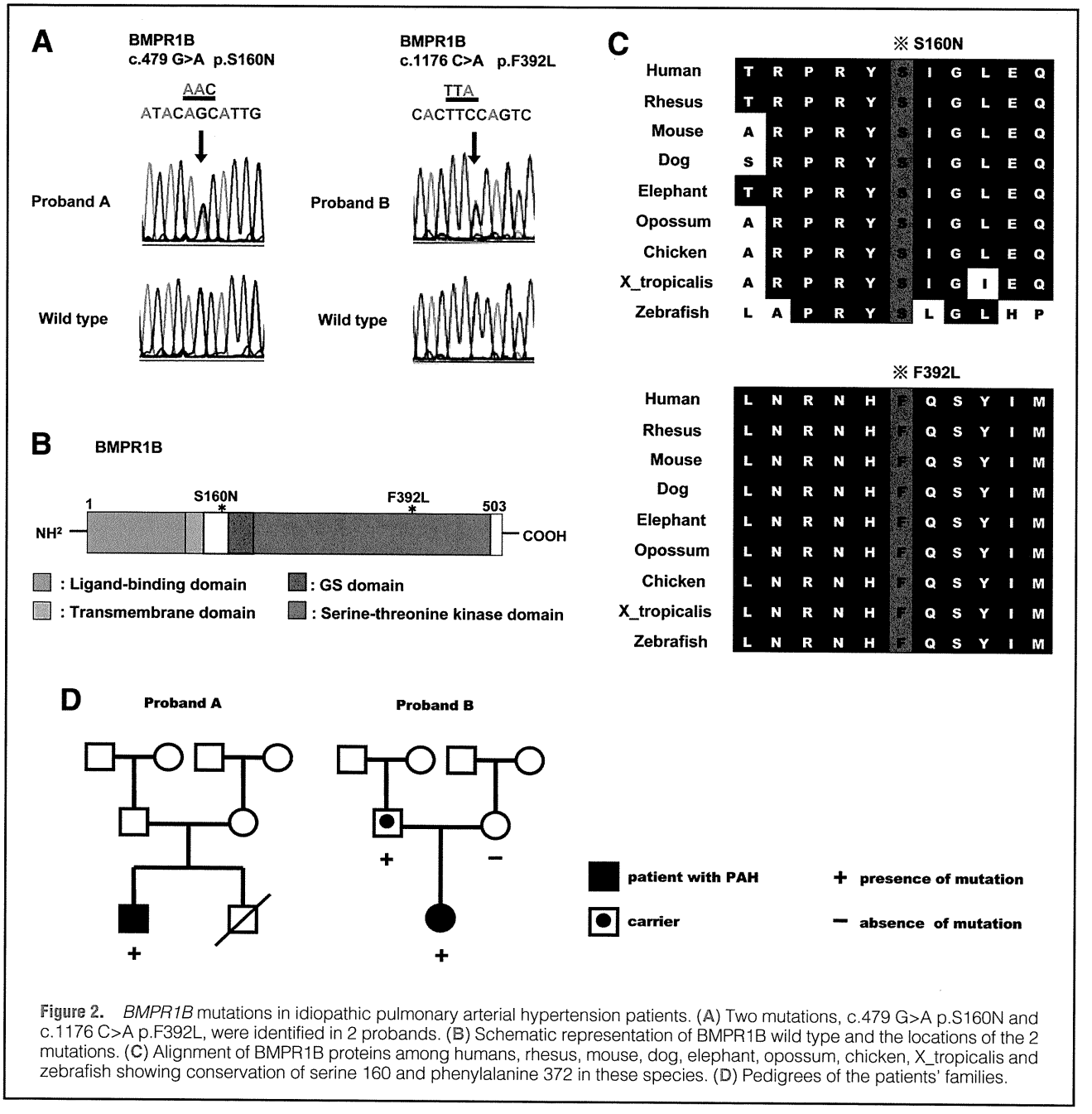

Figure 2. *BMPR1B* mutations in idiopathic pulmonary arterial hypertension patients. (A) Two mutations, c.479 G>A p.S160N and c.1176 C>A p.F392L, were identified in 2 probands. (B) Schematic representation of BMPR1B wild type and the locations of the 2 mutations. (C) Alignment of BMPR1B proteins among humans, rhesus, mouse, dog, elephant, opossum, chicken, X_tropicalis and zebrafish showing conservation of serine 160 and phenylalanine 372 in these species. (D) Pedigrees of the patients' families.

fade reagent with 46'-diamidino-2-phenylindole-2 HCl (DAPI) (Invitrogen). Fluorescence digital images were recorded with an LSM 5 PASCAL Laser Scanning Microscope (Carl Zeiss, New York, NY, USA).

Statistical Analysis

All results are expressed as mean±SD. For the statistical comparison of 2 samples, a 2-tailed Student's t-test was used where applicable. Values of $P<0.05$ were considered significant. Statistical analyses were performed using JMP for Windows (version 8; SAS Institute, NC, USA).

Results

Sequence Analysis

We screened mutations in *SMAD7*, *BMPR1A* and *BMPR1B* genes in 43 patients with IPAH/HPAH who had no mutations in *BMPR2*, *ALK1* and *SMAD8* (Figure 1). In this study, no mutations were identified in *SMAD7* and *BMPR1A*.

We identified, however, 2 *BMPR1B* missense mutations in 2 independent probands with IPAH. In proband A, c.479 G>A p.S160N was identified (Figure 2A). In proband B, c.1176 C>A p.F392L was identified (Figure 2A). As depicted in Figure 2B, BMPR1B consists of an extracellular ligand-binding domain, a transmembrane domain, an amino acid stretch involved in phosphorylation called GS domain and a serine-threonine kinase domain. Mutation S160N is located outside

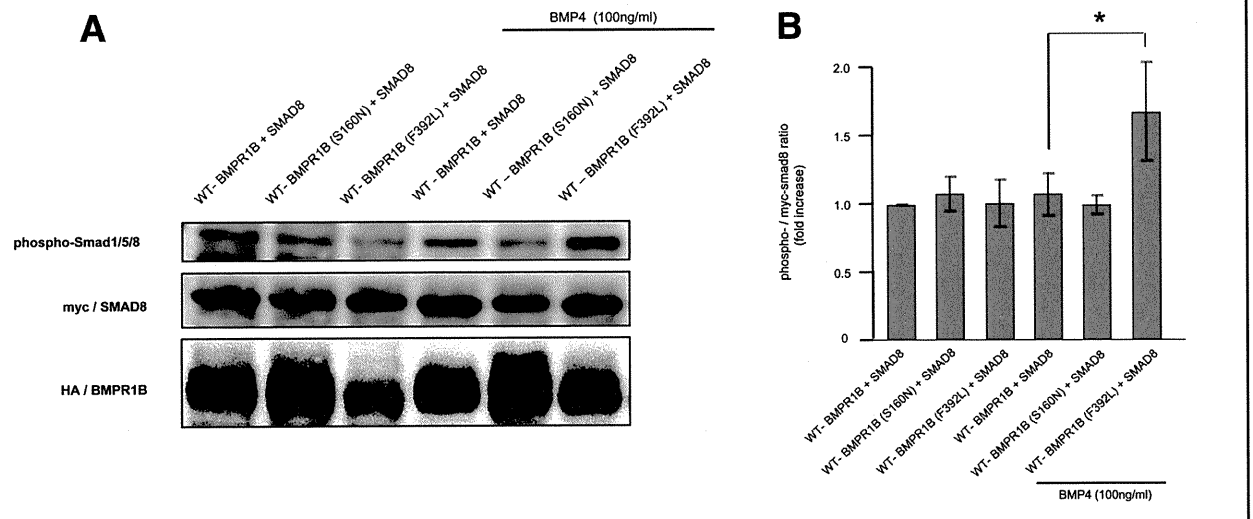

Figure 3. SMAD8 is strongly phosphorylated by BMPR1B F392L with BMP4 in Western blotting. (**A**) The addition of BMP4 induced SMAD8 phosphorylation in the presence of wild-type or mutant BMPR1B equally. Confluent cells were stimulated with 100 ng/ml BMP4 in DMEM/0.1% FBS for 60 min, followed by lysis for total protein. (**B**) The ratio of phospholylated-Smad8 densitometry to myc-Smad8 densitometry expressed in-fold increases in the presence of BMPR1B F392L with BMP4. Values represent mean ± SD of 3 independent experiments. Statistical differences between groups were assessed by using a Student's t-test. *P<0.05.

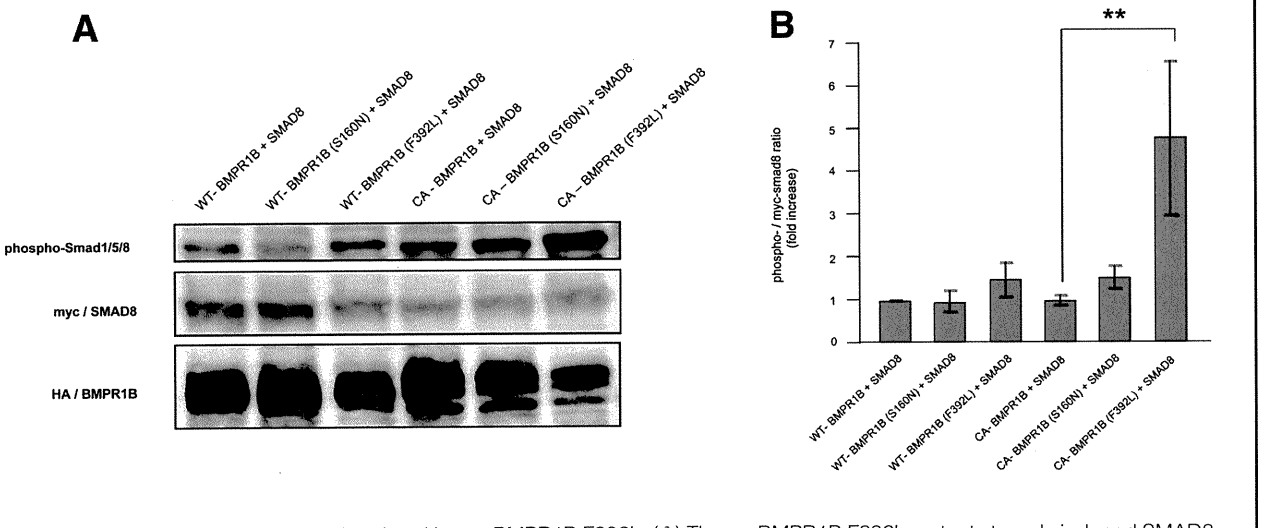

Figure 4. SMAD8 is strongly phosphorylated by ca-BMPR1B F392L. (**A**) The ca-BMPR1B F392L mutant strongly induced SMAD8 phosphorylation more than ca-BMPR1B and ca-BMPR1B S160N. (**B**) The ratio of phospholylated-Smad8 densitometry to myc-Smad8 densitometry expressed in-fold increases in the presence of ca-BMPR1B F392L. Values represent mean ± SD of 3 independent experiments. Statistical differences between groups were assessed by using a Student's t-test. **P<0.005.

of these functional domains, and mutation F392L is located in the serine-threonine kinase domain. The alignment of the BMPR1B protein between 9 distantly related species showed that these amino acids are highly conserved (Figure 2C).

