and determine the extent of RV myocardial remodeling is of utmost importance. Furthermore, patients currently receive standard left ventricular (LV) failure therapies which are often ineffective in the right ventricle (7,8). A potential reason for this failure is the differences in molecular signaling pathways between the right and the left ventricle which cause diverse adaption strategies to pathologic conditions (6,7). To date, data referring to molecular pathways contributing to RV remodeling and RVI in patients with CCHD are scarce. The aim of this study was to identify differential gene expression over time in affected patients and to define molecular signaling pathways which might play a pivotal role in the development of RVI in patients with CCHD.

We present the following article in accordance with the MDAR reporting checklist (available at http://dx.doi.org/10.21037/cdt-20-894).

Methods

The study protocol is depicted in *Figure 1*. Briefly, RV tissue was obtained from patients with a primary diagnosis of tetralogy of Fallot (TOF) at the time of surgical treatment after informed consent. Surgeries included corrective repair for infants or elective replacement of right ventricle to pulmonary artery (RV-PA) conduit for adult patients. Clinical, laboratory, electrocardiogram and imaging data were analyzed by medical chart review. The criteria for RVI were selected based on the International Right Heart Foundation Working Group recommendations (9) and the scientific statement of the American Heart Association (10). Patients with syndromes and any other organ failure were excluded. Total RNA was isolated, RNA quantity was measured, and RNA quality was assessed. Whole transcriptome analysis was performed by total RNA sequencing of cardiac tissue samples as previously described (11). RNA library was prepared, quality and quantity of the RNA library were estimated, and RNA sequencing of 100 bp paired-end runs was performed with Illumina HiSeq 4000 platform (Illumina, San Diego, California, United States). The number of reads mapping to annotated genes was quantified after alignment and differential gene expression between groups was performed. Pathway and Gene Set Enrichment Analysis, which are common approaches to interpret gene expression data based on functional annotation, were performed. Bioinformatical tools used to validate pathway analysis, biological activity and allocation of individual genes to Gene Ontology categories are depicted in *Figure 2* and selected suitable genes are shown in Figure S2.

The total of 3,010 differentially expressed genes were compared to 50 most significant genes, regulating cardiac development and heart maturation, which were identified by RNA sequencing of murine cells during embryonic and postnatal period. Overlapping genes were not included in further interpretation of results (12) (Figure S1 and Table S9). Western blot was performed as previously described (11) for selected proteins isolated from RV myocardial tissue of affected patients.

Statistic evaluation was implemented by applying R Bioconductor package DEseq2 to transcriptome profiles and tested for differential gene expression between adult and infant patients. The P values were corrected for the purpose of multiple testing by Benjamini and Hochberg procedure. The level of significance was set at a P value of less than 0.01 and a fold-change value of greater than 2 or less than –2. Based on that, all significant differentially

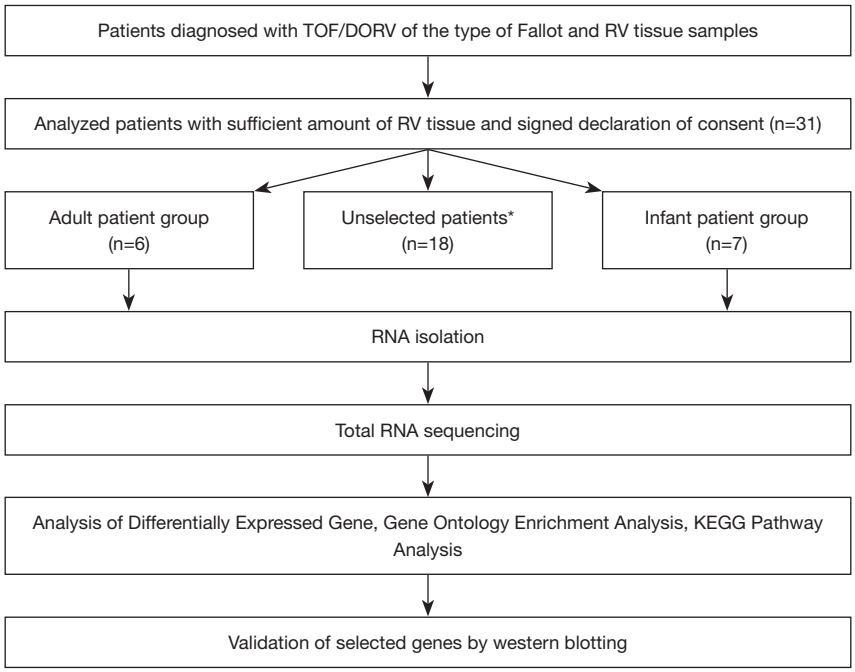

Figure 1 Flow chart of the performed process of the study design. Patient enrollment with underlying anatomical diagnosis, group classification and process of sample preparation with analysis of results. *, unselected patients due to genetic disorders, lacking clear clinical characterization and insufficient signs of RV impairment. DORV, double outlet right ventricle; KEGG, Kyoto Encyclopedia of Genes and Genomes; RNA, ribonucleic acid; RV, right ventricular; TOF, tetralogy of Fallot.

expressed genes were selected for further analysis. The results of Gene Ontology (GO) and Pathway analysis with an adjusted P value less than 0.01 (GO) and a P value less than 0.05 (KEGG) were assigned as significant.

The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by institutional ethics committee (approval 10/16/2017, number 242/17S, and approval 01/11/2017, number 592,16S) and individual consent for this retrospective analysis was taken from all patients.

Please see Supplementary file (Appendix 1) for detailed description of Material and Methods.

Results

Myocardial tissue samples were collected from a group of 6 adult patients with signs of chronic RVI undergoing RV-PA conduit surgery ("cases") and from a group of 7 infant patients undergoing elective TOF correction ("controls"). Patients in the "case" and the "control" group were in median 34 and 0.5 years, respectively, old at the time of sample collection (range, 29 to 62 years and 4.8 to 5.8 months, respectively). Follow-up time since corrective surgery was 34 years in median (range, 27 to 50 years) in adult patients with CCHD and RVI. Underlying structural heart disease was TOF in 12 patients and double outlet right ventricle (DORV) of the type Fallot in 1 patient (*Tables 1,2*). Clinical, laboratory, cardiopulmonary exercise testing and imaging parameters of adult patients are depicted in *Table 3* (13,14). All patients of the case group had progressive exercise intolerance and were in New York Heart Association functional class II and higher. Most patients showed engorgement of their jugular veins or enlarged liver on physical examination. Three of 6 patients showed severe stenosis of the pulmonary valve and 3 of 6 patients showed severe pulmonary regurgitation. There was RV hypertrophy and enlargement in all patients on transthoracic echocardiography and/or cardiovascular magnetic resonance tomography (CMR). Exemplary imaging is depicted in *Figure 3*.

The statistical evaluation with Principal Component Analysis visualized well separated samples, demonstrating difference in the compared groups and homogeneity (*Figure 4*). Transcriptome profiling of RV specimens identified in total

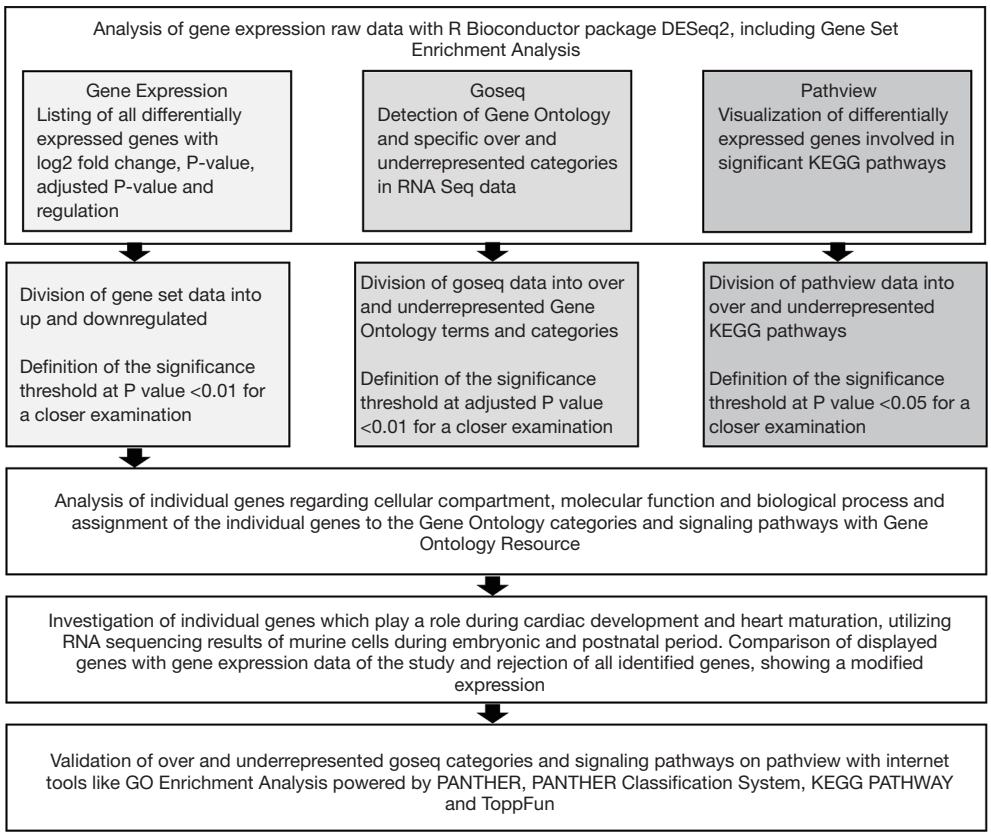

Figure 2 Bioinformatical analysis of whole transcriptome differential expression data. Bioinformatical analysis performed to evaluate significant biological pathways derived from whole transcriptome differential gene expression data. GO, Gene Ontology; goseq, gene ontology for RNA Sequencing; KEGG, Kyoto Encyclopedia of Genes and Genomes; PANTHER, Protein Analysis Through Evolutionary Relationships; RNA, ribonucleic acid; RNA Seq, RNA Sequencing.

