Genetic disturbances in patients with bodily isomerism from a single center: clinical implications of affected genes and potential impact of ciliary dyskinesia

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Abstract

So-called heterotaxy or isomerism is characterized by abnormal lateralization and malformations of the bodily organs. The mechanism is unclear, although there is growing evidence that ciliary dyskinesia is involved. We reviewed genetic findings from patients with isomerism to determine if affected genes were known to be associated with isomerism and ciliary dyskinesia and determine associations between genotype and clinical findings. We identified patients with isomerism cared for over a 16-year period. Characteristics were compared between those with and without identified mutations. A total of 83 patients with isomerism were identified. Of those who had genotyping, 14/27 had mutations identified, most frequently involving the *CFC1 and NODAL* genes. Specific mutations were associated with clinical findings, with *NODAL* mutations often portending need for increased clinical support. Genes associated with isomerism and/or ciliary dyskinesia were identified in the cohort. Specific gene mutations may help predict clinical course.

Introduction

Isomerism is a unique clinical entity in which there is mirror imagery of the thoracic organs within the same individual, which has also been characterized by the more ambiguous term *heterotaxy*.[1,2] As a result, both bronchuses are either long and hyparterial, making them morphologically left, or short and eparterial, meaning that they are morphologically right. The lungs usually share the same morphological sidedness as the bronchuses, with either bilobed or trilobed lungs being present bilaterally.[3-7] In the heart, isomerism is unequivocally present with the atrial appendages. The atrial appendages are the most constant of the atrial morphological features, meaning that they can be used, from the stance of the heart, for segregating isomerism into its right and left isomeric subsets.[8-10] Examination of large numbers of autopsied specimens shows that this is a more reliable method of segregating *heterotaxy* than use of splenic anatomy.[11,12] Isomerism in the heart, furthermore, is also found in the conduction system, with sinus nodes known to be bilaterally present in the setting of right isomerism.[13] Twin atrioventricular nodes may also be present in either subset, this potentiating in many cases to arrhythmias.[14,15] The arrangement of the abdominal organs, however, although markedly different from the usual or mirror-images lateralized arrangements, shows minimal evidence of isomerism.[8]

The mechanism underscoring an isomeric formation of the thoracic organs is unclear, although emerging data indicates that isomerism may be the result of ciliary dyskinesia, with dyskinesia being found in at least two-fifths of afflicted patients.[16] While the ciliary ultrastructure can be normal, the ciliary beat is abnormal. This may alter leftward differentiation of embryonic flow at the node during early development. Additionally, formation of non-motile cilia may impact intracellular signaling, which could lead to functional abnormalities not only during embryogenesis, but also later in life.[17,18] In patients with isomerism, the presence of ciliary dyskinesia can result in significant sinupulmonary disease and may also mediate splenic dysfunction, increasing the risk of bacteremia and thrombocytosis.[19-21] Hepatic, renal, and central nervous system malformations and dysfunction may also be a result of ciliary dyskinesia.[20,22-32]

Ciliary dyskinesia and isomerism are now known to be associated with specific genetic mutations, some being common to both. Genes such as *Lefty1* and *Pitx2* were initially demonstrated to be associated with isomerism, with additional genes subsequently identified.[33,34] The primary aim of this study, therefore, was to determine if mutations were present in a cohort of patients from a single center known to have isomeric features, and to quantify what proportion of affected genes have been previously associated with either isomerism, ciliary dyskinesia, or both. Additionally, we sought to assess the association of specific affected genes with anatomic and clinical outcomes.

Materials and Methods

Selection of patients

We reviewed medical records of all patients cared for since 1998 with isomerism at Children's Hospital of Wisconsin. This was the year that electronic medical records were implemented at our institution. The dataset included patients born in or after 1998, as well as those born earlier who transitioned their care to our heart center after 1998. Patients were identified through multiple searches so as to ensure the highest yield of patients. Medical records, medical billing data, and the echocardiography database, cardiac catheterization database, cardiothoracic surgical database were queried for *isomerism*, *heterotaxy*, *asplenia*, *multiple spleens*, and *polysplenia*. The resulting lists of patients were then combined, and redundant entries removed. The remaining patients were reviewed, and included if they had both congenital heart disease and recognized features of isomerism. Congenital heart disease was defined as any intracardiac lesion and/or an abnormality of venous return to the heart. We considered isomerism to be present when there was a cardiovascular malformation in addition to evidence of abnormal arrangement of the abdominal organs, including the spleen, or overt evidence of pulmonary isomerism. The diagnosis of right or left isomerism was based on our subsequent review of the clinical and anatomic data.