Clinical Characteristics

Proband A (Patient No. 11) When the patient was 6 years old, right ventricular hypertrophy was identified by electrocardiography during a health examination and he was diagnosed with IPAH. The first symptom was mild dyspnea on exercise at 7 years of age. His hemodynamic data at 7 years of age revealed a mean pulmonary arterial pressure (mPAP) of 66 mmHg, right atrial pressure (RAP) of 12 mmHg and cardiac index (CI) of 2.0 $L \cdot min^{-1} \cdot m^{-2}$. His condition progressed to World Health Organization (WHO) functional class III at 7 years of age. He has been receiving epoprostenol, home oxygen therapy, a cardiotonic drug, vasodilator, anticoagulant and diuretics since the age of 7 years. His current condition is WHO functional class III at 17 years old. There is no family history of PAH (Figure 2D). His younger brother died of viral encephalitis at 2 years of age. The other family members have not been screened for *BMPR1B* mutations because their blood

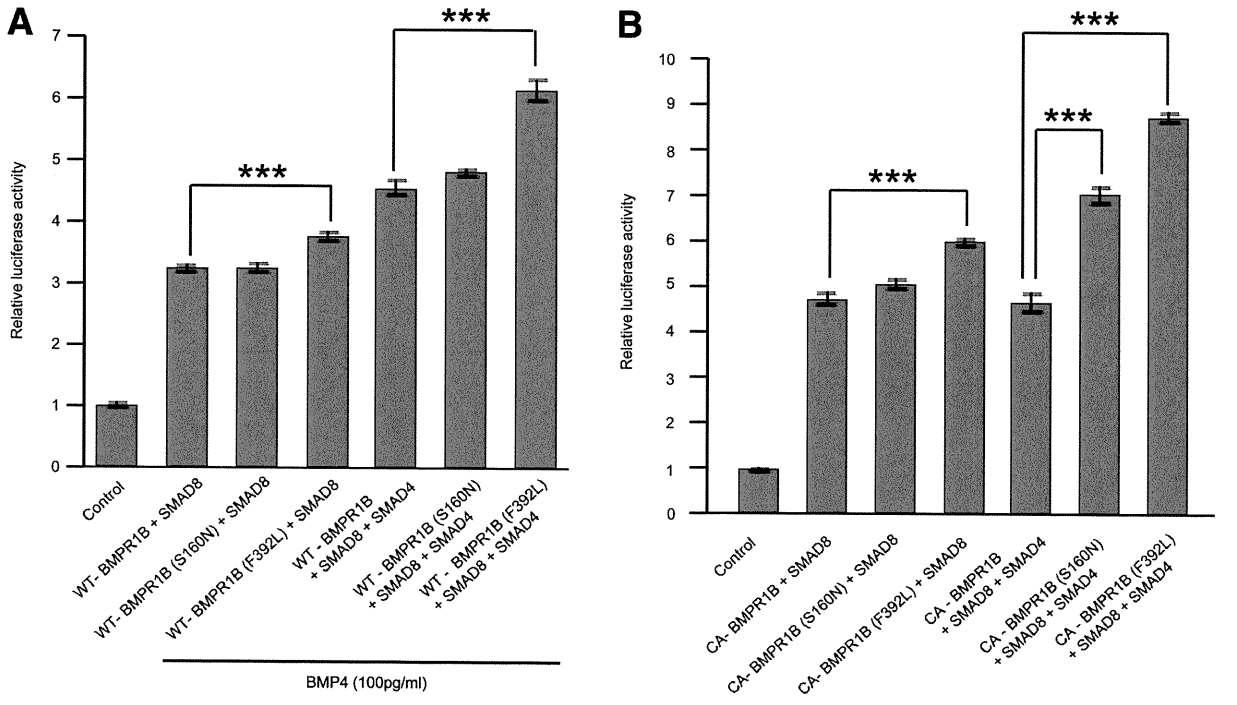

Figure 5. Luciferase activity induced by BMPR1B mutants. (A) Both ca-BMPR1B S160N with SMAD8 and ca-BMPR1B F392L with SMAD8 increased BMP responsive activity, and both ca-BMPR1B mutant with SMAD8 and SMAD4 induced higher activity than ca-BMPR1B with SMAD8 and SMAD4. (B) After stimulation with human BMP4 (100 pg/ml) for 12 h, BMPR1B F392L and SMAD8 with or without SMAD4 induced higher activity than wild-type BMPR1B and SMAD8 with or without SMAD4. Values represent mean ± SD of 3 independent experiments. Statistical differences between groups were assessed by using a Student's t-test. ***P<0.0001.

samples were not obtainable. He has no malformation of the digits, limbs and genitalia.

Proband B (Patient No. 18) This patient's first symptom was syncope at 12 years of age. Her hemodynamic data at 7 years of age revealed a mPAP of 111 mmHg, RAP of 11 mmHg, CI of 3.5 L·min^{-1}·m^{-2} and a pulmonary artery wedge pressure of 9 mmHg. Her WHO functional class was III at 13 years of age. She has been receiving epoprostenol, home oxygen therapy, vasodilator, anticoagulant and diuretics since the age of 13 years. Her current condition is WHO functional class II at 22 years old. There is no family history of PAH, but the patient's 39-year-old father was identified as having the same mutation (Figure 2D,S1). The patient's mother did not have the same mutation. The other family members have not been screened for *BMPR1B* mutations because their blood samples were not obtainable. She has no malformation of the digits, limbs and genitalia.

Western Blotting Analysis

The addition of BMP4 induced Myc-SMAD8 phosphorylation in the presence of wild-type or mutant BMPR1B. In addition, BMPR1B F392L with BMP4 increased Myc-SMAD8 phosphorylation more than wild-type BMPR1B and BMPR1B S160N (Figures 3A,B).

As shown in Figures 4A and B, additional Western blotting analysis revealed that the ca-BMPR1B F392L mutant induced Myc-SMAD8 phosphorylation more strongly than ca-BMPR1B and ca-BMPR1B S160N.

Luciferase Assay

We investigated the transcriptional activity mediated by wild-type or mutant BMPR1B and SMAD8 with or without SMAD4 to determine whether mutant BMPR1B could increase BMP-responsive promoter-reporter activity.

The first luciferase assay showed that, after stimulation with human BMP4, *BMPR1B* F392L and *SMAD8* with or without *SMAD4* induced higher activity than wild-type *BMPR1B* and *SMAD8* with or without *SMAD4* (Figure 5A).

The second luciferase assay showed that both ca-BMPR1B S160N with SMAD8 and ca-BMPR1B F392L with SMAD8 increased BMP responsive activity, and both ca-BMPR1B mutant with SMAD8 and SMAD4 induced higher activity than ca-BMPR1B with SMAD8 and SMAD4. In particular, ca-BMPR1B-F392L with SMAD8 and SMAD4 induced approximately 2-fold higher activity than ca-BMPR1B with SMAD8 and SMAD4 (Figure 5B).

Immunocytochemistry

COS1 cells transfected with the wild-type or mutant BMPR1B were subjected to immunofluorescence staining. Wild-type and 2 mutants exhibited intense and equal staining of plasma membrane and cytoplasm (Figure S2), suggesting that intracellular production and transportation of BMPR1B were not affected by these mutations.

Discussion

In this study, we first describe 2 missense mutations in *BMPR1B*

in IPAH patients. BMPR1B is a member of the BMP family that belongs to the TGF-β superfamily. The TGF-β/BMP signal pathway has 2 types of receptors. There are 7 type 1 receptors (ALK1, ALK2, BMPR1A known as ALK3, ALK4, ALK5, BMPR1B known as ALK6 and ALK7) and 5 type 2 receptors (ActR2A, ActR2B, TGF-β R2, AMHR2 and BMPR2).[25] BMPs bind independently to both type 1 and type 2 receptors. For example, BMP4 can bind to one of the type 1 receptors, BMPR1B, and one of the type 2 receptors, BMPR2. Upon ligand binding, the type 2 receptors phosphorylate and activate the type 1 receptors. The activated type 1 receptors propagate the signal by phosphorylating a family of transcription factors, called Smads. BMPR1B activates SMAD1, SMAD5 and SMAD8 by phosphorylation. These activated Smads complex with a common partner Smad, SMAD4, and accumulate in the nucleus where they interact with transcriptional regulators for target genes.[25,26]

BMPR1B mutations are known to be associated with brachydactyly type A2 and type C/symphalangism-like phenotype.[27,28] Acromesomelic chondrodysplasia with genital anomalies is also associated with *BMPR1B* mutations.[29] Four missense mutations (p.I200K, p.Q249R, p.R486Q and p.R486W) and 1 small deletion (c.del 359-366) have been reported to date.[27–29] In addition, functional analysis of 3 of 4 missense mutations revealed a loss of function in all.[27,28] However, both mutations that we identified in IPAH revealed a gain-of-function in our experimental condition.

It has been hypothesized that an imbalance of increased TGF-β levels and decreased BMP signals induced by *BMPR2* mutation leads to PAH.[30] In addition, ALK1 mutations in HHT revealed the down-regulation of SMAD1/5/8 signaling,[31] and SMAD8 nonsense mutation that we identified revealed loss of BMP signals.[14]

Our findings conflict with the above hypothesis because the *BMPR1B* mutations caused promotion of BMP signals. BMPR1B is expressed in human pulmonary arterial smooth muscle cells (PASMCs) at higher levels than in human microvascular endothelial cells and human pulmonary artery endothelial cells.[32] A previous report suggested that BMPR1B plays an important role in PASMC mitosis of PAH patients.[33] Quantification using real-time PCR revealed that the BMPR1B expression in PASMCs of PAH patients was more than 10-fold higher than in PASMCs of controls.[33] This report suggests that BMP signals through BMPR1B are promoted in PASMCs of IPAH patients. Because BMPR1B S160N and F392L promoted BMP signals in the present study, they might be associated with the pathogenesis of PAH. Furthermore, another study reported that disruption of BMPR2 led to diminished signaling by BMP2 and BMP4, and augmented signaling by BMP6 and BMP7 in PASMCs.[34] Thus, not only inhibition but also promotion of BMP signals might be associated with the onset of PAH.

In this study, 1 *BMPR1B* mutant, F392L, strongly induced SMAD8 phosphorylation and increasing transcriptional activation in the presence of SMAD8 or SMAD8/SMAD4. In contrast, another mutant, S160N, did not induce them so markedly. This difference of effect might depend on the position of mutation in the *BMPR1B* gene. Mutation F392L is located in one of the functional domains, the serine-threonine kinase domain, but mutation S160N is located outside of the functional domains. However, mutation S160N might also cause gain-of-function because ca-*BMPR1B* S160N with *SMAD8* and *SMAD4* induced higher activity than ca-*BMPR1B* with *SMAD8* and *SMAD4* in the luciferase assay. We guess that coexistence with *SMAD8* and *SMAD4* is more physiological than with *SMAD8* only in a living body, so these results might be appropriate. Several studies have revealed that BMPR2 mutations that are located outside of the functional domains were identified in IPAH/HPAH patients, PVOD patients and associated-PAH patients.[4,35–37] There might be an unknown special function outside of the already-known functional domains in both BMPR2 and BMPR1B. Further functional analysis on mutant S160N is needed.

The age at onset of our patients with a *BMPR1B* mutation was younger than that of other IPAH patients. It was difficult to identify other differences in their phenotypes. The father of Proband B has the same *BMPR1B* mutation, but he has no clinical signs of PAH to date. This is not surprising because *BMPR2* mutations have very low penetrance in familial PAH.

It is necessary to study an increased number of subjects to investigate the phenotype of PAH with *BMPR1B* mutation in detail. In addition, to undertake further analysis of the function of *BMPR1B* in the pathogenesis of PAH, further investigations using human PASMCs and/or animal models with *BMPR1B* mutation will be necessary.

Acknowledgments

We are grateful to the patients and their family members. We thank Dr Kohei Miyazono for providing the plasmids. We thank Dr Hisato Yagi, Dr Yoshihisa Matsushita and Dr Maya Fujiwara for their excellent technical assistance. We also thank Dr Rumiko Matsuoka for her supervision.

Funding Sources

None.

Disclosures

None.

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Supplementary Files

Supplementary File1

Table S1. Baseline Characteristics and Hemodynamic Parameters of 43 IPAH/HPAH Patients

Figure S1. Sequence analysis of *BMPR1B* mutation in the family of proband B.

Figure S2. Subcellular distribution of wild-type BMPR1B, BMPR1B S160N and BMPR1B F392L in COS1 cells.