Table 1 Patients characteristics of the adult patient group

Adult patients	Identification number					
	111087	111088	111089	111091	111092	111094
Age (years)	31	43	29	37	61	31
Sex (M/F)	M	F	F	F	F	F
Height (cm)	172	174	165	156	162	150
Weight (kg)	66.3	75.9	63.1	68.5	73.1	44.8
BMI (kg/m^2)	22.3	25.1	23.1	28.4	27.8	20.0
Anatomical diagnosis	DORV	TOF	TOF	TOF	TOF	TOF
Type of surgery	Allograft implantation	RVOTO resection	Conduit change	Conduit change	RVOTO resection, conduit change	Conduit change
Age at corrective repair (years)	4	3.5	0.4	4	11	5

BMI, body mass index; DORV, double outlet right ventricle; RVOTO, right ventricular outflow tract obstruction; TOF, tetralogy of Fallot.

Table 2 Patients characteristics of the infant patient group

Infant patients	Identification number						
	111093	111095	111096	111097	111099	111100	111198
Age (months)	5	5	5	5	5	5	4
Sex (M/F)	M	M	F	M	M	M	M
Height (cm)	64	62	63	62.5	63	68	63
Weight (g)	7,605	5,950	5,960	7,405	5,900	6,960	5,700
Anatomical diagnosis	TOF	TOF	TOF	TOF	TOF	TOF	TOF
Type of surgery	Corrective repair	Corrective repair	Corrective repair	Corrective repair	Corrective Repair	Corrective repair	Corrective repair

TOF, tetralogy of Fallot.

Table 3 Clinical parameters, cardiorenal and cardiohepatic serum markers, electrocardiogram as well as imaging parameters and preoperative medication

Adult patients	Patient identification number					
	111087	111088	111089	111091	111092	111094
Clinical parameters						
Functional capacity (NYHA classification)	II	III	II	III	IV	III
Peripheral edema	0	0	0	0	+	0
Engorgement of jugular veins	0	+	+	+	0	0
Enlargement of liver	0	0	+	+	+	0
Dyspnea	0	+	+	+	+	+
Cyanosis	0	0	0	0	0	0
Cardiorenal serum markers						
GFR (mL/min)	–	69	120	106	44	118
Creatinine (mg/dL)	1.60	1.00	0.67	0.66	1.24	0.61
Blood urine nitrogen (mg/dL)	85.5	31.0	20.7	33.9	68.4	27.9
Cardiohepatic serum markers						
Bilirubin (mg/dL)	0.591	1.25	0.430	0.25	2.49	0.33
γ-GT (U/L)	53.6	21.6	57.5	24.7	480	38.4
AP (U/L)	38.2	56.7	84.4	46.0	128	56.5
Electrocardiogram						
Right-axis deviation	0	+	+	0	0	0
Incomplete/complete right bundle branch block	+	+	+	+	+	+
Length of QRS interval (ms)	200	100	110	100	120	120
Abnormal repolarization	+	+	+	0	+	+
Atrial/ventricular tachyarrhythmias	+[a]	0	0	+[b]	+[c]	0
Implantable cardioverter-defibrillator implantation	0	0	0	0	+[d]	0

Table 3 (*continued*)

Table 3 (*continued*)

Adult patients	Patient identification number					
	111087	111088	111089	111091	111092	111094
Cardiac magnetic resonance imaging						
RVEF (%)	50	52	67	57	–	52
LVEF (%)	51	58	59	68	–	60
Pulmonary regurgitation fraction (%)[A]	52	10	21	11	–	–
RVSV (mL)	121	87	92	48	–	58
RVEDVI (mL/m^2)	138	89	84	51	–	80
Increased RVEDV[B]	+	+	+	0	–	+
RVESVI (mL/m^2)	68	42	28	22	–	39
Increased RVESV[C]	+	0	0	0	–	0
Echocardiography						
Decreased RVEF[D]	0	0	0	0	+	0
Pulmonary regurgitation[E]	+	0	+	0	+	0
PV Vmax (m/s)	3.90	–	3.48	4.53	1.94	4.05
PV mean PG (mmHg)	–	–	–	54.52	11.24	–
PV max PG (mmHg)	60.78	85	48.5	82.07	15.07	65.73
RV hypertrophy	+	+	+	+	+	+
Increased RVP[F]	0	+	+	–	+	+
Preoperative medication						
Beta-blocker	+	0	0	0	+	0
Diuretic	+	0	0	0	+	0
Angiotensin converting enzyme inhibitor	+	0	0	0	0	0

0, not present; +, present; –, data not available. The electrocardiogram criteria for right ventricular impairment were taken from the scientific statement of the American Heart Association (10). The imaging reference values used were taken from the Guidelines for the Echocardiographic Assessment of Right Heart in Adults reported from the American Society of Echocardiography (13). The cut of value for moderate to severe pulmonary regurgitation were based on the publication of the Department of Cardiology and the Toronto Congenital Cardiac Care Centre for Adults (14). [a], atrial fibrillation; [b], non-sustained ventricular tachycardia on exercise testing; [c], atrial flutter and non-sustained ventricular tachycardia in invasive electrophysiological examination; [d], primary prophylactic implantable cardioverter-defibrillator with adequate shocks later on; [A], moderate to severe pulmonary regurgitation fraction >20%; [B], right ventricular enddiastolic volume index >80 mL/m^2; [C], right ventricular end-systolic volume index >46 mL/m^2; [D], RVEF <44%; [E], moderate to severe pulmonary regurgitation; [F], increased RVP: right ventricular pressure >40 mmHg. NYHA, New York Heart Association Class; GFR, glomerular filtration rate; ɣ-GT, gamma-glutamyl transpeptidase; AP, alkaline phosphatase; RVEF, right ventricular ejection fraction; LVEF, left ventricular ejection fraction; RVSV, right ventricular stroke volume; RVEDVI, right ventricular enddiastolic volume index; RVESVI, right ventricular end-systolic volume index; PV Vmax, maximum velocity over pulmonic valve; PV mean PG, mean pressure gradient over pulmonic valve; PV max PG, maximum pressure gradient over pulmonic valve; RV, right ventricular.

23,398 genes to be differentially expressed in the case group of adult patients with RVI as compared to the control group of infant TOF patients. Thereof, 12,626 genes were upregulated, and 10,772 genes were downregulated. Considering a P adjusted value of less than 0.01 and a fold change value of greater than 2 and less than –2, a total of 3,010 genes were significantly differentially expressed. Of those, 1,703 genes were upregulated, and 1,307 genes were downregulated (*Figure 5*). Hierarchical clustering of the 100 most significantly differentially expressed genes is shown in *Figure 6*.

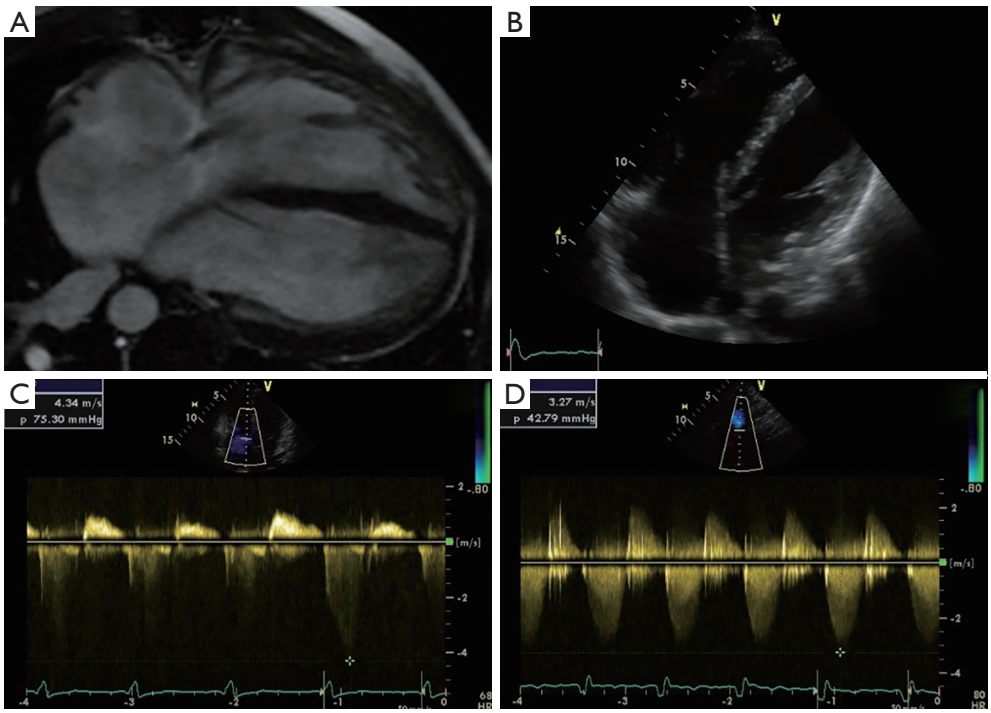

Figure 3 Exemplary imaging from patient 111087 showing right ventricular enlargement on cardiac magnetic resonance imaging (A) and echocardiography (B) with elevated right ventricular pressure estimated by pressure gradient between the right ventricle and the right atrium on Doppler imaging (C). Echocardiographic continuous wave Doppler imaging illustrates severe stenosis and regurgitation of the right-ventricular-to-pulmonary-artery (RV-PA) allograft (D).

