Functional Zebrafish Studies Based on Human Genotyping Point to Netrin-1 as a Link Between Aberrant Cardiovascular Development and Thyroid Dysgenesis

Robert Opitz,* Marc-Philip Hitz,* Isabelle Vandernoot, Achim Trubiroha, Rasha Abu-Khudir, Mark Samuels, Valérie Désilets, Sabine Costagliola,* Gregor Andelfinger,* and Johnny Deladoëy*

Institute of Interdisciplinary Research in Molecular Human Biology (R.O., I.V., A.T., S.C.), Université Libre de Bruxelles, 1070 Brussels, Belgium; Research Center of Centre Hospitalier Universitaire Sainte-Justine (M.-P.H., I.V., R.A.-K., M.S., G.A., J.D.), Department of Pediatrics, Université de Montréal, Montréal, Québec, Canada H3T 1C5; and Department of Medical Genetics (V.D.), Centre Hospitalier Universitaire de Sherbrooke, Sherbrooke, Canada J1H 1P8

Congenital hypothyroidism caused by thyroid dysgenesis (CHTD) is a common congenital disorder with a birth prevalence of 1 case in 4000 live births, and up to 8% of individuals with CHTD have co-occurring congenital heart disease. Initially we found nine patients with cardiac and thyroid congenital disorders in our cohort of 158 CHTD patients. To enrich for a rare phenotype likely to be genetically simpler, we selected three patients with a ventricular septal defect for molecular studies. Then, to assess whether rare de novo copy number variants and coding mutations in candidate genes are a source of genetic susceptibility, we used a genome-wide single-nucleotide polymorphism array and Sanger sequencing to analyze blood DNA samples from selected patients with co-occurring CHTD a congenital heart disease. We found rare variants in all three patients, and we selected Netrin-1 as the biologically most plausible contributory factor for functional studies. In zebrafish, ntn1a and ntn1b were not expressed in thyroid tissue, but ntn1a was expressed in pharyngeal arch mesenchyme, and ntn1a-deficient embryos displayed defective aortic arch artery formation and abnormal thyroid morphogenesis. The functional activity of the thyroid in ntn1a-deficient larvae was, however, preserved. Phenotypic analysis of affected zebrafish indicates that abnormal thyroid morphogenesis resulted from a lack of proper guidance exerted by the dysplastic vasculature of ntn1a-deficient embryos. Hence, careful phenotyping of patients combined with molecular and functional studies in zebrafish identify Netrin-1 as a potential shared genetic factor for cardiac and thyroid congenital defects. (Endocrinology 156: 377–388, 2015)
being less frequent. Congenital hypothyroidism caused by thyroid dysgenesis exists in the familial (2%) and sporadic (98%) forms (3), with a discordance rate of 92% between monozygotic twins (4) and a female predominance (2); congenital heart disease is also predominantly sporadic (5) with a discordance rate of 90% between monochorionic twins (6).

Germline mutations in thyroid-related transcription factors NKX2.1, FOXE1, and PAX8 have been identified in 3% of patients with sporadic CHTD and no cardiac defects (7, 8). For NKX2.1 and PAX8, all mutations reported to date were monoallelic, and patients presented with orthotopic thyroid gland hypoplasia. Conversely, FOXE1 mutations have been found in the biallelic state in patients presenting with athyreosis, cleft palate, and spiky hair (7). The lack of linkage to these genes in some multiplex families with CHTD points to considerable genetic heterogeneity in this disorder (9). Two other genes, NKX2.5 and HHEX, have been implicated in thyroid and cardiac phenotypes based on human and genetic mouse models (10–12). In transcriptome analysis of ectopic thyroids, none of these transcription factors exhibited decreased expression (13). Altogether these findings underline the importance of identifying new genes associated with CHTD and congenital heart disease (CHD) by considering the following: 1) sporadic de novo germline genetic events [ie, either de novo copy number variants (CNVs) or de novo point mutations], 2) the possibility of multiple hits (de novo or inherited) in modifier genes, 3) the possibility of low-penetrance variants, and 4) somatic epigenetic or genetic events (14).

CNVs have been recognized as a major source of genetic variability (15, 16) and have been shown to confer susceptibility to sporadic diseases (15, 17) such as autism (17) and CHD (18). An association between CHTD and chromosomal variants was demonstrated in two previous studies performed with lower resolution than that used in the present work (19, 20).

Like CHTD, CHD is a sporadic condition (5). An 8% co-occurrence of CHD in CHTD is greater than expected by chance alone, given a global prevalence of CHD of 0.6%–1% (21, 22). Therefore, CHD and CHTD might share common genetic or epigenetic etiologies. A link between cardiovascular and thyroid development has previously been demonstrated in mouse and zebrafish studies revealing coordinated morphogenetic processes (23) as well as the occurrence of thyroid anomalies in mice and zebrafish models with defective cardiovascular development (23, 24). Therefore, we performed a pilot study by selecting patients with co-occurring CHD and CHTD to look for shared genetic factors.

Subjects and Methods

Ethics statement

This study was approved by the Sainte Justine Ethics Committee (Research Ethic Board number 94). All of the parents provided written informed consent.

