

Genetic determinants of right-ventricular remodeling after tetralogy of Fallot repair

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BACKGROUND: Hypoxia-inducible factor (*HIF1A*) regulates the myocardial response to hypoxia and hemodynamic load. We investigated the association of *HIF1A* variants with right-ventricular (RV) remodeling after tetralogy of Fallot (TOF) repair.

METHODS: Children with TOF were genotyped for three single-nucleotide polymorphisms in *HIF1A*. Genotypes were analyzed for association with RV myocardial protein expression and fibrosis at complete repair ($n = 42$) and RV dilation, fractional area change, and freedom from pulmonary valve/conduit replacement on follow-up.

RESULTS: In 180 TOF patients, mean age at repair was 1.0 ± 0.8 y with follow-up at 9.0 ± 3.5 y; 82% had moderate to severe pulmonary insufficiency. Freedom from RV reinterventions at 5, 10, and 15 y was 92, 84, and 67%, respectively. Patients with more functioning *HIF1A* alleles had higher transforming growth factor $\beta 1$ expression and more fibrosis at initial repair as compared with controls ($P < 0.05$). During follow-up, patients with more functioning *HIF1A* alleles showed less RV dilation, better preservation of RV function, and greater freedom from RV reinterventions ($P < 0.05$). This was confirmed in a replication cohort of 69 patients.

CONCLUSION: In children who have had TOF repair, a lower number of functioning *HIF1A* alleles was associated with RV dilation and dysfunction, suggesting that hypoxia adaptation in unrepaired TOF may influence RV phenotype after repair.

Tetralogy of Fallot (TOF) is the most common cyanotic congenital heart defect, occurring in 4 of every 10,000 births (1). “Classic” TOF comprises pulmonary stenosis, ventricular septal defect, aortic override, and right-ventricular (RV) hypertrophy. In the unrepaired state, the right ventricle is exposed to hypoxia and pressure overload. Even after complete surgical repair (typically performed in early infancy), persistent pulmonary regurgitation after repair is common and contributes to progressive RV dilation often culminating in RV failure, ventricular arrhythmias, and reduced survival (2). Recent attempts at preserving pulmonary valve function at primary repair have not translated into long-term benefits on RV dilation and dysfunction (3,4). Similarly, pulmonary valve replacement after the onset of RV dilation facilitates reverse

RV remodeling but does not restore normal ventricular function and exercise capacity (5). Therefore, the strategy of relying on RV dilation as a primary indication for reintervention has met with limited success. In order to identify at-risk patients early, before significant RV modeling has occurred, requires a better understanding of the genes and biologic pathways that mediate RV remodeling.

One such pathway is the hypoxia-response pathway, which is activated in response to both hypoxia and mechanical or hemodynamic stress. The right ventricle is exposed to chronic hypoxia and pressure overload during fetal life and infancy before TOF repair, and to ongoing hemodynamic stresses related to volume overload from pulmonary insufficiency and/or pressure overload from conduit stenosis after TOF repair. A central mediator of cardiac adaptation to hypoxia and mechanical stress is hypoxia-inducible factor 1 α (*HIF1A*). In acute hypoxia, *HIF1A* is cardioprotective by virtue of its ability to induce angiogenic, metabolic, and erythropoietic genes (6,7). Chronic *HIF1A* upregulation however can promote transforming growth factor $\beta 1$ (TGF $\beta 1$)-mediated organ fibrosis (8,9). TGF $\beta 1$ promotes the transformation of endothelial and smooth muscle cells to fibroblasts. With ongoing stress, fibroblasts convert to proliferating myofibroblasts that induce extracellular matrix deposition and fibrosis. This mechanism for cardiac fibrosis was first reported by Zeisberg *et al.* (9) in mouse hearts in response to pressure overload. Importantly, this process of fibroblast transformation was reversible by TGF $\beta 1$ inhibition. The process of active fibroblast transformation may therefore reflect a reversible protective response to environmental stress that facilitates the transition of stressed endothelial or smooth muscle cells into fibroblasts to help them survive in adverse environmental conditions. These processes have not been evaluated in TOF, in which the right ventricle is exposed to chronic hypoxia and hemodynamic stresses. Our previous study showed that several TOF patients with severe hypoxia showed impaired RV adaptation, with downregulation of vascular endothelial growth factor (VEGF) and antioxidant enzymes and upregulation of collagen and plasma lactate levels postoperatively (10). However, the cause of

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impaired myocardial adaptation to hypoxia in some patients and the long-term impact of chronic hypoxia on RV remodeling were not studied.