Please find supplemental file(s);
http://dx.doi.org/10.1253/circj.CJ-11-1281

Advance Publication by J-STAGE

Circulation Journal
Official Journal of the Japanese Circulation Society
http://www.j-circ.or.jp

Genetic Analysis of Essential Cardiac Transcription Factors in 256 Patients With Non-Syndromic Congenital Heart Defects

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Background: The genetic basis of most congenital heart defects (CHDs), especially non-syndromic and non-familial conditions, remains largely unknown.

Methods and Results: DNA samples were collected from immortalized cell lines and original genomes of 256 non-syndromic, non-familial patients with cardiac outflow tract (OFT) defects. Genes encoding NKX2.5, GATA4, GATA6, MEF2C, and ISL1, essential for heart development, were analyzed using PCR-based bidirectional sequencing. The transcriptional activity of proteins with identified sequence variations was analyzed using a luciferase assay. A novel sequence variant (A103V in *MEF2C*) was identified, in addition to 4 unreported non-synonymous sequence variants in 3 known causative genes (A6V in *NKX2.5*, T330R and S339R in *GATA4*, and E142K in *GATA6*) in 5 individuals. None of these was found in 500 controls without CHDs. In vitro functional assay showed that all proteins with identified sequence variations exhibited significant changes in transcriptional activity and/or synergistic activity with other transcription factors. Furthermore, overexpression of the A103V MEF2C variant in a fish system disturbed early cardiac development.

Conclusions: New mutations in the transcription factors NKX2.5, GATA4, GATA6, and MEF2C that affect their protein function were identified in 2.3% (6/256) of patients with OFT defects. Our results provide the first demonstration of *MEF2C* mutation and suggest that disturbances in the regulatory circuits involving these cardiac transcription factors may cause a subset of non-syndromic and non-familial CHDs.

Key Words: Congenital heart defects; Genetics; Genotype; Pediatrics; Screening

Congenital heart defects (CHDs) account for the majority of human birth defects, with an incidence of 4–10 per 1,000 live births, and are the leading non-infectious cause of mortality in newborns.[1] Despite their clinical importance, little is known about the genetic basis of CHDs.

Results from recent studies in animal models suggest that changes in the functioning of transcription factors essential for cardiac progenitor lineages (eg, NKX2.5, MEF2C, ISL1, T-box, and GATA families) may result in various CHDs.[2–11] These transcription factors exhibit overlapping expression patterns, to some degree, and intricate cross-talk during heart development, suggesting that they constitute a core regulatory network for normal cardiac morphogenesis, and making them good candidates as the genetic cause of variable CHDs.[12–15]

Based on knowledge gained from linkage analyses and animal models, causative genes have been identified for several syndromes and/or familial conditions. For example, Basson et al identified mutations in *TBX5*, as autosomal dominant traits, that are frequently associated with atrial septal defects (ASD), ventricular septal defects (VSD), and upper limb defects in Holt-Oram syndrome.[16] Others have demonstrated that *TBX1* is a major genetic determinant of CHDs associated with 22q11.2 deletion syndrome.[17] In non-syndromic conditions, using linkage analysis of familial cases, Scott et al have iden-

Received November 30, 2011; revised manuscript received March 5, 2012; accepted March 12, 2012; released online April 13, 2012
Time for primary review: 6 days

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ISSN-1346-9843 doi:10.1253/circj.CJ-11-1389

tified mutations in *NKX2.5* in four families with ASD and atrioventricular conduction disturbance,[18] whereas Garg et al have identified mutations in *GATA4* in 2 families with ASD and VSD.[19] However, the genetic basis of most types of CHDs is yet to be determined because the overwhelming majority of CHDs are non-syndromic with no segregation in Mendelian ratios.

Recently, we identified 2 mutations in *GATA6* by screening DNA from 21 non-syndromic patients with persistent truncus arteriosus (PTA), which is the most severe outflow tract (OFT) defect.[20] We also demonstrated a molecular basis for the pathogenesis of PTA, whereby mutations in *GATA6* resulted in a disturbance of direct regulation of semaphorin 3C (SEMA3C) and plexin A2 (PLXNA2), which mediate essential neurovascular signaling in the development of the OFT.[20] To further explore the genetic causes of non-syndromic and non-familial CHDs, we undertook a genetic analysis of selected essential cardiac transcription factors, focusing on lesions involving the OFT. OFT defects constitute approximately 30% of CHDs[21] and are often difficult to repair surgically because of complex morphological abnormalities, resulting in an unfavorable prognosis. In the present study, we report our findings in 256 non-syndromic patients with OFT defects.

Methods

A detailed description of the methodology used is available as online supplemental information for this paper (Data S1).

Mutation Analysis and Clinical Evaluation of Patients

The establishment of a genomic bank with different cell lines and the extraction of genomic DNA samples have been described previously.[22] All exons and flanking introns of *NKX2.5*, *GATA4*, *GATA6*, *MEF2C*, and *ISL1* were amplified by polymerase chain reaction (PCR) and were sequenced using direct, bidirectional sequencing (Data S1). The mutations identified were subsequently confirmed using original genomic DNA extracted from the patients' peripheral blood leukocytes. Primer sequences are given in Table S2. In all, 256 samples were collected from patients with CHDs involving OFT defects. Phenotypic data for the affected individuals and their family members were obtained from detailed clinical evaluations based on echocardiography, cardiac catheterization, and/or surgical findings. Genomic samples from available family members, 206 healthy Japanese volunteers with no CHDs (controls), and 294 samples of control genomic DNA (BioChain, Hayward, CA, USA) were analyzed using the same protocol. This investigation conformed with the principles outlined in the Declaration of Helsinki, and the clinical evaluations and genetic studies of the patients and their families were approved by the Internal Ethics Committee of Tokyo Women's Medical University and were undertaken only after the patients and their family members had provided informed consent.

Plasmid Construction and Site-Directed Mutagenesis

Site-directed mutagenesis was performed using a QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA) according to the manufacturer's instructions. *GATA6*-pcDNA3.1,[20] *NKX2.5*-pcDNA3.1, *GATA4*-pcDNA3.1 or *Mef2c*-pcDNA were used for mutagenesis. *Mef2c*-pcDNA was kindly provided by Dr Brian Black (Cardiovascular Research Institute, University of California, San Francisco, CA, USA). Mouse Mef2c wild-type (WT) and A103V mutant cDNA were subcloned into a pCS2+ plasmid.[23] All vectors constructed were verified by sequencing.

Luciferase Assay

HeLa cells were transfected using Lipofectamine LTX and Plus reagent (Invitrogen, Carlsbad, CA, USA) with 400 ng reporter vector, 800 ng expression vectors, and 0.25 ng pSV-*Rluc* internal control vector, as described previously.[20] Luciferase activity was measured 36 h after transient transfection by using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA) according to the manufacturer's instructions. Experiments were repeated at least 3 times.

Co-Immunoprecipitation Assays

COS-1 cells were transiently transfected with lipofectamine LTX (Invitrogen), collected after 48 h in lysis buffer (Tris-buffered saline (TBS), 0.4% Nonidet P-40 (NP-40), and EDTA-free complete protease inhibitor cocktail; Roche Diagnostics, Basel, Switzerland), incubated with monoclonal anti-FLAG M2 antibody (Sigma-Aldrich, St. Louis, MO, USA) and Dynabeads protein G beads (Invitrogen), immunoblotted with monoclonal anti-GATA6 (R&D systems, Minneapolis, MN, USA) or monoclonal anti-FLAG M2 antibodies, and detected by western blotting.

Overexpression of Mef2c mRNA in Medaka Fish

The plasmid mouse *Mef2c* WT or A103V mutant-pCS2+ was digested with Not I, and RNA samples of the *Mef2c* with polyA were prepared using the mMessage mMachine Kit (Invitrogen) for injections. Medaka embryos were fertilized in vitro and injected at the 1-cell stage (215 eggs) as previously described.[24] Pressure was adjusted to inject approximately 1 nl at a concentration of 0.2 μg/μl. Injected embryos were transfered into heat type of container where they were held at 28.5 degrees celsius. Embryos were observed, scored daily, and investigated for cardiac phenotypes at 5 days post-fertilization.

Statistical Analysis

For luciferase assays, all experiments were performed at least in triplicate and data are reported as normalized relative light units (fold activation) together with the SEM. For promoter activity assays, all experiments were performed at least in triplicate and data are reported as the ratio of normalized relative light units for coexpression with NKX2.5, GATA4, GATA6, or Mef2c to that with mock (pcDNA3.1). In all Figures, error bars indicate the SEM. Data were analyzed by 2-tailed unpaired t-test or chi-square test. $P \leq 0.05$ was considered significant. For non-synonymous GATA4 and GATA6 nucleotide changes found in patients and controls, frequencies were compared between patients and controls by chi-square test. $P \leq 0.05$ was considered significant.

Results

Novel Sequence Variants of Genes Encoding Cardiac Transcription Factors Identified in Patients With Non-Syndromic CHDs

In our genetic analysis of selected essential cardiac transcription factors (*NKX2.5*, *GATA4*, *GATA6*, *MEF2C*, and *ISL1*) in 256 patients with non-syndromic OFT defects, including 125 with tetralogy of Fallot (TOF), 84 with pulmonary atresia with VSD, 23 with double outlet the right ventricle, and 24 with PTA, we identified 5 new sequence variants in 6 individuals (6/256; 2.3%). These variants included an *NKX2.5* variant (A6V), 2 *GATA4* variants (T330R and S339R), a *GATA6* variant (E142K), and a *MEF2C* variant (A103V; Figure 1) that were not found in the 500 normal controls without CHDs, suggesting that these variants are potentially involved in the etiol-