Subjects

To determine whether rare CNVs are associated with syndromic cases of CHTD, we selected patients with CHTD and CHD. First, the Zoom Endo database (Endocrinology Service, Centre Hospitalier Universitaire Sainte-Justine) containing 158 patients with CHTD (diagnosis established by ¹²³I scintigraphy) was merged with a cardiac echocardiography database containing echographies of 20,000 different patients from the same institution. Nine patients were found to overlap, representing a prevalence of 5.5% (9 of 158) for CHD among patients with CHTD, a prevalence consistent with an earlier survey by our group (2). To enrich for a rare phenotype likely to be genetically simpler, we selected three patients with a ventricular septal defect (VSD) for molecular studies. The other six patients, with transient patent ductus arteriosus (PDA) and transient atrial septal defect (ASD) type II (ASD II), were excluded because transient PDA and ASD II are benign and observed in high proportion of otherwise normal newborns; one case (patient 3) also had developmental delay and arthrogryposis (Figure 1). Clinical characteristics for each patient are reported in Table 1. The control cohort consisted of 203 ethnically matched individuals with no evidence of thyroid or heart disease after a medical history review, physical examination, electrocardiogram, and echocardiography. Informed consent was obtained from all participants. Cardiac and endocrinological phenotyping of patients are reported in Table 1, and complete clinical case reports are available in Supplemental Data.

Direct sequencing

Blood was obtained from patients and parents using peripheral venipuncture. DNA was extracted using a pureLink genomic DNA minikit (Life Technologies). Exons and intron-exon junctions were sequenced for NKX2.5, HHEX, NKX2.1, and PAX8. The single exon, including the polymorphic region encoding the alanine stretch, was sequenced for FOXE1. Primers and amplification conditions are available upon request.

Post hoc whole-exome sequencing

For the three patients, exome sequencing was performed subsequently at the McGill University and Genome Québec Innovation Center using the Agilent SureSelect oligo capture library and Illumina HiSeq 2 × 100 paired end reads. Details for exome sequencing and variant analysis were performed as described in our previous paper (25).

CNV detection analysis

Samples were genotyped on the Affymetrix genome-wide single-nucleotide polymorphism (SNP) Array 6.0 according to the manufacturer’s specifications. To increase specificity, we used a merge procedure of two different algorithms (ie, genotype console software 3.0.2 from Affymetrix and Birdsuite 1.5.3 from the Broad Institute (Cambridge, Massachusetts) to call CNVs, as published previously by our group (18) and as further described in the Supplemental Data.
Quantitative PCR validation

CNVs found by genome-wide SNP array were validated using TaqMan gene copy number assays (Applied Biosystems). Probes were designed using publicly available software (http://www5.appliedbiosystems.com/tools/cnv/). The TaqMan assay identifications are listed in Table 1 and a detailed protocol is provided in the Supplemental Data.

Zebrafish embryo culture

Zebrafish (Danio rerio) embryos were raised at 28.5°C and staged in hours postfertilization (hpf) as described (26). Transgenic zebrafish lines tgl(gmCherry) (23) and tgl(kdrl:EGFP) (27) were used in this study. Embryos were anesthetized in 0.016% tricaine (Sigma), fixed in 4% phosphate-buffered paraformaldehyde (PFA; Sigma) overnight at 4°C, washed in PBS containing 0.1% Tween 20, gradually transferred to 100% methanol, and stored at −20°C until used for in situ hybridization or immunofluorescence analyses. All zebrafish work at the Institute of Interdisciplinary Research in Molecular Human Biology followed protocols approved by the Institutional Animal Care and Use Committee.

Morpholino injections

For inhibition of ntn1a and ntn1b function, zebrafish embryos were injected with morpholino antisense oligonucleotides

Table 1. Phenotypic and Genotypic Characterization of the Three Patients

| Case | Sex | Thyroidal Phenotype | Cardiac Phenotype | Other Phenotype | Direct Sequencing NXX2.5a | Copy | Loss/Gain | Chromosome | Start | End | Size, kb | Genes | Classification | Validation | Tag Man Assay |
|------|-----|---------------------|------------------|----------------|--------------------------|------|-----------|------------|-------|-----|---------|-------|---------------|-----------|--------------|
| 1    | F   | Ectopy             | VSD, ASD         | α               | R25C                    | N/A  | N/A       | N/A        | N/A   | N/A | N/A     | N/A   | N/A           | N/A       | N/A          |
| 2    | M   | Athyreosis         | VSD, ASD         | wt              | R25C                    | 1    | Loss      | 16p12.3    | 19    | 21   | 333     | SYT17 | Rare Inherited | Confirmed by qPCR | Hs01544221_cn |
| 3    | M   | Ectopy             | VSD, ASD         | Arthrogryposis  | R25C                    | 1    | Loss      | 17p13.3    | 6     | 90   | 157     | X111          | Rare de Nova   | Confirmed by qPCR | CHL14  |
| 2    | G   | Gain               | Y                |                 |                          | 2    | Gain      | 22q11.21   | 19    | 90   | 202     | NTN1           | Rare de Nova   | Confirmed by qPCR | CHL14  |

Abbreviations: F, female; M, male; N/A, not applicable; PDA, patent ductus arteriosus; qPCR, quantitative PCR; VSD, ventricular septal defect; wt, wild type.

Only positive results for NXX2.5 are presented. No mutations or rare variants were found in NXX2.1, FOXE2, PAX8, or HHEX.

