Biallelic Pathogenic *GFRA1* Variants Cause Autosomal Recessive Bilateral Renal Agenesis

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ABSTRACT

Background Congenital anomalies of the kidney and urinary tract (CAKUT) are one of the most common malformations identified in the fetal stage. Bilateral renal agenesis (BRA) represents the most severe and fatal form of CAKUT. Only three genes have been confirmed to have a causal role in humans (*ITGA8*, *GREB1L*, and *FGF20*).

Methods Genome sequencing within a diagnostic setting and combined data repository analysis identified a novel gene.

Results Two patients presented with BRA, detected during the prenatal period, without additional recognizable malformations. They had parental consanguinity and similarly affected, deceased siblings, suggesting autosomal recessive inheritance. Evaluation of homozygous regions in patient 1 identified a novel, nonsense variant in *GFRA1* (NM_001348097.1:c.676C>T, p.[Arg226*]). We identified 184 patients in our repository with renal agenesis and analyzed their exome/genome data. Of these 184 samples, 36 were from patients who presented with isolated renal agenesis. Two of them had loss-of-function variants in *GFRA1*. The second patient was homozygous for a frameshift variant (NM_001348097.1:c.1294delA, p.[Thr432Profs*13]). The *GFRA1* gene encodes a receptor on the Wolffian duct that regulates ureteric bud outgrowth in the development of a functional renal system, and has a putative role in the pathogenesis of Hirschsprung disease.

Conclusions These findings strongly support the causal role of *GFRA1*-inactivating variants for an autosomal recessive, nonsyndromic form of BRA. This knowledge will enable early genetic diagnosis and better genetic counseling for families with BRA.

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Congenital anomalies of the kidney and urinary tract (CAKUT) are one of the most common malformations identified in the fetal stage. They comprise a broad phenotypic spectrum of renal and urinary tract malformations, ranging from milder presentations such as duplex ureter, to severe fatal phenotypes of bilateral renal agenesis (BRA). Renal agenesis is defined as the complete absence of renal tissue, and can be unilateral or bilateral.[1] Whereas unilateral renal agenesis is compatible with life, BRA usually results in death *in utero* or during the perinatal period.[1,2] Syndromic and nonsyndromic forms of renal agenesis are known. The most common syndromic associations include branchio-oto-renal syndrome (*EYA1*, *SIX5* genes; MIM 113650), Fraser syndrome (*FRAS1*, *FREM2*, *GRIP1* genes; MIM 219000), Pallister–Killian syndrome (*GLI3* gene;

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MIM 146510) and Townes–Brock syndrome (*SALL1* gene; MIM 107480).

To date, mutations in about 40 genes are known to lead to CAKUT.[3] However, so far, only three genes—*ITGA8*,[4] *GREB1L*,[5] and *FGF20*[6]—have been shown to cause renal agenesis in humans. Identification of candidate genes for BRA remains a challenge, not only due to the rarity of the condition but also due to its early mortality.

GFRA1 is an important receptor component for glial cell line–derived neurotrophic factor (GDNF) protein. GDNF/GFRA1 and tyrosine kinase RET signaling complex are crucial for initiation of the ureteric bud (UB) during renal morphogenesis. Defects in the UB formation result in renal agenesis and can cause perinatal lethality.[7–11] Therefore, the *GFRA1* gene is considered a likely causal candidate for BRA.

We present two unrelated families with recurrent, nonsyndromic BRA and homozygous loss-of-function (LoF) variants in *GFRA1*. The gene has been shown to have a crucial role in renal morphogenesis in animal models,[12,13] supporting a causal role for BRA in humans.

Significance Statement

Bilateral renal agenesis (BRA) represents the most severe form of congenital anomalies of the kidney and the urinary tract. Currently, only three genes are known to cause nonsyndromic BRA in humans. The rarity and the high mortality of BRA make it challenging to identify additional loci. Genome sequencing identified a novel causal association of *GFRA1* variants with BRA in humans. Two homozygous, putative, loss-of-function variants (p.Arg226* and p.Thr432Profs*) were found in index cases with BRA from two unrelated consanguineous families by prioritizing homozygous variants and conducting a dedicated database search. These findings have implications for early genetic diagnosis and genetic counseling for families with BRA.

