The genetics of *situs inversus totalis* without primary ciliary dyskinesia

Merel C. Postema[1], Amaia Carrion-Castillo[1], Simon E. Fisher[1,2], Guy Vingerhoets[3], Clyde Francks[1,2 *]

[1] Department of Language & Genetics, Max Planck Institute for Psycholinguistics, Nijmegen, Netherlands
[2] Donders Institute for Brain, Cognition and Behaviour, Radboud University, Nijmegen, Netherlands
[3] Department of Experimental Psychology, Ghent University, Belgium

*Correspondence to: clyde.francks@mpi.nl

Abstract

Situs inversus totalis (SIT), a complete left-right mirror reversal of the visceral organs, is usually described as a recessive disorder. SIT can occur with Primary Ciliary Dyskinesia (PCD). However, most people with SIT do not have PCD, and the etiology of their condition remains poorly studied. Those without PCD may have an elevated rate of left-handedness, implying developmental mechanisms linking brain and body laterality. We sequenced the genomes of 15 people with SIT, of which six had PCD, and 15 controls. The SIT subjects with PCD all had likely recessive mutations in genes already known to cause PCD. Two non-PCD SIT cases also had recessive mutations in known PCD genes, suggesting reduced penetrance for PCD in some SIT cases. One non-PCD SIT case had a recessive mutation in *PKD1L1*, which has previously been linked to SIT without PCD. However, six of the nine non-PCD SIT cases, including most of the left-handers in this dataset, had no obvious candidate genes or significant pathways affected by the mutations that they carried. While we cannot exclude a monogenic basis, more complex genetic models must also be considered, as well as environmental influences or random effects in early development.

Keywords: *Situs inversus totalis*, primary ciliary dyskinesia, left-handedness, whole genome sequencing

Main text

Roughly 1:6,000-8,000 people have SIT (1). SIT is classically described as a recessive genetic condition (2), which can occur alone or in combination with Primary Ciliary Dyskinesia (PCD), a recessive disorder which involves mutations that disrupt motile cilia (1). Cilia within an early embryonic structure called the 'node' normally generate a leftward fluid flow, which helps to create the left-right body axis, but often people with PCD have SIT, presumably due to disrupted left-right axis formation when the nodal ciliary mechanism does not work. The combination of SIT with PCD is known as Kartagener syndrome, and is seen in 20-25% of SIT cases (3). Nonetheless, the majority of SIT cases (75-80%) do not have PCD (1). Few genes have been reported to be involved in SIT without PCD, and some genes that have, can also cause partial disruptions of visceral laterality, known as Heterotaxy or *Situs ambiguus* (SA), including *ZIC3* [MIM:300265] (4), *CCDC11* [MIM:614759] (5), *WDR16* [MIM:609804] (6), *NME7* [MIM:613465] (7), and *PKD1L1* [MIM:609721] (8).

Approximately 85-90% of people are right-handed, and this is not altered in Kartagener's syndrome (9), implying a developmental dissociation between brain laterality for handedness, and nodal-ciliary visceral patterning. Consistent with this, Vingerhoets *et al*. (10) studied fifteen people with SIT, six of whom had Kartagener's syndrome, and found that only one with Kartagener's was left-handed: however, at least five of the nine SIT cases without PCD were left-handed. Although based on a small sample, this suggests developmental mechanisms which might link handedness and visceral laterality, but independently of genes involved in PCD. A study based on common genetic variation also reported a possible link between a continuous measure of left-versus-right hand motor skill and genes involved in visceral laterality (11), although the sample size was limited for complex-trait genome-wide association analysis.

Here we performed whole genome sequencing in the same set of fifteen SIT subjects studied by Vingerhoets *et al*., as well as 15 healthy controls matched for age, sex, education, and handedness (Table 1, Table S1). We focused initially on non-synonymous coding variants predicted to affect protein function, and variants which affect splice donor and acceptor sites (see Supplementary Methods). As SIT is classically described as a recessive disorder, we considered homozygous mutations and potentially compound heterozygous mutations (i.e. when at least two different mutations were present in a given gene in heterozygous form). Additionally, variants were only considered as potentially causative mutations when they had minor allele frequencies below 0.005, according to population databases (Supplementary Methods), or were novel to the current dataset. Genes recessively mutated in any of the 15 controls were excluded. According to these criteria, the number of recessively mutated, potentially causative genes per subject ranged from 5 to 15 in the fifteen SIT cases.