Copy number, chromosome location with the start, end, and length of the rare CNVs with their classification, and major encompassing gene (University of California, Santa Clara, genome browser; HG18 assembly).

No rare deletion or duplication was validated in patient 1.
Whole-mount in situ hybridization (WISH)

DNA templates for synthesis of ntr1a, ntr1b, tg, and nkx2.1a riboprobes were generated by PCR (see Supplemental Table 1 for primer sequences). Plasmids for myl7 and kdr1 riboprobes have been used as described (32, 33). Single-color WISH was performed essentially as described (34). For dual-color WISH, riboprobes labeled with digoxigenin (DIG) and dinitrophenol were used and sequential alkaline phosphatase staining was performed with BM Purple and Fast Red (Sigma) as described (23). Fluorescent WISH (FISH) using a DIG-labeled riboprobe for tg was performed as described (23). Antibodies in WISH and FISH experiments are listed in Supplemental Table 2. Stained embryos were postfixed in 4% PFA (Sigma) and embedded in 90% glycerol for whole-mount imaging or in 7% low melting point agarose (Lonza) for vibratome sectioning. Tissue sections at 50–60 μm thickness were cut on a Leica VT1000S vibratome and mounted in Glycergel (Dako). Images of stained sections were acquired using an AxioCam digital camera mounted on an AxioPlan 2 microscope (Zeiss).

Whole-mount immunofluorescence

Whole-mount immunofluorescence (WIF) staining was performed essentially as described (23). Specificities of primary and secondary antibodies used to detect green fluorescent protein (GFP), mCherry, cardiac troponin T, and Tα in zebrafish embryos are provided in Supplemental Table 2. After WIF staining, specimens were incubated in 4′,6′-diamino-2-phenylindole (DAPI) to label cell nuclei and postfixed in 4% PFA. Combined FISH and WIF staining was performed as described (23) Confocal images were acquired using an LSM 510 confocal microscope (Zeiss). Three-dimensional reconstruction of confocal stacks was performed using Zen 2010 D software (Zeiss).

Statistical analyses

Data sets from thyroid cell number measurements in zebrafish were first analyzed for normal distributions (Kolmogorov-Smirnov test) and homogeneity of variances (Bartlett’s test). If measurements of a response attribute, or a log-transformation of it, were found to be normally distributed with equal variances, then an unpaired Student’s t test was used for pair-wise comparison of stage-matched experimental groups. Statistical analyses were performed using the software package GraphPad Prism 4.0 (GraphPad). Differences were considered significant at $P < .05$.

Results

Clinical ascertainment

By merging data from our endocrinology and cardiology clinical databases, we identified nine patients with congenital hypothyroidism caused by thyroid dysgenesis (CHTD) and congenital heart defects (CHD). From these nine, a subset of three patients was selected for molecular studies, based on the clinical severity (ie, patients with ventricular septum defects). Parental DNAs were available for all three patients.

Direct sequencing of HHEX, NKX2.1, NKX2.5, FOXE1, and PAX8 and post hoc whole-exome sequencing

To exclude mutations in genes known to be associated with isolated CHTD (NKX2.1, FOXE1, and PAX8) or with combined thyroid and cardiac defects (HHEX, NKX2.5), we sequenced these genes in all three patients. No candidate pathogenic mutations were detected in HHEX, NKX2.1, FOXE1, and PAX8. All patients were homozygous for the FOXE1 14 alanine stretch polymorphism (35). Subsequently, we performed whole-exome sequencing as described elsewhere (25) and no additional pathogenic variants were found in these patients.

Identification of a rare inherited heterozygous variant of NKX2.5 in patient 1

In patient 1, we identified a c.73C>T transition in NKX2.5 (rs28936670, minor allele frequency (0.003), resulting in a pArg25Cys change. This variant was inherited from the unaffected father (Table 1). No rare deletion or duplication was validated in that patient.

CNV detection

CNV detection in patients and their parents was performed using the Affymetrix genome-wide SNP Array 6.0. All detected variants were not found in 203 ethnically matched controls. No rare CNV were found in patient 1. CNV found in patients 2 and 3 are described below.

Identification of a rare inherited deletion of synaptotagmin-17 (SYT17) in patient 2

In patient 2, we identified a deletion of 76 kb encompassing SYT17 and the undefined locus UNQ5810 on chromosome 16p13.2 (Table 1). This variant was inherited from the unaffected father. SYT17 is ubiquitously expressed, with high levels in the thyroid and heart. SYT17 belongs to the group of synaptotagmin-soluble N-ethylmaleimide sensitive fusion factor attachment protein receptor interactors, although its possible involvement in CTHD and CHD have not been examined to date.
Identification of two rare de novo CNVs and a 47, XYY karyotype in patient 3

In patient 3 (Figure 1), we identified two rare de novo CNVs as well as a 47, XYY karyotype (Table 1). The first de novo deletion encompassed a total of 855 kb of sequence on chromosome 22q11, corresponding to an atypical 22q11 deletion syndrome (36). This interval contains 33 genes that would thus be haploinsufficient, including CRKL. Somewhat surprisingly, a routine FISH performed with the usual TUPLE1 (TUP-like enhancer of split gene 1) probe revealed no 22q11 deletion. However, FISH using BAC RP11-801020 confirmed the deletion in the distal DiGeorge syndrome (DGS) region in the index case, whereas parental FISH results were normal. The second CNV was a de novo 12-kb deletion between the second and third exons of netrin-1 (NTN1) on chromosome 17p13.1. NTN1 is a laminin-related secreted protein that acts as an axon guidance molecule during neural development (37).