METHODS

Genome Sequencing

Written and informed consent, as per the institutional protocols, was obtained from both families for genetic studies. DNA was extracted using standard methods from dried blood spots from the index case and parents (family 1) submitted on filter cards (CentoCard). For family 2, the genomic analysis was performed on DNA obtained from fetal liver tissue (taken during autopsy), and from blood DNA from both parents.

To carry out genome sequencing (GS), DNA was fragmented by sonication and Illumina (San Diego, CA) adapters were ligated to generate fragments for sequencing, on a HiSeqX platform (Illumina), to yield an average coverage depth of at least 30×. For the index case of family 1, an average coverage depth of 55.8× was obtained, with 99.4% of the target DNA covered at least 10×. For the index case of family 2, an average coverage depth of 32.7× was obtained, with 99.0% of the target DNA covered at least 10×. Raw sequence data analysis, including base calling, de-multiplexing, alignment to the hg19 human reference genome (Genome Reference Consortium GRCh37), and variant calling were performed using the HiSeq Analysis Software version 2.0 pipelines (Illumina). The short reads were aligned to the GRCh37 (hg19) build of the human reference genome using the Isaac aligner algorithm. Variant calling was performed on the alignment files for single nucleotide variants and insertion-deletions using Starling Small Variant Caller.[14] Canvas and Manta were used for detecting structural variants and copy number variants.[15,16] Variants were annotated using SnpEff and in-house, *ad hoc* bioinformatics tools.[17]

Variant Analysis

After variant annotations, filtering and prioritization were performed with a tool developed in-house (CENTOGENE). The system allows for annotated variants to be imported and customized filtering that takes into account variant- and phenotype-related parameters (frequencies, zygosity, type of variant, Human Phenotype Ontology terms, mode of inheritance, among others).

Variants were selected for reporting by considering the compatibility with the suspected phenotype and expected disease mechanism. All provided clinical data, family history, consanguinity, disease onset/course, available test results, and clinical suspicions were considered. For variant interpretation, the type of variant and frequency in public databases, such as gnomAD, ExAc, and disease-centered databases, such as Human Gene Mutation Database and CentoMD, were considered.

Data Repository Analysis

A search was conducted in CENTOGENE data repository[18] using the term "renal agenesis." Patient clinical data were individually curated on the basis of the provided phenotype (isolated unilateral renal agenesis or BRA) and syndromic renal agenesis. Exome/genome data were analyzed to search for rare, high-effect biallelic variants in *GFRA1*.

Sanger Validation and Cosegregation Analysis

The variant-containing exons of the *GFRA1* gene (NM_005264.5) were amplified (primers available upon request) and Sanger sequenced from both forward and reverse orientation on a 3730xl sequencer (Thermo Fisher Scientific, Waltham, MA) in available family members. Exon numbering was used according to reference sequence NM_005264.5 (11 exons). Variant nomenclature followed standard Human Genome Variation Society recommendations.[19]

Detected *GFRA1* variants were submitted to the Leiden Open Variation Database repository under the variant identifiers 0000663717 (family 1) and 0000663718 (family 2).

RESULTS

Family 1

The index patient was a male newborn from healthy, consanguineous parents from the United Arabs Emirates. Family history was positive for renal agenesis in a female sibling who died