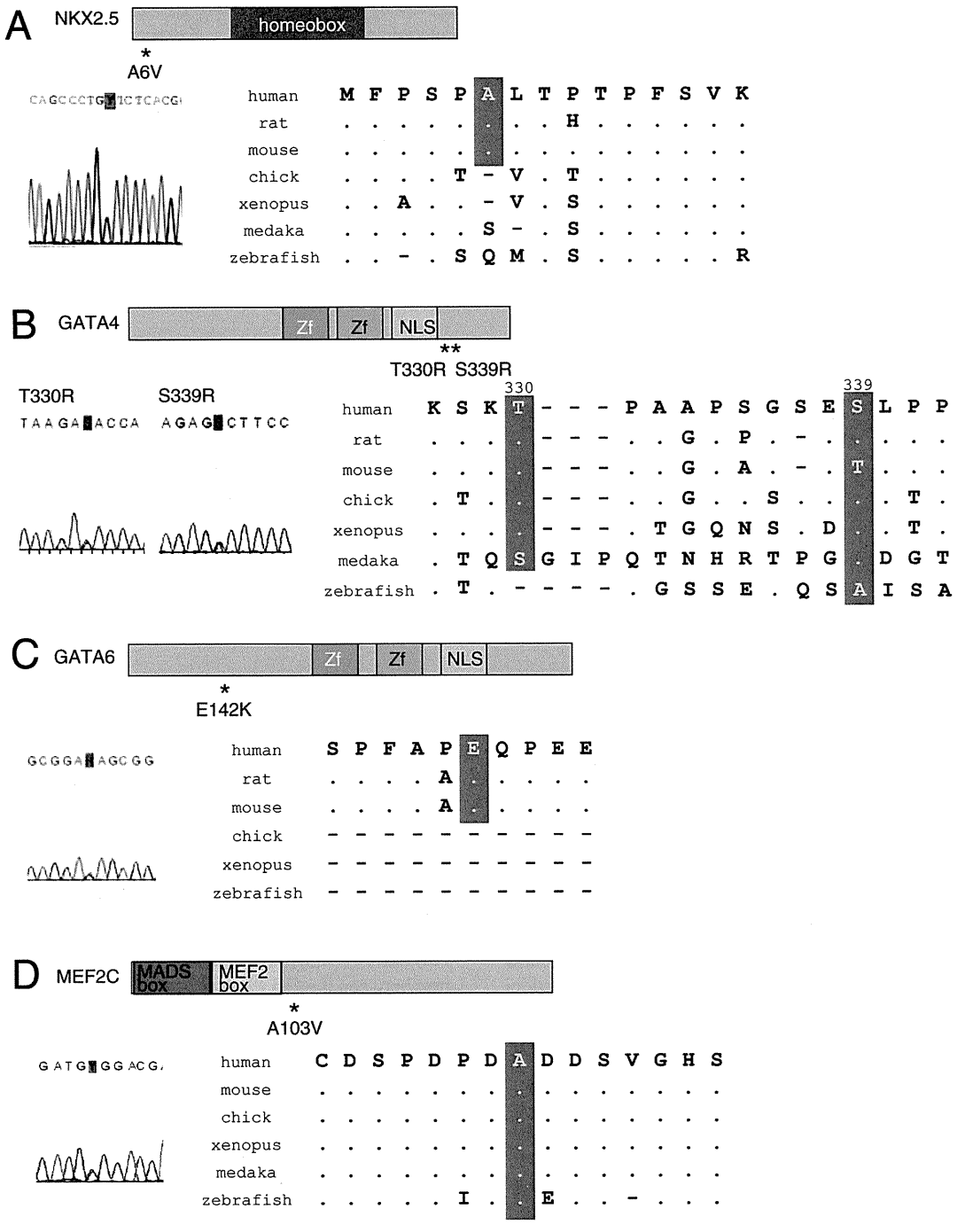

Figure 1. Identification of novel sequence variants in cardiac transcription factors in patients with outflow tract (OFT) defects. The structures of the human NKX2.5 (A), GATA4 (B), GATA6 (C) and MEF2C (D) genes, the positions of the novel variants (*), and the conservation of amino acids between species are shown. Zf, zinc finger; NLS, nuclear localization signal. Changes in amino acids are highlighted in red.

ogy of OFT defects. The results of the genetic analyses and the associated phenotypes are summarized in Table 1. As indicated in Table 1, the *GATA6* variant E142K was found in 2 unrelated patients with OFT defects whose phenotypes were somewhat variable. We also identified non-synonymous variations in the coding regions of *GATA4* and *GATA6* in both patients and controls (Table 2), as well as numerous known sequence polymorphisms in all genes, although none of these non-synonymous sequence variants showed significant difference in the frequency between patients and controls. We did not find any new sequence variants of *ISL1* in our patient group.

Table 1. Unique Non-Synonymous Sequence Variants Identified in Patients With Congenital Heart Defects

Gene	GenBank accession number	Patient no.	Nucleotide change	Amino acid change	Transcriptional activation	Cardiac phenotype	Extracardiac anomalies	Race	Sex
NKX2.5	NM_001166175	1	17C>Y	A6V	Loss of function	TOF	None	Japanese	Male
GATA4	NM_002052	2	989C>S	T330R	Loss of function	PTA	None	Japanese	Female
		3	1017C>S	S339R	Loss of function	PAVSD	None	Japanese	Female
GATA6	NM_005257	4	424G>R	E142K	Loss of function	PAVSD, MAPCA	None	Japanese	Female
		5	424G>R	E142K	Loss of function	DORV, VSD, PS, dextrocardia	None	Japanese	Male
MEF2C	NM_002397	6	308C>Y	A103V	Gain of function	PAVSD	None	Japanese	Female

TOF, tetralogy of Fallot; PTA, persistent truncus arteriosus; PAVSD, pulmonary atresia with ventricular septal defect (VSD); MAPCA, major aorticopulmonary collateral arteries; DORV, double outlet right ventricle; PS, pulmonary stenosis.

Table 2. Non-Synonymous GATA4 and GATA6 Nucleotide Changes Found in Patients and Controls

Gene	Nucleotide change	Amino acid change	No. of alleles with nucleotide changes		Significance*	Reference SNP
			Patients	Controls		
GATA4	1128C>S	H376Q	2/256	1/500	NS	rs116414842
	1138G>R	V380M	5/256	6/500	NS	rs114868912
	1220C>M	P407E	8/256	18/500	NS	rs115099192
GATA6	43G>S	G15R	38/256	30/500	NS	Unreported
	151G>R	E51K	1/256	1/519	NS	Unreported
	551G>R	S184N	3/256	4/500	NS	Unreported
	584G>R	G195A	1/256	1/500	NS	Unreported

NS, not significant.
*Statistical significance in frequency between patients and controls.

Functional Analyses of Cardiac Transcription Factors With the Identified Sequence Variations

To examine the clinical relevance of the identified sequence variants to non-syndromic OFT defects, luciferase assays were performed to evaluate the transcriptional activity of each factor exhibiting a sequence variation. First, we analyzed basal transcription activities using the common cardiac-specific promoter *NPPA*. The NKX2.5 variant A6V exhibited a 50% decrease in transcription activity compared with the WT protein (Figure 2A; P=0.0066, 2-tailed unpaired t-test; n=3). In addition, this variant exhibited a significant decrease in synergistic effects when it was cotransfected with *GATA4* compared with WT *NKX2.5* cotransfected with *GATA4* (Figure 2A; P=0.011, 2-tailed unpaired t-test; n=3).

The 2 GATA4 variants T330R and S339R exhibited significant decreases in synergistic effects on the *NPPA* promoter compared with WT GATA4 when they were cotransfected with *NKX2.5* (Figure 2B; P=0.015 and P=0.026, respectively 2-tailed unpaired t-test; n=3), although, alone, neither variant exhibited any significant decrease in transcription activity on the *NPPA* promoter. The GATA4 variant S339R also exhibited a significant decrease in its synergistic effect with GATA6 (Figure 2B; P=0.044, 2-tailed unpaired t-test; n=3), whereas the GATA4 variant T330R did not.

The GATA6 variant E142K exhibited significantly decreased transcriptional activity on the *NPPA* promoter (Figure 2C; P=0.0045, 2-tailed unpaired t-test; n=3). Furthermore, the GATA6 variant E142K exhibited significantly decreased synergistic activity with GATA4 on the *NPPA* promoter compared with WT GATA6 (Figure 2C; P=0.0010, 2-tailed unpaired t-test; n=3). In addition to synergistic effects between GATA6 and GATA4 on the *NPPA* promoter, we also found synergistic effects for GATA6 with TBX5 on the *NPPA* promoter (Figure 2C; P=0.034 for GATA6 WT with TBX5 v. GATA6 alone; P=0.031 for TBX5 alone, 2-tailed unpaired t-test; n=3). Furthermore, results from the co-immunoprecipitation assay revealed that the GATA6 protein was able to physically associate with the TBX5 protein (Figure 2D; WT), suggesting that the strong synergistic effect on the expression of *NPPA* was due to a direct interaction between GATA6 and TBX5. The GATA6 variant E142K exhibited significantly decreased synergistic activity with TBX5 on the *NPPA* promoter compared with WT GATA6 (Figure 2C; P=0.00092, 2-tailed unpaired t-test; n=3), although the interaction between the GATA6 variant E142K and TBX5 was unaffected (Figure 2D; E142K). Moreover, compared with WT GATA6, the GATA6 variant E142K exhibited significantly decreased transcription activity on the *SEMA3C* promoter (Figure 2E; P=0.029, 2-tailed unpaired t-test; n=3) and the *WNT2* promoter (Figure 2F; P=0.040, 2-tailed unpaired t-test; n=3), both of which are downstream targets of GATA6.

Although no disease-associated mutation in *MEF2C* has been reported to date, we identified a non-synonymous *MEF2C* sequence variant (A103V) in the present study. A recent animal study reported that Mef2c directly regulates *Smyd1*, which is essential for early cardiomyocyte differentiation during development of the OFT.[25] To investigate whether the *MEF2C* variant A103V results in a disruption of *Smyd1* regulation, we used a *Smyd1* promoter that included an Mef2c-dependent consensus sequence[25] (Figure 2G) in our luciferase assay. The results suggest that the MEF2C variant A103V has a significant gain-of-function activity on the *Smyd1* promoter compared with WT MEF2C (Figure 2H; P=0.040, 2-tailed unpaired t-test; n=3). The results of all functional analyses are summarized in Table 1.

Abnormal Heart Development in Medaka Fish With Overexpression of Mef2c

MEF2C is a highly conserved protein across species (Figure 3A)

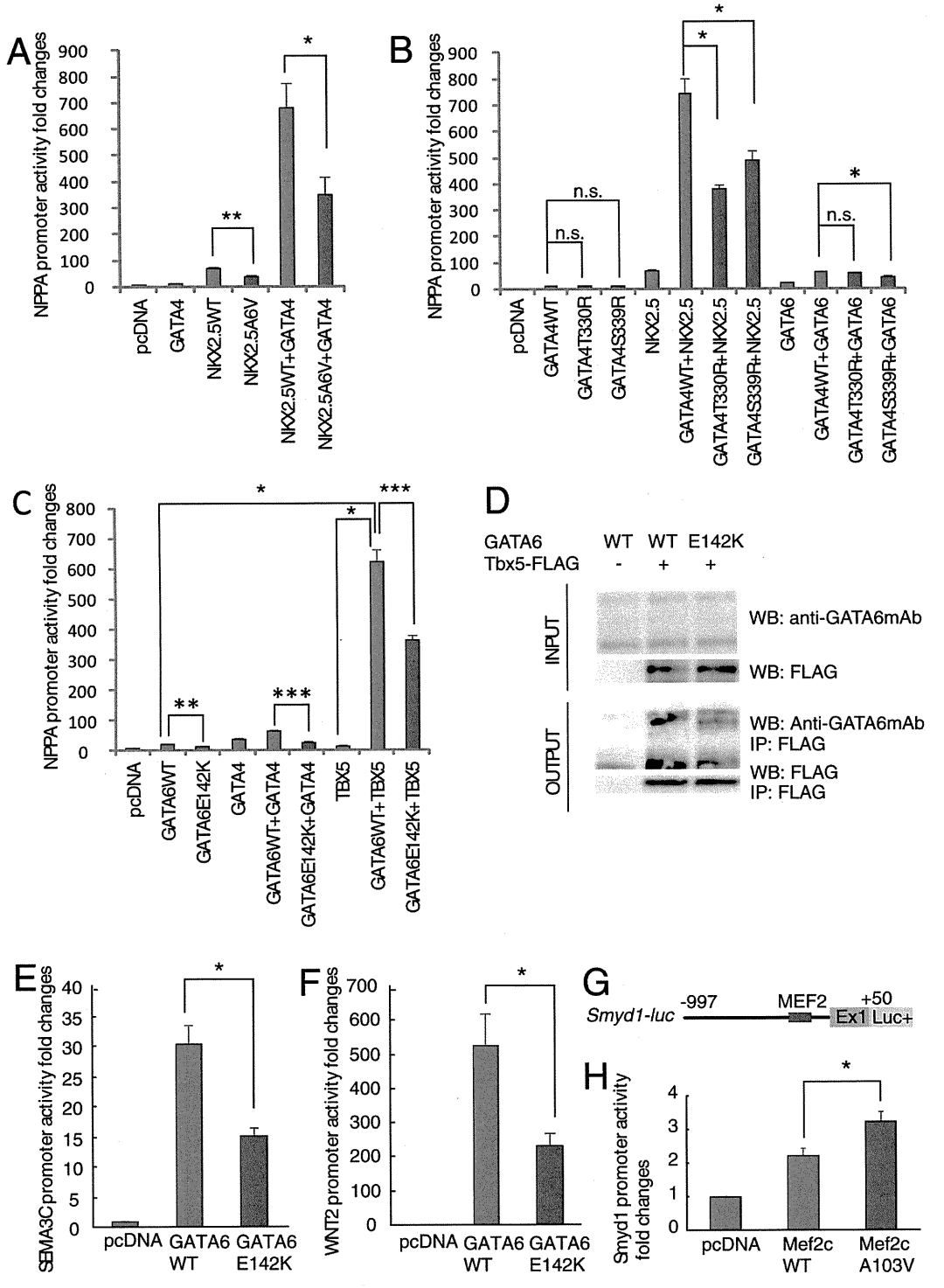

Figure 2. Transcriptional activities of proteins with identified sequence variants. (**A**) Relative luciferase activity in HeLa cells transfected with NKX2.5 wild-type (WT) or NKX2.5 variant A6V expression constructs and *NPPA-luc* with or without coexpression of GATA4 WT. (**B**) Relative luciferase activity in HeLa cells transfected with GATA4 WT or the GATA4 variants T330R or S339R expression constructs and *NPPA-luc* with or without coexpression of NKX2.5 WT or GATA6 WT. (**C**) Relative luciferase activity in HeLa cells transfected with GATA6 WT or GATA6 mutant (E142K) expression constructs and *NPPA-luc* with or without coexpression of GATA4 WT or TBX5. (**D**) Interaction between TBX5 and GATA6 WT or mutant protein (E142K). Co-immunoprecipitation of GATA6 demonstrates an association of both WT and E142K with FLAG-tagged TBX5. (**E**,**F**) Relative luciferase activity in HeLa cells transfected with GATA6 WT or GATA6 mutant (E142K) expression constructs and *SEMA3C-luc* (**E**) or *WNT2-luc* (**F**). (**G**) Structure of *Smyd1-luc* with a consensus MEF2 binding site. Genomic organization of a 1-kb section of the 5′ end of the mouse *Smyd1* locus subcloned upstream of *luc*+ reporter gene. (**H**) Relative luciferase activity in HeLa cells transfected with Mef2c WT or Mef2c mutant (A103V) expression constructs and *Smyd1-luc*. *P<0.05, **P<0.01, ***P<0.005.