Netrin1 mRNA expression in zebrafish embryos

Because Netrin1 is implicated in the regulation of various developmental processes including angiogenesis, nonneuronal cell migration, and epithelial morphogenesis (38, 39), we decided to use zebrafish embryos as a model to characterize the expression and function of Netrin1 with respect to thyroid and cardiovascular development. In zebrafish, two paralogous homologs of human NTN1 are expressed, ntn1a (40) and ntn1b (41). Zebrafish ntn1a and ntn1b act as axon guidance molecules, and ntn1a has also been implicated in vascular development (30, 42). We first used WISH to examine spatiotemporal patterns of ntn1a and ntn1b expression in the thyroid/pharyngeal region for which detailed expression data were not yet available. For this purpose, embryos and larvae were fixed at various developmental stages throughout their development between 24 and 100 hpf. Thyroid specification in zebrafish occurs around 24 hpf (43) and the thyroid primordium can be stained by WISH using a nkd2.1a riboprobe (Figure 2A). For ntn1a, we did not detect any notable expression in the thyroid/pharyngeal region of zebrafish embryos at 24, 26, 30, and 34 hpf (Figure 2B and data not shown). Robust ntn1a expression became detectable in lateral pharyngeal regions at 72 hpf (Figure 2O). Dual-color WISH showed no detectable ntn1b expression in thyroid cells at any stage examined (Figure 2, P–R, and data not shown), and vibratome sections of stained embryos revealed no ntn1b mRNA expression in the pharyngeal arch region (Figure 2S). However, between 46 and 55 hpf, the thyroid primordium was transiently apposed to ntn1b-expressing cardiac tissue (Figure 2, N and T, and data not shown).

ntn1a knockdown causes defective cardiovascular and thyroid development

Although ntn1a-morphants have been reported for nervous system and vascular development (28, 30, 31), no data have been available concerning thyroid development in ntn1a-deficient embryos. To test whether ntn1a is required for normal thyroid development, we knocked down ntn1a function in zebrafish embryos using a previously validated ntn1a sb-MO (28–30). Injection of 5–6 ng ntn1a sb-MO efficiently prevented normal ntn1a mRNA splicing and closely recapitulated previously described effects patterns on the trunk vasculature (Supplemental Figure 1). All results reported below have been obtained using this MO concentration.

To examine early thyroid and cardiac development in ntn1a-deficient embryos, we performed dual-color WISH for cardiac and thyroid markers in 28- and 55-hpf embryos. WISH staining of embryos with the myocardium-specific myl7 probe revealed cardiac laterality defects in ntn1a-morphants. The first bilateral symmetry breaking event during zebrafish heart development is a leftward displacement of the cardiac cone, a process called cardiac jogging, which results in a leftward positioning of the venous pole relative to the midline. The direction of cardiac jogging is regulated by left-right signaling (44), and a normal leftward positioning of the venous pole (left jogging) was observed in noninjected (NI) embryos (NI-controls) and embryos injected with control-MO (MO-controls) at 28 hpf (Figure 3, A and B). In contrast, the direction of cardiac jogging was randomized in ntn1a-morphants (Figure 3U) with 42%, 28%, and 30% of ntn1a-morphants displaying left heart jogging (Figure 3C), a no-jog phenotype (Figure 3D), and right heart jogging (Figure 3E), respectively. A second important event involved in establishing laterality of the zebrafish heart is the process of cardiac looping occurring between 36 and 48 hpf (44). When examined at 55 hpf, hearts of NI-controls (Figure 3F) and MO-controls (Figure 3G) showed correct D-looping (ventricle positioned right to the atrium), whereas ntn1a-morphants displayed abnormal heart looping (Figure 3V). Only 47% of ntn1a-morphants showed D-looped
26% had unlooped midline hearts (Figure 3H), 26% had unlooped midline hearts (Figure 3I) and 27% showed a reversed looping with the ventricle positioned left to the atrium (Figure 3J).

Although cardiac jogging and subsequent cardiac looping are discrete processes, the direction of cardiac jogging is generally considered a good predictor for cardiac looping phenotypes (45). Our experiments were not designed to address specifically the relationship between jogging directionality and subsequent cardiac looping for individual embryos, but the observed frequencies of jogging and looping anomalies in ntn1a-morphants are consistent with a model in which left jogging (42%) is expected to be accompanied by D looping (47%), right jogging (30%) by reversed looping (27%), and a no-jog phenotype (28%) would result in unlooped midline hearts (26%).

