ARTICLE | Genetics inMedicine

Pathogenic mosaic variants in congenital hypogonadotropic hypogonadism

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Purpose: Congenital hypogonadotropic hypogonadism (CHH) is a rare disorder resulting in absent puberty and infertility. The genetic architecture is complex with multiple loci involved, variable expressivity, and incomplete penetrance. The majority of cases are sporadic, consistent with a disease affecting fertility. The current study aims to investigate mosaicism as a genetic mechanism for CHH, focusing on de novo rare variants in CHH genes.

Methods: We evaluated 60 trios for de novo rare sequencing variants (RSV) in known CHH genes using exome sequencing. Potential mosaicism was suspected among RSVs with altered allelic ratios and confirmed using customized ultradeep sequencing (UDS) in multiple tissues.

Results: Among the 60 trios, 10 probands harbored de novo pathogenic variants in CHH genes. Custom UDS demonstrated that three of these de novo variants were in fact postzygotic mosaicism —two in *FGFR1* (p.Leu630Pro and p.Gly348Arg), and one in *CHD7* (p.Arg2428*). Statistically significant variation across multiple tissues (DNA from blood, buccal, hair follicle, urine) confirmed their mosaic nature.

Conclusions: We identified a significant number of de novo pathogenic variants in CHH of which a notable number (3/10) exhibited mosaicism. This report of postzygotic mosaicism in CHH patients provides valuable information for accurate genetic counseling.

Genetics in Medicine (2020) 22:1759–1767; https://doi.org/10.1038/s41436-020-0896-0

Keywords: hypogonadotropic hypogonadism; postzygotic mosaicism; copy-number variation; *FGFR1*; *CHD7*

INTRODUCTION

Congenital hypogonadotropic hypogonadism (CHH) is a rare genetic endocrine disorder resulting from a defect in gonadotropin-releasing hormone (GnRH) secretion or action, which leads to absent puberty and infertility (see Supplementary Table S1 for complete list of associated OMIM numbers). CHH is diagnosed in patients with (1) absent or partial puberty at age 17 years, (2) low or normal gonadotropin levels in the context of low serum sex steroids, (3) a normal hypothalamus and pituitary on imaging, and (4) otherwise normal anterior pituitary function. When a lack of sense of smell (anosmia) is present, the condition is termed Kallmann syndrome (KS). Additionally, there is growing evidence that male patients with micropenis and/or cryptorchidism can be diagnosed with neonatal CHH by evidence of hypogonadotropic hypogonadism during minipuberty (ages 2 weeks to 6 months).[1–3]

To date, pathogenic variants in over 30 genes underlie CHH,[4,5] however variants are only present in 51% of patients.[6] Originally, CHH was thought to be a simple Mendelian disorder. However, studies have demonstrated oligogenic pathogenic variants in the known CHH genes in 11–15% probands,[6,7] partially explaining the variable expressivity and reduced penetrance observed in some families. Clearly, other factors are likely to be involved.

Between 66% and 82% of CHH cases are sporadic,[8] which is not unexpected given the reproductive failure of the phenotype. Recessive pathogenic variants can appear to be sporadic, especially when extended family members are unable to be evaluated. However, only approximately 10%

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Submitted 27 April 2020; revised 25 June 2020; accepted: 25 June 2020
Published online: 29 July 2020

of CHH cases have biallelic variants in the known recessive CHH genes.[6]

De novo pathogenic variants are another important factor that may contribute to the sporadic CHH cases. High-throughput sequencing has demonstrated a mutation rate of 1.0 to 1.8×10^{-8} de novo mutations per nucleotide, per generation—roughly correlating to 40–82 mutations across the genome of an individual, with 1 to 2 present in coding sequence.[9] De novo mutations were thought to occur primarily as germline events, however high-throughput sequencing has confirmed a significant contribution of nongermline events to this process.[10] Specifically, postzygotic mutations result in distinct cell populations with different genetic components within a single individual, i.e., mosaicism. Approximately 3–7% of de novo point mutations are detectable in leukocyte DNA as mosaic mutations likely resulting from early postzygotic events.[9,11] However, the overall rate of mosaicism is predicted to be even greater, as prior studies have mainly used blood leukocyte DNA rather than from a wider spectrum of cell types.

Increasingly, postzygotic mosaic variants have been found to underlie a multitude of human diseases, including McCune–Albright syndrome—another rare but important endocrine disorder.[12] In most cases, the phenotypes resulting from either germline or de novo variants are mostly similar, however mosaicism can result in milder, attenuated phenotypes depending on the distribution and frequency in different tissues, as it has been shown in Alport syndrome[13] or pyruvate dehydrogenase complex deficiency.[14]

In this study, we evaluate 60 CHH trios to estimate the frequency de novo pathogenic variants in known CHH genes. In addition, we hypothesize that at least some of these variants will prove to be mosaic.

MATERIALS AND METHODS

Subjects and clinical studies

The Lausanne University Hospital cohort consists of 60 trios (affected proband and unaffected parents): 39 probands (27 male and 12 female) with CHH and anosmia (KS), and 21 probands (16 male, 5 female) with normosmic CHH (nCHH). CHH was diagnosed according to the European Consensus Statement on Congenial Hypogonadotropic Hypogonadism[15] and as described in the introduction. The clinical trial registry number is NCT01601171.

