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## Abstract

Defects of situs are associated with complex sets of congenital heart defects in which the normal concordance of asymmetric thoracic and abdominal organs is disturbed. The cellular and molecular mechanisms underlying the formation of the embryonic left-right axis have been investigated extensively in the past decade. This has led to the identification of mutations in at least 24 different genes in humans with heterotaxy and situs defects. Those mutations affect a broad range of molecular components, from transcription factors, signaling molecules, and chromatin modifiers to ciliary proteins. A substantial overlap of these genes is observed with genes associated with other congenital heart diseases such as tetralogy of

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Fallot and double outlet right ventricle, d-transposition of the great arteries, and atrioventricular septal defects. In this chapter, we present the broad genetic heterogeneity of situs defects including recent human genomics efforts.

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## 38.1 Introduction

Situs inversus is found in about 0.01 % of the population, or about 1 person in 10,000. In the most common situation, the relationship between the organs is unchanged, and most people with situs inversus have no medical symptoms or complications [1]. In cases of situs ambiguus, the arrangement of the thoracoabdominal organs across the left-right (LR) axis of the body is variable. Frequently, the term heterotaxy is used synonymously for situs ambiguus. This broad term includes patients with a wide variety of very complex cardiac lesions. A right-sided heart position is called dextroposition. If the apex of the heart points to the right, it is called dextrocardia.

Heterotaxy shows a high 79 % recurrence risk ratio among first-degree relatives, which underlines a strong genetic basis of the disease [2]. Recent studies concerning its etiology have yielded new insights into the genetic architecture and will be summarized here [3]. A number of specific gene studies in patients were based on insights obtained by the study of left-right patterning in the early embryo of model organisms. However, multiple genetic variations, RNA and protein expression, and the interaction of these molecules impacts on the ultimate phenotype. Furthermore, this individual-specific mix of genes and expression patterns collides with the environment (teratogens, maternal exposures, and infectious agents; see Chap. 16). We will focus on the genetic causes and provide insight into the extensive genetic heterogeneity of situs defects.

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## 38.2 Copy Number Variations

Copy number variations (CNVs) are sub-chromosomal changes in genome structure and generally comprise structural variants of intermediate size that range from about 1000 to  $5 \times 10^6$  bases of DNA [3, 4]. These deletions, duplications, and inversions lead to gain or loss of chromosomal segments often containing multiple genes. Recent studies have identified multiple CNVs that contribute to non-syndromic CHD [3].

Fakhro et al. analyzed more than 250 patients with heterotaxy with high-resolution genotyping and estimated from these data copy number frequencies. They found 45 CNVs in 39 cases of which five were further assessed and confirmed by Morpholino knockdown in *Xenopus tropicalis*, the clawed frog [5]. Knockdown of these genes, *NEK2* (NIMA-related kinase 2), *ROCK2* (Rho-associated, coiled-coil containing protein kinase 2), *TGFBR2* (transforming growth factor, beta receptor II), *GALNT11* (polypeptide N-acetylgalactosaminyltransferase 11), and *NUP188* (nucleoporin 188 kDa), leads to strongly disrupted morphological

LR development and altered expression of *Pitx2* (paired-like homeodomain 2), a molecular marker of LR patterning [5].

Glessner et al. studied several hundred CHD trios with two complementary high-resolution techniques, single nucleotide polymorphism (SNP) arrays and whole exome sequencing [6]. A significant increase in CNV burden was observed when comparing CHD trios with healthy trios. Rare *de novo* CNVs were identified in 51 probands, among them three patients with heterotaxy. The CNVs of these patients were located on chromosomes 6q21.2, 17p11.2, and 22q13.2, and size ranged between 5 and 680 kilobases. They affected genes such as *CDKN1A* (cyclin-dependent kinase inhibitor 1A), *FAM27L* (family with sequence similarity 27-like), and *CYP2D6* (cytochrome P450, family 2, subfamily D, polypeptide 6), respectively [6].