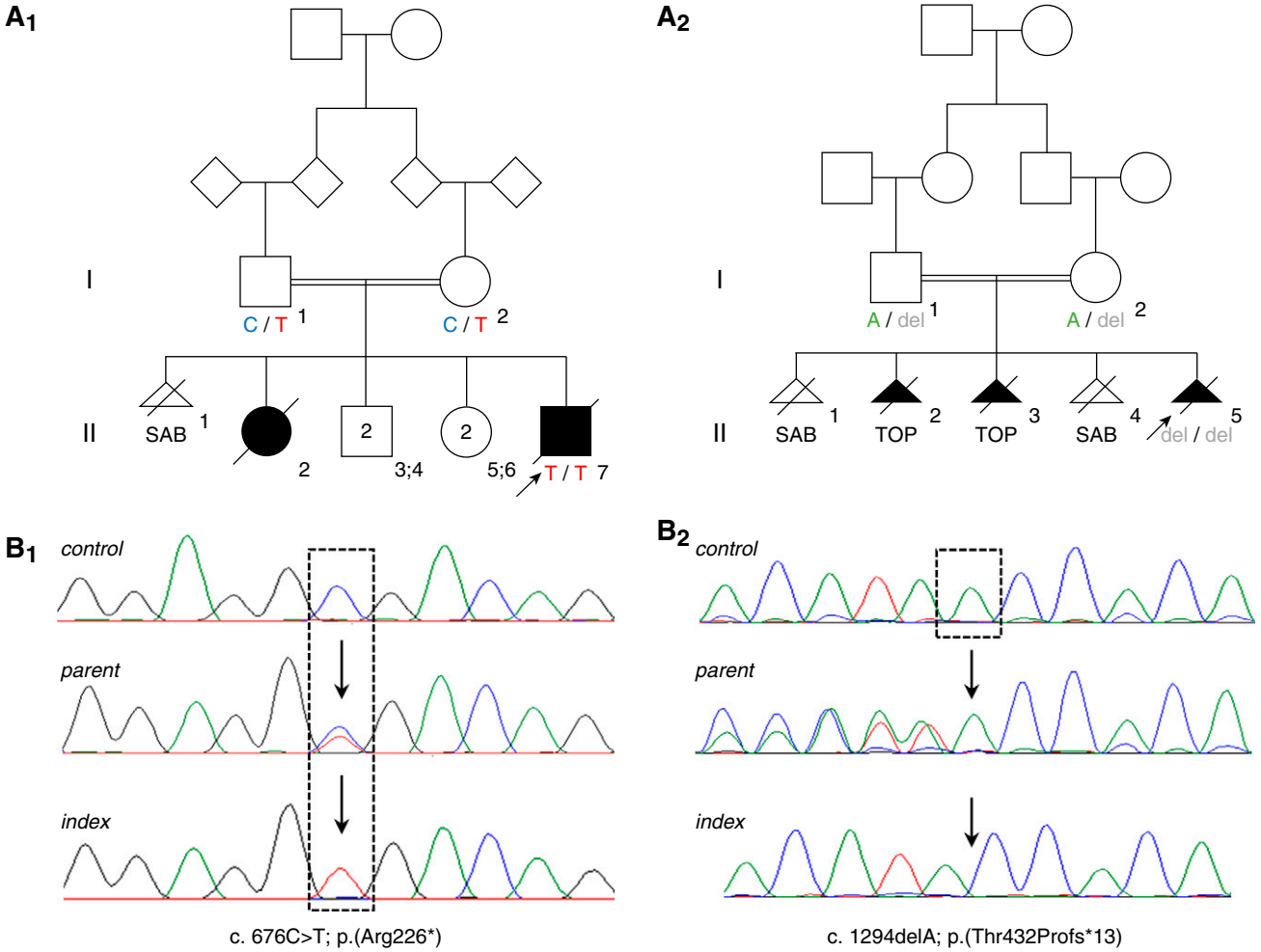

Figure 1. Pedigree analysis and variants (sanger) found in the two families. (A) Simplified pedigree of (A_1) family 1 and (A_2) family 2. The shaded symbols represent individuals affected with BRA. Genotypes are indicated below tested individuals. (B) Corresponding chromatograms showing a substitution of cytosine (blue) to thymine (red), leading to (B_1) a premature stop codon (family 1), and (B_2) deletion of one adenine (green) residue (family 2), with introduction of a frameshift and premature stop codon. A control sample (wild type), parent (heterozygote), and index patient (homozygote) are shown for each family. SAB, spontaneous abortion; TOP, termination of pregnancy.

in the neonatal period. Additionally, there were four siblings who were asymptomatic (Figure 1, A_1). The index patient presented with BRA, Potter facies, lung hypoplasia, and hypoxic respiratory failure. He died soon after birth.

Due to the clinical suspicion of an underlying autosomal recessive disease, GS was performed for the index patient and parents.