Ethics statement

Written informed consent was obtained from all participants, and the study was approved by the ethics review committee of the Lausanne University Hospital in accordance with the Declaration of Helsinki.

DNA extraction and genetic studies

DNA was extracted from peripheral whole blood leukocytes using the PureGene kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. Exome sequencing was performed on blood DNA for all probands according to previously described methods,[6] as well as the parents from 33 of the trios. For the parents from the remaining 27 trios, variants in the known CHH genes were evaluated using bidirectional Sanger sequencing to evaluate inheritance.

For exome sequencing, Agilent V2 or V5 probes (Agilent, Santa Clara, CA, USA) were used to isolate exome fragments which were then sequenced to an average depth of >50× on the HiSeq 2000 instrument (Illumina, San Diego, CA, USA). FASTQ files were analyzed following the Genome Analysis Toolkit (GATK) best practices and annotated using SnpEff v4. Paternity was confirmed for all trios. Control frequencies were obtained from gnomAD populations (http://gnomad.broadinstitute.org, last accessed 4 March 2020). The results from the exome and Sanger sequencing were then filtered to include nonsynonymous and splice-site (±6 bp from intron–exon boundary) variants in 24 known CHH genes (*ANOS1*, *AXL*, *CHD7*, *FEZF1*, *FGF17*, *FGF8*, *FGFR1*, *GNRH1*, *GNRHR*, *HS6ST1*, *IL17RD*, *KISS1*, *KISS1R*, *LEP*, *LEPR*, *NSMF*, *PCSK1*, *PROK2*, *PROKR2*, *SEMA3A*, *SOX10*, *TAC3*, *TACR3*, and *WDR11*)[6] with minor allele frequencies (MAF) of <0.1%, i.e., rare sequence variants (RSVs). GenBank gene reference numbers (RefSeq) are noted in Supplementary Table S2. Variants were filtered further using InterVar (http://wintervar.wglab.org/, last accessed 4 March 2020) to include those classified as pathogenic (P), likely pathogenic (LP), or variation of unknown significance (VUS) according to the American College of Medical Genetics and Genomics (ACMG) standards.[16] Both gnomAD and Database of Genomic Variants (http://dgv.tcag.ca/dgv/) were used for copy-number variation (CNV) controls. Based on previous publications,[10,17] potential allelic imbalance indicative of either mosaicism or CNV was identified in samples with variant frequencies <40%.

De novo variants were identified using the VariantMaster software.[18] All de novo and potential mosaic variants were visualized using the Integrative Genomics Viewer (IGV, https://software.broadinstitute.org/software/igv/), and verified using bidirectional Sanger sequencing.

Ultradeep sequencing

DNA was extracted from hair follicles, buccal swabs, and urine representing different embryological tissues (i.e., ectoderm, endoderm, and mesoderm) using the PureGene kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. A targeted AmpliSeq DNA panel (Illumina) for the mosaic and CNV variants and single-nucleotide polymorphisms (SNPs) in the surrounding genomic regions was designed using the Illumina DesignStudio software for sequencing (https://www.illumina.com/informatics/sample-experiment-management/custom-assay-design.html). It also included assays from regions on chromosomes 3, 8, 10, 20, and 22 that were used as controls for evaluating true heterozygote read frequencies. In addition to the probands ($n = 4$) and parents ($n = 8$) from the study, an additional 26 samples ($n = 15$ blood DNA, $n = 11$ other DNA samples) were included as controls.

In vitro assay

The in vitro FGFR1 cell signaling assay was performed as previously described.[19] L6 myoblasts (ATCC, Manassas, VA, USA) were transiently transfected with either wild type (WT) or mutant FGFR1 complementary DNA (cDNA) along with the osteocalcin FGF-response element (OCFRE) luciferase reporter. Cells were then treated with increasing doses of FGF8, with FGFR1 mutant values expressed as the percentage of maximal WT. Prism software (version 7; GraphPad) was used to plot three-parameter sigmoidal dose–response curves. Mutant activity was compared with WT in terms of EC_{50} dose and maximal FGF8 response. The assays were performed in triplicate and repeated three times.

Statistical analysis

Exome sequencing reads were evaluated for a deviation from the expected 50% heterozygote frequency using a chi-square (X^2) test with 1 degree of freedom. Read frequencies for true heterozygotes were calculated as mean ± standard deviation and 95% confidence interval (CI). Variation across tissue types from an individual subject was evaluated using a 2×4 two-tailed Fisher's exact test. Post hoc pairwise analysis was performed using a 2×2 two-tailed Fisher's exact test. P values of < 0.01 were considered significant.

RESULTS

De novo analysis for CHH genes

Exome sequencing for 60 trios shows 28 probands with RSVs in the known CHH genes classified as either P, LP, or VUS and a MAF of <0.1% (Table 1). Ten probands carry de novo variants that are also not present in the gnomAD controls. All are classified as pathogenic or likely pathogenic according to ACMG standards. Notably, five of these variants (*FGFR1* p. Leu630Pro, *CHD7* p.Trp908Arg, *CHD7* p.Cys989Tyr, *CHD7* p.Tyr1412Asp, and *ANOS1* p.Cys86Ser) would have been classified as VUS had parental testing not demonstrated them as de novo. Additionally, the de novo *FGFR1* p.Gly348Arg is present in two unrelated CHH probands, thus indicating a potential hotspot for recurrent variants within the gene.