### 38.3 Single Gene Defects

A number of different genes have been implicated in situs defects indicating that there is an extensive genetic heterogeneity. To date, 24 genes have been shown to be involved in situs abnormalities; but this number should be seen as preliminary and is likely to rise with future genetic studies. Genes playing a role in heterotaxy frequently encode cardiac transcription factors, components of signaling pathways, or histone-modifying proteins, along with other proteins (Table 38.1 lists all known genes).

**Table 38.1** Genes where mutations in patients with heterotaxy and situs defects have been described

Gene	Protein function	Phenotype	Status	References
<i>Transcription factors (TF)</i>				
<i>NKX2-5</i>	Homeobox TF	Heterotaxy, situs inversus	Two or more independent reports	[7, 8]
<i>GATA4</i>	GATA binding TF	Dextrocardia	Single case report	[9]
<i>ZIC3</i>	Zink finger TF	Heterotaxy, situs ambiguus, situs inversus, dextrocardia	Two or more independent reports	[10–12]
<i>Genes involved in signaling pathways</i>				
<i>ACVR2B</i>	Activin receptor (TGFbeta family)	Heterotaxy, dextrocardia	Two or more patients	[13]
<i>GDF1</i>	Ligand (TGFbeta family)	Situs inversus, situs ambiguus, dextrocardia	Two or more patients	[14]
<i>CFC1</i>	Ligand (EGF-CFC family)	Heterotaxy, dextrocardia	Two or more independent reports	[15–17]
<i>LEFTY A</i>	Ligand (TGFbeta family)	Dextrocardia	Two or more patients	[18]

(continued)

**Table 38.1** (continued)

Gene	Protein function	Phenotype	Status	References
<i>NODAL</i>	Ligand (TGFbeta family)	Dextrocardia, situs inversus	Two or more patients	[19]
<i>Histone-modifying genes</i>				
<i>RNF20</i>	Histone-modifying gene	Heterotaxy, dextrocardia	Single case	[20]
<i>SMAD2</i>	Histone-modifying gene	Heterotaxy, dextrocardia	Two or more patients	[20]
<i>MED20</i>	Histone-modifying gene	Heterotaxy, dextrocardia	Single case report	[20]
<i>NAA15</i>	Histone-modifying gene	Heterotaxy, dextrocardia	Single case report	[20]
<i>Ciliary genes</i>				
<i>DNAI1</i>	Dynein arm	Situs inversus	Two or more independent reports	[21, 22]
<i>DNAH5</i>	Dynein arm	Situs inversus	Two or more patients	[23]
<i>NPHP2</i>	Ciliary gene	Situs inversus	Two or more patients	[23]
<i>NPHP3</i>	Ciliary gene	Situs inversus	Two or more patients	[24]
<i>NPHP4</i>	Ciliary gene	Dextrocardia, situs inversus	Two or more patients	[25]
<i>CCDC11</i>	Ciliary gene	Situs inversus	Single case report	[26]
<i>Other genes</i>				
<i>PKD2</i>	Polycystin 2	Dextrocardia, situs inversus	Two or more independent reports	[27, 28]
<i>SHROOM3</i>	Cytoskeletal protein	Dextrocardia, situs inversus	Two or more patients	[29]
<i>CRELD1</i>	Cell adhesion protein	Heterotaxy	Two or more patients	[30]
<i>MKRN2</i>	E3 ubiquitin ligase	Situs inversus	Single case report	[20]
<i>OBSCN</i>	Sarcomeric protein	Heterotaxy, dextrocardia	Single case report	[20]
<i>UMODL1</i>	Urinary protein	Heterotaxy, dextrocardia	Single case report	[20]

### 38.3.1 Cardiac Transcription Factors

The expression of cardiac transcription factors occurs in highly specified temporal-spatial patterns throughout development [3] (see Chap. 12). Transcription factors orchestrate heart development, and many of them are associated with isolated CHD [3, 31] (see, e.g., Chap. 30). Transcriptional focal points include NK2 homeobox 5

(*NKX2-5*) and GATA binding protein 4 (*GATA 4*) which are known to be involved in situs defects.