The purpose of this study was to evaluate the influence of genetic variations in *HIF1A* on RV angiogenic factor expression and fibrosis at the time of surgical repair in infancy and on RV remodeling during serial follow-up after surgery. Specifically, we investigated the association of three common variants in the *HIF1A* gene with RV fibrosis at the time of complete repair and on RV size, RV function, and freedom for RV reinterventions during follow-up.

RESULTS

Primary Cohort

One hundred and eighty eligible patients were included; 55% were male. Clinical and echocardiographic characteristics are described in **Table 1**. Median age at repair was 0.6 y, mean age at follow-up was 9 y, 84% had “classic” TOF, and 82% had moderate to severe pulmonary regurgitation at last follow-up.

HIF1A Allele Frequencies

Frequencies of functioning *HIF1A* alleles were: *HIF1A*145T 74%, *HIF1A*1326C 82%, and *HIF1A*1744C 89%. Cumulative frequencies were as follows: 1% of the cohort had one, 2% had two, 16% had three, 15% had four, 14% had five, and 51% had six functioning alleles. Genotype frequencies were comparable with the ventricular septal defects controls as well as the general population (data not shown). All genotypes were in Hardy–Weinberg equilibrium with no gender-based differences in single-nucleotide polymorphism (SNP) frequencies. Whites had a higher frequency of functioning alleles as compared with non-whites (92 vs. 74%, $P = 0.01$). The analysis was therefore adjusted for race to address racial differences in SNP frequencies. There were no associations between year of birth and frequency of high-risk alleles ($P = 0.11$). Analysis was also adjusted for TOF subtype, age at surgery, year of primary repair, type of repair, and duration of follow-up.

HIF1A Genotype and RV Phenotype Associations

A higher number of functioning *HIF1A* alleles was associated with lower RV outflow tract z-scores at last follow-up (estimated effect size (EST): $-0.365(0.118)z$ per functioning allele, $P = 0.002$) (**Figure 1a**), slower progression of pulmonary valve regurgitation (EST: $+0.015/y^2$ for zero to three functioning alleles, $+0.009/y^2$ for four or five functioning alleles and $0.006/y^2$ for six functioning alleles, $P = 0.005$), and slower progression of RV dilation over time (EST: $+0.021/y^2$ for zero to three functioning alleles, $+0.013/y^2$ for four or five functioning alleles and $+0.090/y^2$ for six functioning alleles, $P = 0.02$) despite similar degrees of pulmonary regurgitation across genotypes early after repair ($P = 0.26$). At last follow-up, a higher number of functioning *HIF1A* alleles were also associated with a higher RV fractional area change (EST: $+1.2(0.6)\%$ per functioning allele, $P = 0.04$) (**Figure 1b**), lower grade of tricuspid valve regurgitation (EST: $-0.066(0.037)$ per functioning allele, $P = 0.07$), and lower RV end-systolic area indexed to

Table 1. Patient characteristics

Variable	Value
Male gender	99 (55%)
Race ^a	
Asian	33 (20%)
Black	9 (6%)
White	111 (69%)
Other	8 (5%)
Additional cardiac anomalies	
Pulmonary artery anomalies	35 (19%)
Patent ductus arteriosus	27 (15%)
Atrial septal defect	37 (21%)
Right aortic arch	3 (2%)
Aorto-pulmonary collaterals	7 (4%)
Coronary artery abnormalities	6 (3%)
Other cardiac anomalies	5 (5%)
Chromosome abnormalities/genetic syndrome	15 (8%)
Systemic pulmonary arterial shunt before repair	12 (7%)
Year of repair	
1991–1995	36 (20%)
1996–2000	54 (30%)
2001–2005	37 (21%)
2006–2009	53 (29%)
Median age at repair (y, interquartile range)	0.58 (0.37–1.11)
Neonatal repair (<2 mo of age)	11 (6%)
Type of repair	
No patch repair	66 (37%)
Transannular patch	60 (33%)
Valve-sparing	54 (30%)
RV reinterventions	25 (14%)
Pulmonary valve replacement	16 (9%)
Conduit replacement	9 (5%)

RV, right-ventricular.