WISH staining of the thyroid marker nkx2.1a in 28 hpf embryos did not reveal gross differences in size, shape, and location of the thyroid primordium between NI-controls (n = 43 of 45) and MO-controls (n = 83 of 86), thyroid tissue of ntn1a-morphants was not limited to the midline and showed irregular lateral expansions (Figure 3, M–O). At later stages, ntn1b was expressed in lateral pharyngeal regions (arrowheads in panel O). Sagittal sections of dual-color-stained embryos revealed that ntn1b expression is absent in the thyroid primordium (marked by nkx2.1a staining, arrowhead in panels F–H). Parasagittal sections (panels I and J) show strong ntn1a expression in the pharyngeal mesenchyme (arrows in panel I) surrounding the aortic arch arteries (marked by kdrl staining and indicated as numbers 1, 3, 4, 5, and 6 in panel J). Sagittal (panels F and G), transverse (panel H), and parasagittal sections (panels I and J) are shown. Scale bar, 100 μm (A–E and (K–O); 50 μm (F–J and P–T).
in zebrafish embryos with defects in pharyngeal vasculature morphogenesis (23). Using transgenic \textit{tg(kdrl:EGFP)} embryos expressing enhanced green fluorescent protein (EGFP) in endothelial cells, we detected gross malformations of the pharyngeal vasculature after injection of \textit{ntn1a} sb-MO but not control-MO or \textit{ntn1b} tb-MO (Figure 4, A–C). Similar to mammalian embryos, the zebrafish aortic arch artery (AA) network consists of paired bilateral arteries that connect the heart OFT to the dorsal aortae. Confocal microscopy of 55 hpf \textit{tg(kdrl:EGFP)} embryos and three-dimensional (3D) reconstruction of the pharyngeal vasculature revealed a spectrum of defects in AA morphogenesis in 63% of \textit{ntn1a}-morphants (Figure 4, D–H). Although the AA1 and the branchial AAs 3–6 were clearly formed in 55-hpf control embryos, perturbed AA morphogenesis in \textit{ntn1a}-morphants ranged from AA hypoplasia to severe underdevelopment of the entire AA system (Figure 4, K–M). AA malformations in \textit{ntn1a}-morphants...
were further accompanied by hypobranchial artery (HA) dysplasia and failure to form a paired ventral aorta connecting each AA to the heart OFT.

Confocal microscopy of double transgenic \textit{tg}(tg:mCherry;kdrl:EGFP) embryos, expressing mCherry in thyroid and EGFP in endothelial cells, showed that aberrant lateral thyroid expansion occurred predominantly in \textit{ntn1a}-morphants displaying abnormal HA morphologies (Figure 4, I–M, and Figure 5). Confocal analyses of 80-hpf embryos showed that lack of a normal AP expansion of thyroid tissue in \textit{ntn1a}-morphants was correlated with an overall poorly developed hypobranchial vasculature in-
including the defective AA and HA formation (Figure 4, N–R). When counting the number of thyroid cells (cells double positive for mCherry and DAPI) in 80 hpf tg(tg:mCherry) embryos, no differences were detected between MO-controls and ntn1a-morphants (Figure 6). At 100 hpf, however, thyroid tissue of ntn1a-morphants contained significantly fewer thyroid cells than MO-controls (Figure 6). On the contrary, the functional maturation of thyroid tissue appeared largely unaffected in ntn1a-morphants as judged by their capacity to form functional follicles producing the thyroid hormone T4 (Figure 4, S–V).

Discussion

The etiology of CHTD is one of the remaining enigmas in the pathophysiology of thyroid diseases (46). CHTD is a sporadic condition that has a discordance rate of 92% between monozygotic twins (4) and a significant association with congenital heart defects (2, 47). This prompted us to consider de novo germline genetic events (ie, de novo CNVs and/or point mutations) as the underlying cause. In this pilot study, we used a combination of targeted candidate gene sequencing and high-density CNV analysis to assess the role of rare alleles with major effects in CHD and CHTD. We found rare variants in all three patients, and we selected netrin-1 as the biologically most plausible contributory factor for functional studies in zebrafish. This study also underlines the value of combining phenotype and genotype profiling with zebrafish functional studies to uncover new pathogenic genes from a small cohort of patients (11, 48).

In patient 3 we found three potentially relevant rare structural variants: an 47, XYY karyotype; an atypical 22q11 deletion; and an 17q deletion. First, standard karyotyping revealed a 47, XYY chromosome constitution that was corroborated by high-resolution karyotyping. An association between congenital hypothyroidism and sex chromosome aneuploidy (ie, especially in 47, XXY) has been previously reported (49). Thorwarth et al (20) also reported a wide Y duplication in a patient with thyroid hypoplasia and a VSD.

The 22q11 deletion in patient 3 overlaps with deletions associated with DGS in which thyroid dysfunction is a variable component of the phenotype. However, genotype-phenotype correlations have been restricted to classic deletions as defined by the TUPLE1 probe (50). The TUPLE1 standard probe did not detect the deletion in our patient even though the clinical picture was typical for DGS. According to studies in mice, deletion of Tbx1 fully replicates the thyroid phenotype seen in DGS (51), yet this gene was not deleted in patient 3. A role for Crkl in anteroposterior patterning of the pharyngeal apparatus has been described; this process requires the cooperation with

Figure 5. Three-dimensional reconstruction of confocal images of the heart OFT region of 55 hpf tg(tg:mCherry;kdr:EGFP) embryos expressing mCherry (red) in thyroid cells and EGFP (green) in endothelial cells and endocardium. A, In embryos injected with a co-MO, the endothelial cells of the HA embrace the thyroid primordium, which is present as a compact ovoid midline structure located rostral to the heart OFT. B and C, In contrast, embryos injected with a ntn1a-MO displayed irregular thyroid morphologies. Uni- or bilaterally expanding thyroid tissue was predominantly observed along the course of an abnormally bifurcated HA. AA1, aortic arch artery 1. Scale, 20 μm.