Of the ten pathogenic variants in *FGFR1*, four are de novo. Similarly, four of ten pathogenic variants in *CHD7* are de novo (Table 1). Additionally, one of two pathogenic variants in *ANOS1* and the single pathogenic variant identified in *SOX10* are de novo.

For exome sequencing with an average read depth of >30×, true heterozygotes will typically have allelic ratios (percentage of reads with the variant present relative to total reads) of 40–60%,[10,17] and nearly all of the heterozygous variants in our data set fall within this range (Table 1). However, three of the de novo variants have allelic ratios <40% and differ from the expected range: *FGFR1* p.Leu630Pro (37%, $p < 0.09$), *FGFR1* p.Gly348Arg (21%, $p < 0.0001$), and *CHD7* p.Arg2428* (32%, $p < 0.01$). The proband with the *FGFR1* p.Leu630Pro also carries an RSV in *PROKR2* (p. Asp34Asn) that is inherited maternally and classified as VUS. As noted above, the *FGFR1* p.Gly348Arg variant is also present as a true heterozygote (54%), thus limiting the odds that the reduced frequency is due to an amplification bias. Based on published reports on similar data,[10,11,17,20] postzygotic mosaicism is a potential source of these reduced allelic ratios.

Additionally, a paternally inherited variant (*CHD7* p. Tyr1325His) has an allelic ratio of 37% and a MAF of 0.00007 in controls. The SNP rs77454387 is located 14 kb from this variant and also shows a variant frequency of 35%. Therefore, the *CHD7* p.Tyr1325His reduced ratio is likely the result of an inherited CNV in this region—in this case the presence of three copies.

Postzygotic mosaicism analysis using ultradeep sequencing

As mosaicism can be confirmed by detecting significant variation of the variants across different tissue types, the subjects and family members were recontacted to collect additional samples. Of the three probands exhibiting potential mosaicism, two agreed to the collection of additional tissues (buccal, hair, and urine) for DNA extraction. The remaining potentially mosaic proband refused collection, as well as the proband with the likely CNV. Concurrently, a targeted panel of assays for ultradeep sequencing (UDS) was designed to increase the sensitivity to detect variant frequencies.

To establish a reference range of allelic ratios for true heterozygote calls in the UDS panel, regions on chromosomes 3, 8, 10, 20, and 22 were evaluated, excluding the observed potential mosaic and CNV targets. Blood DNA from 27 samples ($n = 15$ controls, $n = 8$ parents, $n = 4$ probands) was sequenced, as well as buccal, hair follicle, and urine DNA samples ($n = 11$ samples from 5 controls, $n = 6$ samples from 3 probands).

UDS shows an average depth of 5094 reads for 113 SNPs (Supplementary Table S3) across the target regions. For 1284 heterozygote calls across all samples, the mean allelic ratio is 49.7% ± 2.2 (95% CI 49.6–49.9%). Considering only individuals for which multiple samples are available, 754 heterozygote calls are detected with a mean allelic ratio of 49.8% ± 2.3 (95% CI 49.7–50.0%). Thus, UDS proves to be a sensitive method to detect smaller changes in allelic ratios relative to exome sequencing.

Turning to the potential mosaic and CNV variants, the frequencies of the *FGFR1* p.Leu630Pro variant range from 23% to 47% in the tissue samples (blood, buccal, hair follicle, urine) of the proband S374 from family 113 (Fig. 1c and Supplementary Table S5). Each allelic ratio is statistically different than the expected heterozygote call ratio ($p < 0.0001$), and the variation across tissues is also significant ($p = 0.0021$). Similarly, the frequencies of the *FGFR1* p. Gly348Arg variant range from 5% to 21% in tissue samples from proband S439 from family 178. Each allelic ratio is statistically different than the expected heterozygote call ratio ($p < 0.0001$), and the variation across tissues is also significant ($p = 0.0024$). As noted above, proband S412 (family 151) harbors a true heterozygote *FGFR1* p.Gly348Arg variant by exome sequencing. UDS sequencing of this sample confirms this with an observed variant frequency of 48%. Attempts to

Table 1 Rare variants (MAF < 0.1%) from exome sequencing of known CHH genes in KS and nCHH probands from trios.