Family 2

A consanguineously married couple was evaluated for antenatally detected malformations on level-2 ultrasound studies. This was their fifth pregnancy, with the first and fourth having miscarried in the late first trimester due to unidentified causes. The second and third pregnancies were terminated due to BRA at the 18th and 20th week, respectively. A genetic etiology was not evaluated (Figure 1, A_2). The current pregnancy was conceived spontaneously. The first-trimester maternal serum screen was low risk for aneuploidies and the nuchal translucency scan did not show any abnormal findings. The specialized ultrasonography scan at 20 weeks revealed oligohydramnios (amniotic fluid index, 3.1 cm). Because of this, fetal details could not be evaluated, and the urinary bladder and kidneys could not be visualized. Fetal growth was normal and the ductus venosus showed reduced flow. Another ultrasonographic scan was performed because of suspected BRA, and it showed anhydramnios with absent kidneys, consistent with the suspicion of BRA. The parents were counseled about the nature of the abnormality and the fatal outcome linked to BRA. After this, the parents opted for a therapeutic termination of the pregnancy and a fetal autopsy was performed for the detailed phenotype of the malformation. Nonsyndromic, isolated renal agenesis was confirmed *via* the autopsy. Microscopic

examination of the colon was not performed. The fetal chromosomal microarray was normal. Targeted exome sequencing did not identify a causative gene for BRA. After this, genome sequencing was performed to ascertain a genetic basis for the renal agenesis.

Genome and Database Analyses

In family 1, an analysis focusing on homozygous regions identified a nonsense variant in the *GFRA1* gene (NM_005264.5: c.676C>T, p.Arg226*). The variant is located in exon 6, and it is very rare—present in only one out of 251,436 alleles in gnomAD (last accessed March 24, 2020) and detected for the first time in the CentoMD database.[11,12] The variant was confirmed by Sanger sequencing in the index patient (homozygous) and parents (heterozygous) (Figure 1, B_1). This variant was classified as pathogenic according to the revised American College of Medical Genetics and Genomics criteria: PVS1 (LoF variant in a gene where LoF is a known mechanism of disease), PM2 (absent or very rare in controls from gnomAD, ExAc, and CentoMD) and PS3 (well-established *in vivo* functional studies supportive of a damaging effect).

We then queried the CENTOGENE data repository with the aim of identifying additional cases with LoF variants in *GFRA1*. The gene was considered a casual candidate on the basis of the previous reports on the function of the mouse ortholog.[13,20,21] From approximately 40,000 patient samples with exome sequencing/GS data, 184 samples were identified as having renal agenesis (Human Phenotype Ontology term), provided as part of the clinical information. A detailed evaluation of the clinical information allowed for the identification of 148 patients with a syndromic presentation, including malformations in other systems, intellectual disability, or other neurologic features. Moreover, 36 patients presented with isolated renal agenesis, two of these patients had LoF variants in *GFRA1* (family 1 and family 2).

In family 2, GS performed for diagnostic purposes did not reveal any clinically relevant variant in the established diagnostic genes. A frameshift variant was identified in exon 11 of *GFRA1* (NM_005264.5: c.1294delA, p.Thr432Profs*), which introduces a premature stop codon 12 positions downstream. The variant is not known in gnomAD (last accessed March 24, 2020) and was detected for the first time in CentoMD. The variant was confirmed by Sanger sequencing in the index patient (homozygous) and parents (heterozygous) (Figure 1, B_2). This variant was classified as pathogenic according to the revised American College of Medical Genetics and Genomics criteria: PVS1 (LoF variant in a gene where LoF is a known mechanism of disease), PM2 (absent or very rare in controls from gnomAD, ExAc, and CentoMD), and PS3 (well-established *in vivo* functional studies supportive of a damaging effect). Additional rare, coding, homozygous variants identified are shown in the Supplemental Table 1. These variants were excluded from further analysis on the basis of lower evolutionary conservation and lack of evidence showing a link to renal development/agenesis. Given the previous report of heterozygous *GFRA1* and *RET* variants in a patient and a possible oligogenic inheritance,[22] we also analyzed the *RET* gene in detail, but no relevant variants were identified. The sequence chromatogram of the variants identified is shown in Supplemental Figures 1 and 2.