Figure 6. Determination of thyroid cell number in tg(tg:mCherry) embryos injected with a ctrl-MO and ntn1a sb-MO. Embryos were fixed at 80 and 100 hpf, and thyroid cells were labeled by immunofluorescence using an anti-mCherry antibody. Counterstaining with DAPI was used to label cell nuclei. Confocal z-stacks (1 μm sections) comprising the whole thyroid tissue were acquired, and the number of all cells double positive for mCherry and DAPI was measured for each embryo (see insert for a single confocal section from a control thyroid). N denotes the number of embryos analyzed. Asterisk denotes the significant differences between treatment means. *, P < .05 (unpaired t test). Ctrl, control; n.s., not significant.
Tbx1 and local retinoic acid signaling (52). Of note, even if up to 50% of classical DGS present with thyroid hypoplasia (53), thyroid ectopy is not reported in DGS, and in a series of atypical DGS (ie, CRKL deletion), no patients were reported to have hypothyroidism (36). Therefore, CRKL deletion is associated with neither thyroid ectopy nor hypothyroidism, and we did not select CRKL for further validation with functional assays.

More importantly, patient 3 carries a de novo 12-kb deletion on chromosome 17p13.1 within the NTN1 gene, which is predicted pathogenic because two consecutive exons are deleted. NTN1 is of great interest in light of findings that place this gene in the Sonic hedgehog signaling pathway and implicate it as a physical and functional interactor of the Down syndrome cell adhesion molecule, a gene implicated in the cardiac phenotype of Down syndrome patients (54–56). NTN1 is crucial for normal brain development but recent studies have implicated NTN1 signaling also in the regulation of nonneuronal cell migration and survival and vascular development as well as epithelial cell adhesion and migration during lung and pancreas morphogenesis (57, 58). Similar to lungs and pancreas, the thyroid is derived from foregut endoderm, but a role of netrin-1 action during thyroid development has not yet been reported.

Although the clinical significance of the detected variants at the NTN1 locus remains to be determined, our functional studies in zebrafish embryos provide significant evidence linking netrin-1 action with pharyngeal vessel and thyroid morphogenesis. First, we detected dynamic expression of zebrafish homologs of NTN1 in the pharyngeal region, although neither ntn1a or ntn1b was expressed in the developing thyroid itself. Instead, ntn1a was expressed in pharyngeal tissue surrounding the developing AAs, and defective AA formation was one hallmark of the ntn1a loss-of-function phenotype. Although defective thyroid morphogenesis became apparent at 55 hpf, after thyroid separation from the pharyngeal floor, perturbed AA formation was already evident at earlier stages. Another key observation was that the presence of irregularly shaped and ectopically located thyroid tissue was strictly associated with aberrant morphogenesis of pharyngeal vessels (eg, the HA) that are important for guiding late thyroid relocalization (23). Together these observations suggest that abnormal pharyngeal vessel formation might be the primary effect of ntn1a deficiency, whereas the thyroid anomalies most likely represent a secondary response to the lack of a proper guidance function exerted by dysplastic pharyngeal vessels. In this regard, our data reinforce the concept that embryonic blood vessels play a critical role in thyroid organogenesis (24, 59) and that vascular anomalies may account for certain cases of CHTD. Consistently, there are clinical observations of vascular malformations (eg, hypoplasia or agenesis of thyroid arteries) in some cases of ectopic thyroids in humans (60, 61). Ntn1a-deficient zebrafish embryos also displayed cardiac laterality defects, but neither abnormal heart looping nor heart looping was correlated with the presence of thyroid anomalies.

Two other inherited variants were found. First, the NXK2.5 p.R25C variant identified in patient 1 was previously found in patients with both CHD and CHTD (11). In addition, functional assays suggested that the R25C mutant exhibits impaired binding and transactivation properties (11, 62). Because this variant was also found in the father of patient 1 as well as at low frequency in some control series with a minor allele frequency of 0.003 on dbSNP, we postulate that it constitutes a reduced penetrance risk allele. Second, we found a deletion in SYT17 in patient 2, again inherited from an unaffected father. Given that SYT17 deletion was inherited from the healthy father, we did not select this gene for further validation with functional assays (48).

Our study implicates known pathways of thyroid and heart development and replicates previous results suggesting a possible contributory role for the NXK2.5 p.R25C variant. The incomplete penetrance of this variant in our patient 1 is compatible with either genetic or environmental modifiers. These observations suggest a complex mode of inheritance in CHTD and CHD, which are now genetically traceable using modern high-resolution platforms (63).

Based on the high yield of rare variants identified in this pilot analysis, future studies in patients with CHTD and CHD are warranted, and these studies should include a comprehensive analysis of protein-coding mutations and structural genomic variation. High-resolution chip platforms and whole genome sequencing in trios with affected CHTD/CHD children will yield insight into complex inheritance patterns affecting interacting pathways of embryonic development of the thyroid and heart.