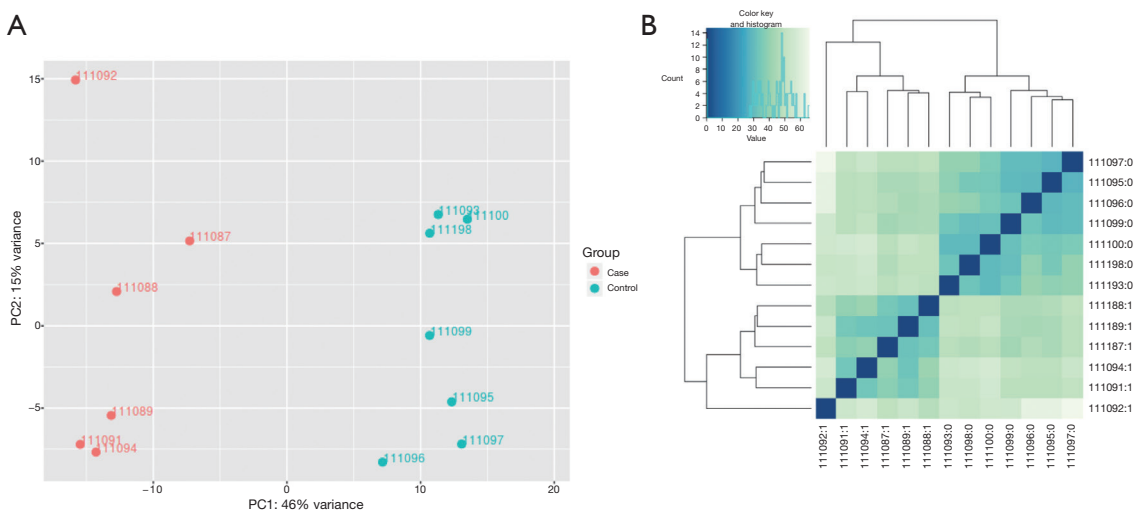

Figure 4 Principal component analysis of adult TOF patients and RVI secondary to chronic RV volume and/or pressure overload compared to infant TOF patients. The diagram charted both groups as clusters, at which red dots symbolize the adult patients ("cases") and blue dots the infant patients ("controls") (A). The samples are well separated, demonstrating difference in the compared groups (B). Low variance and homogeneity within both clusters is the base for comparability. TOF, tetralogy of Fallot; RVI, right ventricular impairment; RV, right ventricular.

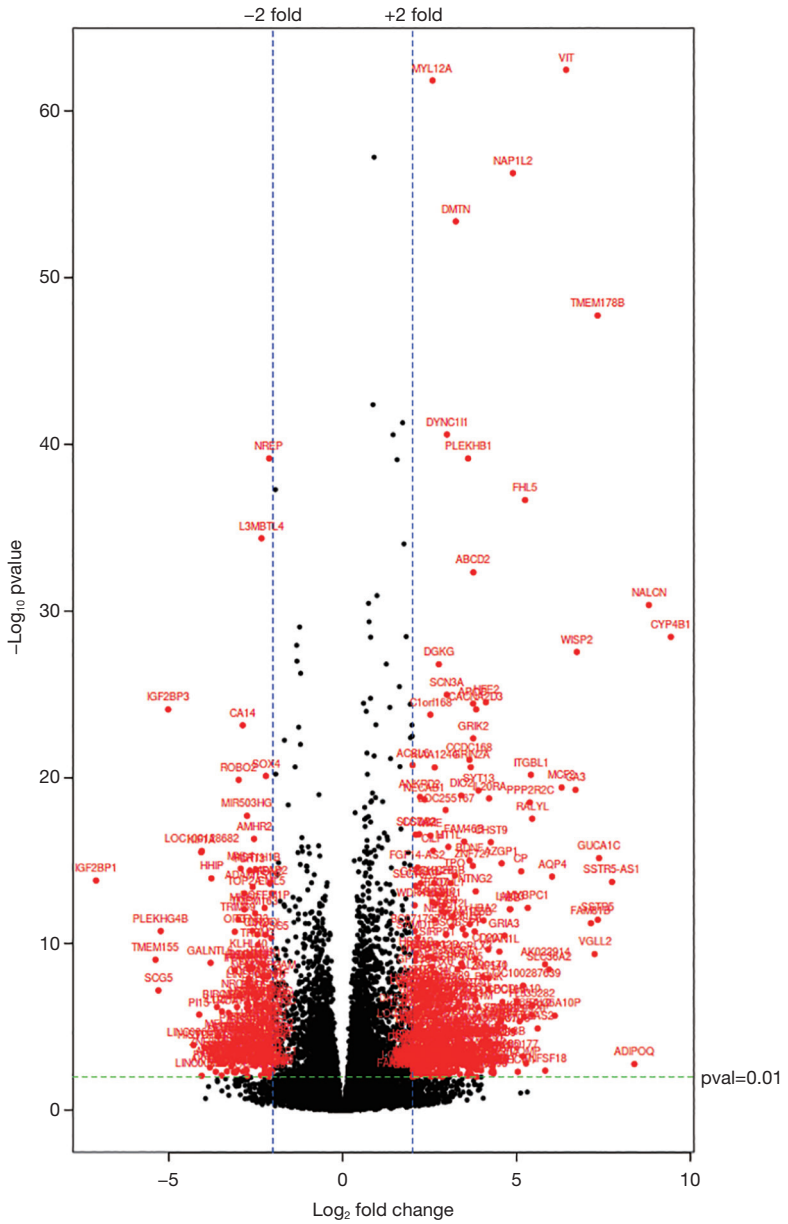

Figure 5 Volcano Plot visualizes detected significantly dysregulated genes from gene expression analysis. The log 2-fold change is displayed on the x axis and the significance is shown on the y axis as the negative logarithm (log10scale) of the FDR corrected P value. The significance cutoff (P value <0.01) is highlighted with a green dashed line. Dysregulated genes which lie within the defined significant limits are shown in red.

Pathway and gene set enrichment analysis revealed 162 GO terms to be significantly upregulated by the expressed genes. The highly expressed GO terms for cellular components indicated cytoplasmic part and cytoplasm, including contractile fiber as well as contractile fiber part with myofibril, sarcomere, I Band and Z Disc, to be dysregulated in adult patients with CCHD. Additionally, extracellular space, extracellular organelle inclusive of extracellular vesicle and extracellular exosome were highlighted to be involved in dysregulated pathways. Cellular component genes, associated with vesicle, intracellular vesicle, cytoplasmic vesicle, secretory vesicle and secretory granule rank among the most significantly upregulated pathways (Tables S1,S7). Most significant

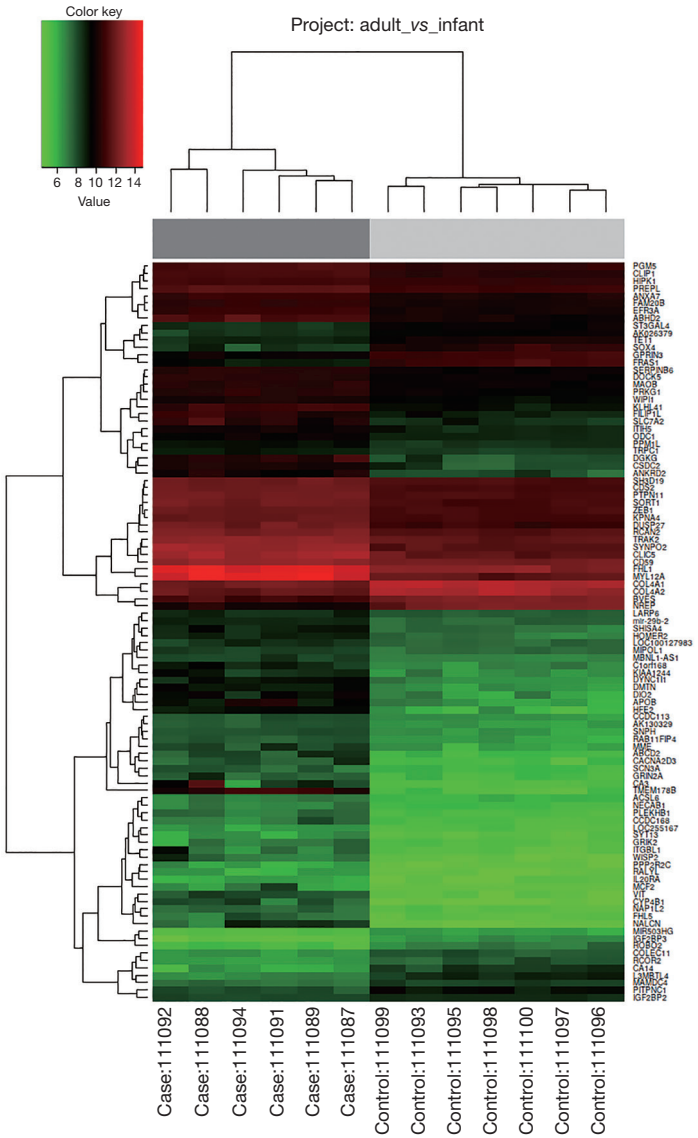

Figure 6 Hierarchical clustering of the 100 most significantly dysregulated genes. Heatmap classified the samples into two major clusters of upregulated (red colored) and downregulated (green colored) genes according to the condition ("cases/adult" versus "controls/infant" patients) of the samples. By using the heatmap, raw gene expression data were visualized and grouped, based on the similarity of their gene expression pattern. Each column signifies different patient samples, and each row represents a significantly dysregulated gene. The variation in color and intensity of the boxes symbolizes the changes in gene expression, entailing red represents upregulated genes, green represents downregulated genes and black implies unchanged gene expression. The appositional heatmap features the 100 most significant dysregulated genes of the gene expression raw data.

increase of molecular function included protein binding as well as oxidoreductase, catalytic peptidase regulator, peptidase inhibitor activity (Tables S2,S7). Significantly increased biological processes included oxidation reduction process, vesicle mediated transport and secretion by cells, as well es regulation of exocytosis (Tables S3,S7).

