

ARTICLE

De novo microduplications at 1q41, 2q37.3, and 8q24.3 in patients with VATER/VACTERL association

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The acronym VATER/VACTERL association describes the combination of at least three of the following congenital anomalies: vertebral defects (V), anorectal malformations (A), cardiac defects (C), tracheoesophageal fistula with or without esophageal atresia (TE), renal malformations (R), and limb defects (L). We aimed to identify highly penetrant *de novo* copy number variations (CNVs) that contribute to VATER/VACTERL association. Array-based molecular karyotyping was performed in a cohort of 41 patients with VATER/VACTERL association and 6 patients with VATER/VACTERL-like phenotype including all of the patients' parents. Three *de novo* CNVs were identified involving chromosomal regions 1q41, 2q37.3, and 8q24.3 comprising one (*SPATA17*), two (*CAPN10*, *GPR35*), and three (*EPPK1*, *PLEC*, *PARP10*) genes, respectively. Pre-existing data from the literature prompted us to choose *GPR35* and *EPPK1* for mouse expression studies. Based on these studies, we prioritized *GPR35* for sequencing analysis in an extended cohort of 192 patients with VATER/VACTERL association and VATER/VACTERL-like phenotype. Although no disease-causing mutation was identified, our mouse expression studies suggest *GPR35* to be involved in the development of the VATER/VACTERL phenotype. Follow-up of *GPR35* and the other genes comprising the identified duplications is warranted.

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INTRODUCTION

The VATER/VACTERL association (OMIM #192350) refers to the rare, non-random co-occurrence of the following component features (CFs): vertebral defects (V), anorectal malformations (A), cardiac defects (C), tracheoesophageal fistula with or without esophageal atresia (TE), renal malformations (R), and limb defects (L).¹ Population-based epidemiological studies have reported a prevalence among infants of 1 in 10 000 to 1 in 40 000 with male predominance.^{2–4} Since this multisystem malformation complex has been associated with reduced reproduction, it is reasonable to assume that a significant proportion of cases are caused by *de novo* mutations. In accordance with this, previous studies have identified several *de novo* chromosomal microaberrations, which are likely to be disease causing.^{5–16} However, none of these *de novo* chromosomal microaberrations has led to the identification of a disease-causing gene and the etiology in most cases is still unknown.

Using a SNP-array-based genome-wide approach, we systematically screened 41 patients with VATER/VACTERL association and 6 patients with a VATER/VACTERL-like phenotype, including all of the patients' parents, in order to identify causative *de novo* copy number variations (CNVs). Pre-existing data from the literature were used to choose candidate genes within the identified CNVs for *in situ* hybridization (ISH) on sections from mouse embryos at embryonic days (E) 12.5–14.5. Based on the ISH, candidate genes were prioritized for sequencing analysis in an extended cohort of 192 patients with VATER/VACTERL association and VATER/VACTERL-like phenotype in order to identify further disease-causing mutations.

MATERIALS AND METHODS

Subjects and DNA isolation

This study was approved by the local ethics committee and all families provided written informed consent. Families were contacted through the

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German self-help organization for patients with anorectal malformations (SoMA e.V.) and various pediatric surgical departments throughout Germany. Among the patients was one aborted female fetus, enrolled through the Department of Paidopathology, University of Bonn. All patients and their families were personally recruited by one of four experienced physicians starting in 2009. None of the patients used for array-based analysis has been reported in our previous studies.^{15,16} To optimize for detection of *de novo* CNVs, we only included patients with a negative family history of the disease. Forty-one patients presented with three or more CFs of the VATER/VACTERL association, while six patients presented with only two, with or without further anomalies (VATER/VACTERL-like phenotype).¹ For the mutation-sequencing analysis, an additional 59 patients with VATER/VACTERL association and 60 patients with VATER-/VACTERL-like phenotype recruited within Germany were added to the patient sample, of which 18 patients were previously described in Schramm *et al.*^{15,16} An additional 26 patients with VATER/VACTERL association were recruited within the AGORA project and were added to the sample for the mutation-sequencing analysis. The AGORA project is run within the Netherlands and patients are being recruited by pediatric surgeons and clinical geneticists of the Radboud University, Nijmegen Medical Centre, Nijmegen, the Netherlands. The complete sample for mutation-sequencing analysis comprised 192 patients ($n=132$ patients with VATER/VACTERL association; $n=60$ patients with VATER-/VACTERL-like phenotype). As all of the recruited patients in Germany and the Netherlands were part of research projects on the genetics of anorectal malformations, all patients had anorectal malformations. Blood or saliva was taken from the patient and from both parents for DNA isolation of genomic DNA, carried out using the Chemagic Magnetic Separation Module 1 (Chemagen, Baesweiler, Germany) or, in case of saliva samples, the Oragene DNA Kit (DNA Genotek Inc., Kanata, ON, Canada).