Family	Proband	Sex	Dx	Gene	cDNA change	Protein change	Inheritance	gnomAD frequency	ACMG classification	Variant reads (%)
28	S289	F	KS	*FGFR1*	2058delC	Phe686Leufs*28	De novo	-	P	41
66	S327	F	KS	*SOX10*	267delC	Met90Cysfs*19	De novo	-	P	56
113	S374	M	nCHH	*FGFR1*	1889T>C	Leu630Pro	De novo	-	P	37
				PROKR2	100G>A	Asp34Asn	Maternal	0.000004	VUS	49
151	S412	F	KS	*FGFR1*	1042G>A	Gly348Arg	De novo	-	P	54
				IL17RD	1136A>G	Tyr379Cys	Maternal	0.00009	VUS	43
178	S439	M	KS	*FGFR1*	1042G>A	Gly348Arg	De novo	-	P	21
301	S735	M	KS	*ANOS1*	256T>A	Cys86Ser	De novo	-	LP	100
313	S771	F	KS	*CHD7*	2722T>C	Trp908Arg	De novo	-	P	46
				WDR11	1066G>A	Val356Ile	Maternal	0.002	VUS	46
463	S1247	F	KS	*CHD7*	7282C>T	Arg2428*	De novo	-	P	32
485	S1315	M	KS	*CHD7*	2966G>A	Cys989Tyr	De novo	-	LP	46
				HS6ST1	652C>T	Pro218Ser	Paternal	0.002	VUS	58
756	S1871	M	KS	*CHD7*	4234T>G	Tyr1412Asp	De novo	-	LP	55
22	S283	M	KS	*CHD7*	4914T>G	Asp1638Glu	Paternal	0.00001	VUS	44
43	S304	M	KS	*SEMA3A*	2201G>A	Arg734Gln	Paternal	0.00001	VUS	56
72	S333	F	KS	*CHD7*	4847A>G	Tyr1616Cys	Maternal	-	VUS	47
				SEMA3A	196C>T	Arg66Trp	Maternal	0.0005	VUS	52
75	S336	M	KS	*FGF17*	287G>A	Arg96Gln	Paternal	0.000008	VUS	45
110	S371	M	nCHH	*GNRH1*	87delA	Leu30Cysfs*12	Biallelic	-	P	100
133	S394	M	nCHH	*TACR3*	443A>T	His148Leu	Biallelic	0.000004	VUS	100
184	S445	M	KS	*AXL*	1549G>A	Gly517Ser	Maternal	0.004	VUS	58
190	S451	M	nCHH	*AXL*	1549G>A	Gly517Ser	Paternal	0.004	VUS	55
				FGFR1	1368G>T	Met456Ile	Paternal	0.0004	VUS	57
				GNRHR	350T>G	Leu117Arg	Paternal	-	LP	56
				GNRHR	266T>A	Leu89*	Maternal	-	P	47
237	S498	M	KS	*FEZF1*	697A>G	Ser233Gly	Maternal	-	VUS	52
435	S1106	M	KS	*FGFR1*	2233C>T	Pro745Ser	Maternal	-	P	51
448	S1175	F	KS	*CHD7*	8188G>A	Ala2730Thr	Paternal	0.00002	VUS	48
				FGFR1	1093_1094dupAG	Pro366Glyfs*4	Maternal	-	P	58
454	S1188	M	KS	*GNRH1*	101G>A	Gly34Glu	Maternal	-	VUS	48
464	S1251	M	KS	*FGFR1*	1977+1G>A	?	Paternal	-	P	48
605	S1647	M	KS	*SEMA3A*	458A>G	Asn153Ser	Paternal	0.002	LP	45
608	S1655	M	nCHH	*FGFR1*	1306_1307dupTC	Met437Profs*2	Maternal	-	P	41
627	S1682	F	nCHH	*CHD7*	8497C>G	Gln2833Glu	Paternal	-	VUS	48
667	S1722	M	KS	*ANOS1*	1890C>A	Tyr630* (HEMI)	Maternal	-	P	100
761	S1879	M	nCHH	*CHD7*	3973T>C	Tyr1325His	Paternal	0.00007	VUS	37

ACMG American College of Medical Genetics and Genomics, *CHH* congenital hypogonadotropic hypogonadism, *Dx* diagnosis, *F* female, *KS* Kallmann syndrome, *LP* likely pathogenic, *M* male, *MAF* minor allele frequency, *nCHH* normosmic congenital hypogonadotropic hypogonadism, *P* pathogenic, *VUS* variant of unknown significance, - not detected in controls.

collect additional samples on proband S1247 (family 463) were unsuccessful, however, the UDS on the original blood DNA sample confirms an allelic ratio 28% for the *CHD7* Arg2428* variant, which deviates significantly from the expected ($p < 0.0001$). UDS on samples from the parents of all trios confirms their de novo nature as no variant reads are present (data not shown).

Altogether, these results confirm the mosaic nature of the three of ten de novo pathogenic variants in these probands. Overall, mosaic pathogenic variants were observed in three of the 60 trios.

The UDS allelic ratio for the *CHD7* p.Tyr1325His variant in proband S1879 (family 761) confirms as 33% (Fig. **1c** and Supplementary Table S5) indicating the presence of three genomic copies. Additionally, SNPs rs77454387 and rs4342642 also show an altered allelic ratio, and the maximal CNV region between rs4509369 to rs3763591 (Fig. **2**) encompasses exons 10–28 of the gene. There are no corresponding CNVs in controls from either the gnomAD database or the Database of Genomic Variants (DGV), even if the exact breakpoints are smaller. The CNV is also present in the same proportion in the father (data not shown) and is absent in the mother.