DISCUSSION

By combining GS, prioritization of homozygous regions, and data repository analysis, we have identified biallelic LoF variants in *GFRA1* as causal of BRA in humans. The gene has been considered a candidate for BRA due to its function and existing studies in animal models.[12,13]

This gene encodes a member of the GDNF receptor family of proteins. GDNF is a structurally related, potent neurotrophic factor that plays key roles in the control of neuron survival and differentiation.[12,23,24] GFRA1 is a receptor for GDNF and mediates activation of the RET tyrosine kinase receptor.[25] GDNF binds GFRA1, which is expressed in early kidney development and throughout branching morphogenesis, and forms a signaling complex with the receptor tyrosine kinase RET.[25] GDNF-GFRA1-RET signaling activates cellular pathways that are required for normal induction of the UB from the Wolffian duct.[24,25] Targeted disruptions of the *Gdnf* and *Ret* genes result in very similar phenotypic defects in the kidneys, along with the complete loss of the enteric neurons throughout the digestive tract.[8–10,26] Similarly, Gfra1-deficient mice demonstrate agenesis of the kidney and absence of enteric neurons.[27,28] Even conditional loss of the Gfra1 protein in the Wolffian duct epithelium before ureteric branching is enough to cause renal agenesis in mice.[12] Additionally, conditional ablation of *Gfra1* in mice during late gestation induces rapid and widespread neuronal death in the colon, leading to colon aganglionosis reminiscent of Hirschsprung disease (HD).[23]

Patients with HD are three- to 18-fold more likely to have CAKUT. *RET* and *GDNF* mutations have been described in both patient groups,[29–31] in agreement with the observation in mice. *RET* mutations have been identified in a spectrum of congenital malformations involving the RET axis, including isolated HD, isolated CAKUT, or both together.[32] Whereas Skinner *et al.*[30] reported 30% of *RET* variants in their series of 29 fetuses with renal agenesis, Jeanpierre *et al.*[31] reported *RET* variants in only 7% of their larger series of 105 patients.

In humans, missense variants in *GFRA1* have been reported in patients with HD.[33,34] Despite the screening of *GFRA1* in patients with CAKUT, no causative variants were identified until now.[29,30] In this study, we identified two unrelated patients out of 36 cases with unilateral renal agenesis or BRA. In both cases, parental consanguinity and positive family history with affected siblings suggested an autosomal recessive disease. These characteristics are probably the main differences from the patients that were investigated in previous studies.

On the other hand, *GFRA1* variants have been reported in patients with HD, but not in cases with vesicoureteral reflux[35] or CAKUT in general (Human Gene Mutation Database). There is, however, one exception: a rare, missense variant in *GFRA1* inherited with other two *RET* variants has been reported in a patient with right cystic dysplasia, left dysplasia, bilateral megaureter, and cryptorchidism. The authors postulated a possible oligogenic effect of the variants. Although inherited from an unaffected parent, *RET* variants were shown to influence activity of mitogen-activated protein kinase *in vitro*.[22] The *GFRA1* variant (NM_001348097.1: c.1328G>A, p.Gly443Asp) is currently of unknown clinical significance (PM2, absent from controls in gnomAD, accessed March 24, 2020; and BP4, multiple lines of computational evidence suggest no effect on gene or gene product). Other variants in *GFRA1* have been detected in patients with central hypoventilation syndrome and HD; similarly, their pathogenicity is, so far, unclear.[34,36]

Various additional morphogenetic pathways have previously been implicated to be essential for proper renal morphogenesis. These signaling pathways include the BMP- (TGFB), NOTCH-, Hedgehog-GLI-, and FGF-related cascades, and canonical WNT/β-catenin signaling that converge and partially modulate one another during the development of the kidneys and the urinary tract.[37–39] Thus, other candidate genes would deserve attention.

In conclusion, our study shows that biallelic LoF variants in *GFRA1* lead to autosomal recessive, nonsyndromic BRA. Our findings have implications for early genetic diagnosis and counseling for families with BRA.