A total of 218 GO terms were significantly downregulated in adult compared to infant patients on functional annotation of differentially expressed genes. The most significant GO terms for cellular components included intracellular organelles, chromosome and protein-DNA complexes (Tables S4,S8). Significantly downregulated genes of

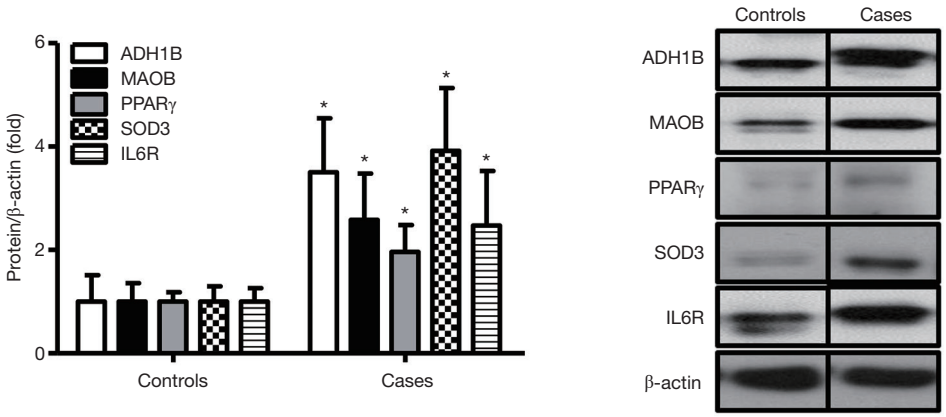

Figure 7 Western blot validation of selected dysregulated target proteins. Total proteins were isolated from the right ventricular myocardium of infant- ("controls") and adult ("cases") patients. Western blot analyses were performed using antibodies against alcohol dehydrogenase 1B (ADH1B), monoamine oxidase B (MAOB), peroxisome proliferator-activated receptor gamma (PPARγ), superoxide dismutase 3 (SOD3), and interleukin 6 receptor (IL6R). β-actin served as a loading control. Representative blots are shown. Two-tailed Student's *t*-test was used (n=3; *, P<0.05 vs. corresponding control).

molecular function were mostly associated with DNA and transcription processes (Tables S5,S8). In line Biological processes involving cell cycling and regulation of gene expression were significantly downregulated (Tables S6,S8).

KEGG analysis showed retinol metabolism (hsa00830) and PPAR signaling pathway (hsa03320) to be significantly upregulated, considering a P value of less than 0.05 (Table S7). Cell cycle (hsa04110), DNA replication (hsa03030) and ribosome (hsa03010) were most significantly downregulated on KEGG analysis, based on a P value of less than 0.05 (Table S8).

Western blot analyses on proteins extracted from RV tissues were performed for validation of RNA sequencing results. Protein levels of *ADH1B*, *PPAR gamma*, *MAOB* and *SOD3* representing critical genes involved in the retinol metabolism, PPAR signaling, and response to oxidative stress pathways, respectively, were significantly increased (*Figure 7*).

Discussion

Differential gene expression and molecular signaling pathways contributing to pathologic myocardial remodeling differ between the left (6,15) and the right ventricle (5,6,15,16). Knowledge about the molecular fundamentals and cellular changes which are involved in the development of RV remodeling secondary to chronic pressure and/or volume overload in CCHD patients is limited (7). The present study evaluated differentially expressed genes of RV tissue from adult patients with TOF and chronic pressure and/or volume overloaded right ventricle compared to infants with TOF by whole transcriptome profiling. Detailed bioinformatic and statistical analysis identified signaling pathways potentially contributing to pathologic RV remodeling in affected patients.

GO Enrichment Analysis and KEGG pathway analysis identified significant dysregulation of genes encoding for structural cellular component and of genes involved in certain biological and functional processes to be significantly dysregulated in RV tissues of adult patients with TOF and chronic hemodynamic RV stressors. Genes associated to the contractile fiber part and actin cytoskeleton of the cell, in particular genes of myofibril, I Band, Z Disc and the contractile units of myocytes, the sarcomere, were particularly upregulated. Increase of contractile protein expression was also described by others in infants with pressure overloaded right ventricles (17). Those findings might explain diastolic RV dysfunction reported by others in patients with TOF (18,19). Analogous findings of alterations in gene expression of cytoskeleton proteins and extracellular matrix proteins were also made in myocardium of failing left ventricles (3,7,15,16). Additionally, upregulation of oxidation-reduction processes was identified as critical biological process involved in RV remodeling of affected patients in the current study. Mechanical pressure is one of the main cardiac stress factors, leading to dysfunctional mitochondria, resulting in increased

reactive oxygen species (ROS) production. Dysfunctional mitochondria and imbalance of the redox system contribute to HF (20). Furthermore, upregulation of genes encoding for proteins involved in cell-cell-communication and several molecular pathways, implicating extracellular exosome, extracellular vesicle, vesicle mediated transport and secretory vesicle, were found to be upregulated in adults with RVI in the current study. There is evidence that ROS can stimulate the production of exosomes and affect the molecular content of exosomes, among various other external stimulation factors, of which are some still unknown (21,22). Exosomes are extracellular 40 to 150 nm diameter lipid vesicles secreted by cells (22,23) to facilitate intercellular communication by transferring miRNA, mRNA and proteins between cells (24). It is hypothesized that exosomes might play a role in cardiac remodeling in the response to cardiac stress by influencing inflammation, interstitial fibrosis, ventricular hypertrophy and changes in contractility (22). Due to the unique function of exosomes to promote intercellular communication, they are currently of interest as therapeutic agents and potential biomarkers for various diseases, including cardiovascular diseases (25,26).

The current study also showed the upregulation of the peroxisome proliferator-activated receptor (PPAR) signaling pathway. PPARs play a role as nuclear receptor transcription factors, activated by specific ligands such as long chain fatty acids (27). The expression of genes, regulated by the three PPAR isoforms, contribute to cardiac metabolic processes, in particular lipid, fatty acid and glucose homeostasis, cell differentiation and control of inflammation (27-30). Upregulation of the PPAR signaling pathway in the current study is consistent to results of previous studies which report dysregulated PPAR signaling pathway in the context of cardiac remodeling, myocardial hypertrophy and HF (27,31-33). PPARs regulate gene expression by forming a nuclear heterodimeric complex with retinoid X receptor (RXR) and binding to specific promoter regions of the target genes to be regulated (29,30). *RXR* is one of the major genes involved in retinol metabolism. In line with this, KEGG bioinformatical analysis revealed a highly significant involvement of the retinol pathway in RV tissues of affected patients. In addition to *RXR* (P value 0.0051), genes such as *RDH10* (P value 0.00076), *ADH1B* (P value 4.93E-11), *ADH1C* (P value 0.00037) and *CYP1A1* (P value 1.01E-05) were significantly upregulated. Activation of retinoic acid signaling in left ventricular tissue in conjunction with myocardial ischemia and remodeling after myocardial infarction in mice was reported by others (34). The retinol metabolism utilizes vitamin A and its derivates to ensure cardiac supply of various forms of all trans retinoic acids (atRA) (34). AtRA function as ligands via binding to nuclear receptors like RAR or RXR (34). The receptors form heterodimers and bind to DNA regulatory sequences, whereby regulating the frequency of gene expression, plus modifying interconnection with other signaling pathways (34). The results of the current study concur with previous studies, investigating dysregulation of retinol metabolism in ventricular remodeling and HF. We speculate that not only the left ventricle, but also the right ventricle shows a dysregulation of retinol metabolism in the development of RVI, which is also supported by our results. The activation of metabolites of retinol metabolism may play a previously unimagined role in RV remodeling and in response to cardiac damage and repair. The simultaneous overexpression of genes involved in fatty acid metabolism, PPAR signaling pathway as well as retinol metabolism indicate the interdependence of the individual signaling pathways (31) and promote the presumption of contributing to RV remodeling in stages of RVI in adult patients with CCHD.

During the development of RV remodeling and RVI, RNA sequencing revealed a marked adjustment of gene expression, regarding mitotic cell cycle, cell division and DNA replication. Downregulation of differentially expressed genes, associated with DNA binding transcription factor activity, transcription regulator activity as well as RNA polymerase 2 transcription factor activity, support the assumption of decreased cell cycling in stressed RV myocardium of adult patients with signs of RVI. In line with above findings, reduction of gene expression with respect to nucleus, chromosome, protein-DNA complex, spindle and replication fork strengthen suspicion of declined cell proliferation and cell maintenance in cardiac remodeling. Downregulation of cell cycle leads to a fatal lack of myocardial regeneration, due to failing proliferation of normal functioning cardiomyocytes (35). Experimental research determined cardiac loss of ability to reentry cell cycle, since differentiated cells such as adult cardiomyocytes typically become post-mitotic and exit the cell cycle postnatal (36,37). The present study indicates that stressed myocardium retains the incapability to re-enter cell cycle in patients with CCHD and RVI, facing adverse cardiac remodeling. Cardiac tissue consequently encounters difficulties in adaption to pathologic conditions such as pressure and volume overload, which results in impaired

cardiac injury repair and hypertrophy, leading to increased risk of cardiac morbidity and mortality (38,39). The incapacity to enter cell cycle, guaranteeing cell proliferation, injury regeneration and preservation of ventricular function, aggravate pathophysiologic conditions and encourage development of RV remodeling and RVI.