^aRace known in 162 cases.

body size (EST: $-0.663(0.287)cm^3$ per functioning allele, $P = 0.02$). There was no association between number of functioning alleles and RV systolic pressure ($P = 0.38$) or degree of RV hypertrophy ($P = 0.29$). The overall freedom from RV reinterventions including pulmonary valve or conduit replacement was 92, 84, and 67% at 5, 10, and 15 y, respectively. Freedom from pulmonary valve reinterventions was significantly higher in patients with four or more functioning *HIF1A* alleles as compared with those with fewer than four functioning *HIF1A* alleles, with a decrease in hazard ratio for reinterventions by 0.77 for every additional functioning *HIF1A* allele ($P = 0.04$) (**Figure 2**).

RV Myocardial Protein Expression and Fibrosis

The expression levels of *HIF1A* ($P = 0.003$), VEGF ($P = 0.001$), and TGF β 1 ($P = 0.001$) were significantly higher in the RV of

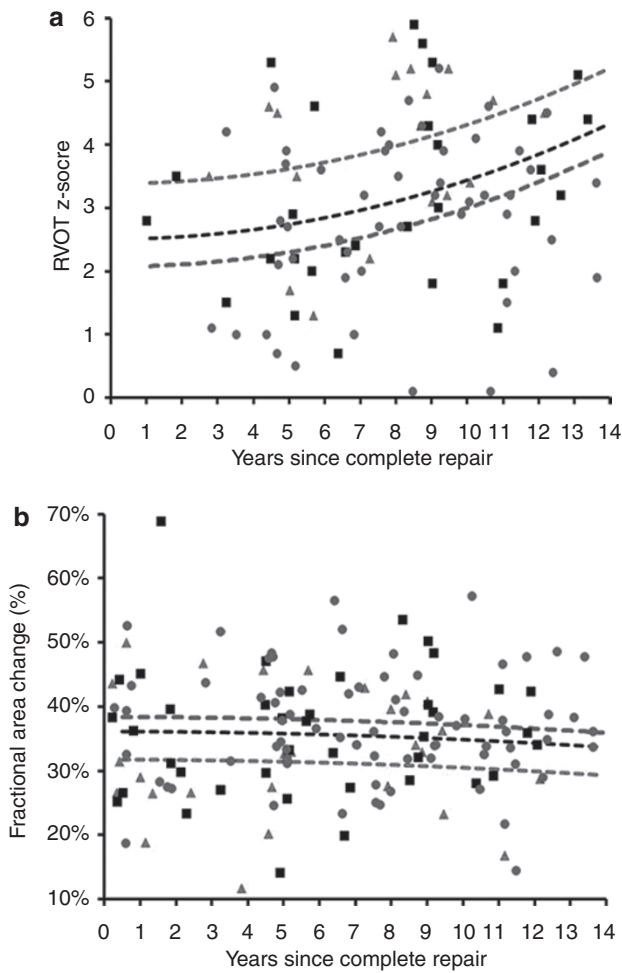


Figure 1. HIF1A genotyping and RV remodeling. (a) *HIF1A* genotype and RVOT dilation: RVOT z-score increased progressively after TOF repair but was lower in patients with a higher number of functioning *HIF1A* alleles ($P = 0.002$) ($n = 180$). (b) *HIF1A* genotype and RV function: RV systolic function assessed using fractional area of change decreased with time, but the rate of decline of systolic function was lower in TOF patients with a higher number of functioning *HIF1A* alleles ($P = 0.04$). Red triangles, one to three functioning *HIF1A* alleles; black squares, four or five functioning *HIF1A* alleles; blue circles, six functioning *HIF1A* alleles. The red, black, and blue lines are regression lines showing the relationship between RVOT z-score or fractional area change and number of functioning *HIF1A* alleles. *HIF1A*, hypoxia-inducible factor 1 α ; RVOT, right-ventricular outflow tract; TOF, tetralogy of Fallot.

patients undergoing TOF repair ($n = 42$, median age 6 mo) as compared with patients undergoing ventricular septal defect repair ($n = 20$, median age 5.3 mo) (Figure 3). Fibrosis area was also significantly greater in TOF patients as compared with controls ($P < 0.001$). Strong red nuclear staining indicated persistent nuclear localization of *HIF1A* in TOF, suggestive of chronic hypoxia. Representative images are shown in Figure 4.

