Screening Chromosomal Aberrations by Array Comparative Genomic Hybridization in 80 Patients with Congenital Hypothyroidism and Thyroid Dysgenesis

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Objective: Congenital hypothyroidism occurs in 1:3500 live births and is therefore the most common congenital endocrine disorder. A spectrum of defective thyroid morphology, termed thyroid dysgenesis (TD), represents 80% of permanent congenital hypothyroidism cases. Although several candidate genes have been implicated in thyroid development, comprehensive screens failed to detect mutation carriers in a significant number of patients with nonsyndromic TD. Due to the sporadic occurrence of TD, *de novo* chromosomal rearrangements are conceivably representing one of the molecular mechanisms participating in its etiology.

Methods: The introduction of array comparative genomic hybridization (CGH) has provided the ability to map DNA copy number variations (CNVs) genome wide with high resolution. We performed an array CGH screen of 80 TD patients to determine the role of CNVs in the etiology of the disease.

Results: We identified novel CNVs that have not been described as frequent variations in the healthy population in 8.75% of all patients. These CNVs exclusively affected patients with athyreosis or thyroid hypoplasia and were nonrecurrent, and the regions flanking the CNVs were not enriched for segmental duplications.

Conclusions: The high rate of chromosomal changes in TD argues for an involvement of CNVs in the etiology of this disease. Yet the lack of recurrent aberrations suggests that the genetic causes of TD are heterogenous and not restricted to specific genomic hot spots. Thus, future studies may have to shift the focus from singling out specific genes to the identification of deregulated pathways as the underlying cause of the disease. **(*J Clin Endocrinol Metab* 95: 3446–3452, 2010)**

Congenital hypothyroidism (CH) occurs in 1:3000–4000 live births and therefore represents the most common congenital endocrine disorder. Its incidence diverges in ethnic groups, *e.g.* 1:2,000 in Hispanic and 1:10,000 in African-American newborns in the United States, arguing for the involvement of genetic modifications in the etiology of CH (1). Defects of thyroid hormone biosynthesis are present in only 15–20% of cases, whereas a spectrum of defective thyroid morphology—*i.e.* 1) hypoplasia, 2) ectopic thyroid tissue, 3) hemithyroidea, or 4) athyreosis—represents 75–85% of all cases of permanent CH (2–5). The latter condition, termed thyroid dysgenesis (TD; OMIM 218700), is considered to result from defects in early organ development, although the underlying mechanisms remain unknown. The emergence of familial cases, although rare, is significantly elevated, as docu-

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Abbreviations: BAC, Bacterial artificial chromosome; CGH, comparative genomic hybridization; CH, congenital hypothyroidism; CNV, copy number variant; TD, thyroid dysgenesis.

mented in several studies (1, 2, 6, 7). By contrast, preponderantly sporadic occurrence and observed discordance in monozygotic twins (8) are suggesting nongenetic factors or stochastic events in early embryogenesis to be involved in the majority of cases. A significant contribution of environmental factors, however, is highly unlikely because neither seasonal nor geographic differences in prevalence or association to maternal infections are obvious (9).

In the past decade, a group of transcription factors involved in early thyroid organogenesis has been implicated in normal thyroid organogenesis. In mice, the thyroid anlage appears from embryonic day 8–8.5, and coexpression of the associated genes, in particular *Nkx2-1*, *Foxe1*, and *Pax8*, could be shown in thyroid precursor cells in these initial steps of morphogenesis.

Therefore, these genes appeared to be obvious candidates in the etiology of TD. But corresponding to the critical role of these candidates in organs other than the thyroid, affected patients were only identified as additional phenotypic characteristics were considered. The extrathyroidal clinical spectrum in these patients comprises neurological and pulmonary (*NKX2-1*), kidney (*PAX8*), hair and palatal (*FOXE1*), and heart malformations (10–16). Moreover, mutations in the TSH receptor gene, which is expressed during later stages of organ growth (17), result in thyroid hypoplasia and are recessively inherited (18). However, comprehensive screens failed to detect mutation carriers in a significant number of patients with nonsyndromic TD, with the percentage of positive findings not exceeding 5% of all cases.