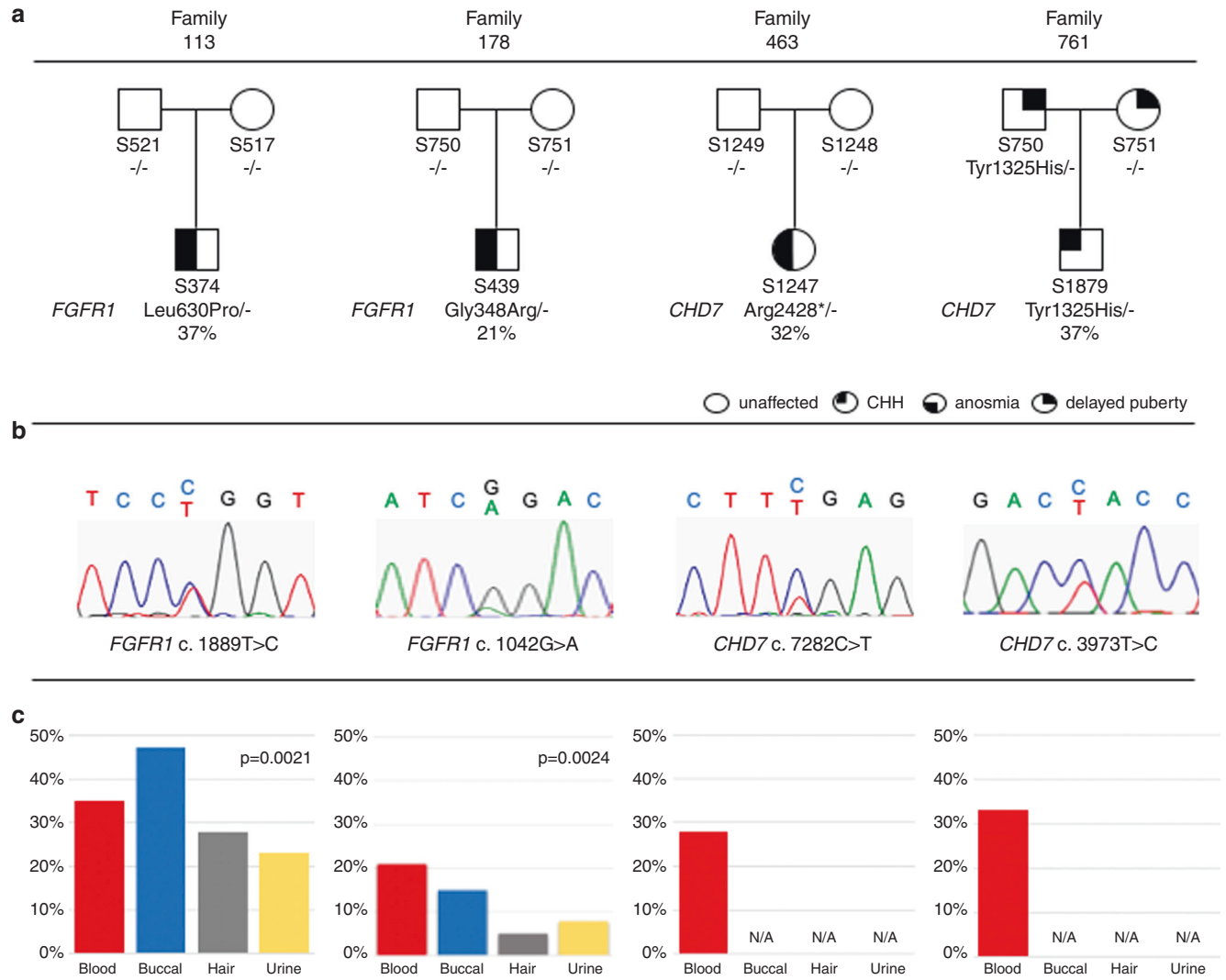

Fig. 1 Pathogenic mosaic variants and copy-number variants (CNVs) in congenital hypogonadotropic hypogonadism (CHH) patients. (**a**) Pedigrees with de novo variants in known CHH genes. Percentages indicate variant reads from exome sequencing. Square, male; circle, female; arrow, proband; -, wild type. (**b**) Sanger chromatograms of corresponding variants. (**c**) Ultradeep sequencing of DNA from multiple tissues. When indicated, the *p* value corresponds to a 2 × 4 two-tailed Fisher's exact test.

Functional validation of pathogenic variants

To confirm that the mosaic CHH variants are pathogenic, we investigated their functional impacts in vitro. The *FGFR1* p.Leu630Pro variant has been previously reported in this patient by our group,[3] and in vitro studies clearly demonstrated that the variant results in complete loss of receptor function.

The *FGFR1* Gly348Arg variant has previously been reported in a different patient with KS and split hand/foot malformation (SHFM);[19] however, in vitro validation was not performed. Therefore, the effect of this variant on downstream MAPK (mitogen activated protein kinase) signaling was evaluated using the osteocalcin FGF response element (OCFRE) luciferase assay (Fig. 3). Stimulation of the WT FGFR1 receptor with increasing doses of fibroblast growth factor 8 (FGF8) shows a typical sigmoidal dose–response curve with an approximate fivefold induction of luciferase activity. In contrast, the Gly348Arg mutant is unable to induce luciferase activity and confirms that this variant is loss-of-function.