The major limitation of the current study is the lack of whole transcriptome profile of RV tissue gained from healthy patients without structural heart disease and without myocardial disease state as a control group. The use of a donor heart, which is discarded for organ transplantation, would have provided an opportunity to remove healthy tissue from the right ventricle and use it as a reference material. Nevertheless, it must be noted, that not-transplantable donor hearts are often not completely healthy with exposition to abundant factors, typically changing gene expression. Besides, the rejection of a donor heart is a rarity. Also, the falsification of the results of gene sequencing by temporal issue and processing complications should also not be underestimated. Because of altered myocardial gene expression after organ ischemia and organ processing outside the body it is not ideal to use not-transplantable donor hearts as healthy reference tissue (40). The focus of the current study was therefore the alteration of gene expression patterns contributing to RV remodeling in chronic volume and/or pressure overloaded RVs over lifetime in a cohort of CCHD patients with similar structural heart disease, namely TOF. The control group consistent of infants TOF where RV tissue was collected at the time of surgical repair at the age of 4–5 months. Limited data of developmentally regulated genes in myocardial tissue, showing a switch during aging process, hamper the identification and exclusion of all genes, being essential in heart development during early childhood. By comparing most significant single-cell RNA sequencing results of murine cells to our results, genes associated with cardiac development were identified and excluded for further interpretation. However, according to few studies there are indications that genes play a variety of roles in both heart development and pathological cardiac remodeling (41). It has also been described that embryonic patterns and fetal gene programs, which are crucial for heart development and maturation, get reactivated in pathological conditions, leading to HF and death (42). Studies have shown that members of the myocyte enhancer factor 2 (*MEF2*) family are expressed in both embryonic cardiac tissue and postnatal hypertrophic cardiac remodeling (43). MEF2 proteins such as MEF2C function as transcription factors, increasing expression of certain fetal and cardiac genes, including natriuretic peptide A (*NPPA*), skeletal alpha actin (*ACTA1*), desmin (*DES*) and dystrophin (*DMD*). Concomitant to previous observations expression of *MEF2C*, *NPPA*, *ACTA1*, *DES* and *DMD* were found to be upregulated in our study, reinforcing the suggestion of reactivation of fetal gene programs in pathological heart conditions (Table S10). Considering these emerging evidences, important information of gene expression changes during cardiac remodeling might get missed by excluding alleged developmentally regulated genes. Another limitation is the unequal distribution of female and male patients in the infant and adult patient group. While the infant patient group is dominated by males (6/7), the adult patient group is composed of more females (5/6). The gender disparity might have an impact on gender specific alterations in gene expression, which makes transferability more difficult. However, whereas there are contradictory data regarding gender differences in gene expression of the left ventricular myocardium in human (44) and animal (45) studies, to our knowledge the impact of gender on mRNA expression in human RV heart tissue has not been described so far in the literature. Two of the six adult patients were on cardiac medication at the time of study. The influence of this on gene expression in unknown and due to small sample size, we were not able to focus on this in the current analysis. Lastly, as this is a single-center study with retrospective data analysis, the results cannot simply be generalized and extrapolated to other centers.

Conclusions

There is altered RV gene expression over lifetime in adult patients with RVI due to long-term sequelae of CCHD. Analysis of different gene expression profiles identified molecular pathways involved in cell-cell communication, exosomes, extracellular vesicles, oxidation reduction process, contractile fiber and retinol metabolism to be involved in pathologic RV remodeling secondary to chronic volume and/or pressure overload. The identification of molecular pathways leading to pathological RV remodeling and failure is crucial for the development of new therapies and upcoming biomarkers, screening assays for risk stratification and disease monitoring. The current study contributes to a better understanding of the molecular characterization of RV remodeling and RVI, which significantly increases morbidity and mortality in patients with CCHD, surviving into adulthood.

Acknowledgments

Funding: This study was supported by the German Centre for Cardiovascular Research (Deutsches Zentrum für Herz-Kreislauf-Forschung e. V., DZHK) Excellence Program Rotation Grant (Cordula Maria Wolf), Berlin, Germany.

Footnote

Provenance and Peer Review: This article was commissioned by the Guest Editors (Yskert von Kodolitsch, Harald Kaemmerer, Koichiro Niwa) for the series "Current Management Aspects in Adult Congenital Heart Disease (ACHD): Part IV" published in *Cardiovascular Diagnosis and Therapy*. The article has undergone external peer review.

Reporting Checklist: The authors have completed the MDAR reporting checklist. Available at http://dx.doi.org/10.21037/cdt-20-894

Data Sharing Statement: Available at http://dx.doi.org/10.21037/cdt-20-894

Peer Review File: Available at http://dx.doi.org/10.21037/cdt-20-894

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at http://dx.doi.org/10.21037/cdt-20-894). The series "Current Management Aspects in Adult Congenital Heart Disease (ACHD): Part IV" was commissioned by the editorial office without any funding or sponsorship. The authors have no other conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by institutional ethics committee (approval 10/16/2017, number 242/17S, and approval 01/11/2017, number 592,16S) and individual consent for this retrospective analysis was taken from all patients.

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Cite this article as: Pollmann K, Raj Murthi S, Kračun D, Schwarzmayr T, Petry A, Cleuziou J, Hörer J, Klop M, Ewert P, Görlach A, Wolf CM. Molecular signaling pathways in right ventricular impairment of adult patients after tetralogy of Fallot repair. Cardiovasc Diagn Ther 2021;11(6):1295-1309. doi: 10.21037/cdt-20-894

Supplementary

Appendix 1

Table of contents:

http://dx.doi.org/10.21037/cdt-20-894

Methods

Patients Characteristics

Of all patients with a primary diagnosis of tetralogy of Fallot (TOF) undergoing surgical treatment at the German Heart Center Munich between April 2009 and May 2016, RV tissue and a signed informed consent were available in the institutional biomaterial bank from 31 patients (Figure 1). Surgeries included corrective repair for infants or elective replacement of the right ventricular to pulmonary artery conduit for adult patients, due to long-term consequences of hemodynamic abnormalities. Demographic, clinical, electrocardiographic data, transthoracic echocardiography (TTE), chest x-ray, cardiovascular magnetic resonance imaging (CMR), computed tomography (CT) scans and catheterization data were collected by retrospective chart review and were analyzed. Based on clinical information, clinical status with distinct signs of RVI and RV function was assessed, following the definition of adult and infant patient groups.

The criteria for RVI were selected based on the International Right Heart Foundation Working Group recommendations (1) and the scientific statement of the American Heart Association (2). These included clinical parameters such as functional capacity (New York Heart Association (NYHA) classification), peripheral edema, and engorgement of jugular veins, enlargement of liver, dyspnea and cyanosis. Serum markers like reduced GFR, increased creatinine and blood urea nitrogen were selected, in order to evaluate the cardiorenal abnormalities. For cardiohepatic abnormalities, elevated blood levels of bilirubin, ɣ-glutamyl transpeptidase (ɣ-GT) and alkaline phosphatase (AP) were chosen. The assessment of RV size and function was examined with (TTE) and CMR. Parameters included right ventricular ejection fraction (RVEF), left ventricular ejection fraction (LVEF), pulmonary trunk regurgitation fraction, right ventricular stroke volume (RVSV), right ventricular enddiastolic volume index (RVEDVI), right ventricular endsystolic volume index (RVESVI), evidence of moderate to severe pulmonary regurgitation, maximum velocity over pulmonic valve (PV V max), mean pressure gradient over pulmonic valve (PV mean PG), maximum pressure gradient over pulmonic valve (PV max PG), right ventricular hypertrophy and increased right ventricular pressure (RVP).

RV tissue from infants with TOF obtained at the time of corrective surgery during infancy was selected for comparison due to the similar underlying structural heart defect in this group and due to the lack of long-standing right ventricular hemodynamic stressors. Patients with confirmed genetic diseases, additional syndromes and any other organ failure were excluded.

RNA Isolation and Quality Assessment

RV tissue was collected during surgical procedures. Immediately after tissue removal, all samples were frozen in liquid nitrogen and stored at -80 °C until RNA isolation. Following manufacturer's instructions, total RNA was isolated using miRNeasy Mini Kit and QIAcube robotic workstation (Qiagen, Hilden, Germany) at the Institute of human genetics at Helmholtz Centre Munich, Germany. Quantity of RNA was assessed by measuring the concentration of isolated total RNA, using NanoDrop 2000 spectrophometer (Thermo Fisher Scientific, Waltham, Massachusetts, United States). RNA purity was estimated by examining the 260/280 ratio, as recommended by the manufacturer (Thermo Fisher, Scientific, Waltham Massachusetts, Unites States). Quality of the isolated RNA was estimated by determination of RNA integrity number (RIN), according to manufacturer's specifications.

Total RNA Sequencing

Whole transcriptome analysis was performed by total RNA sequencing of cardiac tissue samples as previously described (3). RNA library was prepared by using 1 µg of RNA, which was poly (A) selected, fragmented and reverse transcribed with Elute, Prime and fragment mix adhered to Illumina's information (Illumina, San Diego, California United Stated). Subsequently, tailing, adaptor ligation and library enrichment was done, following manufacturer's recommendation of TruSeq Stranded mRNA Sample Prep Guide (Illumina, San Diego, California, United States). Quality and Quantity of the RNA library were estimated by Agilent 2100 Bioanalyzer and Quant-iT PicoGreen dsDNA Assay Kit (Thermo Fisher Scientific, Waltham, Massachusetts, United States). RNA sequencing of 100 bp paired-end runs was performed with Illumina HiSeq 4000 platform (Illumina, San Diego, California, United States). A proximate alignment against human genome assembly hg19 (GRCh37) and UCSC known gene annotation was done by using STAR aligner (v2.4.2a) (4). Quantification of the number of reads mapping to annotated genes was accomplished by using HT-seq count (v0.6.0), in which fragments per kilobase of transcript per million fragments mapped (FPKM) were selected as the unit

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of measurement (5). Utilizing R Bioconductor package DESeq2 differential gene expression analysis was completed (6), followed by pathway- and Gene Set Enrichment Analysis with R Bioconductor package gage (7), pathview (8) and goseq (9). Tools like Gene Ontology (GO) (Department of Genetics, Stanford University School of Medicine, Stanford, California, United States.) (10,11), Kyoto Encyclopedia of Genes and Genomes (KEGG) (Institute for Chemical Research, Kyoto University, Uji, Kyoto, Japan) (12), ToppFun (Division of Biomedical Informatics, Cincinnati Children's Hospital Medical Center, Cincinnati, Ohio, United States) (13) and PANTHER Classification System (14) were applied to validate pathway analysis, biological activity and allocation of individual genes to Gene Ontology categories (Figure 2 and Figure S2).