Meanwhile, the identification of new genes in TD with classical genetic tools is hampered by the mainly sporadic occurrence of the disease. One of the pathogenetic mechanisms conceivably involved in the etiology of TD is the high rate of *de novo* mutations as frequently seen in common genomic disorders (*e.g.* 22q11 microdeletion syndrome, Williams-Beuren syndrome, Smith-Magensis syndrome). These mostly complex malformation-retardation syndromes are due to submicroscopic chromosomal gains and losses, and only the recent advances in molecular cytogenetics gave increasing ability in localizing the causative alterations. Even more, copy number variants (CNVs) could recently be shown to occur also in various organic malformations without mental retardation (19, 20). The applied technique, namely array-based comparative genomic hybridization (array CGH), is dedicated to the identification of copy number changes in a test genome relative to a reference genome. In this study, we performed a comprehensive screen of 80 TD patients with genome-wide array CGH technology on a submegabase resolution to determine the role of CNVs in the etiology of the disease.

Patients and Methods

Patients

We selected 57 females and 23 males (n = 80) with TD for array CGH analysis. All individuals were detected in neonatal screening programs, and abnormal thyroid gland morphology was subsequently confirmed by ultrasound examination. Associated extrathyroidal malformations were present in 13 cases (15.9%). Distribution of various forms of TD and details of associated malformations in the cohort are given in Table 1. Additionally, two pairs of monozygotic, discordant twins were included in the screening analysis. Intragenic mutations in *NKX2-1*, *FOXE1*, and *NKX2.5* had been previously excluded in phenotype characteristic individuals. *PAX8* mutations were excluded in all patients with hypoplastic thyroids by direct sequencing of the coding exons 1–11. The study was approved by the local ethics committee.

TABLE 1. Epidemiological composition of cohort

	No. of individuals	%
Gender		
Females	58	70.7
Males	24	29.3
Total	82	
Female/male ratio		2.4
Thyroid morphology		
Athyreosis	37	45.1
Ectopy	5	6.1
Hemithyroidea	4	4.9
Hypoplasia[a]	34	41.5
Normal[b]	2	2.4
Total	82	
Extrathyroidal congenital malformations		
Cardiac[c]	7	53.8
ASD	2	15.4
ASD and PFO	1	7.7
VSD	3	23.1
PDA	1	7.7
Cleft palate and VSD	1	7.7
Trisomy 21[d]	1	7.7
xxy/xy mosaicism and hearing loss	1	7.7
Goldenhaar syndrome	1	7.7
Mental retardation syndrome[e]	1	7.7
Neurological impairment	1	7.7
Total	13	15.9[f]

ASD, Atrial septic defect; PFO, patent foramen ovale; VSD, ventricular septic defect; PDA, patent ductus arteriosus.

[a] All patients with diagnosed hypoplastic thyroids were screened for PAX8 mutations.

[b] Healthy discordant twin sibling (n = 2).

[c] Individuals with associated cardiac defects were screened for NKX2.5 mutations.

[d] Karyotype 46, XX, +21.

[e] Male patient with CH, severe psychomotor retardation, and several congenital anomalies.

[f] Proportion of extrathyroidal malformations (n = 80) corresponding to those reported in CH caused by TD that are elevated compared to CH of different pathogenesis (6).

TABLE 2. Chromosomal copy number changes detected by array CGH screening analysis