Array genomic hybridization analysis

For molecular karyotyping, we used the Illumina HumanOmni1-Quad-v1 BeadChip (marker content, 1140419; median marker spacing 1.5 kb) (San Diego, CA, USA). A DNA sample was considered to have failed if less than 98% of the markers were generated on the respective BeadChip.

Paternity testing

Paternity testing was performed using the GenomeStudio (v2010.3, www.illumina.com/) genotyping module in accordance with the manufacturer's recommendations.

CNV analysis

To identify potential CNVs, the SNP fluorescence intensity data from all patients and their parents were analyzed with QuantiSNP (v2.1 and v2.2, www.well.ox.ac.uk/QuantiSNP/) using an Objective Bayes Hidden-Markov model for calling putative CNVs.¹⁷ CNVs with a log Bayes factor below 7 were discarded.¹⁸ The results were also checked visually using the GenomeStudio genotyping module (v2010.3, www.illumina.com/) for marker and signal quality and the occurrence of mosaic aberrations. The Cartagenia Bench software (Cartagenia n.v., Leuven, Belgium) was used to filter detected CNVs for *de novo* events, variable regions in our control cohort of 531 unaffected controls, and gene content. The remaining aberrations were checked for gene content according to UCSC human genome browser assembly build 18, and for their presence in the databases DGV (<http://dgvbeta.tcag.ca/dgv/app/home?ref=NCBI36/hg18>) and DECIPHER (<http://decipher.sanger.ac.uk/>). CNVs identified to exist in either of these databases, with at least 10 full overlapping reports in DGV and at least five full overlapping reports in DECIPHER, were excluded. However, we did not exclude CNVs although they were fully overlapping by at least five reports in DECIPHER, if one of the associated phenotypes comprised any of the classical component features of the VATER/VACTERL association: e.g. esophageal atresia (TE), anorectal malformation (A), cardiac defects (C).

Quantitative PCR

For verification of the filtered CNVs, qPCR was performed as described previously using SYBR Green for detection.¹⁹ All primer sequences are listed in Supplementary Table 2.

In situ hybridization of mouse embryo sections

To prioritize candidate genes within the remaining CNVs for further mutation analysis in larger patient cohorts, ISH on sections from mouse embryos at E12.5–14.5, the crucial embryonic timeframe for the VATER/VACTERL association-relevant tissues, was performed as previously described.²⁰ Antisense probes were generated by PCR from a total cDNA library from E10.5 where PCR products contained a 3' T7 RNA polymerase-binding site for *in vitro* transcription. Probes were purified using G-50 Sephadex columns (GE Healthcare, Munich, Germany). The 903-bp probe for *Gpr35* (starting 13 bp downstream of the ATG start site) spans nearly the entire 924-bp coding region and recognizes both known mRNA variants. *Eppk1* is encoded by a single, large exon (>20 kb) consisting of 16 plakin repeat domains, which are the hallmark of all plakin family members.²¹ Thus, the unique 647-bp probe for *Eppk1* spans the 3'UTR. Detection of alkaline phosphatase activity was visualized using BM Purple (Roche Diagnostics, Mannheim, Germany). Following staining, slides were quickly dehydrated in 80% and then 100% ethanol, cleared twice for 1 min in xylene (Roth, Karlsruhe, Germany) and coverslips were mounted with Entellan mounting medium (Merck, Darmstadt, Germany). Photographs were obtained using AxioVision software (Zeiss, Göttingen, Germany) with a Zeiss AxioCam and SteREO DiscoveryV12 microscope. Three sections from at least two different embryos were analyzed for each stage shown and representative images are provided.

Sequence analysis

Analysis of the protein-coding exons 5 and 6 of the human *GPR35* gene was performed using polymerase chain reaction (PCR), and PCR-amplified DNA products were subjected to direct automated sequencing (3130XL Genetic Analyzer, Applied Biosystems, Foster City, CA, USA) according to the manufacturer's specifications. Both strands of each amplicon were sequenced. All primer sequences are available upon request.