We compared RV expression patterns in patients with four or more ($n = 30$, median age 6 mo) vs. those with fewer than four functioning *HIF1A* alleles ($n = 12$, median age 6.4 mo). Patients with four or more functioning alleles had higher nuclear *HIF1A* expression ($P = 0.001$) and more fibrosis ($P = 0.02$) as compared with patients with fewer than four functioning alleles (Figures 3 and 4). There was a direct correlation

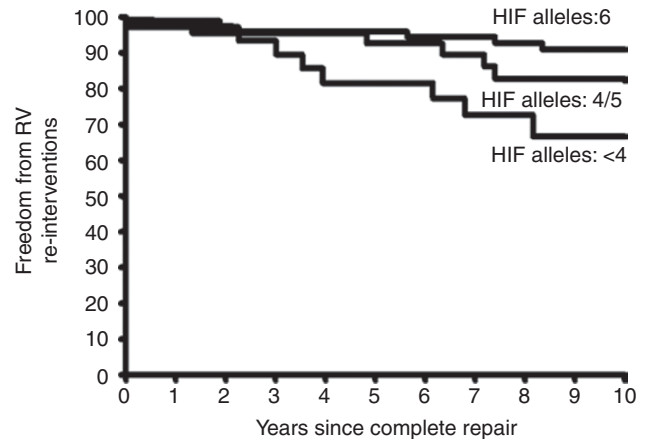


Figure 2. *HIF1A* genotype and freedom from RV reinterventions. Kaplan–Meier survival curve showing freedom from RV reinterventions (pulmonary valve or conduit replacement) during follow-up. Freedom from reinterventions was significantly lower in patients with four or more functioning *HIF1A* alleles as compared with those with fewer than four alleles. The hazard ratio for reintervention decreased by 0.78 for every additional functioning allele ($P = 0.04$). *HIF1A*, hypoxia-inducible factor 1 α ; HR, hazard ratio; RV, right-ventricular.

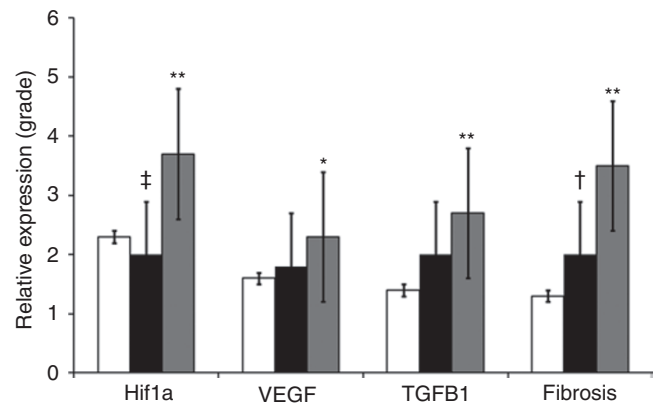


Figure 3. *HIF1A* genotype and RV protein expression. The relative intensity of protein expression in the RV before repair showed that *HIF1A*, VEGF, and TGF β 1 expression and fibrosis area were significantly higher in TOF patients with four or more functioning *HIF1A* alleles undergoing repair (gray) ($n = 42$) as compared with ventricular septal defect controls undergoing repair (white) ($n = 20$) ($*P < 0.01$, $**P < 0.001$ vs. non-TOF controls). In the TOF cohort, *HIF1A* expression and fibrosis area were significantly higher in patients with four or more functioning *HIF1A* alleles (gray) as compared with patients with fewer than four alleles (black) ($†P < 0.05$, $‡P < 0.001$ vs. TOF with four or more functioning alleles). *HIF1A*, hypoxia-inducible factor 1 α ; RV, right-ventricular; TGF β 1, transforming growth factor β 1; TOF, tetralogy of Fallot; VEGF, vascular endothelial growth factor.

between the number of functioning alleles and the intensity of nuclear *HIF1A* expression ($r^2 = 0.45$, $P = 0.003$) and the fibrosis area ($r^2 = 0.69$, $P = 0.005$). There was no difference in VEGF expression between genotype groups.

Replication Cohort

Sixty-nine additional eligible TOF patients were genotyped for replication analysis. The replication cohort was comparable to the primary cohort—57% male, 68% white, 19% Asian,