Case no.	Gender	Age (yr)	Aberration	Size (Mb)	Phenotype	Genes[a]	Inheritance	Start[b]	End[b]	Conrad/ Shaikh/ DoGV[c]
I	Female	16	del 5q12.1	0.17	Athyreosis	Mimitin	Mother	60277014	60448818	0/0/0
			dup 15q12	0.26		GABRB3	*De novo*	24204064	24502829	0/0/0
II	Female	17	del 20p12.1	0.075	Athyreosis	MACROD2	*De novo*	14439297	14515214	0/0/0
III	Male	9	dup 2p21	0.54	Hypoplasia	PKRCE SRBD1	Mother Grandmother	45295875	45824967	0/0/1
IV	Male	8	del Y p11.2-q11.23	19.7	Hypoplasia, VSD	TTTY12/11//8/9/10/13, TSPY1/2, USP9Y, DDX3Y, UTY, TMSB4Y, VCY1B, NLGN4Y, CDY, HSFY1, Cyorf14, CD24, CYorf15A/B, SMCYY, E1F1AY, RBMY1A1, RBMY1D, PRY2, BPY2, DAZ1/2/3, CDY1	*De novo*	7501428[d]	27191703[d]	
V	Female	19	dup 2q14.2	2.85	Athyreosis	EN1, MARCO, INSIG2, DDX18, DPP10	Father	115723718	119525663	0/0/0
			del 10q23.1	0.45		NRG3	*De novo*	83826723	84069756	0/0/0
VI	Female	1.5	dup 4q31.1	0.36	Athyreosis	LRBA, RPS3A, SNORD73A, SH3D19, ESSPL	Father	151892900	152223483	0/0/1
			dup 22q11.21	2.34		DGCR 2/6/8/14, PRODH, BC069275, TSSK2, GSCL, SCL25A1, CLTCL1, HIRA, MRPL40, UFD1 liter, CDC45 liter, CLDN5, Y11593, GP1BB, TBX1, FLJ21125, DKFZp761P1121, TXNRD2, COMT, ARVCF, CR592601, ZDHHC8, RANBP1, RTN4R, DGCR6 liter, ZNF74, KLHL22, SCARF2, PCQAP, PIK4CA, SERPIND1, SNAP29, CRKL, THAP7, P2RXL1, SLC7A4, GGT2, HIC2	Mother	17086045	20127468	0/0/1
VII	Male	6	dup 22q12.3	0.27	Athyreosis	LOC730090, MB, APOL6, BC047864	Father	34303814	34429542	0/0/1

Genes within affected genomic regions according UCSC Genome Browser (HG18; NCBI36); protein-coding status genes only, pseudogenes and miscRNAs not mentioned.

[a] RefSeq genes within aberrant intervals (in cases IV and VI, only selected genes are listed).

[b] Genomic position according to 244 k oligo array results.

[c] Number of corresponding CNVs in the cohort analyzed by Conrad *et al.* (28)/2026 healthy individuals published by Shaikh *et al.* (27)/number of corresponding CNVs in the Database of Genomic Variants (DoGV; including those from Shaikh *et al.*) as of Aug. 21, 2009.

[d] Confirmatory results by conventional karyotyping; aberrant genomic position according to BAC array result.

Array CGH

Genomic DNA of all patients as well as of healthy controls was isolated from peripheral blood leukocytes using the QIAGEN DNA blood mini kit (QIAGEN, Hilden, Germany). Array CGH was carried out as described previously (21, 22). In brief, sonicated patient and control DNA was labeled by random priming with Cy3-dUTP and Cy5-dUTP (Bioprime Array CGH; Invitrogen, Carlsbad, CA), respectively, and hybridized onto a submegabase resolution tiling path bacterial artificial chromosome (BAC) array, consisting of approximately 36,000 BAC clones obtained from several sources as described elsewhere (23–25). Step-by-step protocols are also provided at http://www.molgen.mpg.de/~abt_rop/molecular_cytogenetics/. Details concerning this platform have been submitted to the Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/; GLP: 5000 and 5114). Arrays were scanned with the G2565BA Agilent Microarray Scanner System (resolution, 10 μm; photomultiplier tube, 100% for Cy3/Cy5, respectively) (Agilent Inc., Santa Clara, CA) and analyzed using GENEPIX Pro 5.0 software (Molecular Devices, Sunnyvale, CA). Analysis and visualization of array CGH data were performed with our software package CGHPRO (26). For the assessment of copy number gains and losses, we used conservative log2 ratio thresholds of 0.3 and −0.3, respectively. Deviant signal intensity ratios involving three or more consecutive BAC clones were considered to be potentially pathogenic, unless they were covered more than once by a CNV present in a reference set of 600 healthy individuals and patients suffering from diseases other than TD. These 600 samples have been analyzed in our laboratory using the same BAC array platform and data interpretation parameters as those used for the TD samples in this study. The resulting set of CNVs was verified by array CGH on a 244k oligonucleotide array from Agilent, following the manufacturer's instructions (protocol no. G4410-90010) and tested for inheritance by cohybridization of parental DNA on BAC arrays as described above. Conventional karyotyping was carried out as a verification experiment in case IV. Furthermore, we mirrored these CNVs against recently published datasets, including 2026 clinically well-characterized healthy individuals (27) and the recent results of a whole genome screen for CNVs at a 500-bp resolution (28). CNVs were considered identical if they shared at least 50% of the genomic sequence. All chromosome coordinates are referring to the UCSC Genome Browser Assembly May 2004 (HG18/NCBI Build 36; http://genome.ucsc.edu/). Cytoscape (29) was used for the elucidation of potential interactions between genes within the intervals of interest.