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Address all correspondence and requests for reprints to: Johnny Deladoey, MD, PhD, Centre Hospitalier Universitaire Sainte-Justine, 3175 Côte Sainte-Catherine, Montréal Québec, Canada H3T 1C5. E-mail: johnny.deladoey@umontreal.ca; or Gregor Andelfinger, MD, PhD, Centre Hospitalier Universitaire Sainte-Justine, 3175 Côte Sainte-Catherine, Montréal, Québec, Canada H3T 1C5. E-mail: gregor.andelfinger@umontreal.ca; or Sabine Costagliola, Institute of Interdisciplinary Research in Molecular Human Biology, Université Libre de Bruxelles, Route de Lennik 808, 1070 Brussels, Belgium. E-mail: scostag@ulb.ac.be.

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References

1. Deladoey J, Belanger N, Van Vliet G. Random variability in congenital hypothyroidism from thyroid dysgenesis over 16 years in Quebec. J Clin Endocrinol Metab. 2007;92(8):3158–3161.
2. Devos H, Rodd C, Gagne N, Laframboise R, Van Vliet G. A search for the possible molecular mechanisms of thyroid dysgenesis: sex ratios and associated malformations. J Clin Endocrinol Metab. 1999;84(7):2302–2306.
3. Castanet M, Polak M, Bonaiti-Pellie C, Lyonnet S, Czernichow P, Leger J. Nineteen years of national screening for congenital hypothyroidism: familial cases with thyroid dysgenesis suggest the involvement of genetic factors. J Clin Endocrinol Metab. 2001;86(5):2009–2014.
4. Perry R, Heinrichs C, Bourdoux P, et al. Discordance of monozygotic twins for thyroid dysgenesis: implications for screening and for molecular pathophysiology. J Clin Endocrinol Metab. 2002;87(9):4072–4077.
5. Calcagni G, Digilio MC, Sarkozy A, Dallapiccola B, Marino B. Familial recurrence of congenital heart disease: an overview and review of the literature. Eur J Pediatr. 2007;166(2):111–116.
6. Manning N, Archer N. A study to determine the incidence of structural congenital heart disease in monochorionic twins. Prenat Diagn. 2006;26(11):1062–1064.
7. Clifton-Bligh RJ, Wentworth JM, Heinz P, et al. Mutation of the gene encoding human TTF-2 associated with thyroid agenesis, cleft palate and choanal atresia. Nat Genet. 1998;19(4):399–401.
8. Macchia PE, Lapi P, Krude H, et al. PAX8 mutations associated with congenital hypothyroidism caused by thyroid dysgenesis. Nat Genet. 1998;19(1):83–86.
9. Castanet M, Sura-Trueba S, Chautry A, et al. Linkage and mutational analysis of familial thyroid dysgenesis demonstrate genetic heterogeneity implicating novel genes. Eur J Hum Genet. 2005;13(2):232–239.
10. Biben C, Weber R, Kesteven S, et al. Cardiac septal and valvular dysmorphogenesis in mice heterozygous for mutations in the homeobox gene Nkx2–5. Circ Res. 2000;87(10):888–895.
11. Dentice M, Cordeddu V, Rosica A, et al. Missense mutation in the transcription factor NKX2–5: a novel molecular event in the pathogenesis of thyroid dysgenesis. J Clin Endocrinol Metab. 2006;91(4):1428–1433.
12. Martinez Barbera JP, Clements M, Thomas P, et al. The homeobox gene Hex is required in definitive endodermal tissues for normal forebrain, liver and thyroid formation. Development. 2000; 127(11):2433–2445.
13. Abu-Khurdi R, Paquette J, Lefort A, et al. Transcriptome, methylome and genomic variations analysis of ectopic thyroid glands. PLoS One. 2010;5(10):e12420.
14. Deladoey J, Vassart G, Van Vliet G. Possible non-mendelian mechanisms of thyroid dysgenesis. Endocr Dev. 2007;10:29–42.
15. Lafarte AJ, Feuk L, Rivera MN, et al. Detection of large-scale variation in the human genome. Nat Genet. 2004;36(9):949–951.
16. Redon R, Ishikawa S, Fitch KR, et al. Global variation in copy number in the human genome. Nature. 2006;444(7118):444–445.
17. Sebat J, Lakshmi B, Malhotra D, et al. Strong association of de novo copy number mutations with autism. Science. 2007;316(5823):445–449.
18. Hitz MP, Lemieux-Percault LP, Marshall C, et al. Rare copy number variants contribute to congenital left-sided heart disease. PLoS One. 2012;8(9):e1002903.
19. Uccellatore F, Sava I, Giuffrida D, et al. Cytogenetic analysis in congenital hypothyroidism. J Endocrinol Invest. 1990;13(7):605–607.
20. Thorwarth A, Mueller I, Biehmann H, et al. Screening chromosomal aberrations by array comparative genomic hybridization in 80 patients with congenital hypothyroidism and thyroid dysgenesis. J Clin Endocrinol Metab. 2010;95(7):3446–3452.
21. Roller MD, Strickland MJ, Richle-Colarusso T, Mahle WT, Correa A. Prevalence of congenital heart defects in metropolitan Atlanta, 1998–2005. J Pediatr. 2008;153(6):807–813.
22. Best K, Draper E, Kurinczuk J, et al. PPO.17. Is congenital heart disease on the increase in the UK? A register-based study. Arch Dis Child Fetal Neonatal Ed. 2014;99(suppl 1):A155.
23. Optiz R, Maquet E, Huisken J, et al. Transgenic zebrafish illuminate the dynamics of thyroid morphogenesis and its relationship to cardiovascular development. Dev Biol. 2012;372(2):203–216.
24. Alt B, Elsalini OA, Schrumpf P, et al. Arteries define the position of the thyroid gland during its developmental relocalisation. Development. 2006;133(19):3797–3804.
25. Samuels ME, Gallo-Payet N, Schwarztzenburger J, et al. Bioactive ACTH causing glucocorticoid deficiency. J Clin Endocrinol Metab. 2013;98(2):736–742.
26. Kimmel CB, Ballard WW, Ullmann B, Schilling TF. Stages of embryonic development of the zebrafish. Dev Dyn. 1995; 203(3):253–310.
27. Jin SW, Herzog W, Santoro MM, et al. A transgene-assisted genetic screen identifies essential regulators of vascular development in vertebrate embryos. Dev Biol. 2007;307(1):29–42.
28. Suli A, Mortimer N, Shepherd I, Chien CB. Netrin/DCC signaling controls contralateral dendrites of octavolateralis efferent neurons. J Neurosci. 2006;26(51):13328–13337.
29. Stacher Hornrdli C, Chien CB. Sonic hedgehog is indirectly required for intraretinal axon pathfinding by regulating chemokine expression in the optic stalk. Development. 2012;139(14):2604–2613.
30. Wilson BD, Li M, Park KW, et al. Netrins promote developmental and therapeutic angiogenesis. Science. 2006;313(5787):640–644.