DISCLOSURES

C. Beetz, A. Bertoli-Avella, S. Khan, R. Merdzanic, O. Paknia, M. Rocha, and A. Rolfs are employees of CENTOGENE GmbH. P. Bauer is currently employed by CENTOGENE GmbH and has ownership interest in CENTOGENE B.V. All remaining authors have nothing to disclose.

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Dr. Veronica Arora and Dr. Aida M. Bertoli-Avella wrote the manuscript; Dr. Ayman W. El Hattab and Prof. Ratna Dua Puri are treating physicians; Prof. Ishwar C. Verma was the primary treating physician, designed the study, and edited the manuscript; Dr. Suliman Khan, Dr. Maria Eugenia Rocha, and Dr. Rijad Merdzanic analyzed the next-generation sequencing data; Dr. Aida M. Bertoli-Avella, Dr. Omid Paknia, Prof. Arndt Rolfs, and Prof. Peter Bauer supervised the diagnostic workflows, procedures, and database analyses; Dr. Suliman Khan, Dr. Christian Beetz, and Prof. Peter Bauer contributed to the study design and made substantial contributions to the manuscript.

SUPPLEMENTAL MATERIAL

This article contains the following supplemental material online at http://jasn.asnjournals.org/lookup/suppl/doi:10.1681/ASN.2020040478/-/DCSupplemental.

Supplemental Table 1. Homozygous variants found in raw data analysis.

Supplemental Figure 1. Chromatograph for the variant detected in family 1 NM_005264.5: c.676C>T, (p.Arg226*).

Supplemental Figure 2. Chromatograph for the variant detected in family 2 NM_005264.5: c.1294delA, (p.Thr432Profs*).

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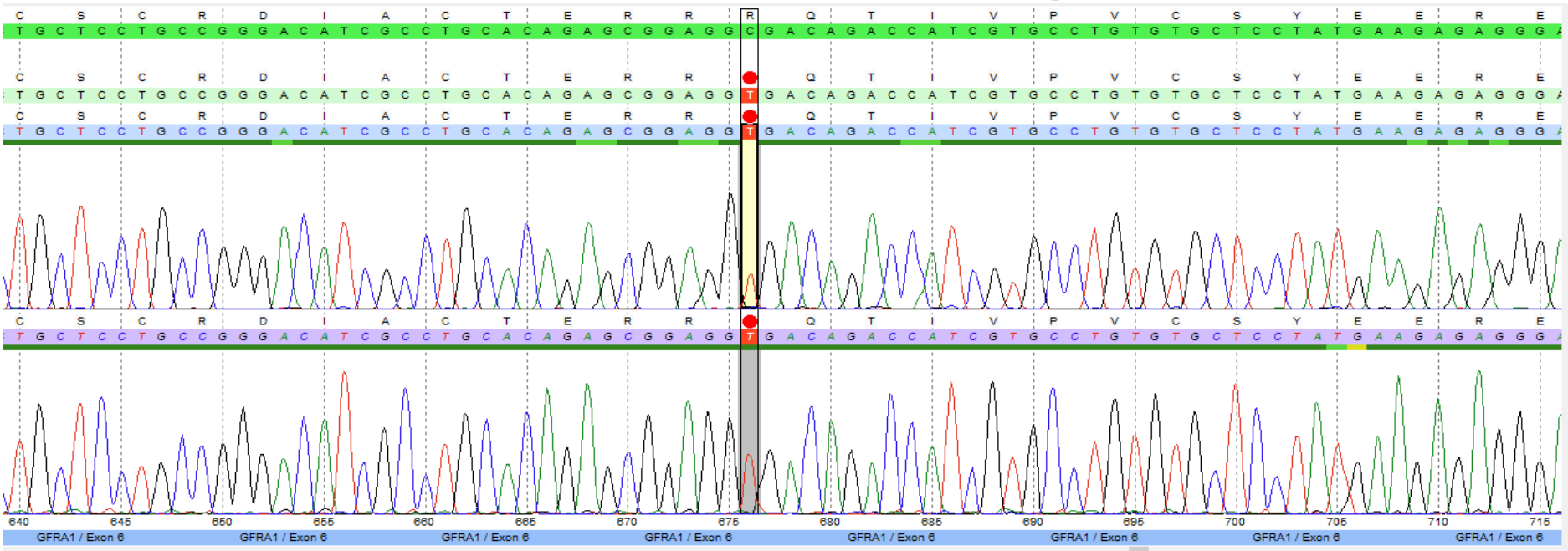
Supplementary table: Homozygous variants found in raw data analysis. Rare homozygous coding variants remaining as candidates in family 1 and 2. Homozygous variants detected in other samples in our or external data repositories (healthy individuals or affected with a distinct phenotype) were excluded.