Currently, there are no reliable in vitro assays for evaluating the functional impact of *CHD7* pathogenic variants. A single study using an in vivo model in which variant morpholinos were injected into zebrafish has been published.[21] The results were subtle and this method has not been utilized in subsequent studies. Despite the lack of in vitro methods to validate CHD7 variants, the Arg2428* variant is located in exon 34 of this 38 exon gene and thus likely undergoes nonsense-mediated decay[22] resulting in haploinsufficiency. Three CHARGE syndrome (OMIM 214800) patients have been reported with this identical variant in the CHD7 database (https://molgenis51.gcc.rug.nl/), and this de novo variant is classified as pathogenic by ACMG standards. The Tyr1325His variant has not been reported in the literature,

and is not present in the CHD7 database; however, it is located in the critical helicase C domain of the protein. It is classified as VUS by ACMG, thus the functional implication of this variant remains unconfirmed.

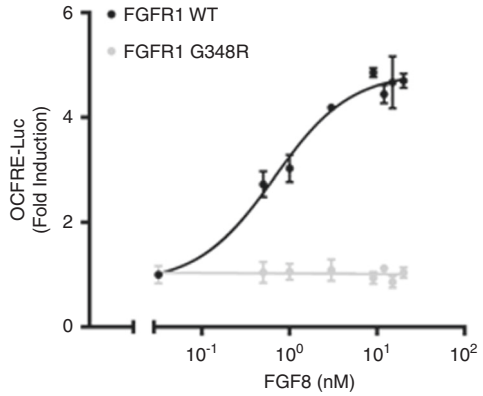

Fig. 2 Transcription reporter activity of FGFR1 WT and G348R. L6 myoblasts were transiently transfected with either wild type (WT) or mutant FGFR1 complementary DNA (cDNA) along with the osteocalcin FGF response element (OCFRE) luciferase reporter. FGFR1 mutant values expressed as the percentage of maximal WT. The assays were performed in triplicate and repeated three times.

Phenotypes in mosaic and CNV patients

Given the limited number of patients with these pathogenic variants in *FGFR1* and *CHD7*, it is challenging to detect any changes in the severity of the phenotypes. In our study (Table 2) and a previous publication describing patients with CHH and split hand/foot malformation,[19] a total of three patients harbor the *FGFR1* p.Gly348Arg pathogenic variant. The true heterozygote from our study also carries an RSV in *IL17RD* (p.Asp34Asn) that is classified as VUS. All three probands have absent puberty, however our mosaic patient and the SHFM patient exhibit severe GnRH deficiency as indicated by their micropenis and cryptorchidism—features absent in our heterozygous proband. Both of our probands are anosmic (KS), whereas the SHFM malformation proband is normosmic.

The proband mosaic for the *CHD7* p.Arg2428* was originally diagnosed with CHH; however, following the identification of a pathogenic variant in *CHD7* was re-evaluated for additional CHARGE syndrome features. Coloboma, semicircular canal hypoplasia, and pulmonary artery stenosis were identified and confirmed the diagnosis of CHARGE. Puberty was absent, and there are no indications of a milder phenotype in this individual.

Finally, proband S1879 harboring the CNV and *CHD7* Tyr1325His variant had a partial puberty that did not progress