Watanabe et al. identified a deletion frameshift mutation in *NKX2-5* in a familiar CHD case with heterotaxy as well as atrial septal defect (ASD) [7]. Izumi et al. described a further deletion frameshift mutation in *NKX2-5* (along with three other variants of unknown significance in genes associated with ciliary disease; see Sect. 38.3.4) in a patient with complex CHD including heterotaxy using clinical exome sequencing [8]. In *GATA4*, a frameshift mutation was identified by Hirayama-Yamada et al. in a family with multiple ASD cases of which one showed additional dextrocardia [9]. However, affection of other contributing genes might be likely in this case.

The X-linked form of heterotaxy is caused by mutations in the zinc finger transcription factor *ZIC3* (Zic family member 3) and affects approximately 1 % of sporadic heterotaxy cases [1]. Mutations often cause loss of function and, in some cases, result in abnormal subcellular localization and trafficking [1]. *ZIC3* was the first gene unequivocally associated with human situs abnormalities [32, 33]. To date, a number of different *ZIC3* point mutations (missense, nonsense, and frameshift) have been described in X-linked familial heterotaxy cases as well as in sporadic heterotaxy and isolated CHD cases [10–12, 32, 34]. Patients from these studies show the whole spectrum of situs defects such as situs inversus, heterotaxy (situs ambiguus including asplenia and polysplenia), and dextrocardia. In summary, *ZIC3* is the most frequent disease gene for laterality defects.

### 38.3.2 Genes Involved in TGF $\beta$ Signaling Pathways

Heart development involves coordination of a number of signaling pathways [31]. Several mutations in signaling molecules have been detected in different forms of CHD associated with situs defects.

Activins and their receptors are members of the transforming growth factor beta (TGF $\beta$ ) family of signaling molecules. Two missense mutations in *ACVR2B* encoding the activin receptor type II B were found in three patients showing heterotaxy and complex CHD [13]. Kaasinen et al. described a family with right atrial isomerism associated with situs inversus, situs ambiguus, dextrocardia, and asplenia showing mutations in the growth differentiation factor 1 (*GDF1*) [14]. Two truncating mutations of *GDF1* were observed to segregate with the phenotype in an autosomal recessive manner [14]. Of note, this study also identified 11 carriers of heterozygous truncating mutations in *GDF1* in control subjects without CHD indicating a high frequency and compatibility with normal development and health.

*CFC1* encoding CRYPTIC protein is a member of the EGF (epidermal growth factor)-CFC (Cripto, Fr11, and Cryptic) family encoding extracellular proteins important for intercellular signaling pathways during vertebrate embryogenesis. Bamford et al. were the first to describe loss-of-function mutations in human *CFC1* in patients with heterotaxic phenotypes [15]. They identified nine patients carrying four different missense mutations and one deletion with various forms of

heterotaxy associated with CHDs such as d-transposition of the great arteries (d-TGA), ventricular septal defect (VSD), and ASD; five of the patients showed dextrocardia. The mutant proteins had aberrant cellular localization in transfected cells and showed functional effects in a zebrafish model [15]. Selamet Tierney et al. found three non-synonymous variants in *CFC1* in patients with laterality defects and CHD and suggested that these may act as susceptibility alleles in conjunction with other genes and/or environmental factors [16]. Further, Roessler et al. screened *CFC1* in a cohort of 251 patients with laterality defects and identified two mutations [17].

A further member of the TGF $\beta$  family, the left-right determination factor 2 (*LEFTY2*, also known as *LEFTYA*), was shown to be mutated in heterotaxy patients and is well known for its role in left-right patterning during mouse development [18]. Kosaki et al. found one nonsense and one missense mutation in *LEFTYA* in two patients with LR-axis malformations and CHD [18]. However, they stated that the *LEFTYA* mutant alleles may be necessary, but not sufficient, to give an LR phenotype in these affected individuals, because each mutation was found to be carried by one of the parents [18].