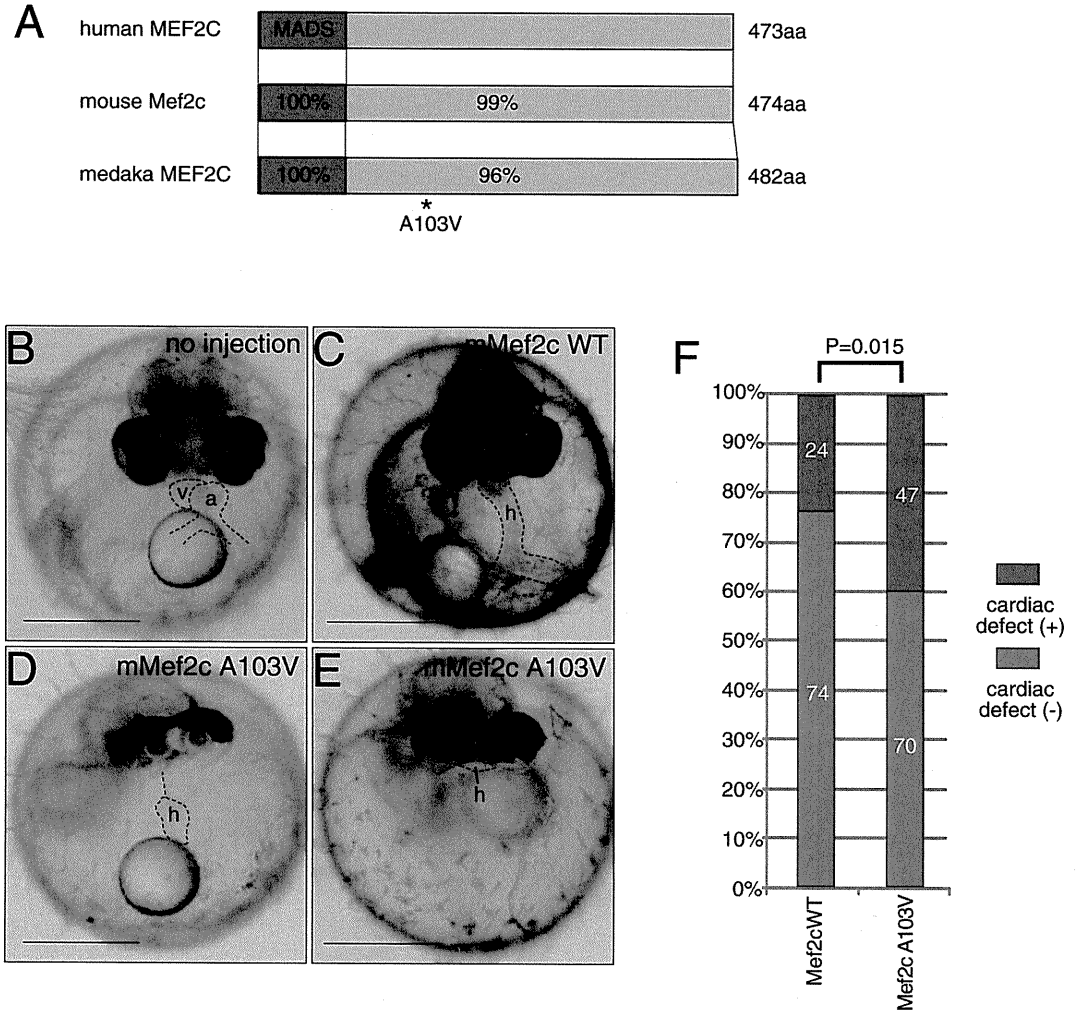

Figure 3. Overexpression assay of Mef2c in medaka. (**A**) Conservation and similarity of MEF2C among human, mouse and medaka. Red box indicates MADS box domain and percentage shows amino acid similarity across species. (**B–E**) Frontal view of medaka embryos at 5 days after fertilization. The heart morphogenesis is shown in illustration to see the phenotype of cardiac development in each embryo. In control embryos without injection, atrium (a) and ventricle (v) are segmented in the looped heart tube (**B**). On the other hand, cardiac formation was disturbed in medaka with Mef2c wild-type (WT; **C**) or A103V mRNA injection (**D**,**E**). h, premature heart. Scale bars=500μm. (**F**) The frequency of abnormal cardiac phenotype in medaka embryos injected with Mef2c WT and A103V mutant. Numbers of observed embryos are indicated in white. Medakas injected with Mef2c A103V mRNA showed abnormal cardiac phenotype more frequent than those injected with Mef2c WT (P=0.015, chi-square test).

and alanine 103 is well conserved (**Figure 1D**). In order to assess the gain-of-function of *MEF2C* A103V variant protein during heart development in vivo, we used a fish system. Overexpression of Mef2c WT or A103V protein in medaka by mRNA injections showed a range of abnormal cardiac development (**Figures 3B–E**; **Movies S1,S2**). Approximately 25% of embryos injected with WT Mef2c and 40% of embryos injected with A103V variant Mef2c developed cardiac defects ranging from disorganized cardiac chamber formation in the straight heart tube to ectopic beating tissues with no formation of the heart tube, suggesting that overexpression of Mef2c may disturb early cardiac development. Consistent with A103V variant having a gain-of-function effect on the transcriptional activity of Mef2c, abnormal cardiac development was observed more frequently in transgenic medaka embryos injected with the variant Mef2c than those with the WT (**Figure 3F**; P=0.015, chi-square test).

Discussion

Herein we report on the identification and characterization of multiple sequence variants of the transcription factors, that are essential for cardiac development, in non-syndromic patients with cardiac OFT defects. Each variant exhibited changes, to varying degrees, in the transcriptional activity of its protein products on downstream target genes involved in heart development. The results suggest that the sequence variations identified in *NKX2.5*, *GATA4*, *GATA6*, and *MEF2C* in the present study are likely to be involved in some aspect of the etiology of OFT defects, accounting for approximately 2.3% of cases of non-syndromic OFT defects (6 cases of 256 patients in the present series). These findings suggest that a subset of CHDs

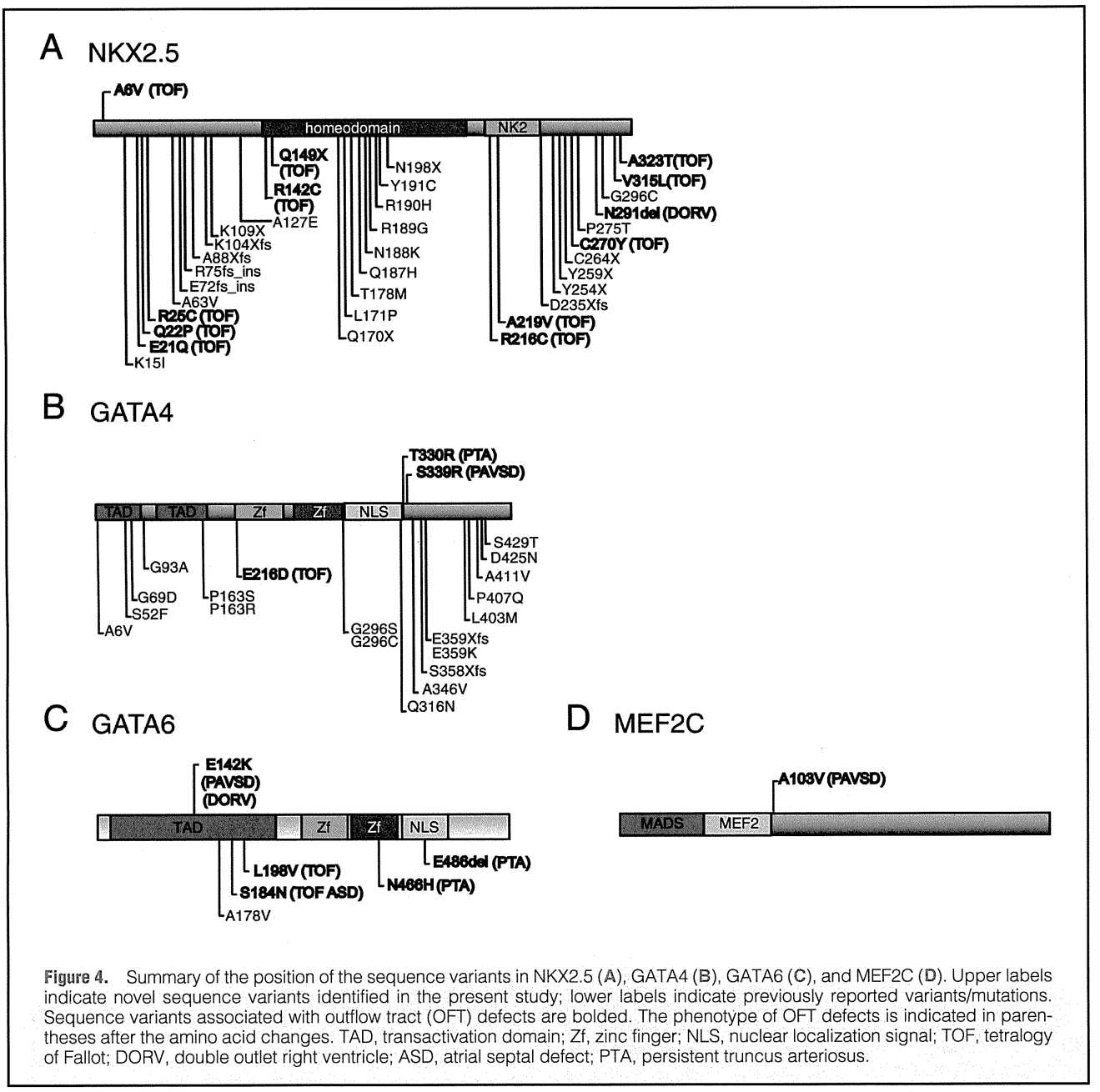

Figure 4. Summary of the position of the sequence variants in NKX2.5 (A), GATA4 (B), GATA6 (C), and MEF2C (D). Upper labels indicate novel sequence variants identified in the present study; lower labels indicate previously reported variants/mutations. Sequence variants associated with outflow tract (OFT) defects are bolded. The phenotype of OFT defects is indicated in parentheses after the amino acid changes. TAD, transactivation domain; Zf, zinc finger; NLS, nuclear localization signal; TOF, tetralogy of Fallot; DORV, double outlet right ventricle; ASD, atrial septal defect; PTA, persistent truncus arteriosus.

may be caused by disruption of cardiac transcription factor regulation. The approach we used in the present study may prove useful in identifying new genetic causes and exploring unknown molecular mechanisms underlying non-syndromic and non-familial CHDs, despite the known difficulties in determining the genetic basis of most such cases of CHDs.