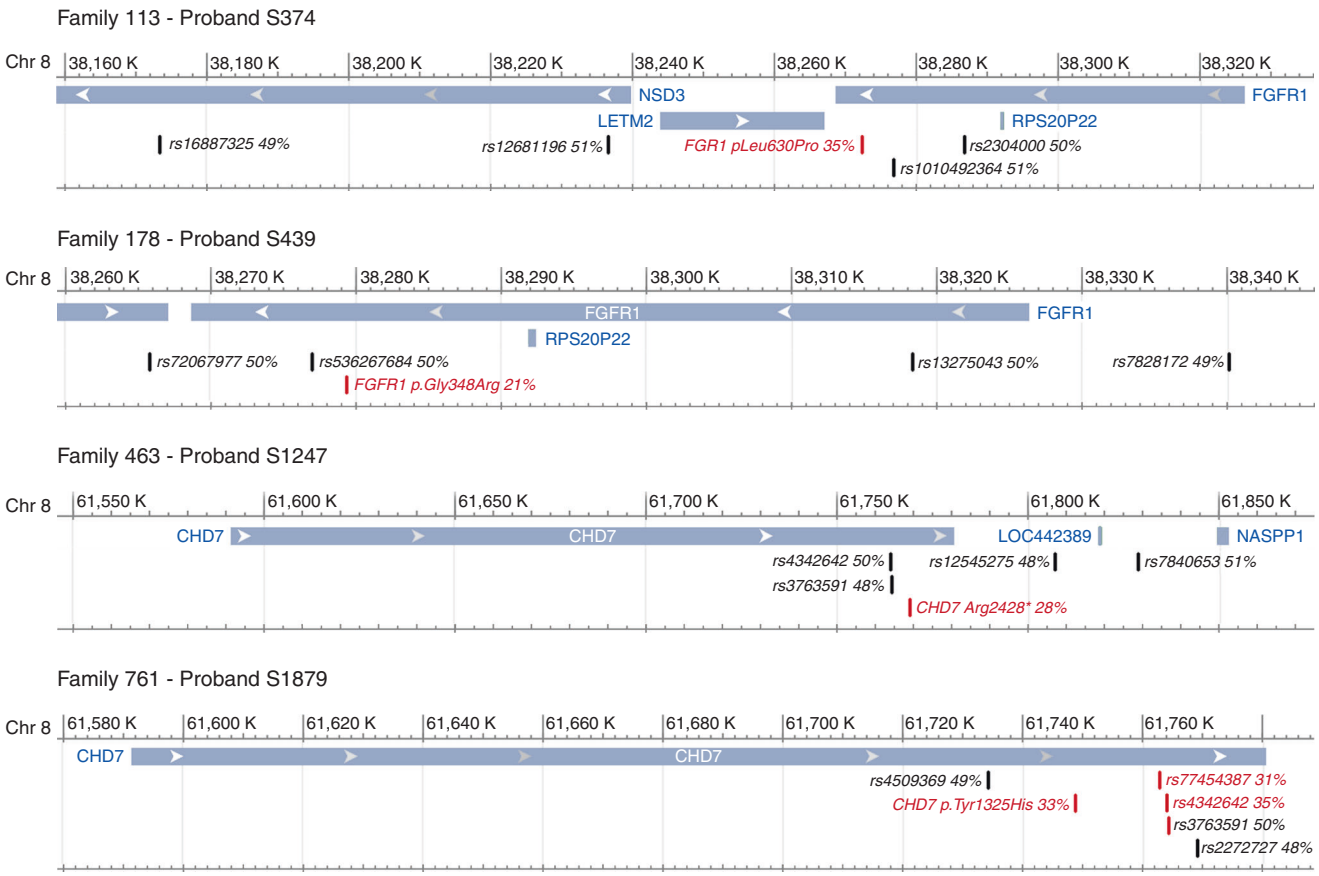

Fig. 3 Gene schematic for regions surrounding mosaic variants and copy-number variants (CNVs). Scale of *x*-axis is in thousands of base pairs, and allelic ratios are noted after each variant. Variants in red indicate statistical deviation from expected heterozygote calls. *WT* wild type.

Table 2 Phenotypes of probands with mosaic, CNV, and heterozygous pathogenic variants.

Family	Proband	Gender	Diagnosis	Olfaction	Puberty	Genital phenotypes	Associated phenotypes	Variants	Inheritance
113	S374	M	CHH	Normal	Absent	Micropenis and bilateral cryptorchidism	Atrial septal defect	*FGFR1* p.Leu630Pro *PROKR2* p.Asp34Asn	De novo (mosaic) Maternal
178	S439	M	KS	Anosmia	Absent	Micropenis and bilateral cryptorchidism	-	*FGFR1* p.Gly348Arg	De novo (mosaic)
463	S1247	F	KS (CHARGE)	Anosmia	Absent	-	Coloboma, semicircular canal hypoplasia, pulmonary artery stenosis	*CHD7* p.Arg2428*	De novo (mosaic)
761	S1879	M	CHH	Normal	Partial	-	Mild right ventricular dilation, aortic dilation	*CHD7* p.Tyr1325His	Paternal
151	S412	F	KS	Anosmia	Absent	-	Hearing loss, abnormal dentition, low bone mass	*FGFR1* p.Gly348Arg *IL17RD* p.Tyr379Cys	De novo Maternal

CHARGE coloboma, heart defects, choanal atresia, retardation of growth and development, genital hypoplasia and ear anomalies, *CHH* congenital hypogonadotropic hypogonadism, *F* female, *KS* Kallmann syndrome, *M* male.

to completion, and also exhibited mild ventricular and aortic dilation. Combined, the reproductive and cardiac features may be milder forms of CHH or atypical CHARGE syndrome, although patients with similar attenuated phenotypes and heterozygous pathogenic variants have been reported.[23]

DISCUSSION

In this study of 60 CHH trios, we identified de novo pathogenic variants in the known CHH genes in 10 probands. Although previous studies have shown de novo variants in CHH,[3,6,23–27] the current report is a systematic evaluation to investigate their overall prevalence. Up to 82% of CHH cases are sporadic,[8] and the current study helps to explain at least part of the sporadic nature of this disorder. The data also underscores the importance of evaluating parental samples when variants are detected. Indeed, half of the variants would only be classified as pathogenic or likely pathogenic according to ACMG standards when they are known to be de novo, implying that the de novo status of a pathogenic variant is critical information for genetic counseling.

Importantly, four of the ten pathogenic variants in either *FGFR1* or *CHD7* are de novo. Although we also detected de novo variants in *ANOS1* and *SOX10*, the small number of variants in these genes overall prevent an accurate estimation of de novo frequencies. Indeed, the frequencies of pathogenic variants in most CHH genes other than *FGFR1* and *CHD7* (the genes most commonly involved in CHH[4,6,28]) are low. However, they collectively represent the majority of CHH cases, thus a comprehensive study across multiple centers will be necessary to accurately assess the de novo frequency for other CHH genes.

When exome sequencing identified disrupted allelic ratios, UDS confirmed mosaicism or CNV in all four cases. The high number of reads (average of 5094 reads) yields accurate heterozygote calls with minimal variation (mean allelic ratio is 49.7% ± 2.2 [95% CI 49.6–49.9%]). Additionally, UDS was able to detect statistically significant variation across blood, buccal, hair follicle, and urine samples in two of our probands.