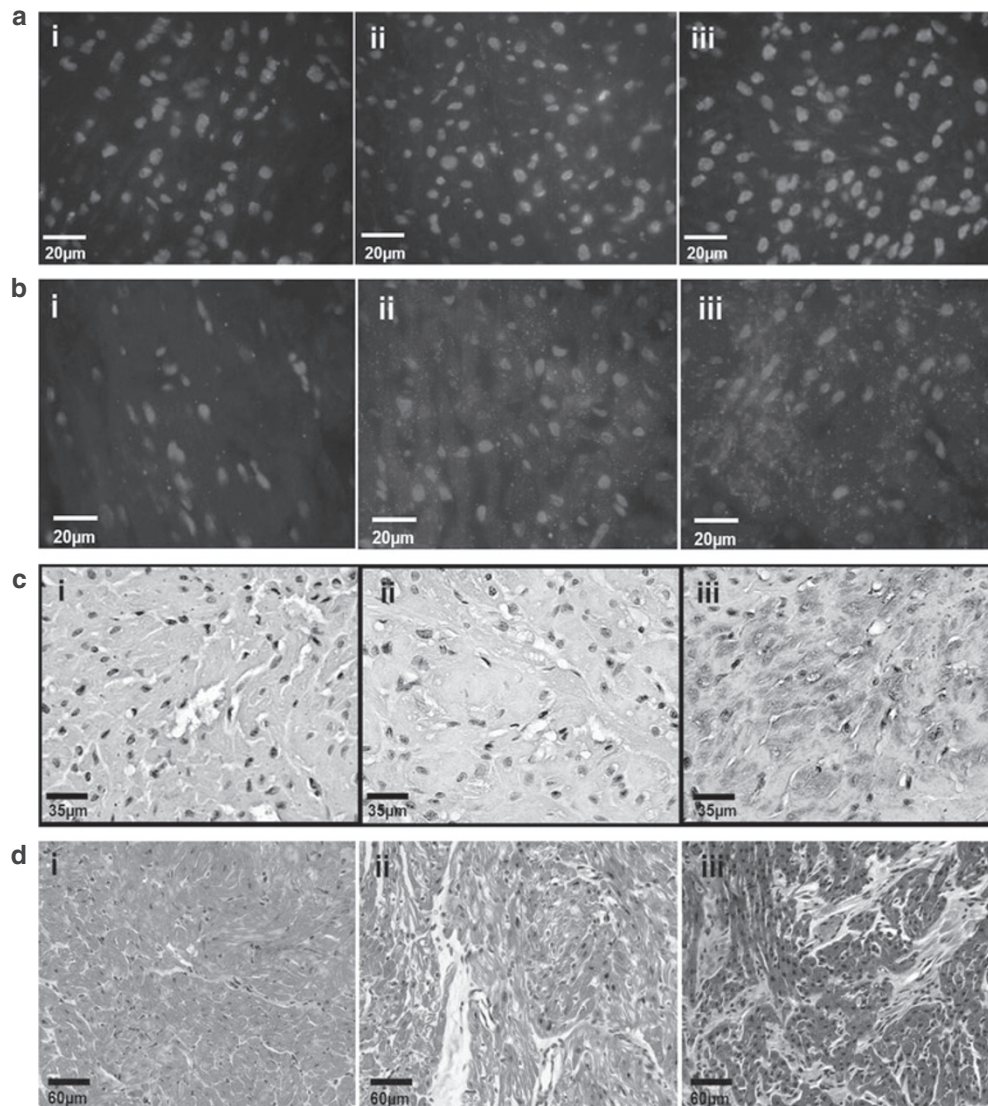


Figure 4. *HIF1A* genotype and RV histology. Representative images of RV immunostaining in RV from (i) unrepaired VSDs ($n = 20$), (ii) unrepaired TOF with fewer than four functioning *HIF1A* alleles ($n = 12$), and (iii) unrepaired TOF with four or more functioning alleles at complete repair ($n = 30$). DAPI represents blue nuclear staining. (a) Nuclear *HIF1A* expression (red) ($\times 600$) was stronger in TOF patients with four or more functioning alleles (iii) as compared with controls (i) or TOF patients with fewer than four alleles (ii). (b) Cytoplasmic VEGF expression (red) ($\times 600$) was higher in TOF patients as compared with controls but was not significantly different between genotype groups. (c) Cytoplasmic TGF β 1 expression (brown) ($\times 400$) was higher in TOF patients with four or more functioning alleles (iii) as compared with controls (i). (d) RV myocardial fibrosis area (blue) ($\times 200$) was higher in TOF patients with four or more functioning alleles (iii) as compared with controls (i) or TOF patients with fewer than four alleles (ii). DAPI, 4',6'-diamidino-2-phenylindole; *HIF1A*, hypoxia-inducible factor 1 α ; RV, right-ventricular; TGF β 1, transforming-growth factor β 1; TOF, tetralogy of Fallot; VEGF, vascular endothelial growth factor; VSD, ventricular septal defect.

