

Expanding the genetic spectrum of *ANOS1* mutations in patients with congenital hypogonadotropic hypogonadism

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STUDY QUESTION: What is the prevalence and functional consequence of *ANOS1* (*KALI*) mutations in a group of men with congenital hypogonadotropic hypogonadism (CHH)?

SUMMARY ANSWER: Three of forty-two (7.1%) patients presented *ANOS1* mutations, including a novel splice site mutation leading to exon skipping and a novel contiguous gene deletion associated with ichthyosis.

WHAT IS KNOWN ALREADY: CHH is characterized by lack of pubertal development and infertility, due to deficient production, secretion or action of GnRH, and can be associated with anosmia/hyposmia (Kallmann syndrome, KS) or with a normal sense of smell (normosmic CHH). Mutations in the *anosmin-1* (*ANOS1*) gene are responsible for the X-linked recessive form of KS.

STUDY DESIGN, SIZE, DURATION: This cross-sectional study included 42 unrelated men with CHH (20 with KS and 22 with normosmic CHH).

PARTICIPANTS/MATERIALS, SETTING, METHODS: Patients were screened for mutations in the *ANOS1* gene by DNA sequencing. Identified mutations were further investigated by RT-PCR analysis and multiplex ligation-dependent probe amplification (MLPA) analysis.

MAIN RESULTS AND THE ROLE OF CHANCE: Hemizygous mutations were identified in three (7.1%) KS cases: a novel splice acceptor site mutation (c.542-1G>C), leading to skipping of exon 5 in the *ANOS1* transcript in a patient with self-reported normosmia (but hyposmic upon testing); a recurrent nonsense mutation (c.571C>T, p.Arg191*); and a novel 4.8 Mb deletion involving *ANOS1* and eight other genes (*VCX3B*, *VCX2*, *PNPLA4*, *VCX*, *STS*, *HDHD1*, *VCX3A* and *NLGN4X*) in KS associated with ichthyosis.

LIMITATIONS, REASONS FOR CAUTION: Objective olfactory testing was not performed in all cases of self-reported normosmia and this may have underestimated the olfactory deficits.

WIDER IMPLICATIONS OF THE FINDINGS: This study further expands the spectrum of known genetic defects associated with CHH and suggests that patients with self-reported normal olfactory function should not be excluded from *ANOS1* genetic testing.

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Introduction

Congenital hypogonadotropic hypogonadism (CHH) is characterized by complete or partial failure of pubertal development due to deficient production, secretion or action of GnRH. Under normal conditions, GnRH is produced by specialized hypothalamic neurons and stimulates the release of gonadotropins (LH and FSH) from the pituitary, which control gonadal maturation and adult reproductive physiology (Boehm *et al.*, 2015).

CHH includes Kallmann syndrome (KS), which is characterized by gonadotropin deficiency with a defective sense of smell (i.e. anosmia or hyposmia), and CHH without olfactory defects (normosmic CHH). Other non-reproductive phenotypes, such as renal agenesis, midline facial defects, dental agenesis, hearing loss, synkinesis and skeletal anomalies, are commonly observed in patients with CHH (Costa-Barbosa *et al.*, 2013; Della Valle *et al.*, 2013).

Up to 50% of patients with CHH reveal a genetic defect in genes that regulate embryonic development or migration of GnRH neurons, or the synthesis, secretion or action of GnRH (Boehm *et al.*, 2015). The identification of genes mutated in patients with CHH has improved the understanding of the neuroendocrine control of reproduction (Forni and Wray, 2015).

The anosmin-1 gene (*ANOS1*; MIM 300836), also known as the Kallmann syndrome 1 (*KAL1*) gene, is one of the most commonly involved genes in CHH and is responsible for the X-linked recessive form of KS (Franco *et al.*, 1991; Legouis *et al.*, 1991). *ANOS1* is located on chromosome Xp22.31, comprises 14 exons and encodes a 680-amino acid extracellular cell adhesion protein, anosmin-1, that is essential for axonal guidance and migration of olfactory and GnRH neurons from the nasal placode to their final destination (Hu and Bouloux, 2011).

The aim of this study was to identify and determine the prevalence of *ANOS1* mutations in a group of men with CHH.

Materials and Methods

Subjects

The study comprised 42 unrelated Portuguese males with CHH, 20 with KS and 22 with normosmic CHH, recruited by Portuguese clinical endocrine centres. Inclusion criteria were male patients with low serum FSH, LH and testosterone levels and failure to enter spontaneous puberty by the age of 18 years or with medically induced puberty below this age. Olfactory function was assessed either by olfaction testing or by self-reporting by the patients, depending on the clinical centre. Patients with a history of an acquired cause of hypopituitarism or with abnormal radiological imaging of the hypothalamic-pituitary region were excluded from the study. In mutation-positive patients, additional family members were also studied. The control population consisted of 85 (34 male, 51 female) Portuguese unrelated volunteers who were recruited among blood donors. Written informed consent was obtained from all subjects and the study was approved by the local research ethics committee (Faculty of Health Sciences, University of Beira Interior, Ref: CE-FCS-2012-012).