Each of the six Kartagener cases had one recessively mutated gene which was annotated 'Kartagener' or 'PCD' in the Clinvar database (12), and was therefore the most likely monogenic cause for their condition (Table 1). These genes were *LRRC6* [MIM:614930], *DNAH11* [MIM:603339], *DNAAF1* [MIM:613190], *CCDC114* [MIM:615038], and *DNAH5* [MIM: 603335] (the latter mutated in two Kartagener cases, consistent with *DNAH5* being the

most common cause of PCD in European-ancestry populations) (13) (Table 1). The Kartagener case with a homozygous *LRRC6* mutation (subject SI06) was the only individual to show an elevated inbreeding coefficient and non-European ancestry (Supplementary Methods; Table S2, Figure S1).

Gene set enrichment analysis using the Gene Ontology (GO) (14, 15), Kyoto Encyclopedia of Genes and Genomes (KEGG) (16), and the Human Phenotype (HP) database (17) (Supplemental Methods), with the total combined list of 54 recessively mutated genes in the six Kartagener cases, produced significant results for various cilia-related pathways, such as ciliary plasm ($p = 0.00307$), ciliary dyskinesia ($p = 0.000121$) and outer dynein arm assembly ($p = 4.32E-05$), as well as the HP term "*situs inversus totalis*" ($p = 0.0101$) (Table S3). As expected, when the single most likely causative gene for each Kartagener subject was removed from this list, there were no longer significant enrichment terms, which further supports that the monogenic causes had been correctly identified. None of the fifteen healthy control subjects had any recessively mutated genes annotated 'Kartagener', 'PCD', 'SA' or 'SIT' in Clinvar, and the list of recessively mutated genes in the fifteen healthy controls did not produce any significant gene set enrichment terms.

Our purpose was not clinical diagnosis or confirmation of already-known Kartagener or SIT genes, and therefore we did not sequence the mutations in Kartagener patients by another technique, nor confirm phase in the compound heterozygotes. Rather, the six Kartagener patients acted as positive controls for our subsequent analysis of non-PCD SIT, by showing that our pipeline for identifying mutations from WGS data was well calibrated, and also that a shared biological pathway was detectable from as few as six subjects in gene set enrichment analysis.

However, gene set enrichment analysis of the 60 recessively mutated genes in the nine subjects with SIT but no PCD produced no significant gene sets, nor did the 42 recessively mutated genes in the subset of five left-handed subjects with non-PCD SIT (Table S3). There were probable monogenic recessive causes for only three of the nine non-PCD SIT subjects, which were the known Kartagener genes *DNAH5* (subject SI12) and *CCDC151* (subject SI16), and the known SIT/SA gene *PKD1L1* (subject SI02) (Table 1). Subjects SI12 and SI16 may therefore have Kartagener's syndrome but were never diagnosed by a physician, perhaps due to low penetrance for PCD diagnostic criteria such as bronchitis. As regards *PKD1L1*, a homozygous missense mutation was previously reported in an individual with SIT and congenital heart disease (CHD) but no PCD, as well as recessive splicing mutations in two individuals with heterotaxy (8). Our subject SI02 had no diagnosis of CHD.

Six non-PCD SIT cases therefore remained 'unsolved', who did not have recessive mutations in genes known to cause human laterality disorders, as annotated in Clinvar. Among these six cases, four were left-handed, and three also had CHD (Table 1). We constructed an extended list of known or suspected laterality genes with reference to the literature and mouse phenotypes (Methods; Table S4), but none of these genes had recessive mutations in the six unsolved non-PCD SIT cases. We performed gene set enrichment analysis for the 41 recessively mutated genes specifically in the six unsolved cases, but saw no significant biological pathways; thus

the biology of their non-PCD SIT could not be linked via the recessive mutations that they carried. Tables S5 and S6 show all recessively mutated genes in the unsolved cases; no clear candidates stand out in terms of their known biology (see also Supplementary Results). For the six unsolved cases we also considered a dominant model using a maximum known mutation frequency of 5.0E-5 (Supplementary Methods), and cross-referenced these with Clinvar and our extended candidate gene list (Table S4), but no likely causative genes emerged (Supplementary Results, Table S7), nor significant pathway-level results. Finally, we also considered larger genomic rearrangements known as Copy Number Variants (CNVs), but no obvious candidate genes emerged (Supplementary Results).