Gonadal (prezygotic) mosaicism for *FGFR1* variants has been reported in unaffected parents of Hartsfield syndrome and CHH probands,[29–31] as well as in unaffected parents of CHARGE syndrome probands with pathogenic variants in *CHD7*.[26] The current study reports postzygotic pathogenic variants in these genes in CHH patients. While 3 of the 60 CHH probands in this study exhibited mosaic pathogenic variants, three of the ten de novo variants were found to be mosaic. This is consistent with recent reports proposing a larger role for postzygotic mosaicism relative to gonadal mosaicism.[9] The presence of mosaic variants is also important to the quality of genetic testing and counseling for CHH patients. Although a hallmark of CHH is infertility with females being anovulatory and males typically unable to produce sperm in the ejaculate, treatment with gonadotropins or GnRH can restore fertility. In this instance, genetic testing of the sperm of male mosaic patients would give a more accurate odds of transmitting a pathogenic variant to their offspring.

The presence of mosaicism in CHH may be underestimated. Studies show that exome sequencing is unlikely to detect mosaicism below 20%,[10,11] and therefore CHH patients with lower levels of mosaicism would have been missed in the current study. Additionally, mosaic patients may have a variant frequency in blood ranging between 40% and 60% and may thus be erroneously considered as heterozygotes. Notably, if a buccal sample (another commonly used sample) had initially been used for the proband with the mosaic *FGFR1* p.Leu630Pro instead of blood DNA, his result (47% by UDS) would have likely been in the typical heterozygote range. Similarly, if only blood and buccal DNA were used in the two patients with multiple tissues, the variation would not be statistically significant (post hoc pairwise analysis, Supplementary Table S4). Therefore, a larger selection of tissues increases the odds of detecting statistically significant variation needed to confirm mosaicism.

High-throughput sequencing is becoming the method of choice for diagnostic testing, especially when multiple genes are involved such as in CHH. However, Sanger sequencing may still be used in certain laboratories. As Sanger is only semiquantitative, mosaicism can go undetected by this method. Indeed, the *FGFR1* p.Gly348Arg and *CHD7* p.Arg2428* variants (Fig. **1b**) in the current study would likely be missed, while the *FGFR1* p.Leu630Pro and *CHD7* p.Tyr1325His variants would be mistakenly called as true heterozygotes. Although exome sequencing accurately detects variants, this is dependent on the bioinformatic parameters used for expected allelic ratios. GATK best practices recommend heterozygote read frequencies of 40–60%, thus variants outside this range would not pass filters. Therefore, it is critical to either review all data including variants not passing filters or widen the range of heterozygote allele ratios to ensure detection of these pathogenic variants.

The *FGFR1* variants detected were demonstrated to be loss-of-function by in vitro testing in the current study (p.Gly348Arg) and in previous publications (p.Leu630Pro),[3] and the truncating nature of the *CHD7* p.Arg2428* variant supports haploinsufficiency. Given their mosaic nature in our patients, it is intriguing to speculate about a potential dose-dependent role in phenotypic expression. However, the limited number of patients observed makes this challenging, and milder phenotypes were not observed. Although the pathogenic variants were detected at varying levels across multiple tissues, it remains unknown whether GnRH neurons in the hypothalamus or cells along their migratory pathway during development have reduced *FGFR1* or *CHD7* content. This challenge is not unique to CHH, as noninvasive sampling of biologically relevant tissue is not feasible in many disorders exhibiting mosaicism. However, larger comprehensive studies may shed light on the contribution of mosaicism to the reduced penetrance, variable expressivity, oligogenicity, and complex genetics of CHH.

We detected a paternally inherited *CHD7* p.Tyr1325His variant within a region of copy-number variation in a CHH proband. The patient has mild cardiac symptoms, but does not meet the criteria for either typical or atypical CHARGE syndrome. A duplication of all or part of the *CHD7* gene has not been detected in other CHH patients, and has been previously reported in only one CHARGE syndrome patient.[32] In our patient, it is unclear whether the duplicated *CHD7* fragment is internal to the native *CHD7* alleles and disrupts transcription, or is located separately (either nearby or in a different part of the genome). Additionally, it is also not known whether the p.Tyr1325His variant is present in the fragment or native alleles, therefore the actual pathogenic mechanism is not known. RNA sequencing would help to answer this question; however, the proband refused collection of additional samples.

In conclusion, the current study demonstrates the role of mosaicism in the complex architecture of CHH. Further, our data provide valuable information needed to improve both the accuracy and content of genetic testing and counseling. Evaluating parental samples for determination of inheritance combined with careful attention to sequencing results in probands and potential follow up using UDS can yield critical insight into the pathology of CHH (i.e., mosaicism).

SUPPLEMENTARY INFORMATION

The online version of this article (https://doi.org/10.1038/s41436-020-0896-0) contains supplementary material, which is available to authorized users.

ACKNOWLEDGEMENTS

We are grateful to the patients and family members who participated in this research study by donating their samples, medical history, and time. We also thank Alexia Boizot for her technical and laboratory assistance. This work was supported by the Swiss National Science Foundation Project SNF 310030_173260 (to N.P.).

DISCLOSURE

The authors declare no conflicts of interest.

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