A previous study has indicated that mutations in the homeodomain of *NKX2.5* are likely to cause ASD, whereas mutations located outside the homeodomain may be associated with OFT defects.[26] Benson et al first identified mutations in the N-terminal region of *NKX2.5* in patients with TOF, and this region may be a hot spot for mutations responsible for TOF[27] (Figure 4A). Consistent with this notion, in the present study we identified the novel sequence variant A6V in the N-terminal region of *NKX2.5* in a patient with TOF. It is known that the N-terminal region is required for NKX2.5 activity essential for cardiomyogenesis.[28] The NKX2.5 variant A6V exhibited significantly decreased transcriptional activity, which likely resulted in TOF in our patient.

Mutations in *GATA4* are known to cause intracardiac septal defects, probably as a result of disruptions to interactions with TBX5.[19] Although only a few reports have demonstrated an association between GATA4 mutations and OFT defects,[29] in the present study we identified 2 novel GATA4 sequence variants in patients with OFT defects (Figure 4B). Both *GATA4* variants (T330R and S339R) were located in a common basic region of the GATA transcriptional factors and both variants resulted in a disruption of the synergistic activity between GATA4 and NKX2.5 or GATA6, although the correlation between the genotype and phenotype was unclear. It is of note that each variant alone did not exhibit any significant decrease in transcription activity. It has been reported that reciprocal

regulation between GATA4 and NKX2.5, as well as a synergistic activity between them, is essential for several cardiac-specific genes.[30–34] Other studies have indicated that GATA4 plays a role in cardiovascular development in collaboration with GATA6.[9,35,36] Loss of both GATA4 and GATA6 blocks cardiac myocyte differentiation, resulting in acardia in mice.[37] GATA4 and GATA6 regulate MEF2C by binding directly to its enhancer and via calcium-dependent pathways;[38,39] in turn, MEF2C plays a role in the differentiation of the OFT myocardium and vascular smooth muscle cells from their progenitor pool (ie, the second heart field).[10] These observations suggest that GATA4 and GATA6 may function redundantly as well as cooperatively in multiple steps of cardiovascular development, ranging from the differentiation of cardiac progenitor cells to the development of the OFT. On the basis of the results obtained in the present study, we believe that a decrease in the synergistic activity of GATA4 with NKX2.5 or GATA6 may result in some type of OFT defect.

The novel GATA6 variant E142K exhibited significantly decreased transcriptional activity, as well as decreased synergistic activity with other cardiac transcription factors, making this variant a strong candidate for a subset of OFT defects. Since our initial report of the first mutations in *GATA6* associated with OFT defects,[20] a further 3 mutations have been reported, with 2 of them associated with OFT defects[40,41] (Figure 4C). These findings indicate that mutations in *GATA6* may be mainly associated with OFT defects, probably as a result of the dysregulation of the SEMA3C–PLXNA2 pathway.[20]

The present study revealed that GATA6 binds directly to TBX5, which is the first demonstration of a direct physical interaction between GATA6 and TBX5 leading to synergistic activity. The GATA6 variant E142K exhibited decreased synergistic activity with TBX5; however, its direct interaction with TBX5 was not altered. These results suggest that TBX5 binds to GATA6 normally via the zinc finger domain, like other cofactors, but the sequence variation E142K in the N-terminal domain of *GATA6* changes transactivation on the target gene independent of binding to TBX5.

The present study also reports the first sequence variation in *MEF2C* associated with CHDs (Figure 4D). MEF2C is essential for the development of the right ventricle and the OFT interacting with NKX2.5.[42] Loss of *Mef2c* in mice results in hypoplasia of the right ventricle and the OFT.[10,11] Furthermore, Mef2c is thought to have an essential role in ventricular cardiomyocyte differentiation,[43] and, recently it was reported that a transduction of Mef2c together with Gata4 and Tbx5 rapidly and efficiently reprogrammed postnatal fibroblasts directly into differentiated cardiomyocyte-like cells.[44] Intriguingly, Mef2c overexpression assay using fish in this current study showed defects of cardiac development, including disorganized cardiac chamber formation and ectopic cardiomyocyte differentiation. These findings indicate that the level of transcriptional activity of Mef2c should be critically controlled for normal cardiac development. In our study, the A103V variant of Mef2c identified in a patient with OFT defect showed gain-of-function in transcriptional activity on the promoter of its downstream target, *Smyd1*, which is essential for early cardiomyocyte differentiation of development of the right ventricle and OFT.[25] Consistently, abnormal cardiac development was observed more frequently in transgenic fish embryos overexpressing the A103V variant of Mef2c than in those with the WT Mef2c. Taken together, our findings suggest that the gene mutation causing the A103V variation of MEF2C is likely to be implicated in the OFT defect as a result of altered transcriptional activity of MEF2C.

Of note, we did not identify any sequence variants in *ISL1*. During development, *Isl1* is expressed in the splanchnic mesoderm comprising the second heart field, which gives rise to the OFT, right ventricle, and most parts of the atria, but is down-regulated in the heart proper.[45] In contrast, *Nkx2.5*, *Gata4*, *Gata6*, and *Mef2c* are expressed continuously in the heart during development.[12–14] One could speculate that this may be why no CHD-associated sequence variant was found in *ISL1* in the present study, although this finding does not rule out the possibility that *ISL1* is responsible for some type of OFT defect in humans. Clearly, in the present study we did not attempt to incorporate the effects of all factors relevant to heart development. Further genetic screening and functional assays for a comprehensive range of transcription factors essential for heart development in patients with all types of CHDs would reveal more precise genotype–phenotype correlations, and eventually provide new insights into the molecular mechanisms underlying CHDs.

Conclusions

Genetic and functional analyses of essential cardiac transcription factors in 256 patients with non-syndromic and non-familial CHDs that could not be evaluated using conventional chromosome and/or linkage analysis identified new gene mutations of *NKX2.5*, *GATA4*, *GATA6*, and *MEF2C* in 6 patients. These mutations may account for approximately 2.3% of OFT defects.

Acknowledgments

The authors are grateful to the patients, their family members, and their referring physicians. The authors thank K. Komatsu and K. Kihara for technical assistance with the sequencing, and M. Ono for technical assistance with the mRNA injection, and M. A. Razzaque and H. Yagi for technical support, and K. Yamamoto for assistance. The authors also thank the members of the Keio Pediatric Cardiology Laboratory for helpful discussions.

This work was supported by the Encouraging Development of Strategic Research Centers, Special Coordination Funds for Promoting Science and Technology, Ministry of Education, Culture, Sports, Science and Technology, Japan.

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Supplemental Files

Supplemental File 1

Data S1. Materials and Methods

Table S1. Human MEF2C Primers for PCR and Sequence Reactions

Table S2. Human ISL1 Primers for PCR and Sequence Reactions

Supplemental File 2

Movie S1. Abnormal cardiac phenotypes of medaka injected with Mef2c A103V mRNA at 5 days after fertilization. Beating premature heart tube was seen in the embryo and blood flow was severely disturbed.

Supplemental File 3

Movie S2. Only beating tissue was seen in ectopic position and no blood flow was detected.

Please find supplemental file(s);
http://dx.doi.org/10.1253/circj.CJ-11-1389

Surgical Results for Functional Univentricular Heart With Total Anomalous Pulmonary Venous Connection Over a 25-Year Experience

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Background. Surgical results for functional univentricular heart with total anomalous pulmonary venous connection (TAPVC) have been unsatisfactory to date.

Methods. During a 25-year period until December 2009, 207 TAPVC patients underwent surgical repair at our institute, including 56 with a univentricular heart. The 10-year survival rate was 51.1% with univentricular heart and 84.7% with biventricular heart ($p < 0.0001$; log-rank, 27.6). Surgical outcomes and risk factors for early and late death after TAPVC repair in univentricular hearts were retrospectively analyzed.

Results. Patients were aged 3.8 ± 4.3 years and weighed 12.3 ± 10.7 kg at operation. Preoperative diagnoses included heterotaxy syndrome in 55, asplenia in 48, preoperative pulmonary venous obstruction in 35, and pulmonary atresia in 20. TAPVC was classified as I in 22, II in 26, III in 5, and IV in 3. Concomitant procedures included Fontan procedure in 29, bidirectional Glenn procedure in 5, systemic–pulmonary shunt in 11, and pulmonary artery banding in 5. There were 17 hospital deaths and 11 late deaths. Fontan completion was undertaken in 31 (55.3%). Postoperative pulmonary venous obstruction was found in 15. Multivariate analysis identified TAPVC III and IV and pulmonary atresia as risk factors for hospital death. Univariate analysis identified postoperative pulmonary venous obstruction and concomitant systemic–pulmonary shunt as risk factors for hospital and late death.

Conclusions. TAPVC III, IV, and pulmonary atresia are risk factors for early postoperative death. Intensive intervention, including perioperative management and operation, is required in these complex patients.

(Ann Thorac Surg 2012;93:606–13)

Operations for functional univentricular heart (univentricle) with total anomalous pulmonary venous (PV) connection (TAPVC) are associated with high rates of morbidity and death. TAPVC or obstructed TAPVC have been reported as risk factors for death in heterotaxy syndrome [1–3] or asplenia [4, 5]. The 10-year survival rate in heterotaxy syndrome has been reported as 39% with TAPVC and 64% without TAPVC [2]. In addition, univentricle has been identified with as a risk factor for death after TAPVC repair [6].

A report of 327 patients undergoing TAPVC repair during a 59-year period found that surgical outcomes have improved over time due to improvements in surgical techniques and perioperative management [7]. The 5-year survival rate after TAPVC repair in the biventricular heart (biventricle) has improved to 97% since 2000 [7]. However, surgical results for univentricle with TAPVC have not yet been satisfactory, with a reported 3-year survival rate of 47% [6]. The purpose of current study was to evaluate, by a retrospective review of medical records, the surgical outcomes and risk factors for death after repair of TAPVC in univentricle.

Patients and Methods

Patient Characteristics

We enrolled 207 consecutive patients who had undergone TAPVC repair at the Tokyo Women's Medical University Hospital from 1985 to December 2009. Of these, 56 had univentricle and 151 patients had biventricle. Actuarial 10-year survival rates after TAPVC repair were 51.1% in univentricle and 84.7% in biventricle ($p < 0.0001$; log-rank = 27.6; Fig 1). We therefore evaluated surgical outcomes and risk factors for death after TAPVC repair in univentricle. A retrospective review of medical records, including operative notes and clinical examination records, were used.

Characteristics of the 56 univentricle patients (30 males, 26 females) are summarized in Table 1. Median age at operation was 3.8 ± 4.3 years, with 12 patients younger than 2 months. Median body weight (BW) was 12.3 ± 10.7 kg, with 10 patients weighing less than 3.5 kg.

Accepted for publication Sept 2, 2011.