The total of 3,010 differentially expressed genes were compared to 50 most significant genes, regulating cardiac development and heart maturation, which were identified by RNA sequencing of murine cells during embryonic and postnatal period. Overlapping genes were not included in further interpretation of results (15) (Figure S1).

Validation of selected genes with Western blot

Total proteins, isolated from RV myocardial tissue from patients, were separated by 8-12% SDS polyacrylamide gel electrophoresis using Mini-Protean 3 system (Biorad), as previously described (16,17). Following separation, proteins were transferred to nitro-cellulose membranes, and incubated with one of the following primary antibodies: alcohol dehydrogenase 1B (ADH1B, Abcam, Cambridge, UK; ab175515, RRID: N/A), monoamine oxidase B (MAOB, Merck, Darmstadt, Germany; ST1582, RRID: AB_10617089), peroxisome proliferator-activated receptor gamma (PPARγ, Cell Signaling, Frankfurt, Germany; S4946, RRID: AB_2166051), superoxide dismutase 3 (SOD3, Merck, Darmstadt, Germany; S4946, RRID: AB_532286), interleukin 6 receptor (IL6R, R&D Systems, Wiesbaden-Nordenstadt, Germany; MAB227, RRID: AB_2127908), or β-actin (Santa Cruz, Heidelberg, Germany, SC-1616, RRID: AB_630836). Goat anti-rabbit, goat anti-mouse or rabbit anti-goat secondary antibodies were used (Merck, 401253, RRID: AB_437779; 401393, RRID: AB_437797; 401515, RRID: AB_437816). Following enhanced chemiluminescence reaction, bands were quantified using ImageJ. Two-tailored student's test was used for statistical evaluation. Data are presented as a mean ± standard deviation.

Ethical Approval

The study was approved by the institution's ethical committee at the Technical University of Munich (approval 10/16/2017, number 242/17S, and approval 01/11/2017, number 592,16S). The study protocol conforms to the ethical standards of the Declaration of Helsinki 1975. All patients or parents gave written informed consent before enrollment.

Statistics

Statistic evaluation was implemented by applying R Bioconductor package DEseq2 to transcriptome profiles and tested for differential gene expression between adult and infant patients. The P values were corrected for the purpose of multiple testing by Benjamini and Hochberg procedure. The level of significance was set at a P value of less than 0.01 and a fold-change value of greater than 2 or less than –2. Based on that, all significant differentially expressed genes were selected for further analysis. The results of Gene Ontology and Pathway analysis with an adjusted P value less than 0.01 (GO) and a P value less than 0.05 (KEGG) were assigned as significant.

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Online Figures:

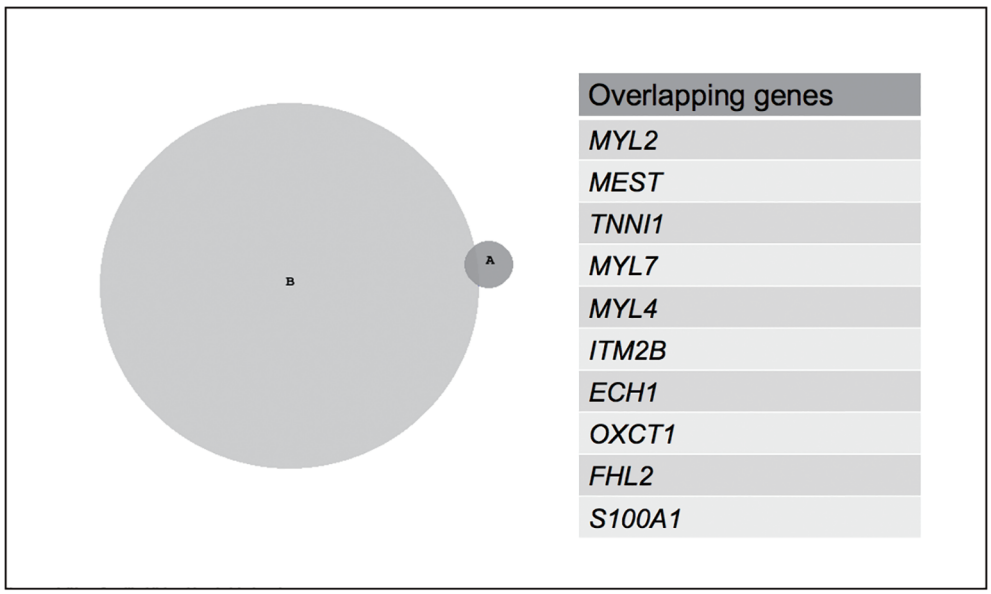

Figure S1 Venn diagram demonstrating the intersection of differentially expressed genes. The total of 3,010 genes differentially expressed between infants and adults with right ventricular impairment are represented by B. The top 50 genes being potential markers for cardiac development and heart maturation are illustrated by A. Genes identical expressed in both studies are listed on the right. These 10 genes were identified as overlapping genes playing a role during heart development and were therefore excluded for further analysis. The complete list of the 50 developmental markers is shown in Table S9.

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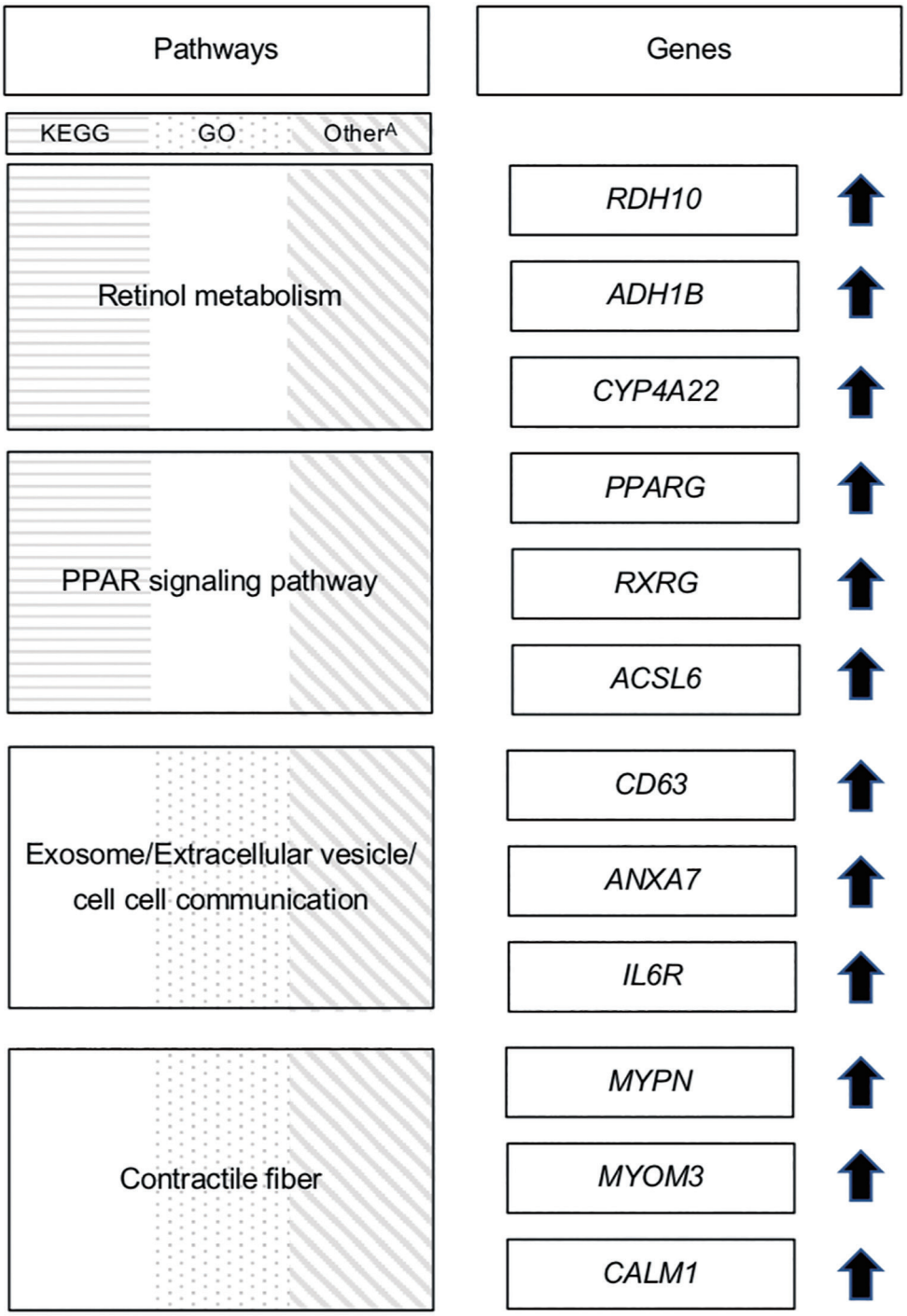

Figure S2 Summary of the results in the form of an overview of the modified signaling pathways with associated genes and their regulation. A, used validation tools like PANTHER Classification, ToppFun. KEGG, Kyoto Encyclopedia of Genes and Genomes; GO, Gene Ontology; PPAR, peroxisome proliferator-activated receptor.

http://dx.doi.org/10.21037/cdt-20-894

Online Tables:

Table S1 Presentation of the 10 most significant upregulated GO terms for cellular component in adult patients with CCHD and RVI

Category	Term	P value	Adjusted P value
GO:0044444	Cytoplasmic part	3.74E-33	8.36E-29
GO:0005737	Cytoplasm	1.23E-29	1.37E-25
GO:0030016	Myofibril	2.61E-18	1.95E-14
GO:0043292	Contractile fiber	2.36E-17	1.32E-13
GO:0030017	Sarcomere	1.24E-16	5.53E-13
GO:0044449	Contractile fiber part	2.68E-16	1.00E-13
GO:0005829	Cytosol	4.18E-15	1.34E-11
GO:0070062	Extracellular exosome	3.81E-14	1.06E-10
GO:1903561	Extracellular vesicle	8.00E-14	1.68E-10
GO:0043230	Extracellular organelle	9.03E-14	1.68E-10

GO, Gene Ontology.