DNA sequence analysis

Genomic DNA was extracted from peripheral blood leucocytes using previously described methods (Miller *et al.*, 1988). Patients were screened for mutations in *ANOS1* by PCR amplification of the 14 coding exons and

exon–intron boundaries, and bidirectional sequencing using CEQ DTCS sequencing kit (Beckman Coulter, Fullerton, CA, USA) and an automated capillary DNA sequencer (GenomeLab TM GeXP, Genetic Analysis System, Beckman Coulter). Primer sequences were previously described (Hardelin *et al.*, 1993), except for the forward primer for exon 1 (5'-TGGGAGGGAGGTCGGCGAGGAGGG-3') and the reverse primer for exon 10 (5'-GTGAATGGATAAAAAGAATGAGTGA-3'), which were designed using Primer 3 Plus (Untergasser *et al.*, 2007). Background amplification of the *KAL* pseudogene (*KALP*) on chromosome Y, which occurred frequently with exons 11–14 of *ANOS1*, was disregarded in the sequence analysis of *ANOS1*. Genomic sequence variants identified in patients were searched in population variant databases (Exome Aggregation Consortium (ExAC) database) (ExAC, 2016) to assess their frequency in the general population. Point mutations were confirmed in patients and excluded in a panel of 85 healthy volunteers (136 alleles) using a sequence-specific restriction enzyme (EcoNI), in the case of the intron 4 mutation, or allele-specific PCR, in the case of the exon 5 mutation. The latter was carried out by multiplex PCR using a modified mutation-specific forward primer (5'-AAGCCCAGAAAAGAGTGAT-3') (modified nucleotides underlined) together with the exon 5 pair of PCR primers. The functional consequences of the observed splice site mutation were predicted using the Mutation Taster (Schwarz *et al.*, 2010) and Human Splicing Finder (Desmet *et al.*, 2009) bioinformatics tools. Mutation nomenclature followed standard guidelines (den Dunnen and Antonarakis, 2001) and was based on the cDNA reference sequence for *ANOS1* (GenBank accession NM_000216.2). Patients with identified *ANOS1* mutations were screened for digenic/oligogenic mutations by sequencing additional genes related to the hypothalamic-pituitary-gonadal axis (*FGFR1*, *GNRH1*, *GNRHR*, *FGF8*, *PROK2*, *PROKR2*, *KISS1R*, *TAC3*, *TACR3* and *CHD7*) (all primer sequences and PCR conditions are available upon request).

RT-PCR analysis

In patients with splice site mutations, RT-PCR was used to investigate mRNA splicing abnormalities, using total RNA extracted from peripheral blood leucocytes from the proband and an unrelated normal individual, as previously described (Infante *et al.*, 2016). RT-PCR was performed using *ANOS1*-specific primers consisting of a forward primer 5'-AATGTTCTAAGTGCCTGGAGCC-3' (spanning the junction of exons 2 and 3) and a reverse primer 5'-CAGAATTTTGAAACCCATCCGTAG-3' (spanning the junction of exons 7 and 8), and using 35 cycles with an annealing temperature of 56°C and with 1.5 mM MgCl₂. The RT-PCR products were then sequenced as described above.

Multiplex ligation-dependent probe amplification analysis

In patients with absence of PCR amplification of the *ANOS1* exons, the multiplex ligation-dependent probe amplification (MLPA) technique was used to confirm the deletion of the gene. *ANOS1* gene dosage analysis was carried out using the commercial SALSA MLPA Probemix P018-F1 SHOX (MRC Holland, Amsterdam, The Netherlands) that comprises probes for several genes including *ANOS1*. Patient and control samples were prepared according to the manufacturer's instructions (MRC Holland). Amplicons were run on an automated capillary DNA sequencer and analysed by fragment analysis software (GenomeLab TM GeXP, Genetic Analysis System, Beckman Coulter).

Identification of deletion boundaries

In patients with a complete *ANOS1* gene deletion, the extent of the deletion was determined by PCR amplification of known genetic markers (NCBI Map Viewer, National Center for Biotechnology Information, US

National Library of Medicine, Bethesda, MD, USA) upstream and downstream of the *ANOS1* locus, and further refined until the closest amplified markers were identified.

Results

Sequence analysis of the entire coding region of *ANOS1*, including exon–intron boundary regions, revealed three different hemizygous mutations: a splice acceptor site mutation in intron 4 (c.542-1G>C), a nonsense mutation in exon 5 (c.571C>T, p.Arg191*), and a complete deletion of *ANOS1* (Figs 1 and 2). Screening of additional CHH genes in these three patients revealed no other mutations.

The c.542-1G>C and c.571C>T mutations were confirmed in patients and excluded in a panel of 85 normal controls (136 alleles), by restriction enzyme (EcoNI) analysis and allele-specific PCR, respectively (Fig. 1C). Furthermore, these variants were not found in any of the population variant databases including the ExAC database (ExAC, 2016).