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Published by Elsevier Inc

0003-4975/$36.00
doi:10.1016/j.athoracsur.2011.09.038

Abbreviations and Acronyms

AVVP = atrioventricular valvular plasty
AVVR = Atrioventricular valve regurgitation
BDG = bidirectional Glenn procedure
BW = body weight
CAVVR = common atrioventricular valve regurgitation
CHF = congestive heart failure
CI = confidence interval
CPB = cardiopulmonary bypass
IVC = inferior vena cava
PA = pulmonary artery
PSVT = paroxysmal supraventricular tachycardia
PVO = pulmonary venous obstruction
RAI = right atrial isomerism
SP shunt = systemic-to-pulmonary shunt
SVC = superior vena cava
TAPVC = total anomalous pulmonary venous connection
TGA = transposition of great artery

Previous cardiac operations included systemic-to-pulmonary (SP) shunt in 18, patent ductus arteriosus ligation in 2, atrioventricular valvuloplasty (AVVP) in 2, bidirectional Glenn procedure (BDG) in 1, and pulmonary artery (PA) banding in 1. TAPVC was classified according to the Darling classification as I in 22, II in 26, III in 5, and IV in 3 (Table 2). Heterotaxy syndrome was diagnosed in 55 patients, including 48 with asplenia and 7 with polysplenia. Functional right ventricle (n = 52) and left ventricle (n = 4) were classified as systemic ventricle. Pulmonary atresia was present in 20, nonconfluent PA in 7, and more than mild atrioventricular valve regurgitation in 27. Preoperative arrhythmia included paroxysmal supraventricular tachycardia in 6 and sick sinus syndrome in 1. Extracardiac abnormalities were found in 8 patients.

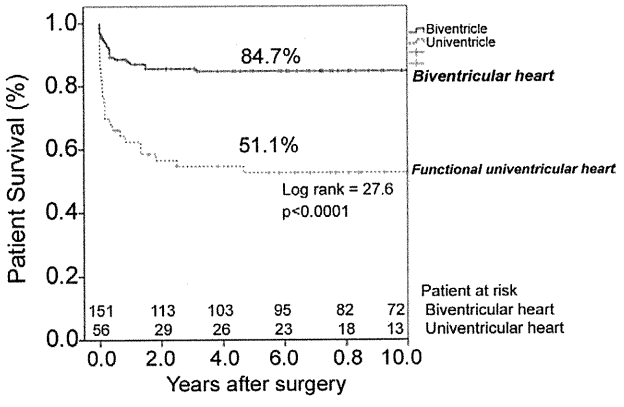

Fig 1. During a 25-year period ending December 2009, 207 total anomalous pulmonary venous connection (TAPVC) repairs were performed at Tokyo Women's Medical University Hospital. Patients with a functional univentricular heart after TAPVC repair had an actuarial survival rate of 51.1% compared with 84.7% in those with a biventricular heart ($p < 0.0001$, log-rank = 27.6).

Table 1. Patient Profiles

Variable	No. or Mean ± SD
Patients, total	56
Sex	
Male	30
Female	26
Age at operation, year	3.8 ± 4.3
Age < 2 months	12
Body weight	
At operation, kg	12.3 ± 10.7
<3.5 kg	10
At birth, kg	2.96 ± 0.4
Arrhythmia	7
PSVT	6
Sinus node dysfunction	1
Extracardiac abnormality	8
Gastric volvulus	2
Intestinal volvulus	1
Esophageal hiatus herniation	3
Pulmonary hypoplasia	1
Previous operation	20
Systemic-to-pulmonary shunt	18
Bidirectional Glenn	1
Patent ductus arteriosus ligation	2
Pulmonary artery banding	1
Atrioventricular valvular plasty	2

PSVT = paroxysmal supraventricular tachycardia; SD = standard deviation.

Definitions

Pulmonary venous obstruction (PVO) was diagnosed by echocardiography or cardiac catheterization. PVO was defined as the flow acceleration more than 2 m/s by the echocardiography [8] or a pressure gradient of more than 4 mm Hg between the PVs and their drainage [6] or less than 50% of normal at the PV by cardiac catheterization [9]. In addition, clinically relevant symptoms of obstruction were taken into consideration.

Hospital death was defined as any death before hospital discharge, and late death was defined as any death after hospital discharge.

Surgical Management

All operations were performed under general anesthesia through a median sternotomy. Cardiopulmonary bypass (CPB) was established with ascending aortic perfusion and bicaval cannulations. Myocardial protection included topical cooling. Deep hypothermic circulatory arrest was used to allow a bloodless field with excellent exposure of the pulmonary vein. For all types of TAPVC, the direct anastomosis between the incised PV and the atrium was performed by continuous suture with 7-0 polypropylene. In addition, the area of the anastomotic orifice was made as large as possible.

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Table 2. Patient Characteristics

Characteristic	No.
Heterotaxy syndrome	
Asplenia	48
Polysplenia	7
TAPVC type	
Type I	22
Ia	6
Ib	16
Type II	26
IIa	0
IIb	26
Type III	5
Type IV	3
Ia + Ib	1
Ib + III	2
Pre-op pulmonary venous obstruction	35
Systemic functional ventricle	
Right ventricle	52
Left ventricle	4
Ventriculoarterial connection	
Double-outlet right ventricle	18
Transposition of great artery	4
Pulmonary outflow	
Pulmonary atresia	20
Nonconfluent pulmonary artery	7
Pulmonary stenosis	21
CAVVR (>mild)	27
Cardiac position	
Dextrocardia	10
Dextroversion	2
Aortocaval juxtaposition	3
Right aortic arch	20
Major aortopulmonary collateral arteries	2
Coronary artery abnormality	
Single coronary artery	1
Coronary arteriovenous fistula	1
Systemic venous connection	
Superior vena cava	
Bilateral	18
Left	6
Interrupted IVC–azygous connection	2

CAVVR = common atrioventricular valve regurgitation; IVC = inferior vena cava; TAPVC = total anomalous pulmonary venous connection.

Statistical Analysis

Statistical analysis was done using SPSS 18.0 software (SPSS Inc, Chicago, IL). Normally distributed continuous variables are expressed as mean ± standard deviation. All p values were two-sided, and values of $p < 0.05$ were considered to be statistically significant. The end point of follow-up was taken as from TAPVC repair until the date of death or the last contact at Tokyo Women's Medical University Hospital.

Actuarial survival rates were analyzed by the Kaplan-Meier curve, and comparisons between groups were made by the log-rank test. The Cox proportional hazard model was used to determine risk factors for death by multivariate analysis. Twenty variables included male sex, age younger than 2 months at TAPVC repair, BW less than 3.5 kg at TAPVC repair, previous operation, asplenia, TAPVC type, pulmonary atresia, nonconfluent PA, concomitant Fontan procedure, concomitant SP shunt, concomitant PA banding, concomitant AVVP, preoperative PVO, postoperative PVO, operation since the year 2000, CPB time exceeding 180 minutes, and use of deep hypothermic circulatory arrest. Each variable that was significantly associated by univariate analysis ($p < 0.1$), was entered into the multivariate analysis.

Results

Concomitant procedures included Fontan in 29 as a definitive procedure, and BDG in 5, SP shunt in 11, and PA banding in 5 as palliative procedures (Fig 2). Other concomitant procedures consisted of AVVP in 22, PA angioplasty in 13, ligation of major aortopulmonary collateral arteries in 2, patent ductus arteriosus ligation in 2, and pacemaker implantation in 1. CPB time was 151.5 ± 48.0 minutes and aortic cross-clamp time was 74.1 ± 33.9 minutes. Deep hypothermic circulatory arrest was used in 12 patients.

There were 17 hospital deaths and 11 late deaths. Causes of hospital death were postoperative PVO in 7, congestive heart failure (CHF) in 6, infection in 2, pulmonary hypertension in 1, and pulmonary bleeding in 1. Morbidities were arrhythmia requiring pacemaker implantation in 4 (asplenia in 3 and polysplenia in 1) and subarachnoid hemorrhage in 1. Persistent pulmonary hypertension in 3 patients resulted in Fontan takedown. Fontan procedure was completed in 31 patients (55.3%).

Actuarial survival rates after TAPVC repair were 60.6% at 1 year and 51.1% at 10 years, with a median duration of follow-up 5.6 years (range, 1 day to 19.8 years).

Postoperative PVO

Postoperative PVO occurred in 15 patients (Fig 3). Of these, 3 patients underwent surgical repair and 2 patients underwent percutaneous transluminal angioplasty (PTA). Four of these 5 patients survived the perioperative period. Eight patients could not undergo surgical repair or PTA due to their poor general condition. All of these died. The remaining 2 patients are still awaiting surgical repair for postoperative PVO.

Postoperative AVVR

Postoperative AVVP for AVVR was performed in 5 patients. Secondary AVVP was required in 3 of 22 patients who underwent concomitant AVVP and TAPVC repair. CHF resulted in 2 hospital deaths and 2 late deaths in this group.

Risk Analysis for Hospital Death

Multivariate analysis identified TAPVC III, IV, and pulmonary atresia as significant independent risk factors for hospital death (Table 3). Univariate analysis identified

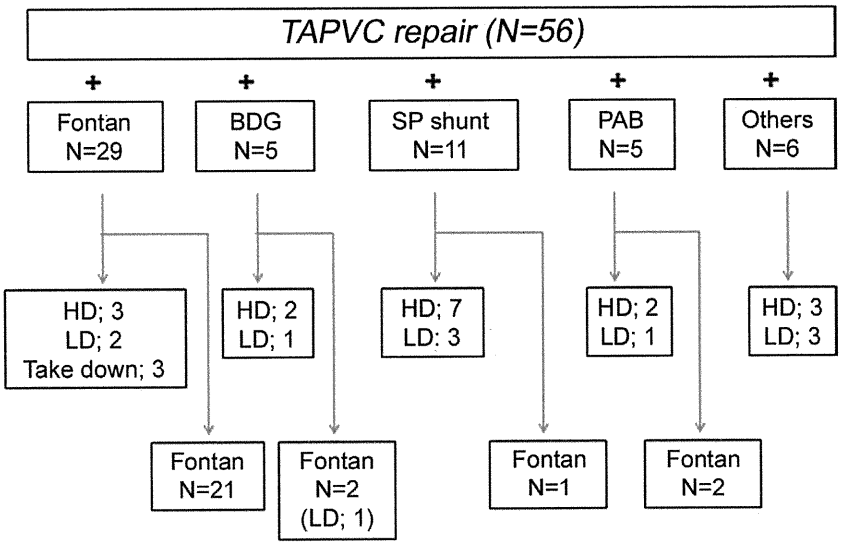

Fig 2. Flow chart shows the clinical course after total anomalous pulmonary venous connection (TAPVC) repair. Concomitant operations consisted of Fontan procedures in 29 as definitive procedures, and the bidirectional Glenn procedure (BDG) in 5, systemic-to-pulmonary (SP) shunt in 11, pulmonary artery banding (PAB) in 5 as palliative procedures, and no palliation in 6. Hospital death (HD) occurred in 17 patients and late death (LD) in 11. Of 34 patients who underwent the Fontan procedure, HD occurred in 3, take down due to Fontan circulation failure in 3, and LD in 3.

age younger than 2 months at operation, BW less than 3.5 kg at operation, concomitant SP shunt, and postoperative PVO as significant risk factors for hospital death, and identified previous operations, TAPVC II, and concomitant Fontan procedure as protective factors for hospital death. Asplenia, preoperative PVO, TAPVC I, and nonconfluent PA were not correlated with hospital death.

Risk Analysis for Late Death

Multivariate analysis did not identify any factors as statistically significant for late death. Univariate analysis identified BW less than 3.5 kg at operation, TAPVC I, concomitant SP shunt, and postoperative PVO as significant risk factors for late death (Table 4), and identified TAPVC II and concomitant Fontan procedure as protective factors for late death. Asplenia and preoperative PVO were not identified as risk factors for late death.

Survival Rates in Asplenia

The actuarial 5-year survival rate of 44.8% with asplenia was lower than the rate of 87.5% without asplenia (p = 0.034, log-rank = 4.51; Fig 4).

Survival Rates in TAPVC I

Survival rates in TAPVC I at 6, 12, and 36 months after TAPVC repair were 63.6%, 54.5%, and 40.9%, respectively (Fig 5). Late deaths in TAPVC I occurred in 7 patients, caused by postoperative PVO in 4, CHF in 2, and pneumonia in 1.