Table S2 Presentation of the 10 most significant upregulated GO terms for molecular function in adult patients with CCHD and RVI

Category	Term	P value	Adjusted P value
GO:0008092	Cytoskeletal protein binding	5.69E-14	1.41E-10
GO:0016491	Oxidoreductase activity	8.96E-12	1.34E-08
GO:0003824	Catalytic activity	3.99E-11	3.72E-08
GO:0005515	Protein binding	2.10E-10	1.45E-07
GO:0003674	Molecular_function	8.28E-09	3.63E-06
GO:0061134	Peptidase regulator activity	4.40E-08	1.61E-05
GO:0003779	Actin binding	1.18E-07	3.66E-05
GO:0005488	Binding	1.36E-07	4.11E-05
GO:0004857	Enzyme inhibitor activity	2.11E-07	6.05E-05
GO:0061135	Endopeptidase regulator activity	2.39E-07	6.69E-05

GO, Gene Ontology.

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Table S3 Presentation of the 10 most significant upregulated GO terms for biological processes in adult patients with CCHD and RVI

Category	Term	P value	Adjusted P value
GO:1901564	Organonitrogen compound metabolic process	8.61E-14	1.68E-10
GO:0044281	Small molecule metabolic process	7.13E-12	1.14E-08
GO:0055114	Oxidation-reduction process	1.44E-11	2.02E-08
GO:0002283	Neutrophil activation involved in immune response	1.72E-11	2.26E-08
GO:0002446	Neutrophil mediated immunity	1.82E-11	2.26E-08
GO:0043312	Neutrophil degranulation	2.33E-11	2.60E-08
GO:0042119	Neutrophil activation	2.69E-11	2.86E-08
GO:0036230	Granulocyte activation	3.56E-11	3.46E-08
GO:0016192	Vesicle-mediated transport	6.82E-11	6.10E-08
GO:0006887	Exocytosis	1.03E-10	8.85E-08

GO, Gene Ontology.

Table S4 Presentation of the 10 most significant downregulated GO terms for cellular component in adult patients with CCHD and RVI

Category	Term	P value	Adjusted P value
GO:0005694	Chromosome	8.57E-28	1.92E-23
GO:0005634	Nucleus	1.05E-26	1.17E-22
GO:0044427	Chromosomal Part	6.44E-24	3.60E-20
GO:0044815	DNA packaging complex	2.95E-23	9.35E-20
GO:0000786	Nucleosome	1.82E-21	3.71E-18
GO:0032993	Protein-DNA complex	4.64E-21	8.66E-18
GO:0044428	Nuclear part	7.83E-19	1.35E-15
GO:0031981	Nuclear lumen	3.36E-18	5.37E-15
GO:0005654	Nucleoplasm	2.77E-17	3.65E-14
GO:0000228	Nuclear chromosome	1.48E-15	1.58E-12

GO, Gene Ontology; DNA, deoxyribonucleic acid.

http://dx.doi.org/10.21037/cdt-20-894

Table S5 Presentation of the 10 most significant downregulated GO terms for molecular function in adult patients with CCHD and RVI

Category	Term	P value	Adjusted P value
GO:0003677	DNA binding	5.29E-22	1.18E-18
GO:0003676	Nucleic acid binding	1.45E-13	9.80E-11
GO:0097159	Organic Cyclic compound binding	2.43E-09	6.63E-07
GO:1901363	Heterocyclic compound binding	3.36E-09	8.73E-07
GO:0003700	DNA binding transcription factor activity	1.14E-07	2.16E-05
GO:0043142	Single-stranded DNA-dependent ATPase activity	7.75E-07	0.00013
GO:0140110	Transcription regulator activity	1.52E-06	0.00023
GO:0000981	RNA Polymerase 2 transcription factor activity, sequence-specific DNA binding	2.39E-06	0.00035
GO:0042393	Histone binding	2.43E-06	0.00036
GO:0031492	Nucleosomal DNA binding	3.18E-06	0.00044

GO, Gene Ontology; DNA, deoxyribonucleic acid; ATP, adenosine triphosphate; RNA, ribonucleic acid.

Table S6 Presentation of the 10 most significant downregulated GO terms for biological processes in adult patients with CCHD and RVI

Category	Term	P value	Adjusted P value
GO:0007049	Cell Cycle	4.93E-26	3.60E-20
GO:1903047	Mitotic cell cycle process	1.33E-23	5.54E-20
GO:0022402	Cell cycle process	1.49E-23	5.54E-20
GO:0051276	Chromosome organization	2.29E-22	6.40E-19
GO:0000278	Mitotic cell cycle	2.88E-22	7.16E-19
GO:0090304	Nucleic acid metabolic process	3.76E-18	5.61E-15
GO:0006259	DNA metabolic process	1.15E-17	1.61E-14
GO:0006261	DNA-dependent DNA replication	1.20E-16	1.49E-13
GO:0140014	Mitotic nuclear division	3.79E-16	4.47E-13
GO:0051301	Cell division	6.13E-16	6.86E-13

GO, Gene Ontology; DNA, deoxyribonucleic acid.

http://dx.doi.org/10.21037/cdt-20-894

Table S7 Upregulated genes matching to the selected differentially expressed signaling pathways in adult patients with CCHD and RVI

	P value	Gene	P value	Adjusted P value	Regulation
Gene Ontology (PANTHER)					
Extracellular exosome; GO:0070062	3.81E-14	APOB	3.69E-25	2.27E-22	Up
		SERPINB6	6.42E-21	2.35E-18	Up
		ACSL4	5,14E-09	2.18E-07	Up
		GPA33	4.73E-09	2.03E-07	Up
		DSC1	2.80E-11	2.00E-09	Up
		MYL12A	1.42E-62	1.66E-58	Up
		SERPINA5	3.32E-09	1.48E-07	Up
		CAB39	2.33E-12	2.16E-10	Up
		CD59	6.34E-19	1.79E-16	Up
		DSTN	8.09E-13	8.49E-11	Up
		SYNC	5.10E-13	5.55E-11	Up
		PPM1L	2.96E-19	8.65E-17	Up
		RNF11	4.70E-15	7.23E-13	Up
		MME	3.12E-17	7.51E-15	Up
		CLIC5	7.40E-22	3.09E-19	Up
		ANXA7	8.92E-18	2.27E-15	Up
		RRAS	6.83E-12	5.63E-10	Up
		RRAS2	2.50E-12	2.30E-10	Up
		RAB5A	3.32E-09	1.48E-07	Up
Extracellular vesicle and cell cell communication; GO:1903561	8.00E-14	APOB	3.69E-25	2.27E-22	Up
		CD59	6.34E-19	1.79E-16	Up
		LGMN	6.89E-05	0.00086	Up
		LGALS3	1,73E-07	4.87E-06	Up
		SERPINB6	6,42E-21	4.87E-06	Up
		CD63	0.0012	0.0092	Up
		DES	5.10E-14	6.67E-12	Up
		HSPA4	5,37E-15	8,21E-13	Up
		PPM1L	2.96E-19	8.65E-17	Up
		RNF11	4.70E-15	7.23E-13	Up
		MME	3.12E-17	7.51E-15	Up
		MYL12A	1.42E-62	1.66E-58	Up
		CLIC5	7.40E-22	3,09E-19	Up
		ANXA7	8.92E-18	2.27E-15	Up

Table S7 (*continued*)

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Table S7 (*continued*)

	P value	Gene	P value	Adjusted P value	Regulation
		SCN3A	1.07E-25	7.34E-23	Up
		SYT13	5.97E-20	2.00E-17	Up
		GRIK2	4.46E-23	2.05E-20	Up
		BDNF	9.87E-16	1.79E-13	Up
		GRIN2A	2.40E-21	9.19E-19	Up
		IL6R	9.89E-13	9.98E-11	Up
		DGKG	1.61E-27	1.21E-24	Up
		DOCK5	4.44E-30	4.72E-27	Up
		PRKG1	8.56E-20	2.82E-17	Up
		MYH7	9.04E-11	5.86E-09	Up
		RCAN2	5.08E-22	2.16E-19	Up
		HOMER2	4.05E-23	1.90E-20	Up
		C1orf168	1.69E-24	8.99E-22	Up
		ITGBL1	6.94E-21	2.50E-18	Up
		SARS	9.46E-06	0.000155	Up
		CHP1	3.64E-08	1.24E-06	Up
		SERPINB1	3.50E-08	1.20E-06	Up
		STAMBP	0,00023	0.0024	Up
Response to oxidative stress; GO:0006979	8.67E-05	HBA2	3.88E-12	3.42E-10	Up
		TPO	7.76E-15	1.16E-12	Up
		BECN1	2.20E-12	2.05E-10	Up
		HBB	8.34E-13	8.67E-11	Up
		MAOB	2.27E-18	6.11E-16	Up
		KPNA4	3.76E-29	3.39E-26	Up
		SOD3	0.00034	0.0033	Up
		TXN2	0.00058	0.0052	Up
Contractile Fiber; GO:0043292	2.36E-17	HABP4	4.01E-16	7.57E-14	Up
		DUSP27	1.55E-27	1.21E-24	Up
		CALM1	3.56E-05	0.00049	Up
		MYBPC1	6.86E-13	7.23E-11	Up
		CMYA5	1.77E-13	2.13E-11	Up
		KLHL41	4.98E-42	1.46E-38	Up
		SYNM	5.47E-16	1.02E-13	Up
		SYNC	5.10E-13	5.55E-11	Up