7% black, and 6% other. Median age at repair was 0.58 y, and median age at enrollment was 5.4 y. Nineteen percent had an identifiable chromosomal anomaly or syndrome, 9% had a palliative shunt before complete repair, and 25% had a transannular patch repair. Seven patients (10%) underwent pulmonary valve replacement, and four (6%) underwent conduit replacement during follow-up. The frequencies of functioning *HIF1A* alleles in the replication cohort were comparable to those in the primary cohort: *HIF1A145T* (rs10873142) 74%, *HIF1A1326C* (rs2057482) 83%, and *HIF1A1744C* (rs11549465) 94%. All genotypes were in Hardy–Weinberg equilibrium. Linear regression analysis comparing patients with homozygous

HIF1A functioning genotypes to heterozygous or nonfunctioning genotypes revealed a protective effect, with patients homozygous for *HIF1A* functioning genotypes demonstrating less RV dilation as measured by RV end-diastolic z-score (Figure 5). Also, patients with three or more functioning alleles had a lower frequency of RV dysfunction as compared with those with fewer than three functioning alleles (0 vs. 16%, respectively, $P < 0.05$).

DISCUSSION

Our study identifies *HIF1A* signaling as an important determinant of myocardial adaptation to hypoxia before repair

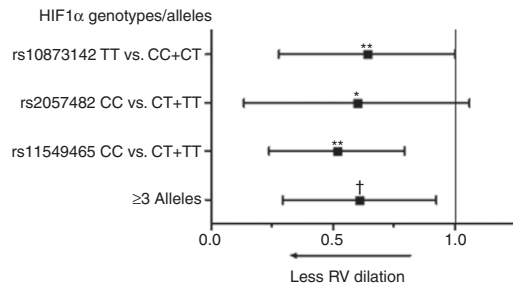


Figure 5. Replication cohort. The graph shows lower odds of right-ventricular (RV) dilation measured using RV end-diastolic z-score in patients homozygous for hypoxia-inducible factor 1α (*HIF1A*) functioning genotypes as compared with heterozygous or nonfunctioning genotypes. * $P = 0.012$ for rs2057482 (odds ratio (OR) 0.6, confidence interval (CI) 0.14–1.06); ** $P = 0.001$ for rs10873142 (OR 0.64, CI 0.28–0.99), and rs11549465 (OR 0.52, CI 0.24–0.79). Patients with three or more functioning *HIF1A* alleles had lower odds of RV dilation as compared with those with fewer than three functioning alleles. † $P < 0.001$ (OR 0.61, CI 0.29–0.92) ($n = 69$).

and to RV remodeling during postsurgical follow-up. Genetic variation in the *HIF1A* gene accounted in part for the inter-individual variability in this response. Patients with more *HIF1A*-functioning alleles had more RV fibrosis at the time of TOF repair but less progression of pulmonary insufficiency, less progression of RV dilation and dysfunction, and greater freedom from RV reinterventions after repair.

HIF1A Genotype and Early RV Phenotype in Unrepaired TOF

Until recently, evaluation of the causes of adverse RV remodeling after TOF repair have focused on anatomic and clinical factors, which have limited sensitivity in predicting which patients are likely to develop progressive RV dilation, dysfunction, and failure and need valve replacement. More recently, the emphasis has shifted to primordial factors that can affect the right ventricle before complete repair, i.e., during the fetal and neonatal periods. Of particular concern is the potential for ongoing hypoxia- and pressure overload-induced injury in the immature right ventricle while awaiting repair. This has led to the adoption of a neonatal repair strategy at several centers in an attempt to protect the RV during this vulnerable period, although the approach has had mixed results (11,12). Identifying which patients may truly benefit from early repair has been challenging. Our data show that patients with a higher number of functioning *HIF1A* alleles had persistent nuclear *HIF1A* expression, upregulation of the profibrotic cytokine TGFβ1, and RV outflow tract fibrosis in the unrepaired state. The ability to correlate genotype with tissue expression from the same patient is a unique strength of this study and provides biologic support for the observed genetic association. One could argue, on the basis of these findings, that chronic hypoxia and/or mechanical stress in the unrepaired state can have an unfavorable impact on the RV phenotype, especially in patients with *HIF1A*-upregulation genotypes, and may provide a marker for patients at-risk for injury, who would benefit most from early repair. However, we need to be cautious and should not interpret all fibrosis as detrimental, given that some fibrosis is reversible and may in fact represent a temporary

protective phenomenon during adverse environmental conditions such as hypoxia. The protective effect on RV remodeling and function on late follow-up indeed provides some support for the latter hypothesis.