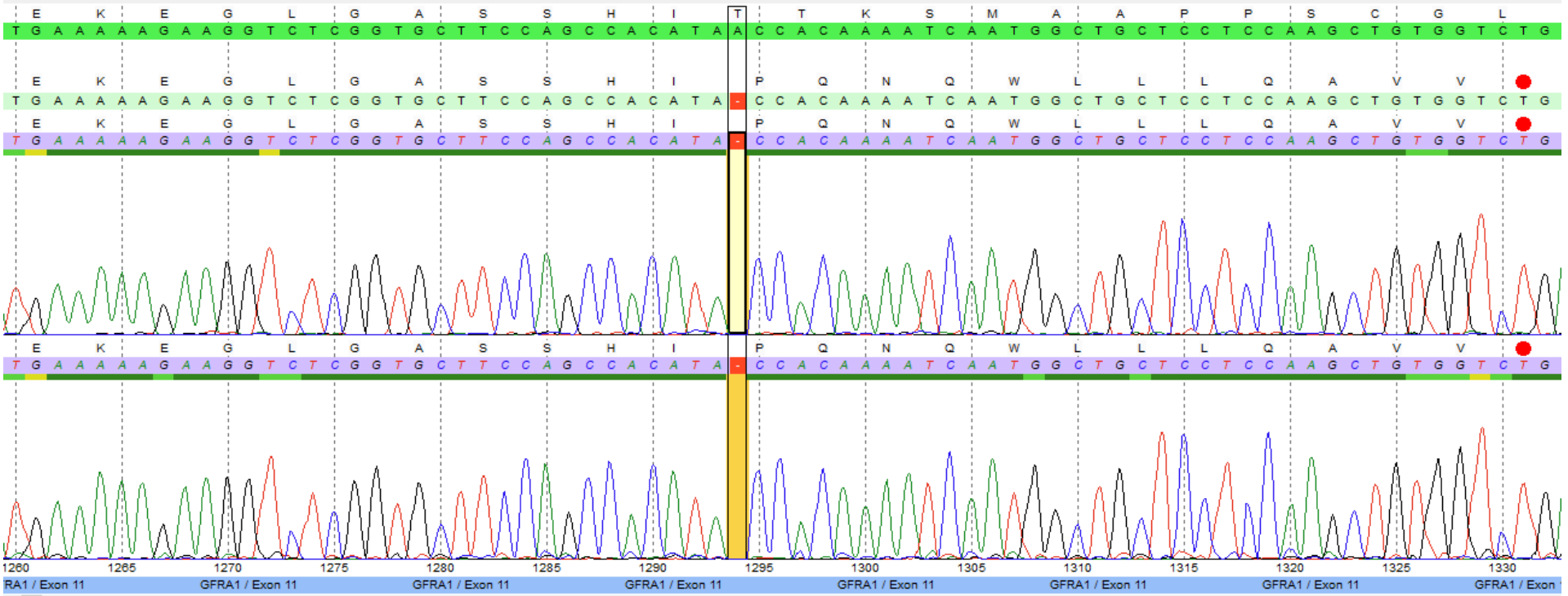
Chr	Genomic coordinate	Gene	Reference seq: nucleotide change	Reference seq.: protein change	Variant_type	dbSNP	OMIM	phyloP	Cadd_raw	PopFreq Max
chr10	**117884826**	***GFRA1***	**NM_005264.5:c.676C>T**	**NM_005264.5:p.Arg226***	**Nonsense**	**rs191814086**	**601496**	**6.762**	**14.5264**	**0.0008**
chr17	3653725	*ITGAE*	NM_002208.4:c.1945A>G	NM_002208.4:p.Met649Val	Missense	rs770556756		-0.126	-3.68351	0
chr1	94685934	*ARHGAP29*	NM_001328664.1:c.220G>C	NM_001328664.1:p.Val74Leu	Missense	rs574016258	610496	3.205	0.932488	0.002
chr10	102849625	*TLX1NB*	NM_001085398.1:c.38G>C	NM_001085398.1:p.Arg13Pro	Missense	rs151326673	612734		1.6072	0.0002
chr10	111886247	*ADD3*	NM_001320591.1:c.1594G>A	NM_001320591.1:p.Asp532Asn	Missense	rs757097119	601568 [Cerebral palsy, spastic quadriplegic, 3]	9.602	6.98095	0.0002
chr10	**117824012**	***GFRA1***	**NM_001348097.1:c.1294delA**	**NM_001348097.1:p.Thr432fs**	**Frameshift**		**601496**			**0**
chr10	124336125	*DMBT1*	NM_007329.2:c.494C>T	NM_007329.2:p.Ala165Val	Missense	rs200063826	601969	0.647	0.619438	0.0031
chr10	124339277	*DMBT1*	NM_007329.2:c.863A>G	NM_007329.2:p.Gln288Arg	Missense	rs764477516	601969	-0.888	-2.18407	0.0044
chr10	127530438	*DHX32*	NM_018180.2:c.1417A>C	NM_018180.2:p.Asn473His	Missense	rs756666817	607960; 611883	6.158	5.70413	0.0011
chr2	21360860	*TDRD15*	NM_001306137.1:c.521C>T	NM_001306137.1:p.Ser174Phe	Missense	rs530287177		0.423	4.85385	0.001
chr2	26717916	*OTOF*	NM_001287489.1:c.791G>A	NM_001287489.1:p.Arg264Gln	Missense	rs370475628	603681 [Auditory neuropathy type 1,Deafness type 9]	6.127	6.54147	0.0002