Analysis of a cohort of 269 patients with heterotaxy and/or isolated cardiovascular malformations revealed four different missense mutations in *NODAL* (Nodal growth differentiation factor) [19]. *NODAL* mutations were found in 14 unrelated subjects consisting of one in-frame insertion/deletion and two conserved splice site mutations. About one third of these patients showed dextrocardia and situs inversus (as well as asplenia in some cases) associated with a wide spectrum of CHD including pulmonary atresia, d-TGA, ASD, and VSD.

### 38.3.3 Histone-Modifying Genes

Histone proteins package chromosomal DNA into structural units called nucleosomes. They act as “spools” around which DNA winds and play an important role in gene regulation. Histone-modifying proteins have been identified for a number of distinct forms of modifications such as acetylation and methylation/demethylation.

Zaidi et al. used exome sequencing in parent-offspring trios to determine and compare the incidence of *de novo* mutations in 362 severe CHD cases (including heterotaxy) and 264 controls [20]. They found an excess of *de novo* mutations in genes involved in the production, removal, and reading of histone 3 lysine 4 (H3K4) methylation, an activating chromatin mark, and H3K27 methylation, an inactivating chromatin mark [20]. In heterotaxy or dextrocardiac patients, they found mutations in *SMAD2* (SMAD family member 2), *MED20* encoding mediator complex subunit 20, and *RNF20* encoding RING finger protein 20, an E3 ubiquitin ligase that regulates chromosome structure by monoubiquitinating histone H2B. The latter case showed additional asplenia as also seen in a further case with a frameshift mutation in *NAA15* (N-alpha-acetyltransferase 15, NatA auxiliary subunit) [20].

### 38.3.4 Ciliary Genes

There are two general types of cilia, namely, primary cilia serving as sensory organelles and motile cilia exerting mechanical force. Motile cilia are critical to the development of proper organ laterality and represent a central pathway disturbed in heterotaxy [35]. Primary ciliary dyskinesia (PCD) is one of the most widely recognized ciliopathies and a genetically heterogeneous disorder resulting from loss of function of different parts of the primary ciliary apparatus, often components of the dynein motor complex [1] (see Chap. 39). In cases where dextrocardia, situs ambiguus, or situs inversus are present in the infant, PCD should be considered as a highly possible diagnosis [36]. To date, PCD-causative mutations in 29 genes are known, and the number of causative genes is bound to rise (as reviewed by Kurkowiak et al. [36]). In this section, we will present a selection of important PCD genes associated with situs defects.

Kartagener syndrome is characterized by the combination of PCD and situs inversus and occurs in approximately 20 % of patients with PCD [35]. The absence of normal ciliary movement results in a lack of definitive patterning; thus whether the viscera take up the normal or reversed left-right position during embryogenesis is driven by stochasticity [1]. The syndrome is caused by mutations in *DNAI1* encoding axonemal dynein intermediate chain 1, an outer dynein arm component that is essential for ciliary function at the node [21]. Guichard et al. identified compound heterozygous *DNAI1* gene defects in three independent patients from a cohort of 34 patients with Kartagener syndrome [21]. A further study in more than 300 PCD patients showed that half of them had situs inversus and at least 6 % of patients with PCD have heterotaxy, and most of those have CHDs such as systemic vein abnormalities, aortic coarctation, atrioventricular septal defect (AVSD), and double outlet right ventricle [22]. Further, the patients carried more mutations in ciliary outer dynein arm genes (*DNAI1* and *DNAH5* encoding axonemal dynein heavy chain 5) as compared with PCD patients with situs solitus [22].

Nephronophthisis (NPHP), an autosomal recessive cystic kidney disease, belongs to the ciliopathies and is characterized by cilia-related defects. Mutations in *nephronophthisis-2* (*NPHP2* also named *INVS* for inversin) can result in NPHP with situs inversus and mild cardiac defects [23]. Bergmann et al. showed that *NPHP3* (also named nephrocystin-3) mutations can cause a broad clinical spectrum of early embryonic patterning defects comprising situs inversus and structural heart defects as well as kidney disease [24]. Interestingly, a genome-wide linkage analysis identified *nephronophthisis-4* (*NPHP4*) mutations in patients with cardiac laterality defects but without NPHP from a consanguineous family [25]. Further sequencing of this gene identified eight additional missense mutations in a cohort of unrelated patients with dextrocardia, situs inversus, and asplenia/polysplenia [25].