HIF1A Genotype and Late RV Phenotype in Repaired TOF

The second important finding was cardioprotection in the form of slower progression of pulmonary insufficiency, less severe RV dilation on follow-up, and better preservation of RV systolic function in patients with a higher number of functioning *HIF1A* alleles in both the primary and replication cohorts. Not surprisingly, this was associated with greater freedom from RV reinterventions. Importantly, these associations persisted after correction for race, TOF subtype, age at repair, year of primary repair, type of repair, and duration of follow-up. Although, overall, this RV phenotype would be considered favorable because it delays the need for reinterventions, the susceptibility to fibrosis in the unrepaired state may suggest that the failure to dilate may be related to stiffer ventricles with more fibrosis. In a study of 92 adults, right and left ventricular fibrosis after TOF repair was common and was associated with ventricular dysfunction, exercise intolerance, and clinical arrhythmia (13,14). Alternatively, *HIF1A* signaling may have a true cardioprotective effect through reversible fibroblast transformation of stressed cells during hypoxia in infancy that results in better long-term myocardial adaptation to hemodynamic load. Unfortunately, given the difficulties in detecting myocardial fibrosis noninvasively and the challenges in quantifying diastolic dysfunction by echocardiography, RV fibrosis and diastolic function were not serially assessed during follow-up. Recently, it has become possible to detect regional myocardial fibrosis noninvasively using cardiac magnetic resonance imaging (MRI) with late gadolinium enhancement (13,14). A limitation of this technique, however, is that it does not detect early fibrosis, is qualitative, and relies on the difference in signal intensity between normal and scarred myocardium. Although this may be useful in evaluating regional fibrosis in patients with myocardial infarction, it appears to be less sensitive in patients with diffuse interstitial fibrosis (15,16). New genetic markers or biomarkers that can identify at-risk patients earlier than current imaging modalities therefore hold promise as risk-stratification tools.

In summary, *HIF1A* is important in the myocardial response to hypoxia before repair and in preserving RV morphology and preventing progressive RV remodeling late after TOF repair. Knowledge of the *HIF1A* genotype may therefore prove useful in identifying patients who are at risk for progressive RV remodeling after repair and who may benefit from earlier pulmonary valve replacement before the onset of significant RV dilation. Whether this subset of patients may also benefit from early neonatal TOF repair to limit hypoxia-induced RV injury remains to be seen. Our study supports the need for additional genomic studies to identify susceptibility genes that can be used to identify at-risk patients early and intervene before RV remodeling and dysfunction become irreversible.

Limitations

We did not assess diastolic dysfunction or severity of fibrosis during follow-up in this study. Although MRI imaging is emerging as an important noninvasive tool to assess fibrosis, its ability to detect early fibrosis is not known, as shown in a recent study of adult hypertrophic cardiomyopathy (15,16). Also, we did not analyze cardiac MRI assessment of pulmonary regurgitant fraction and RV volumes because MRI data were available in only a subset of patients. Our study was not powered to analyze the independent association of myocardial fibrosis with late RV remodeling.

Conclusion

HIF1A genotype may provide a useful adjunct to clinical predictors to identify TOF patients at risk for progressive RV remodeling after TOF repair. Prospective validation of our findings is required. Overall, our findings highlight the importance of identifying genetic modifiers in biologic pathways of RV adaptation as a means of early risk stratification and use of genotype-guided optimization of type and timing of interventions.

METHODS

Patients with TOF <18 y old were prospectively enrolled in the SickKids Heart Centre Biobank Registry, an Ontario province-wide research network aimed at studying the genomic and environmental basis of heart defects (2008–2011). Patients with TOF who had undergone complete repair and were followed at our institution were studied as the primary cohort. Patients with history of TOF repair from other Ontario hospitals were included as a replication cohort. Clinical data were obtained from a review of the medical records. The study was approved by the Hospital for Sick Children Institutional Research Ethics Board, and informed consent was obtained from the parents/legal guardians.