Next, we identified which patients had undergone genetic testing other than a chromosomal karyotype. These included heterotaxy gene panels conducted by polymerase chain reaction, ciliopathy gene panel conducted by

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Ethical standards: this study was done in accordance with the ethical standards of the institutional research committee and with the 1964 Helsinki declaration and its later amendments. As data from human patients was utilized, institutional review board (IRB) approval was obtained. Individual patient consent was waived by the IRB as this is a retrospective study.

Conflict of interest: there are no conflict of interest to disclose for any of the authors.

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polymerase chain reaction, as well as hybridized microarray.

The details of cardiac anatomy were obtained primarily from the echocardiographic findings, although data from computed tomographic, magnetic resonance, and cardiac catheterization studies was also available for some patients. The splenic status was based on findings from abdominal ultrasonography. At our institution, genetic evaluation of patients with isomerism is done on a case by case basis. The proposed methodology was approved by the Institutional Review Board at our institution.

Characteristics of the cohort

For our baseline analysis, we categorized the cardiac diagnoses into primary and secondary lists. The primary diagnosis was the lesion considered to be the most hemodynamically significant, while the secondary diagnosis included other associated lesions. For instance, if a patient had an atrioventricular septal defect with common atrioventricular valvar orifice, double outlet right ventricle, and an interrupted inferior caval vein, then the atrioventricular septal defect was coded as the primary diagnosis, with double outlet right ventricle and interrupted inferior caval vein listed as secondary diagnoses. If a patient simply had interruption of the inferior caval vein, then this was coded as the primary diagnosis. It was implicit that those with bilateral superior caval veins of necessity had a left-sided superior caval vein.

Inferences regarding presence of isomeric right or left atrial appendages

The intracardiac features considered to indicate presence of isomeric right as opposed to left atrial appendages were assessed independently by two authors (RL and RHA), with cognizance taken also of the findings in the other systems of organs. These included, but were not limited to, overall cardiac anatomy, venoatrial connections, and pulmonary and splenic anatomy. The results of the independent assessments were then compared, and any differences discussed, with a consensus being reached by both authors regarding the likely presence of isomeric right as opposed to left atrial appendages. In some instances, the assessors agreed that there was probably usual arrangement of the atrial appendages, despite the presence of known bronchial isomerism.

An aggregate of several features was used to help determine whether isomerism of the right or left atrial appendages was present. Interruption of the inferior caval vein, for example, is known to be associated more with isomerism of the left atrial appendage. In contrast, absence of the coronary sinus and totally anomalous pulmonary venous connection are uniformly present in the setting of right isomerism. The presence of multiple spleens, or absence of a spleen, was also used to help segregate the subsets of isomerism. The bronchial branching pattern was also used when available.

Genetic testing

General microarray testing was done utilizing an array done within the institution itself. Whole genome array-based analysis was done to identify losses or gains of DNA variants (copy number variants) within the human genome using the Affymetrix Cytoscan HD array. Array data was then analyzed using Affymetrix Chromosome Analysis Suite. The array contains 2,696,550 genetic markers, including 743,305 single nucleotide polymorphisms and approximately 1,953,246 non-polymorphic markers for the detection of copy number variants. When the array identified a duplication less than or equal to 500kb or a deletion than or equal to 200kb, quantitative polymerase chain reaction was used to confirm losses or gains. For each segment confirmed with quantitative polymerase chain reaction, an Applied Biosystems TaqMan Gene Copy Number Assay was used. Assay data was analyzed using the Applied Biosystems CopyCaller Software. Each assay is run with positive controls and a non-template control.

Heterotaxy panels were run by Prevention Genetics utilizing a Next General sequencing panel. Full coding regions plus 20 base pairs of non-coding DNA flanking each exon were sequenced for areas of interest. The following genes were sequenced: *ACVR2B, ANKS6, CCDC103, CCD11, CCDC114, CCDC39, CCDC40, DNAAF1, DNAAF2, DNAAF3, DNAH11, DNAH5, DNAI1, DNAI2, DNAL1, FOXH1, GDF1, HEATR2, INVS, LEFTY2, LRRC6, NKX2-5, NME8, NODAL,* and *ZIC3*

Statistical analysis

To identify patients who differed from each other in regard to the arrangement of their organs, we compared the findings between patients as being likely to have isomerism of the right or left atrial appendages as opposed to usual or mirror-imaged atrial arrangement, using chi-squared analysis for categorical data and independent T-tests or Mann-Whitney-U tests where appropriate. Characteristics were also compared between those shown to have isomerism of the right or left bronchuses.