Results

We successfully performed array CGH in 80 TD patients. Altogether, six duplications and four deletions in seven individuals (four female, three male) passed our filtering criteria (see *Patients and Methods*) and were validated on the Agilent platform. Three patients had more than one rearrangement matching the criteria mentioned above (cases I, V, and VI). Array CGH analysis of parental DNA revealed four of the aberrations to occur *de novo* in four patients (cases I, II, IV, and V), whereas six were paternally (cases V, VI, and VII) or maternally (cases I, III, and VI) transmitted. Blood testing for thyroid markers (TSH, free T_4) and ultrasound examination did not reveal abnormalities in any of the parental carriers. Two CNVs overlapped with aberra-

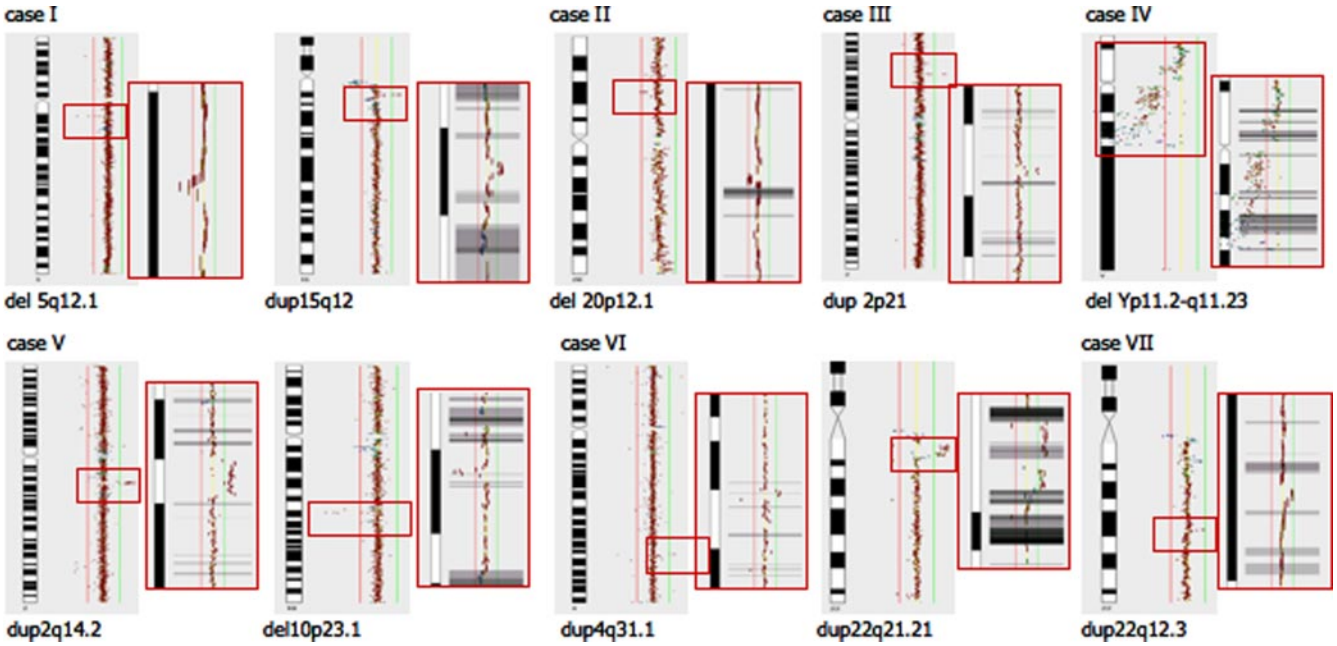

FIG. 1. Whole genome 36K BAC array CGH analysis. Cy3-labeled DNA of patients was cohybridized together with Cy5-labeled DNA from a sex-matched control. Log2 ratios are plotted against chromosomal position; next to true aberrations, outliers might be present as well. Array CGH profiles, whole chromosome, and zoom-in view are shown for cases I–VII: chromosomal overview (*left*) and zoom-in view (*right*) of array CGH profile are shown *red box* indicating zoom-in section; *red and green lines* indicate the log2 ratio thresholds −0.3 (loss) and 0.3 (gain), respectively; *gray bars* indicate CNVs according to the Database of Genomic Variants. BAC clones are colored according to their content of segmental duplications as described previously (26).