RESULTS

Array analysis

Molecular karyotyping identified five possible *de novo* aberrations with gene content in five of the 47 patients. Three of these were confirmed as being *de novo* by qPCR (Table 1, Supplementary Tables 1 and 2, and Supplementary Figure), the others were found to be inherited by one parent (see Supplementary Tables 1 and 2). In the three confirmed cases, there were no other dysmorphic features, congenital anomalies (including structural brain anomalies or evidence of neurologic impairment), or other medical issues except those noted below.

Case 1 is a male patient with butterfly vertebra (V), anal atresia without fistula (A), atrial and ventricular septal defects (C), esophageal atresia IIIb (according to Vogt)²² (TE), bilateral dystopic kidneys, and left-sided renal dysplasia (R). A *de novo* microduplication was found at 1q41 (Table 1) with an estimated size of 132 kb, comprising exons 6–9 of *SPATA17*, encoding the spermatogenesis-associated protein 17.

Case 2 is an aborted female fetus. The pregnancy was terminated at 30 + 1 weeks of gestation because of sonographically proven severe bilateral renal dysplasia. Macroscopic and histological findings obtained at autopsy revealed a cloacal malformation with anal atresia (A), bilateral renal dysplasia (R), urethral agenesis, a secondary bell-shaped thorax, and Potter-sequence facies due to anhydramnion (Figure 1). A *de novo* microduplication was found at 2q37.3 (Table 1) with an estimated size of 25 kb, comprising exons 3–4 of one splice variant of Calpain-10 (*CAPN10*) and exons 3–6 of *GPR35*, encoding the G-protein-coupled receptor 35.

Case 3 is a male patient with butterfly vertebra (V), anal atresia with recto-vesical fistula (A), congenital subvalvular aortic stenosis and ventricular septal defect (C), esophageal atresia IIIb (according to Vogt)²² (TE), high-grade bilateral vesico-ureteral reflux (III° and IV°)

Table 1 Confirmed *de novo* CNVs at 1q41, 2q37.3, and 8q24.3

Case	De novo CNV at	Genomic region duplicated hg 18	First duplicated SNP (flanking non-duplicated SNP)		Last duplicated SNP (flanking non-duplicated SNP)		Minimum/maximum size kb	Affected genes (protein encoded)
			rs10863338 (rs17724824)	rs2953147 (rs2953158)	rs4846444 (rs17041601)	rs4676406 (rs4676403)		
1	1q41	chr1: 215 945 774–216 077 064	rs10863338 (rs17724824)	rs2953147 (rs2953158)	rs4846444 (rs17041601)	rs4676406 (rs4676403)	132–169	<i>SPATA17</i> (spermatogenesis-associated protein 17)
2	2q37.3	chr2: 241 202 666–241 227 781	rs2953147 (rs2953158)	rs6990098 (rs1138597)	rs4676406 (rs4676403)	rs11136345 (rs12545468)	25–33	<i>GPR35</i> (G-protein coupled receptor 35), <i>CAPN10</i> (Calpain-10)
3	8q24.3	chr8: 145 012 210–145 132 100	rs6990098 (rs1138597)		rs11136345 (rs12545468)		120–125	<i>EPPK1</i> (Epiplakin), <i>PLEC-1</i> (Plectin-1), <i>PARP10</i> (poly(ADP-ribose) polymerase 10)



Figure 1 Anal atresia and genital malformations of case 2, a female fetus also presenting with a cloacal malformation with anal atresia (A), bilateral renal dysplasia (R), urethral agenesis, a secondary bell-shaped thorax, and Potter-sequence facies due to anhydramnion.

(R), and bilateral cryptorchidism. A *de novo* microduplication was found at 8q24.3 (Table 1) with an estimated size of 120 kb. This region comprises parts of exon 1 of the epiplakin gene (*EPPK1*), the complete plectin-1 gene (*PLEC-1*), and exons 4–11 of the gene coding for poly(ADP-ribose)polymerase 10 (*PARP10*).

CNVs found in DGV with partial overlap of the three duplicated regions of interest (1q41, 2q37.3, 8q24.3) are listed in Supplementary Table 3. In DECIPHER, no partially but only fully overlapping CNVs were found. Interestingly, the duplicated region 8q24.3 found in case 3 contains partially overlapping CNVs more frequently than the other duplications, most likely due to the adjacent repeat elements mapping to the boundaries of this region.