31. Zhang C, Gao J, Zhang H, Sun L, Peng G. Robo2-slit and Dcc-netrin1 coordinate neuron axonal pathfinding within the embryonic axon tracts. J Neurosci. 2012;32(36):12589–12602.

32. Yelon D, Horne SA, Stainier DY. Restricted expression of cardiac myosin genes reveals regulated aspects of heart tube assembly in zebrafish. Dev Biol. 1999;214(1):23–37.

33. Thompson MA, Ransom DG, Pratt SJ, et al. The cloche and spatial novel genetically differentially affect hematopoiesis and vasculogenesis. Dev Biol. 1998;197(2):248–269.

34. Opitz R, Maquet E, Zoenen M, Dadhich R, Costagliola S. Thymic dysgenesis: multigenic or epigenetic. or both? Endocrinology. 2005;146(12):5035–5037.

35. Thibault M, Castanet M, Sura-Trueba S, et al. Thyroid dysgenesis: multigenic or epigenetic. or both? Endocrinology. 2005;146(12):5035–5037.

36. Levy-Strumpf N, Culotti JG. Robo2-slit and Dcc-netrin1 coordinate neuron axonal pathfinding within the embryonic axon tracts. J Neurosci. 2012;32(36):12589–12602.

37. Zhang C, Gao J, Zhang H, Sun L, Peng G. Robo2-slit and Dcc-netrin1 coordinate neuron axonal pathfinding within the embryonic axon tracts. J Neurosci. 2012;32(36):12589–12602.

38. Yelon D, Horne SA, Stainier DY. Restricted expression of cardiac myosin genes reveals regulated aspects of heart tube assembly in zebrafish. Dev Biol. 1999;214(1):23–37.

39. Vassart G, Dumont JE. Thyroid dysgenesis: multigenic or epigenetic. or both? Endocrinology. 2005;146(12):5035–5037.

40. Lauderdale JD, Davis NM, Kuwada JY. Thyroid dysgenesis: multigenic or epigenetic. or both? Endocrinology. 2005;146(12):5035–5037.

41. Vassart G, Dumont JE. Thyroid dysgenesis: multigenic or epigenetic. or both? Endocrinology. 2005;146(12):5035–5037.

42. Vanderpool RC, Sklar LA, Sura-Trueba S, et al. Thyroid dysgenesis: multigenic or epigenetic. or both? Endocrinology. 2005;146(12):5035–5037.

43. Vanderpool RC, Sklar LA, Sura-Trueba S, et al. Thyroid dysgenesis: multigenic or epigenetic. or both? Endocrinology. 2005;146(12):5035–5037.

44. Vanderpool RC, Sklar LA, Sura-Trueba S, et al. Thyroid dysgenesis: multigenic or epigenetic. or both? Endocrinology. 2005;146(12):5035–5037.

45. Vanderpool RC, Sklar LA, Sura-Trueba S, et al. Thyroid dysgenesis: multigenic or epigenetic. or both? Endocrinology. 2005;146(12):5035–5037.

46. Vanderpool RC, Sklar LA, Sura-Trueba S, et al. Thyroid dysgenesis: multigenic or epigenetic. or both? Endocrinology. 2005;146(12):5035–5037.

47. Vanderpool RC, Sklar LA, Sura-Trueba S, et al. Thyroid dysgenesis: multigenic or epigenetic. or both? Endocrinology. 2005;146(12):5035–5037.

48. Vanderpool RC, Sklar LA, Sura-Trueba S, et al. Thyroid dysgenesis: multigenic or epigenetic. or both? Endocrinology. 2005;146(12):5035–5037.

49. Vanderpool RC, Sklar LA, Sura-Trueba S, et al. Thyroid dysgenesis: multigenic or epigenetic. or both? Endocrinology. 2005;146(12):5035–5037.