Characteristics, including intracardiac and extracardiac anomalies, were compared between those with and without an identified genetic mutation using chi square analysis for descriptive variables. Continuous variables were compared using a Mann-Whitney-U test. On this basis, patients were categorized into groups of *NODAL* mutation only, *CFC1* mutation only, *CFC1* combined with *NODAL* mutation, mutation in another gene, or no mutation. These groups were then compared by chi square analysis for descriptive variables, and analysis of variance for continuous variables.

Results

A total of 83 subjects met criteria for inclusion with the diagnosis of isomerism. All of these patients had karyotypes done. Of these, 27 (32.5%) had undergone genetic analysis other than a karyotype as well (Figure 1). This additional analysis consisted of a general microarray in all 27 of these patients. 8 of these patients also had a heterotaxy panel sent in addition to the karyotype and general microarray. Review of genetic testing identified known mutations in 14 (51.9%) of the 27 patients (Table 1).

Clinical characteristics

When we compared characteristics between those with and without an identified mutation, those with an identified mutation were more likely to have bilaterally right bronchuses (Table 1). There was no statistically significant difference, however, in the inferred morphology of the atrial appendages, or cardiac anatomic features.

The need for mechanical ventilatory support before initial cardiac surgical palliation and the number of days of postoperative mechanical ventilatory support after initial cardiac palliation was higher in those with an identified mutation (Table 2). Mechanical ventilatory support prior to initial cardiac palliation was required in 6 (50%) of those with an identified mutation, but in only 1 (9%) of those without

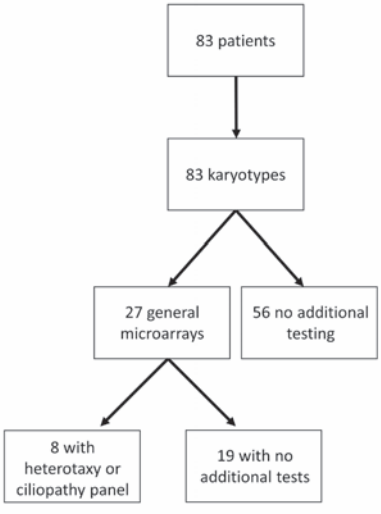

Figure 1. Flowchart illustrating testing done in the cohort.

(P=0.033). Median duration of postoperative mechanical ventilatory support after initial cardiac palliation was 7.5 days in those with an identified mutation, and 3 days in those without (P=0.045). Clinical outcomes of bacteremia, recurrent otitis media, recurrent pneumonia, chronic lung disease, asthma, need for supplemental oxygen at home, need for extracorporeal membrane oxygenation at home, and mortality did not differ between the groups (Table 2).

Genetic characteristics

By combining the positive karyotypes and the microarrays, a total of 13 genes were noted to have mutations. Of the 13 genes, 10 (76.9%) have been demonstrated to have associations with either ciliary abnormalities or isomerism in mice, 8 (61.5%) with ciliary abnormalities, and 8 (61.5%) with isomerism. Of these 13 genes, 5 (38.5%) have been demonstrated to be associated with ciliary abnormalities or isomerism in humans, 2 (15.4%) with ciliary abnormalities, and 3 (23.1%) with isomerism (Table 3).

A *CFC1* mutation was present in 5 (35.7%) of all patients with identified mutations, while a *NODAL* mutation was present in 3 (21.4%), making these the two most commonly affected genes in our cohort. In 2 (14.3%) patients, both *CFC1 and NODAL* were mutated (Table 4). When the cohort was segregated into subgroups of *CFC1* mutation only, *NODAL* mutation only, both *CFC1 and NODAL* mutation, other affected gene, or no mutation, there were statistically significant differences noted between the groups. Those with a *NODAL* mutation only, *CFC1* mutation only, other mutation, or no mutation tended to be associated with right isomerism, while those with

Table 1. Patient characteristics based on isomerism subtype.