Performing homozygosity mapping in a consanguineous family with laterality defects, Perles et al. identified a homozygous splice site mutation in *CCDC11* encoding coiled-coil domain containing 11 protein which is preferentially expressed in ciliated cells [26]. The patient carrying the mutation was characterized by situs inversus and severe cardiac malformations [26].

### 38.3.5 Other Genes

*PKD2*, encoding polycystin 2 and belonging to the superfamily of transient receptor potential channels, causes autosomal dominant polycystic kidney disease. Bataille et al. reported an association of that disease resulting from *PKD2* mutations with left-right laterality defects [27]. One large gene deletion, one single-exon duplication, and one in-frame duplication, respectively, were found in three unrelated patients characterized by dextrocardia or situs inversus in addition to kidney disease [27]. Another patient with complete situs inversus and autosomal dominant polycystic kidney disease caused by a *PKD2* missense mutation (leading to a premature truncation of polycystin 2) was identified by Oka et al. [28].

*CRELD1* encoding the cell adhesion molecule cysteine-rich with EGF-like domains 1 was analyzed in a cohort of 11 patients with AVSD and heterotaxy [30]. One missense mutation was detected in a patient characterized by dextrocardia, right ventricle aorta with pulmonary atresia, and a right aortic arch in addition to partial AVSD [30]. However, Robinson et al. suggested that *CRELD1* mutations might increase the risk of developing a heart defect, rather than being directly causative [30].

Using whole exome sequencing, Tariq et al. found mutations in *SHROOM3* (Shroom family member 3), an actin-binding protein, which is responsible for early cell shape during morphogenesis through a myosin II-dependent pathway [29]. They first identified a mutation in a patient with complex heterotaxy phenotype (including dextrocardia and situs inversus) by exome sequencing and subsequently analyzed a cohort of sporadic heterotaxy patients in *SHROOM3* leading to the identification of further two mutations that were predicted to be pathogenic [29].

Besides alterations in histone-modifying enzymes, the study of Zaidi et al. (see Sect. 38.3.3) identified three *de novo* mutations in *MKRN2* (encoding makorin RING finger protein 2, a probable E3 ubiquitin ligase), in *OBSCN* (encoding obscurin, a giant sarcomeric protein important for myofibrillogenesis), and in *UMODL1* (encoding uromodulin-like 1, a protein similar to uromodulin which is associated with various kidney diseases). Interestingly, Zaidi et al. also described a second *de novo* missense mutation in all of the three genes in a CHD patient without heterotaxy or situs defect [20].

#### Conclusion

As in other forms of congenital heart disease, defects of the situs and heterotaxy have a complex genetic etiology. The findings from the mentioned studies are entirely consistent with a complex, oligogenic disease model. One can speculate that rare heterozygous mutations identified in the sporadic cases have probably an epistatic effect with additional genetic modifiers within other developmental pathways. Even in consanguineous families, there may exist other genetic variants that lead to the phenotype [25, 26]. Further, the observed variety in the phenotype in combination with ciliopathies and kidney disease as well as other CHDs supports this notion.

Point mutations in at least 24 different genes have been described in patients with heterotaxy and situs defects (see Table 38.1). There is a wide genetic heterogeneity including genes encoding for transcription factors, signaling molecules, ciliary



proteins, histone-modifying proteins, and others. There is no doubt that additional genes will be identified based on the technical advances [1, 3, 35]. A substantial overlap of situs genes is observed with genes associated with other CHDs such as tetralogy of Fallot and double outlet right ventricle (see Chap. 32), d-transposition of the great arteries (see Chap. 35), and atrioventricular septal defects (see Chap. 26). To date, there are only a few data pointing to an important role of sub-chromosomal changes such as copy number variation in the etiology of situs defects.

Although there is a strong genetic contribution to situs defects, the majority of situs defects are idiopathic indicating the need for better utilization of novel approaches such as whole exome/genome sequencing to find the genetic variations contributing to this severe congenital heart disease.

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