Gene prioritization and ISH analysis

A database search on expression data and targeted deletions in mice for the genes *Spata17*, *Capn10*, *Gpr35*, *Eppk1*, *Plec1*, and *Parp10* using the Mouse Genome Informatics (MGI) database was performed to narrow down the candidate gene list. Entries provide expression data from the Eurexpress project²³ freely available from the Jackson Laboratory Mouse Genome Informatics database (<http://www.informatics.jax.org/>). As expression of *Spata17*, which encodes a protein related to apoptosis of spermatogenic cells, is restricted to the testis in several species,^{24,25} it was not given high priority for follow-up. The expression of *Capn10* coding for a member of the calpain-like cysteine protease family is moderate and ubiquitous at E14.5. Targeted deletion of *Capn10* resulted in viable mice showing increased adiposity and metabolic defects²⁶ in the homozygous state. Hence, it was not given high priority. Plectin-1 interlinks intermediate filaments with microtubules and microfilaments, and MGI expression data on *Plec1* during development were limited to the retina after E14.0, but are likely to be elsewhere also, considering homozygous knockout embryos exhibiting neonatal death with skin blistering and defects in integument.²⁷ Mutations in *PLEC1* in human have been found to be associated with the epidermolysis bullosa phenotypic

spectrum.²⁸ No expression data were found for *Parp10*, but this gene shares some common exons with *Plec1*.²⁹ In their studies of targeted deletion of *Plec1*, Fuchs *et al*,²⁹ as a consequence of exon sharing between *Plec1* and *Parp10*, generate a *P1c-/-Parp10-/-* mouse line that represents in fact a double knockout (*P1c-/-Parp10-/-*). Although this targeted homozygous deletion was found to affect the nervous system and the motor nerve conduction velocity of the mice model investigated, it can be ruled out that this was due to the targeted deletion of *Parp10*, as the conditional *nes-Cre/plec1/Δ* mouse model (where plectin was knocked down, leaving *Parp10* unaffected) showed comparable phenotypes.²⁹ Owing to the mouse expression data in MGI and lack of congenital malformations in both homozygous murine knockout models of *Plec1* and *Parp10*, both genes were not given high priority for follow-up.

The most promising candidates from the MGI expression data were found to be *Gpr35* and *Eppk1*. *GPR35* is a member of the G-protein-coupled receptor superfamily of transmembrane proteins involved in a variety of physiological pathways. Previous studies showed expression of *GPR35* in tissues of the gastrointestinal tract, nervous system, and lymphoid tissues. It was reported that kynurenic acid might be an endogenous ligand for *GPR35*,³⁰ but more recent data suggested lysophosphatidic acid (specifically 2-oleoyl LPA) to be a more likely candidate.³¹ As Oka *et al*³¹ suggested that RhoA signaling might be activated downstream of *GPR35* (*in vitro*) and RhoA is involved in the non-canonical planar cell polarity (PCP) pathway, it is tempting to speculate that one role of *GPR35* might be cell motility/polarity during embryonic development. According to MGI, *Gpr35* expression has been detected at E14.5 within the stomach, hindgut, rectum,