Genotyping Methods

A blood or saliva sample was obtained for DNA extraction at enrollment in all eligible patients. SNP assays were performed using Applied Biosystems' Taqman SNP genotyping technology (Assays-by-Design) (Life Technologies, Burlington, Ontario, Canada) for the following three SNPs: *HIF1A*145C/T in the 5' untranslated region (rs10873142) (TTAAAACCCCTAAATGTGACACAGTA[C/T]GCATGAGTGA TCATGCATCTCAAGA), *HIF1A*1326C/T (rs2057482) (CCTTTTTTTT GGACACTGGTGGCTCA[C/T]TACCTAAAGCAGTCTATTT ATATTT), and *HIF1A*1744C/T in the 3' untranslated region (rs11549465) (GTTACGTTCCCTTCGATCAGTTGTCA[C/T]CATT AGAAAGCAGTCCGCAAGCCC). "T", "C", and "C" alleles represent functioning alleles, whereas the single-nucleotide substitutions "C", "T", and "T" respectively represent loss-of-function alleles. These candidate SNPs were selected on the basis of previous association studies, known functional effects, allele frequency in the population, and prevalence in published medical literature (17). Loss-of-function SNPs have been associated with impaired adaptation to acute hypoxia or ischemia. However, the influence of these SNPs in chronic hypoxia is not known.

Tissue Studies

RV myocardial samples from the RV outflow tract were obtained at the time of complete repair from a subset of 42 patients with TOF and from 20 patients with isolated ventricular septal defects as controls. Immuno-histochemistry was performed on paraffin-embedded 5- μ mol/l sections with standard techniques using the following primary antibodies: HIF1A, VEGF, and TGF β 1 (Abcam, Cambridge, MA) (18). Immunofluorescence was performed for VEGF and HIF1A using Texas red-conjugated antimouse immunoglobulin G as secondary antibody (Sigma, St Louis, MO). Sections were counterstained with 4',6-diamidino-2-phenylindole. Images were captured using a Nikon Eclipse E1000 microscope (\times 600 magnification) and Openlab 4.02 software (PerkinElmer, Waltham, MA). A horseradish

peroxidase-conjugated streptavidin and di-amino benzidine method (Histostain Kit, Zymed Laboratories, Invitrogen, Carlsbad, CA) was used to evaluate TGF β 1 expression. Images were captured using a Leica DMLB2 microscope (\times 200 magnification). HIF1A expression was quantified in eight fields per section using a GSA image analyzer (GSA Bansemmer & Scheel, Rostock, Germany). The intensity of VEGF and TGF β 1 expression was semiquantitatively graded from 0 (none) to 4 (severe). Fibrosis area was evaluated on Masson's trichrome stain in six sections per sample (\times 200 magnification).

Echocardiographic Data

Serial echocardiographic data were obtained after TOF repair as part of a standardized clinical protocol. A total of 1,046 studies were analyzed, with an average of six per patient and an average time from repair of 4.6 y (maximum 16 y). Data were censored at the time of last follow-up or at pulmonary valve or conduit replacement. All patients underwent two-dimensional echocardiography using a standardized echocardiographic protocol used in the echocardiography laboratory in the Hospital for Sick Children (19). Data were stored on a clinical picture archiving and communication system (Syngo Dynamics, Siemens, Malvern, PA), and measurements were performed off-line by a single observer (A.J.). Echocardiographic analysis included qualitative assessment of the degree of pulmonary regurgitation, which was graded as trivial, mild, moderate, or severe on the basis of qualitative assessment. RV size was assessed using the RV outflow tract dimension and z-score as well as qualitatively with a grading of trivial, mild, moderate, or severe RV dilation. The RV dimensions were obtained by M-mode at the level of the mitral valve from a parasternal long-axis view. The z-scores were calculated on the basis of an institutional database of normal echocardiographic values. RV systolic function was assessed from the fractional area of change, which has been previously shown to correlate with MRI assessment of RV function (20). Fractional area change was derived from the RV end-diastolic area subtracted from the RV end-systolic area, divided by the RV end-diastolic area.

Statistical Analysis

Data are presented as means with SD, medians with minimum and maximum values, and frequencies as appropriate. Linear and logistic univariable regression models (adjusted for repeated measures with an autoregressive covariance structure for serial echocardiogram measurements) with maximum likelihood algorithms for parameter estimation were used to test for associations between genotypes and outcomes using an allelic model with a scale from 0 to 6 functioning alleles. All analyses were adjusted for race, TOF subtype, age at surgery, type of TOF repair, year of primary repair, and duration of follow-up (Table 1). The differences in outcomes between patients with and without risk alleles were reported as EST with 95% confidence intervals for linear regression and hazard ratio with 95% confidence interval for logistic regression models. All models with time-dependent outcomes were adjusted for duration of follow-up. Natural logarithmic transformations were applied to variables with skewed distribution. Tissue results were compared between TOF and ventricular septal defect samples and in TOF patients with fewer than four vs. four or more functioning alleles using Student's *t*-test. All statistical analyses were performed using SAS v.9.2 (SAS Institute, Cary, NC).

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