chr2	186671879	*FSIP2*	NM_173651.3:c.17846T>G	NM_173651.3:p.Leu5949Arg	Missense	rs762024267	615796 [Spermatogenic failure 34]	4.056	3.82643	0.0017
chr2	201501670	*AOX1*	NM_001159.3:c.2383A>C	NM_001159.3:p.Lys795Gln	Missense		602841	3.405	4.68488	0
chr8	11970471	*ZNF705D*	NM_001039615.3:c.707C>G	NM_001039615.3:p.Ala236Gly	Missense	rs773967266		0.666	1.26954	0
chrX	50053245	*CCNB3*	NM_033031.2:c.2076G>C	NM_033031.2:p.Glu692Asp	Missense	rs782385072	300456	1.724	-0.76569	0.0001
chrX	55248225	*PAGE5*	NM_130467.4:c.167G>T	NM_130467.4:p.Arg56Leu	Missense	rs753525619	301009	-1.629	0.638329	0.0004
chrX	78618155	*ITM2A*	NM_004867.4:c.475A>G	NM_004867.4:p.Asn159Asp	Missense	rs780824997	300222	6.368	4.85555	0.0002
chrX	134988296	*SAGE1*	NM_018666.2:c.568A>G	NM_018666.2:p.Ile190Val	Missense	rs782222781	300359	-0.125	-1.31576	0.001

PopMaxFreq (Population maximum frequency): indicates the highest frequency of the variant observed in databases. PhyloP scores indicate evolutionary conserved positions (high positive). CADD (Combined Annotation-Dependent Depletion) ranks genetic variants, including single nucleotide variants (SNVs) and short inserts and deletions (InDels), throughout the human genome reference assembly. RAW score above 4 are considered most as likely pathogenic (ref. PMID: 30371827).

Supplementary figure 1 : Chromatograph of variant detected in family 1 NM_005264.5: c.676C>T, (p.Arg226*)



Supplementary figure 2: chromatograph od variant detected in family 2 NM_005264.5: c.1294delA, (p.Thr432Profs*)



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