In summary, all six Kartagener patients had probable recessive monogenic causes in known Kartagener/PCD genes, while two non-PCD SIT cases also had likely recessive causative mutations in known Kartagener genes, suggesting reduced penetrance for PCD. One non-PCD SIT case had a recessive mutation in a known non-PCD SIT/SA gene (*PKD1L1*). However, six of the nine non-PCD SIT cases in this study remained unsolved, with no clear candidate mutations to take forward for validation or functional analysis. A monogenic model is still possible for the majority of non-PCD SIT cases, but would have to involve genetic heterogeneity across a set of genes which are not currently linked in terms of their known biology, at least to an extent which would have been detectable in this dataset, as it was for the six Kartagener subjects (Table S3). We could not therefore identify a genetic-developmental mechanism that links handedness and visceral asymmetry in this study. Genetic contributions to non-PCD SIT might also involve, for example, non-coding variation, or rare combinations of multiple common variants. *In utero* effects such as prenatal drug exposure might also affect left-right determination (18), as could early embryonic random variation during axis formation. Future studies in larger cohorts may help to further disentangle the genetic contributions to non-PCD SIT. The mutation lists that we provide from the unsolved cases in this study (Tables S5, S6) will be useful to the field in this regard.

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Competing Financial Interests Statement
The authors declare no competing interests.

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Table 1. Overview of most likely causative recessive mutations in each case subject.

Subject	Phenotype	Sex	Age	Hand	CHD	Gene	Location	rs ID	start	end	alt	ref	MAF	impact	impact severity	Clinvar	SIFT/ Polyphen
SI06	Kartagener	Male	46	R	0	*LRRC6*	19q13.3	rs767624733	133687728	133687729	C	T	1.73E-04	splice donor variant	HIGH	Kartagener	NA
SI08	Kartagener	Female	23	R	0	*DNAH11*	19p13.2	rs373706559	21659620	21659621	C	A	6.06E-05	stop gained	HIGH	PCD	NA
SI11	Kartagener	Female	32	R	0	*DNAAF1*	16q24.1	rs569633512	84203963	84203964	T	C	-	splice donor variant	HIGH	Kartagener	NA
						DNAAF1	16q24.1	rs373103805	84193302	84193303	C	G	2.33E-04	Missense variant	MED	Kartagener	deleterious/ probably damaging
SI13	Kartagener	Male	48	L	0	*CCDC114*	7p21	rs779459076	48814907	48814908	C	CACG	9.30E-04	inframe insertion	MED	Kartagener	NA
SI15	Kartagener	Female	31	R	0	*DNAH5*	5p15-p14	None	13786289	13786290	T	A	-	stop gained	HIGH	Kartagener	NA
						DNAH5	5p15-p14	rs397515540	13753397	13753399	GA	G	3.42E-04	frameshift variant	HIGH	Kartagener	NA
SI17	Kartagener	Male	39	R	0	*DNAH5*	5p15-p14	rs548521732	13839638	13839639	T	C	2.09E-04	splice acceptor variant	HIGH	Kartagener	NA
						DNAH5	5p15-p14	rs769458738	13753597	13753598	C	T	3.60E-05	missense variant	MED	Kartagener	Possibly damaging/ NA
SI02	non-PCD SIT	Male	50	R	0	*PKD1L1*	5p15-p14	rs528302390	47870810	47870813	TCA	T	1.09E-03	splice donor variant	HIGH	SA | SIT	NA
SI12	non-PCD SIT	Female	40	R	0	*DNAH5*	5p15-p14	None	13900415	13900416	C	A	-	stop gained	HIGH	Kartagener	NA
						DNAH5	8q24.22	None	13769691	13769693	GC	G	1.79E-05	frameshift variant	HIGH	Kartagener	NA
SI16	non-PCD SIT	Male	21	L	0	*CCDC151*	7p13-p12	rs765121016	11533429	11533433	TCTC	T	1.13E-04	inframe deletion	MED	Kartagener	NA
SI03	non-PCD SIT	Female	26	L	0	Unsolved											
SI04	non-PCD SIT	Male	23	L	1	Unsolved											
SI05	non-PCD SIT	Male	27	R	0	Unsolved											
SI07	non-PCD SIT	Female	35	R	1	Unsolved											
SI09	non-PCD SIT	Female	36	L	0	Unsolved											
SI14	non-PCD SIT	Male	18	L	1	Unsolved											

Subjects shown with two mutations have possible compound heterozygous mutations, otherwise they have homozygous mutations. Hand: Handedness; CHD: Congenital Heart Disease; MAF: Minor Allele Frequency; alt = alternate allele; ref = reference allele.