tions previously reported to be associated with other diseases: 1) dup22q11.21 in case VI is matching the typical deleted region of chromosome 22q11.2 recently reported as 22q11 microduplication syndrome (30, 31); and 2) delYp11.2-q11.23 in case IV (32, 33), respectively. A detailed presentation of the array CGH results is given in Table 2 and Fig. 1; results of 244k hybridizations are available in Supplemental Fig. 1, published on The Endocrine Society's Journals Online web site at http://jcem.endojournals.org. Apart from *TBX1*, a gene located within the 22q11 duplication interval in patient VI, no single gene within our 10 potentially pathogenic CNVs has been implicated in the genesis of TD before.

Furthermore, we employed array CGH in two pairs of monozygotic twins (two females, two males) that are discordant for the disease. Hybridization was carried out in single experiments with each sibling cohybridized with the sex-matched control and, in addition, cohybridization of the twin siblings. We were not able to detect differences in DNA copy number between twin siblings (data not shown) as reported elsewhere (34).

Array CGH data reported here have been deposited in NCBI Gene Expression Omnibus (GEO; Ref. 17) and are accessible through GEO series accession number GSE18152.

Discussion

In this study, 8.75% of patients carried a CNV that has not been observed frequently in the healthy population. This high percentage argues for an involvement of CNVs in the etiology of TD, but proving disease association is challenging. As a result of the widespread application of high-resolution methods such as array CGH, it is increasingly recognized that a considerable proportion of the human genome is copy number variant also in the healthy population. This complicates the interpretation of the biological relevance of a given CNV and requires some sort of selection and prioritization. This process, unfortunately, always represents a compromise between filtering and biasing of information. For example, excluding CNVs present in databases that collect neutral or benign variants proceeds on the risky assumption that those are never disease causing, irrespective of the individual genetic, epigenetic, and environmental background. We have tried to