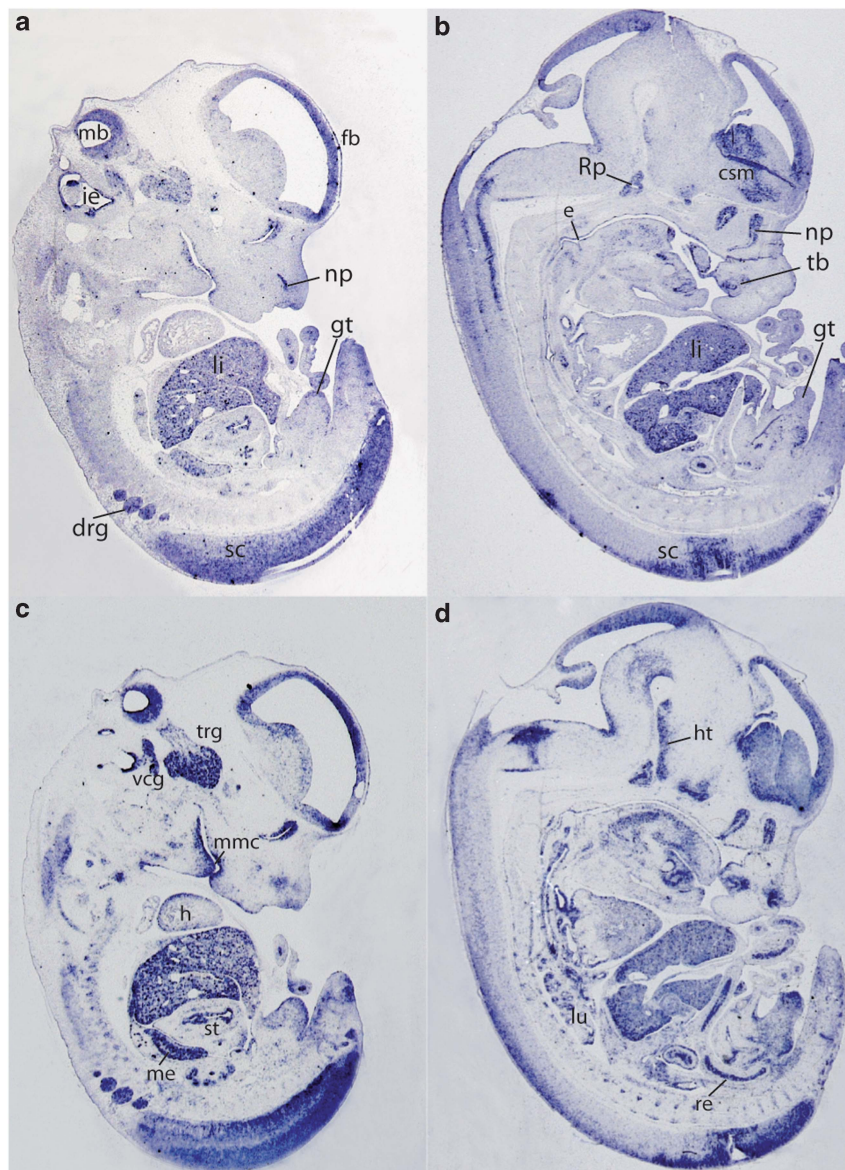


Figure 2 Embryonic mouse expression of *Eppk1* and *Gpr35*. (a) *Eppk1* expression at E12.5 is detectable in the forebrain (fb), midbrain (mb), inner ear (ie), liver (li), nasal passage (np), genital tubercle (gt), dorsal root ganglia (drg), and spinal cord (sc). (b) *Eppk1* expression at E14.5 is detectable in the liver, Rathke's pouch (Rp), esophagus (e), corpus striatum mediale (csm), genital tubercle, tooth bud (tb), spinal cord, and nasal passages. (c) *Gpr35* expression at E12.5. Transcripts are evident in the liver, trigeminal ganglion (trg), vestibulocochlear ganglion (vcg), heart ventricle (h), stomach (st), mesonephros (me), genital tubercle, dorsal root ganglia, and surrounding the maxillo-mandibular cleft (mmc). (d) *Gpr35* expression at E14.5 is detectable in the liver, corpus striatum mediale, hypothalamus (ht), tooth bud, lung (lu), genital tubercle, and rectum.

midgut, bladder fundus region, and urethra. *Eppk1* was expressed at E14.5 in the epidermis, lung, and alimentary canal tissues, including the stomach, esophagus, and gut. However, complete deletion of *Eppk1* results in normal embryonic development,³² whereas there is currently no model for targeted deletion of *Gpr35* in mice. According to these pre-existing data, we decided to validate the expression studies on *Gpr35* and *Eppk1* between E12.5–14.5, which is the equivalent of human gestational weeks 6–8,³³ encompassing the relevant stages of development in mice for organ systems involved in the etiology of VATER/VACTERL.

The expression of *Eppk1* at E12.5 was found within the liver, oral epithelium, nasal passages, brain, and neural tube. At E14.5, it was additionally found in the developing tooth buds and esophageal epithelium (Figure 2). Expression of *Gpr35* at E12.5 was found in the mesonephros, the heart, the esophagus and trachea, the dorsal root ganglia, the genital tubercle, and the rectal region, and a similar expression pattern was detected at E14.5 (Figure 2). This expression not only reflected the affected organ systems of the patient/fetus exhibiting the *GPR35* comprising duplication but also showed expression in tissues relevant to the VATER/VACTERL association in general. Although the genital tubercle and the dorsal root ganglia do not primarily belong to the tissues reflected by the CFs of the VATER/VACTERL association, we point them out because a significant proportion of patients present with genital anomalies/defects arising from the genital tubercle.³ The neurons of the dorsal root ganglia are closely affiliated with vertebral development and are derived from the neural crest cells, which are involved in the formation of tissues including the cardiac outflow tract and enteric nervous system of the gut, suggesting a role in VATER/VACTERL association.