Table S7 (*continued*)

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Table S7 (*continued*)

	P value	Gene	P value	Adjusted P value	Regulation
		FHL5	2.19E-37	3.42E-34	Up
		FHL1	9.46E-35	1.30E-31	Up
		MYH7	9.04E-11	5.86E-09	Up
		MYPN	1.35E-15	2.36E-13	Up
		MYOM3	9.39E-15	1.38E-12	Up
Signaling pathway (KEGG analysis)					
Retinol metabolism (hsa00830)	0.0017	ADH1B	4.93E-11	3.36E-09	Up
		ADH1C	0.00037	0.0035	Up
		RDH10	0.00076	0.0064	Up
		PNPLA4	2.61E-10	1.58E-08	Up
		CYP1A1	1.01E-05	0.00016	Up
		CYP4A22	0.00098	0.0079	Up
		RETSAT	1.18E-08	4.61E-07	Up
PPAR signaling pathway (hsa03320)	0.021	RXRG	0,0051	0,03	Up
		PPARG	2.03E-08	7.46E-07	Up
		APOA1	0.0019	0.014	Up
		ACSL6	1.82E-21	7.33E-19	Up
		ACSL4	5.14E-09	2.18E-07	Up
		CYP4A22	0.00098	0.0079	Up
		AQP7	8.07E-05	0.00099	Up
		ACADM	5.53E-07	1.36E-05	Up

KEGG, Kyoto Encyclopedia of Genes and Genomes; has, Homo sapiens (human); GO, Gene Ontology; PANTHER, Protein Analysis Through Evolutionary Relationships.

http://dx.doi.org/10.21037/cdt-20-894

Table S8 Downregulated genes matching to the selected differentially expressed signaling pathways in adult patients with CCHD and RVI

	P value	Gene	P value	Adjusted P value	Regulation
Gene Ontology (PANTHER)					
Cell Cycle; GO:0007049	4.92E-24	CDT1	5.37E-05	0.00069	Down
		CDC7	2.69E-11	1.94E-09	Down
		MCM2	1.14E-05	0.00018	Down
		PRIM1	3.61E-06	6.85E-05	Down
		ORC6	7.67E-08	2.39E-06	Down
		BCAT1	1.16E-05	0.00018	Down
		MCM3	4.74E-09	2.04E-07	Down
		CDK6	2.20E-09	1.03E-07	Down
		MCM5	4.20E-15	6.55E-13	Down
		RCC1	6.30E-05	0.0008	Down
		E2F7	9.65E-08	2.92E-06	Down
		NASP	8.50E-06	0.00014	Down
		CDK14	0.00021	0.0022	Down
Cell division; GO:051301	6.13E-16	CDT1	5.37E-05	0.00069	Down
		CENPF	3.34E-08	1.15E-06	Down
		NCAPG	1.02E-08	4.03E-07	Down
		STAG2	8.89E-08	2.72E-06	Down
		CDC7	2.69E-11	1.94E-09	Down
		FBXL7	6.42E-08	2.03E-06	Down
		PARD6G	5.22E-05	0.00068	Down
		SPDL1	3.99E-06	7.46E-05	Down
		CASC5	4.48E-11	3.08E-09	Down
		TPX2	5.89E-08	1.88E-06	Down
Mitotic cell cycle; GO:0000278	2.88E-22	DNMT3A	6.29E-09	2.60E-07	Down
		SOX4	8.12E-21	2.88E-18	Down
		WDR62	7.06E-06	0.00012	Down
		NCAPG	1.02E-08	4.03E-07	Down
		AJUBA	6.43E-06	0.00011	Down
		TOP2A	9.27E-14	1.17E-11	Down
		POLA1	3.10E-06	5.98E-05	Down
		SNX30	3.65E-13	4.11E-11	Down
DNA replication; GO:0006260	4.56E-15	POLD1	5.57E-11	3.73E-09	Down
		GINS1	1.59E-07	4.55E-06	Down
		ING4	1.58E-11	1.21E-09	Down

Table S8 (*continued*)

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Table S8 (*continued*)

	P value	Gene	P value	Adjusted P value	Regulation
		HELB	3.29E-07	8.62E-06	Down
		DTL	1.08E-06	2.42E-05	Down
		POLQ	3.04E-05	0.00043	Down
		RBBP4	5.68E-06	0.0001	Down
		FAM111A	7.36E-06	0.00013	Down
Signaling pathway (KEGG)					
Cell cycle (hsa04110)	0.00098	CDK6	2.20E-09	1.03E-07	Down
		ORC6	7.67E-08	2.39E-06	Down
		SKP2	6.68E-08	2.11E-06	Down
		CDC7	2.69E-11	1.94E-09	Down
		MCM5	4.20E-15	6.55E-13	Down
DNA replication (hsa03030)	0.0019	LIG1	5.68E-14	7.38E-12	Down
		POLA1	3.10E-06	5.98E-05	Down
		PRIM1	3.61E-06	6.85E-05	Down
		POLE	2.85E-10	1.67E-08	Down
		RNASEH2C	1.10E-06	2.45E-05	Down
Ribosome (hsa03010)	0.012	RPL18	5.85E-05	0.00075	Down
		MRPS6	0.0086	0.045	Down
		RPS8	0.0004	0.0038	Down

KEGG, Kyoto Encyclopedia of Genes and Genomes; has, Homo sapiens (human); GO, Gene Ontology; PANTHER, Protein Analysis Through Evolutionary Relationships; DNA, deoxyribonucleic acid.

http://dx.doi.org/10.21037/cdt-20-894

Table S9 Results of a separate study representing the top 50 genes identified as potential markers for cardiac development and heart maturation (15). The 50 developmental markers listed below were used for identification of genes with the same expression in our study, symbolizing developmentally regulated genes. The detected overlapping genes were not included for further analysis (Figure S1)

Cluster of the top 50 genes	Developmental markers	Expression
Cluster 1	Myh6	Increased
Cluster 1	Atp2a2	Increased
Cluster 1	Pln	Increased
Cluster 1	Cox6a2	Increased
Cluster 1	Cox7b	Increased
Cluster 1	Ndufa1	Increased
Cluster 1	Uqcrq	Increased
Cluster 1	Atp5e	Increased
Cluster 1	Cox7a1	Increased
Cluster 1	Cox6c	Increased
Cluster 1	Tnni3	Increased
Cluster 1	Fabp3	Increased
Cluster 1	Myl2 *	Increased
Cluster 2	Pgam1	Decreased
Cluster 2	Tubb5	Decreased
Cluster 2	Nme1	Decreased
Cluster 2	Gm5506	Decreased
Cluster 2	Eif5a	Decreased
Cluster 2	Ngfrap1	Decreased
Cluster 2	Cks1b	Decreased
Cluster 2	Cdkn1c	Decreased
Cluster 2	Mest *	Decreased
Cluster 2	Gpc3	Decreased
Cluster 2	H2afz	Decreased
Cluster 2	Tnni1 *	Decreased
Cluster 2	Mif	Decreased
Cluster 2	Hmgn2	Decreased
Cluster 2	Gyg	Decreased
Cluster 2	Myl7 *	Decreased
Cluster 2	Myl4 *	Decreased
Cluster 3	Hadha	Early low expression, then increasing
Cluster 3	Ryr2	Early low expression, then increasing
Cluster 3	Srl	Early low expression, then increasing

Table S9 (*continued*)

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Table S9 (*continued*)

Cluster of the top 50 genes	Developmental markers	Expression
Cluster 3	Nfib	Early low expression, then increasing
Cluster 3	Nfia	Early low expression, then increasing
Cluster 3	Klf6	Early low expression, then increasing
Cluster 3	Itm2b *	Early low expression, then increasing
Cluster 3	Ech1 *	Early low expression, then increasing
Cluster 3	Phyh	Early low expression, then increasing
Cluster 3	Oxct1 *	Early low expression, then increasing
Cluster 3	Gpc1	Early low expression, then increasing
Cluster 3	Fhl2 *	Early low expression, then increasing
Cluster 3	Mt1	Early low expression, then increasing
Cluster 3	Mgst3	Early low expression, then increasing
Cluster 3	Acadl	Early low expression, then increasing
Cluster 3	Lpl	Early low expression, then increasing
Cluster 3	Brp44l	Early low expression, then increasing
Cluster 3	D830015G02Rik	Early low expression, then increasing
Cluster 3	Lars2	Early low expression, then increasing
Cluster 3	S100a1 *	Early low expression, then increasing

*, detected overlapping genes excluded for further analysis.

Table S10 Presentation of overexpressed fetal genes in adult patients with CCHD and RVI

Fetal gene	P value	Adjusted P value	Expression
MEF2C	0.0081	0.043	Increased
NPPA	0.00072	0.0062	Increased
ACTA1	4.55E-09	1.96E-07	Increased
DES	5.10E-14	6.67E-12	Increased
DMD	0.0062	0.035	Increased

http://dx.doi.org/10.21037/cdt-20-894