	Patients with no identified mutation (n=13)	Patients with identified mutation (n=14)	P-value
Common atrioventricular junction	9 (69.2)	9 (64.3)	0.785
Double outlet right ventricle	5 (38.5)	4 (28.6)	0.586
Bilateral superior caval veins	6 (46.2)	7 (50.0)	0.842
Left sided superior caval vein	7 (53.8)	8 (57.1)	0.863
Interruption of the inferior caval vein	4 (30.8)	3 (21.4)	0.580
Atrioventricular connections			
Mixed	8 (61.5)	10 (71.4)	0.557
Double inlet	5 (38.5)	4 (28.6)	
Ventriculoarterial connections			
Concordant	9 (69.2)	11 (78.6)	0.580
Discordant	4 (30.8)	3 (21.4)	
Inferred morphology of the atrial appendages	12 (92.3)	11 (78.6)	0.315
Bronchial morphology			
Bilaterally left	5 (38.5)	0 (0)	0.016
Bilaterally right	8 (61.5)	12 (100)	
Splenic anatomy			
Absence of a spleen	7 (53.8)	8 (57.1)	0.505
Multiple spleens	4 (30.8)	2 (14.3)	
Solitary spleen	2 (15.4)	4 (28.6)	
Stomach and liver sidedness			
Left sided stomach with right sided liver	1 (8.3)	3 (25.0)	0.122
Right sided stomach with left sided liver	9 (75.0)	4 (33.3)	
Left or right sided stomach with midline liver	2 (16.7)	5 (41.7)	
Intestinal malrotation	9 (69.2)	9 (64.3)	0.785
Need for gastrostomy tube	4 (30.8)	5 (35.7)	0.785
Chronic arrhythmia	8 (61.5)	7 (50.0)	0.547
Need for pacemaker	3 (23.1)	0 (0)	0.057
Prenatal diagnosis	10 (76.9)	13 (92.9)	0.244
Repair status at most recent follow-up			
Biventricular repair	5 (38.5)	5 (35.7)	0.883
Univentricular repair	8 (61.5)	9 (64.3)	
Bacteremia	2 (15.4)	4 (28.6)	0.410
Recurrent otitis media	1 (7.7)	0 (0.0)	0.290
Recurrent pneumonia	0 (0.0)	0 (0.0)	--
Chronic lung disease	1 (7.7)	3 (21.4)	0.315
Asthma	2 (15.4)	0 (0.0)	0.127
Need for supplemental oxygen at home	6 (46.2)	3 (21.4)	0.173
Need for extracorporeal membrane oxygenation	3 (23.1)	2 (14.3)	0.557
Mortality	3 (23.1)	5 (35.7)	0.472
Age at death (days)	154 (8 to 1,432)	189 (42 to 235)	0.306
Follow-up times (years)	2.0 (0.5 to 3.8)	1.6 (0.2 to 13)	0.323

Frequencies presented as absolute number (percentage) while numeric values are presented as median (minimum to maximum). The denominator for some variables is lower than the total due to missing data.

both a *Nodal* and *CFC1* mutation tended to be associated with left isomerism (P=0.006). Those with a *NODAL* mutation only, or *CFC1* mutation only, tended to have a right-sided stomach with left-sided liver, while those with a mutation in another gene tended to have right or left sided stomachs with midline livers (P=0.038). Arrhythmias also differed between the various mutations. Supraventricular tachycardia was more commonly noted in those with a *NODAL* mutation only, *CFC1* mutation only, and those with mutations in other genes, but was not present in those with both a *NODAL* and *CFC1* mutation (P=0.024). Sinus nodal dysfunction was most frequently noted in those with only a *CFC1* mutation, while ectopic atrial tachycardia was most frequently noted in those with mutations in other genes (P=0.024).

A mutation in *NODAL* only was associated with increased length of hospitalization for initial cardiac palliation, with a median of 134 days. Those with a mutation in both *NODAL* and *CFC1* had an even greater length of hospitalization for initial cardiac palliation, with a median of 177 days (P=0.031). Mutation in *NODAL* only was also associated with increased length of hospitalization for second cardiac intervention, with a median of 134 days. Those with a mutation in both *NODAL* and *CFC1* had an even greater length of hospitalization for second cardiac palliation, with a median of 235 days (P=0.023). Those with a mutation in *NODAL, CFC1,* or both *NODAL and CFC1*, also required increased length of mechanical ventilatory support after second cardiac palliation (P=0.030).