Candidate gene analysis

We further prioritized *Gpr35* over *Eppk1* as a result of both its expression and available information in the literature, and carried out mutation analysis of *GPR35* in 192 VATER/VACTERL/VACTERL-like patients. Sanger sequencing of the two protein-coding exons (exon 5 and 6) of *GPR35* in these 192 patients (including case 2) did not identify any sequence variant of likely pathological significance. Heterozygous sequence variants with a minor allele frequency less than 1% (rs139197368, rs35146537, rs201441371) were also checked for their *de novo* status. They were found to be inherited by one parent, with the exception of one sequence variant, where the father's DNA was not available for testing. Pathogenicity prediction with four algorithms (PolyPhen-2, SNPs&Go, MutPred, SIFT) predicted none of the three SNPs unanimously as disease causing. Two of these variants have been predicted to be benign in all four prediction algorithms applied. SNP rs201441371 leading to a p.Val237Met substitution was classified as possibly disease causing in two and benign in the other two pathogenicity-prediction algorithms. Hence, incomplete penetrance cannot be excluded for this variant and should warrant functional studies.

DISCUSSION

Although only a few large studies have been published, there are several lines of evidence that *de novo* CNVs are associated with the expression of structural birth defects.³⁴ Depending on the investigated birth defect, *de novo* CNVs have been assumed to be causative in up to 20% of cases.³⁵ In our study of patients with VATER/VACTERL association and VATER/VACTERL-like phenotype, we identified three (6%) likely causative *de novo* CNVs at 1q41, 2q37.3, and 8q24.3. These CNVs contained between one and three genes, including *GPR35* and *EPPK1*, which are known, according to expression data

from MGI, to participate in the development of tissues relevant to the VATER/VACTERL-like phenotype (Table 1). For candidate gene mutation analysis, we prioritized *GPR35* based on its expression in mice in the rectal region, the genital tubercle, and the mesonephros, showing a clear overlap with the malformed tissues in the duplication-carrying patient (case 2) (Figures 1 and 2). Sequence analysis of the protein-coding exons did not identify any disease-causing mutation. It is therefore unlikely that mutations affecting the *GPR35* gene are a frequent cause of VATER/VACTERL association; however, our patient cohort might have been too small to detect rare causal mutational events. Furthermore, we may have missed mutations by not analyzing the non-protein-coding exons or the promoter region. Yet, according to our mouse expression data reflecting both the affected organ systems of the patient/fetus exhibiting the *GPR35* comprising duplication and the tissues relevant to the VATER/VACTERL association, *GPR35* seems to be a promising candidate gene being worth of further follow-up in genetic as well as functional studies. Nevertheless, as we cannot exclude *EPPK1*, *SPATA17*, *CAPN10*, *PLEC1*, or *PARP10* as candidate genes, follow-up of these genes is also warranted.

Our study did not investigate whether the identified microduplications alter the expression of the genes involved or lead to altered gene products. This will require further studies. Particularly, in the case of a negative effect on the gene function, there remains also the possibility that the anomalies seen in patients are the results of autosomal-recessive mutations, with one allele being altered by the *de novo* event and the second allele being altered by an inherited change. We did not exhaustively investigate this possibility. However, the lack of a mutation in *GPR35* in case 2 and the lack of *GPR35* mutations in the extended patients sample render this possibility unlikely for *GPR35*. We can also not exclude the possibility that the expression of a gene residing in a region flanking a microduplication could be affected by means of a position effect. However, thorough screening of the flanking regions failed to detect obvious candidate genes. Finally, it remains possible that there were disease-associated CNVs among the many inherited CNVs and that the full penetrance may only be exerted by interaction with an environmental insult, as has been recently demonstrated for hypoxia and fibroblast growth factor signaling in the etiology of congenital scoliosis.³⁶

In summary, a genome-wide analysis in patients with VATER/VACTERL association or VATER/VACTERL-like phenotype has identified three potentially pathogenic CNVs affecting genes involved in the development of the phenotype-relevant tissues. Although mutation screening of the most promising candidate gene, *GPR35*, did not confirm it as a frequent cause of the disorder, new insights were gained and the identified variant loci warrant further evaluation.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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