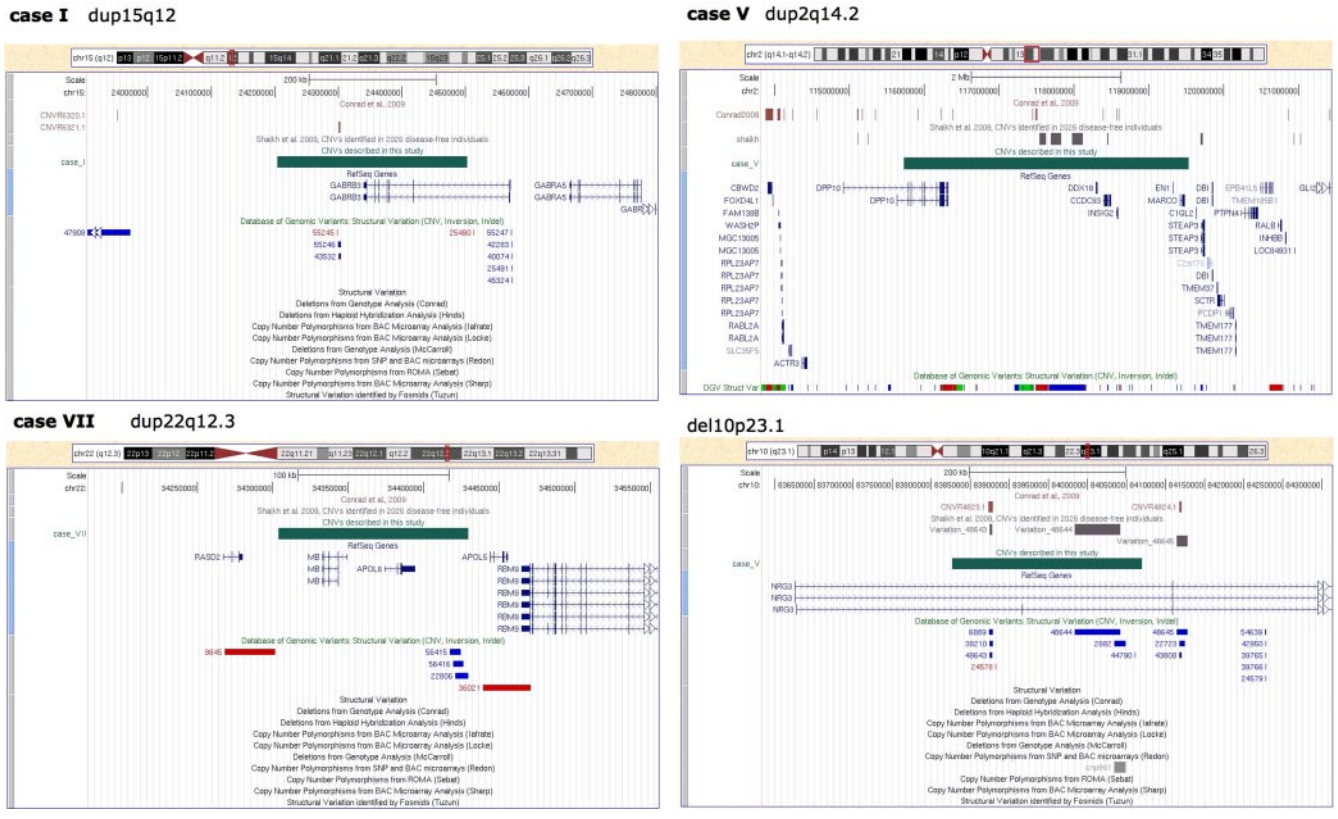

FIG. 2. UCSC screenshots depicting genomic regions (HG18) of CNVs of selected cases identified in this study. The *green bar* indicates the location of the aberrations identified in patients I, V, and VII. *Brown and gray bars* in the custom track above represent CNVs detected in a whole genome screen for CNVs at a 500-bp resolution (28) and CNVs identified in 2026 control individuals by Shaikh *et al.* (27). The specific identifying number is given on the *left*. Genes and their positions are indicated below these. Finally, all variations observed in the Database of Genomic Variants (DoGV) are included at the *bottom of each panel* for reference. These variations are color-coded according to DoGV convention to reflect gain (*red*), loss (*blue*), or gain/loss (*green*).

meet this problem in part by excluding CNVs only if they occurred more than once in the healthy population, reflecting a conservative interpretation of the fact that the likelihood of disease association of a given CNV decreases with its frequency in the healthy population (Table 2, Fig. 2, and Supplemental Fig. 1). It is worth noting that after filtering we have observed an enrichment of CNVs in patients with athyreosis, when compared with the milder forms of TD (Table 2). It will be interesting to see whether this will hold true for independent TD cohorts as well.

One finding that is considered a good indicator of disease relevance is *de novo* appearance. Although the *de novo* rate of benign and neutral CNVs is not finally established yet, the present frequency of 5% of noninherited CNVs appears rather high. Surprisingly, we have identified coincidence of two CNVs in three patients with athyreosis. This could be interpreted in two ways: at least one of the CNVs is not associated with disease, or genes located within the two CNVs act in synergy to produce a phenotypic effect. We have failed to identify interaction partners located on coinciding CNVs.

It is noteworthy that none of the genes with a known role in the etiology of TD were located within the aberrant chromosomal regions of our study. Solely TBX1, duplicated in a patient with athyreosis and located in the DiGeorge critical region on chromosome 22q11, has been described to result in a defect of thyroid development in knockout experiments in mice (35). Although a single patient with DiGeorge syndrome and 22q11 deletion was reported to be affected by minor thyroid abnormalities (36), the few cases with 22q11 duplication did not reveal thyroid abnormalities (37, 38). However, to date a gain of gene dosage by duplication of the critical 22q11 region or the TBX1 gene itself was not investigated regarding its impact on murine thyroid development.

In several other studies, CNVs were linked to disease by recurrence and enrichment in the patient samples. Recurrence in these diseases was mainly promoted by a specific genomic architecture, frequently characterized by a clustering of segmental duplication in the regions flanking the CNV. Apparently, this does not apply to the majority of CNVs identified in this study (Fig. 1). Consequently, the probability of finding identical or overlapping CNVs in TD appears to be quite low. This has direct implications for future CNV screens in TD, especially in regard to the number of patients that have to be analyzed. But even with larger cohorts analyzed, the low chance of recurrent aberrations in independent individuals will require changes in the way of data interpretation. Instead of hoping to find the very same gene affected in separate patients, *in silico* analysis has to expand to the identification of disease-related pathways as highlighted by the candidate genes located in the various CNVs.

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