Comment

Evaluation of Risk Factors for Death

BW AT OPERATION. Of 17 univentricle patients with TAPVC during an 8-year period, BW at operation was identified as a risk factor for death [10]. Of 226 patients with repaired TAPVC in the Society of Thoracic Surgeons database, the surgical outcome in BW ranging from 1 to 2.5 kg at operation was worse than that in BW ranging from 2.5 to 4 kg (risk ratio, 2.95) [11]. Increased BW at operation may also reduce perioperative complications such as intracranial hemorrhage, renal dysfunction, and coagulopathy [11]. However, waiting for an increase in

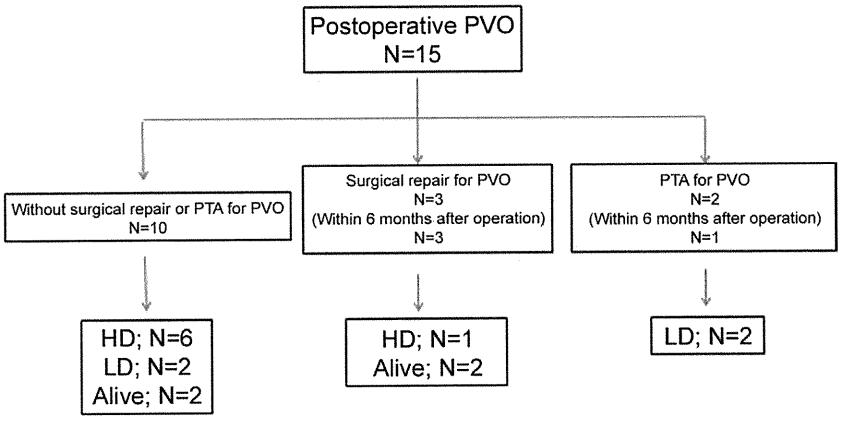

Fig 3. Flow chart shows the clinical course in 15 patients with postoperative pulmonary venous obstruction (PVO). The reoperation and percutaneous transluminal angioplasty (PTA) for postoperative PVO were performed in 3 and 2 patients, respectively. Hospital death (HD) and late death (LD) were found in 6 and 2 patients among 10 patients without surgical repair or PTA for postoperative PVO.

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Table 3. Risk Factors for Hospital Death After Total Anomalous Pulmonary Venous Connection Repair by Cox Proportional Hazard Model

Variable	Univariate Analysis		Multivariate Analysis	
	HR (95% CI)	*p* Value	HR (95% CI)	*p* Value
At operation				
Age < 2 months	5.5 (2.1–14.3)	0.001	...	0.077
Body weight < 3.5 kg	4.21 (1.6–11.1)	0.004	...	0.39
Previous operation	0.208 (0.47–0.91)	0.037	...	0.212
Asplenia	...	0.253	...	...
Pulmonary venous obstruction				
Preoperative	3.36 (8.8–10.8)	0.057	...	0.615
Postoperative	2.88 (1.1–7.5)	0.029	...	0.132
TAPVC type				
I	...	0.553	...	...
II	0.31 (0.1–0.9)	0.042		0.097
III	9.24 (3.1–27.1)	0.001	26.3 (2.9–236)	0.003
IV	3.8 (0.9–16.8)	0.078	15.9 (1.7–147)	0.015
Pulmonary atresia	2.58 (0.9–6.7)	0.051	9.1 (2.2–37.2)	0.002
Nonconfluent pulmonary artery	...	0.53	...	...
Concomitant procedure	...	...	...	...
Fontan	0.15 (0.003–0.15)	0.003	...	0.10
Systemic–pulmonary shunt	3.75 (1.42–9.88)	0.007	...	0.056
Pulmonary artery banding	...	0.77	...	...
Atrioventricular valvular plasty	...	0.141	...	...

CI = confidence interval; HR = hazard ratio; TAPVC = total anomalous pulmonary venous connection.

BW before operation may not be advantageous due to an increase in preoperative morbidities [12].

In our study, there were 6 hospital deaths and 3 late deaths among 10 patients weighing less than 3.5 kg at operation. Univariate analysis identified that BW at operation was a risk factor for both hospital and late death. This indicates that lower BW at operation tends to adversely affect surgical outcome.

AGE AT OPERATION. Of 139 patients with heterotaxy syndrome during a 20-year period, the neonatal procedure was identified as a risk factor for death [1]. In our study, there were 9 hospital deaths, among 12 patients who were aged younger than 2 months at operation, which was a risk factor for hospital death. This indicates that age at operation tends to affect early surgical outcomes, which is consistent with previous reports [1, 10, 13].

PHENOTYPE OF HETEROTAXY SYNDROME. Of 45 patients with heterotaxy syndrome undergoing surgical repair during a 14-year period, the 3-year survival rate of 79% in right atrial isomerism (RAI) was lower compared with the rate of 94% in left atrial isomerism [4].

Our study did not identify asplenia as a risk factor for death, although the 5-year survival rate of 44.8% in patients with asplenia was lower than the rate of 87.5% in patients without asplenia. Investigation for morbidities revealed that infection occurred in 3 patients with asplenia, but did not occur in patients without asplenia. Postoperative pacemaker implantation was undertaken in 3 patients (6.3%) with asplenia.

TAPVC TYPE. Of 31 RAI-repaired TAPVC during an 11-year period, survival rates in TAPVC III and IV were inferior to the rate in TAPVC I and II (hazard ratio, 16.5). Another investigation of 26 univentricle patients with extracardiac TAPVC during a 9-year period identified TAPVC IV as a risk factor for death [15].

In our study, multivariate analysis identified TAPVC III and IV as risk factors for hospital death. This indicates that TAPVC III and IV influence the early surgical outcome.

Univariate analysis identified TAPVC I as a risk factor for late death, although the perioperative outcome was better for TAPVC I. Causes of late death in TAPVC I were postoperative PVO in 4, CHF in 2, and pneumonia in 1. Univariate analysis revealed that postoperative PVO tended to be a risk factor for late death. We consider that the increased rate in late mortality in TAPVC I might be due to the pulmonary hypertension caused by postoperative PVO. One published report indicated that the development of postoperative PVO might be associated with compression by neighboring structures such as the aorta, bronchi, and vertebrae [16]. We therefore consider that careful management and follow-up after hospital discharge are important to improve the surgical outcome in TAPVC I.

VARIATION IN PA STRUCTURE. In 27 patients with heterotaxy syndrome undergoing surgical repair, a nonconfluent PA was identified as a risk factor for death [17]. The authors commented that it was important to regulate the pulmo-

Table 4. Risk Factors for Late Death After Total Anomalous Pulmonary Venous Connection Repair by Cox Proportional Hazard Model[a]

Variable	Univariate Analysis	
	HR (95% CI)	*p* Value
At operation		
Age < 2 months	3.94 (0.81–19.0)	0.088
Body weight < 3.5 kg	8.0 (1.97–32.4)	0.004
Previous operation	...	0.524
Asplenia	...	0.272
PVO		
Preoperative	...	0.229
Postoperative	3.472 (1.0–11.9)	0.048
TAPVC type		
I	5.53 (1.4–22.1)	0.015
II	0.22 (0.05–0.9)	0.029
III	...	...
IV	...	0.674
Pulmonary atresia	...	0.536
Nonconfluent PA	...	0.795
Concomitant procedure		
Fontan	0.07 (0.01–0.3)	0.001
Systemic-to-pulmonary shunt	4.71 (1.2–18.4)	0.026
PA banding	...	0.963
AVVP	...	0.129

[a] None of these variables were significant by multivariate analysis.

AVVP = atrioventricular valvular plasty; CI = confidence interval; HR = hazard ratio; PA = pulmonary artery; PVO = pulmonary venous obstruction; TAPVC = total anomalous pulmonary venous connection.

nary vascular blood flow in heterotaxy syndrome. In our study, multivariate analysis identified that pulmonary atresia affected early surgical outcome, although a nonconfluent PA was not identified as a risk factor. This

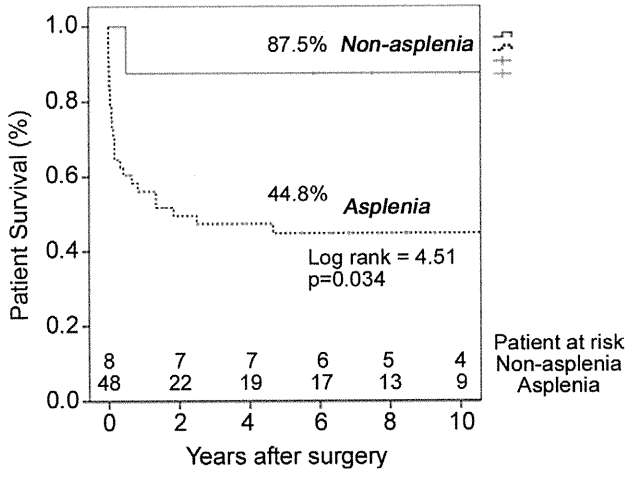

Fig 4. Actuarial 5-year survival rates of 44.8% in patients with asplenia was inferior to that of 87.5% in patients without asplenia (p = 0.034, log-rank = 4.51).

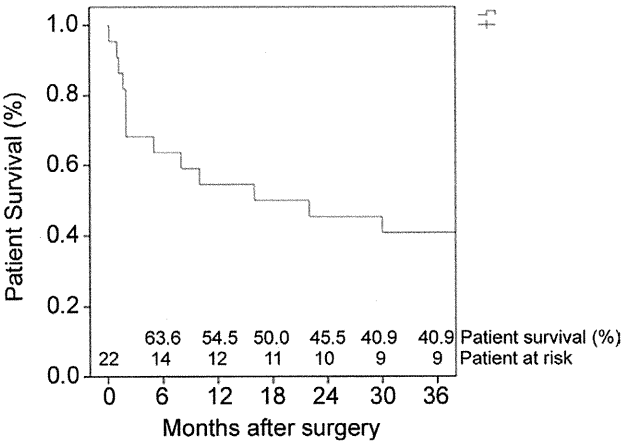

Fig 5. Actuarial survival curve after total anomalous pulmonary venous connection (TAPVC) type I repair shows survival at 6, 12, and 36 months was 63.6%, 54.5%, and 40.9%, respectively.

indicates that patients requiring manipulation of pulmonary blood flow are difficult to manage.

PRESENCE OF AVVR. AVVR is associated with systemic ventricular dysfunction and subsequent pulmonary hypertension. Surgical outcomes are therefore influenced by the ability to control AVVR at TAPVC repair. AVVR has been identified as an independent risk factor for death in heterotaxy syndrome [2] and RAI [4]. After the Fontan procedure, more than mild AVVR influences survival in heterotaxy syndrome [18]. After the extracardiac Fontan procedure, more than mild AVVR in heterotaxy syndrome was 33.9% higher than that of 18.9% in nonheterotaxy syndrome [19].

In our study, 22 patients underwent concomitant AVVP, which was not identified as a risk factor for death. However, 4 of 5 patients requiring postoperative AVVP for AVVR died of CHF. We therefore consider that the patients with preoperative AVVR should undergo concomitant AVVP to avoid postoperative AVVR whenever possible.

REGULATION OF PULMONARY BLOOD FLOW WITH THE CONCOMITANT SP SHUNT OR PA BANDING. Of 44 TAPVC patients with complex cardiac lesions, including univentricle or heterotaxy syndrome, 16 underwent concomitant SP shunt, with a 53% postoperative mortality [13]. Of 17 univentricle with TAPVC, the concomitant palliation to the pulmonary artery with TAPVRC repair was associated with a high risk, which may be due to the adjustment of pulmonary blood flow in those patients [10].

Of the 11 patients in our study who needed an SP shunt, 10 died. Univariate analysis identified a concomitant SP shunt as a risk factor for hospital death, although concomitant PA banding was not identified as risk factor for death. In the management of complex cardiac abnormalities, it is important to consider correlations among the pressure-volume relationships in the heart (preload and afterload) to gain better quality of life [20]. It may be difficult to sustain an adequate circulation in TAPVC repair with concomitant SP shunt, especially in patients