Table 2. Surgical data of cohort based on isomerism subtype.

	Patients with no identified mutation (n=13)	Patients with identified mutation (n=14)	P-value
Underwent first surgery	11 (84.6)	12 (85.7)	0.936
Age at first surgery (days)	9 (4 to 871)	12.5 (0 to 822)	0.469
Need for mechanical ventilation during hospitalization for, but before, first surgery	1 (9.1)	6 (50.0)	0.033
Number of days of mechanical ventilation after first surgery	3 (0 to 8)	7.5 (0 to 114)	0.045
Number of days chest left open postoperatively after first surgery	0 (0 to 6)	1 (0 to 28)	0.110
Total length of hospitalization for first surgery (days)	42 (5 to 154)	94 (7 to 235)	0.166
Underwent second surgery	8 (61.5)	9 (64.3)	0.883
Age at second surgery (days)	155 (103 to 963)	158 (15 to 280)	0.547
Need for mechanical ventilation during hospitalization for, but before, second surgery	1 (12.5)	3 (33.3)	0.312
Number of days of mechanical ventilation after second surgery	2 (0 to 9)	4 (0 to 95)	0.223
Number of days chest left open postoperatively after second surgery	0 (0 to 6)	0 (0 to 9)	0.418
Total length of hospitalization for second surgery (days)	44.5 (14 to 154)	41.5 (8 to 235)	0.733
Underwent third surgery	0 (0.0)	2 (15.4)	0.141
Age at third surgery (days)	--	633.5 (193 to 1074)	--
Need for mechanical ventilation during hospitalization for, but before, third surgery	--	1 (50.0)	--
Number of days of mechanical ventilation after third surgery	--	0 (0 to 0)	--
Number of days chest left open postoperatively after third surgery	-	0 (0 to 0)	--
Total length of hospitalization for third surgery (days)	--	26 (11 to 41)	--

Table 3. Genes identified in cohort and their association with isomerism and ciliary dyskinesia.

Gene	Associated with ciliary abnormalities in mice	Associated with isomerism in mice	Associated with cilia in humans	Associated with isomerism in humans	Inheritance in humans
NODAL	Yes	Yes	No	Yes	Ad
CFC1	Yes	Yes	No	Yes	Ar
CCDC39	Yes	Yes	Yes	No	--
DNAH11	Yes	Yes	Yes	No	--
NKX2.5	Yes	No	No	No	--
TBX1	No	Yes	No	No	--
ZIC3	Yes	Yes	No	Yes	Xl
DNAAF	Yes	Yes	No	No	--
CNTN4	No	No	No	No	--
MCPH1	No	No	No	No	--
TNKS	No	No	No	No	--
SOX7	Yes	No	No	No	--
GATA4	No	Yes	No	No	--

Ad, autosomal dominant; Ar, autosomal recessive, Xl, x-linked.

Discussion and Conclusions

We have identified mutations in approximately half of genotyped patients known to have thoracic isomerism. All but two of these mutations were detected by genetic testing other than chromosome karyotype. Nearly four-fifths of the affected genes are known to be associated with either ciliary dyskinesia or isomerism in mice, with two-fifths known to be associated with these features in humans.

The most frequently affected gene was *CFC1,* which has been noted to be associated with ciliary abnormalities and isomerism in mice, as well as isomerism in humans. There have been 43 cases of *CFC1* mutations in humans reported, with 80% consistent with a diagnosis of left isomerism and the remainder with right isomerism. A majority was described as having a common atrioventricular junction, with double outlet right ventricle also reported in several patients.[33,35-38] Thus far, very few descriptions have provided details of bronchial, pulmonary, or atrial appendage morphology. Our segregation of isomerism, therefore, was based on the aggregate of the other clinical features described. In this regard, previously reported cases have been associated mostly with presumed left isomerism, while the patients making up our current cohort were more likely to have right isomerism.

CFC1 mutations impair normal left-right patterning, as proteins encoded by *CFC1* are cofactors for *NODAL*-related signals.[39] *NODAL* mutations were the second most frequently affected gene in our cohort. Such mutations are associated with ciliary dyskinesia and isomerism in mice, but only with isomerism in humans based on published reports. In one analysis, *NODAL* mutations were associated with a higher occurrence of pulmonary atresia, and 90% of the findings were most consistent with right isomerism.[40] In the mouse, *Nodal* is known to play a role in formation of both endoderm and mesoderm, impacting left-right patterning. The gene encodes a protein that is part of the transforming growth factor beta family, and induces its coreceptor Cripto. Mice deficient in *Nodal* do not have a primitive streak, and thus fail to form any mesoderm.[41-43] Likely due to the lack of mesoderm, there is an increase in endodermal formulation. Mice null for *Nodal* arrest their development during gastrulation, whereas hypomorphic mice have abnormalities in left-right patterning.[44]

In our cohort, we identified several patients with mutations of both *CFC1* and *NODAL* mutation. Those with *NODAL* mutations alone suffered increased morbidity, and those with a combined *CFC1* and *NODAL* mutation had the longest hospitalization of any of our subgroups. To the best of our knowledge, this finding has not been previously described. It is, perhaps, related to underlying ciliary dyskinesia, particularly since need for mechanical ventilatory was increased in the setting of these mutations. We also identified mutations of *CCDC39, DNAH11, NKX2.5, TBX1, ZIC3, DNAAF, SOX7,* and *GATA4* genes. All have previously been described to be associated with ciliary dyskinesia in either mice or humans, and in some cases both.[16,45-63] We find it of significance, therefore, that a large proportion of affected genes are associated with not only isomerism, but also with ciliary dyskinesia. Previous studies have shown that over 40% of patients with isomerism also have ciliary dyskinesia.[16] Nodal cilia in humans have a 9 + 0 arrangement, lacking a central pair of microtubules.[64] These cilia rotate in a rotary pattern, rather than the whiplike pattern of the cilia more traditionally referred to as the motile cilia, which have a 9 + 2 arrangement of microtubules. Studies have shown that mutations in genes affecting the central pair of microtubules can cause laterality abnormalities while mutations in genes affecting portions of the cilia other than the central pair do not affect laterality.[65,66] It is not only flow of embryonic fluid created by this rotary movement of cilia that affects laterality, however, but also impairment in ciliary sensory function. While the motile nodal cilia create the leftward flow of embryonic fluid, there is the requirement of downstream cilia to sense the flow, thus producing the co-called *two-cilia* hypothesis.[67]

Ciliary dyskinesia has effects outside of embryogenesis. Impairment in motile cilia can affect mucociliary clearance, which can increase sinupulmonary symptoms, and may mediate the need for increased mechanical ventilatory support.[19] Impairment of motile cilia has also been implicated in the development of hydrocephalus in patients with isomerism. Impairment in sensory cilia may potentiate hepatic, renal, and splenic dysfunction, although this is yet to be delineated.

We recognize the limitations of our study, which was simply descriptive in demonstrating its inferred associations. We are not able directly to establish causality. While our data has demonstrated trends, future studies, with larger number of patients and additional genetic analysis, will be necessary to establish causality. Our study shows, nonetheless, that future studies focused on isomerism should contain detailed information about the patients, their clinical course, and the genetic data available. This will allow for all associations to be analyzed, and for trends to be noted.

We conclude that at least half of all patients with bodily isomerism are likely to have a mutation if analysed by microarray. Most genes identified in our patients are known to be associated with ciliary dyskinesia or isomerism. Identification of genes may, in the future, be predictive of clinical course.

Table 4. Comparison of gene mutations by genetic subgroup.

Nodal mutation	CFC1 mutation	Mutation in Nodal and CFC1	Other mutation	No mutation identified
- Right isomerism (100%) - Right sided stomach and left sided liver (100%) - Supraventricular tachycardia (100%) - Increased length of hospitalization for first cardiac intervention (median 134 days) - Increased length of hospitalization for second cardiac intervention (median 134 days)	- Right isomerism (67%) - Sinus node dysfunction (67%) and supraventricular tachycardia (33%)	- Left isomerism (100%) - Right sided stomach and left sided liver (100%) - Increased length of hospitalization for first cardiac intervention (median 177 days) - Increased length of mechanical ventilation after second cardiac intervention (median 95 days) - Increased length of hospitalization for second cardiac intervention (median 235 days)	- Right isomerism (100%) - Right or left sided stomach with midline liver (57%) - Ectopic atrial tachycardia (67%) and supraventricular tachycardia (33%)	- Right isomerism (92.3%) - Right sided stomach and left sided liver (75%) - Atrial tachycardia (38%) and complete atrioventricular block (38%)

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