Bioinformatics analysis predicted that the splice site mutation was likely to have a damaging effect on splicing. Further assessment by RT-PCR analysis revealed that the patient's leucocyte *ANOS1* transcript was shorter than the wild-type transcript obtained from a control sample (Fig. 3A). Sequence analysis of the cDNA showed that the mutated transcript was 185 nucleotides shorter than the wild-type (r.542_726del) and that the missing sequence corresponded to exon 5 (Fig. 3B). Thus, the splice acceptor site mutation in intron 4 was found to result in the skipping of exon 5 in the *ANOS1* transcript (Fig. 3C). If translated, the mutated transcript would be expected to result in a truncated protein due to a frameshift and premature termination codon (p.Gly181Aspfs*10). A possible effect of the mutation on nonsense-mediated mRNA decay was not assessed.

A complete deletion of *ANOS1* was suspected in one of the patients due to the repeated failure of PCR amplification of the 14 *ANOS1* exons. MLPA analysis confirmed that a deletion had occurred in the *ANOS1* gene (Fig. 2B) and also in the nearby *NLGN4X* gene (data not shown). PCR amplification of genetic markers in the proximity of the *ANOS1* locus was performed to determine the extent of the deletion

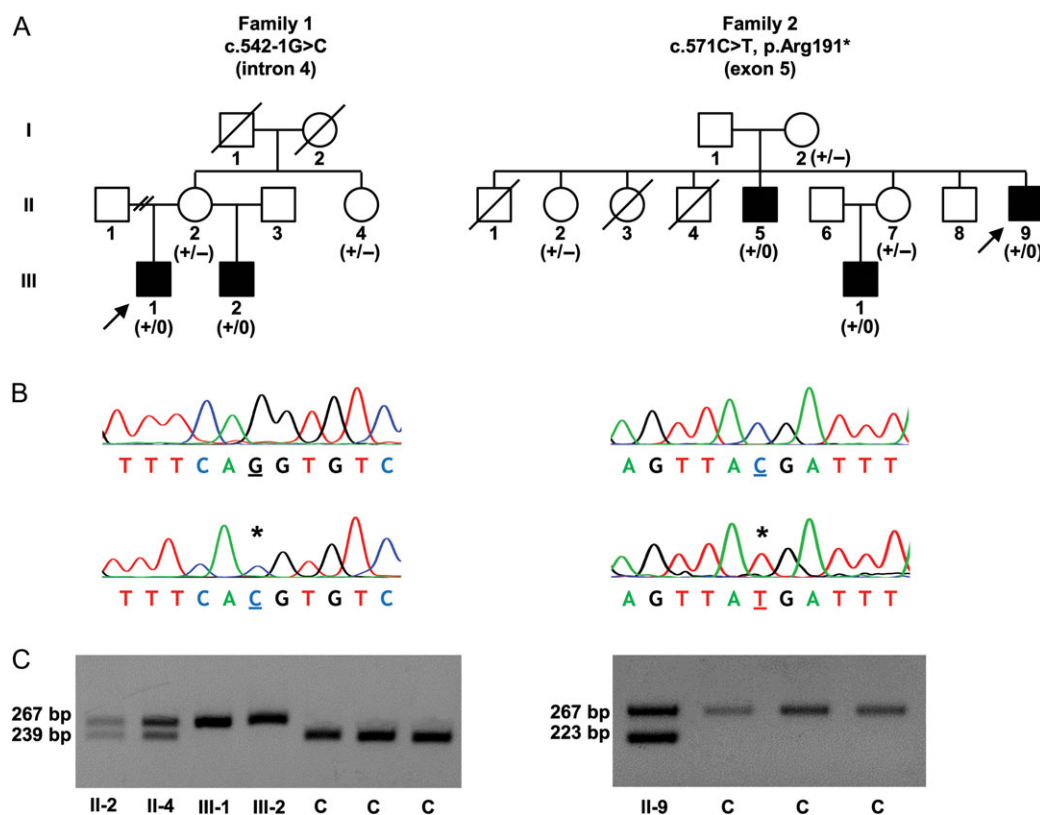


Figure 1 Identification of *ANOS1* point mutations in affected individuals. **(A)** Pedigrees of affected individuals. Squares denote males and circles denote females, filled symbols represent patients with congenital hypogonadotropic hypogonadism (CHH), open symbols represent unaffected individuals, oblique lines through symbols represent deceased individuals, double slash indicates divorce, arrows indicate the index cases. The results of genetic testing are represented under each individual, as hemizygous males with the mutation (+/0), and heterozygous females (+/-). **(B)** DNA sequence analysis of normal individuals (above) and patients (below). The positions of the mutations are indicated by asterisks. Patients are hemizygous for the mutated nucleotides whereas unaffected female carriers (not shown) are heterozygous. **(C)** Confirmation of mutations by agarose gel electrophoresis of EcoNI restriction enzyme digested fragments and allele-specific PCR, respectively. Lanes correspond to family members represented in panel (A) and normal controls (C). bp, base-pairs.

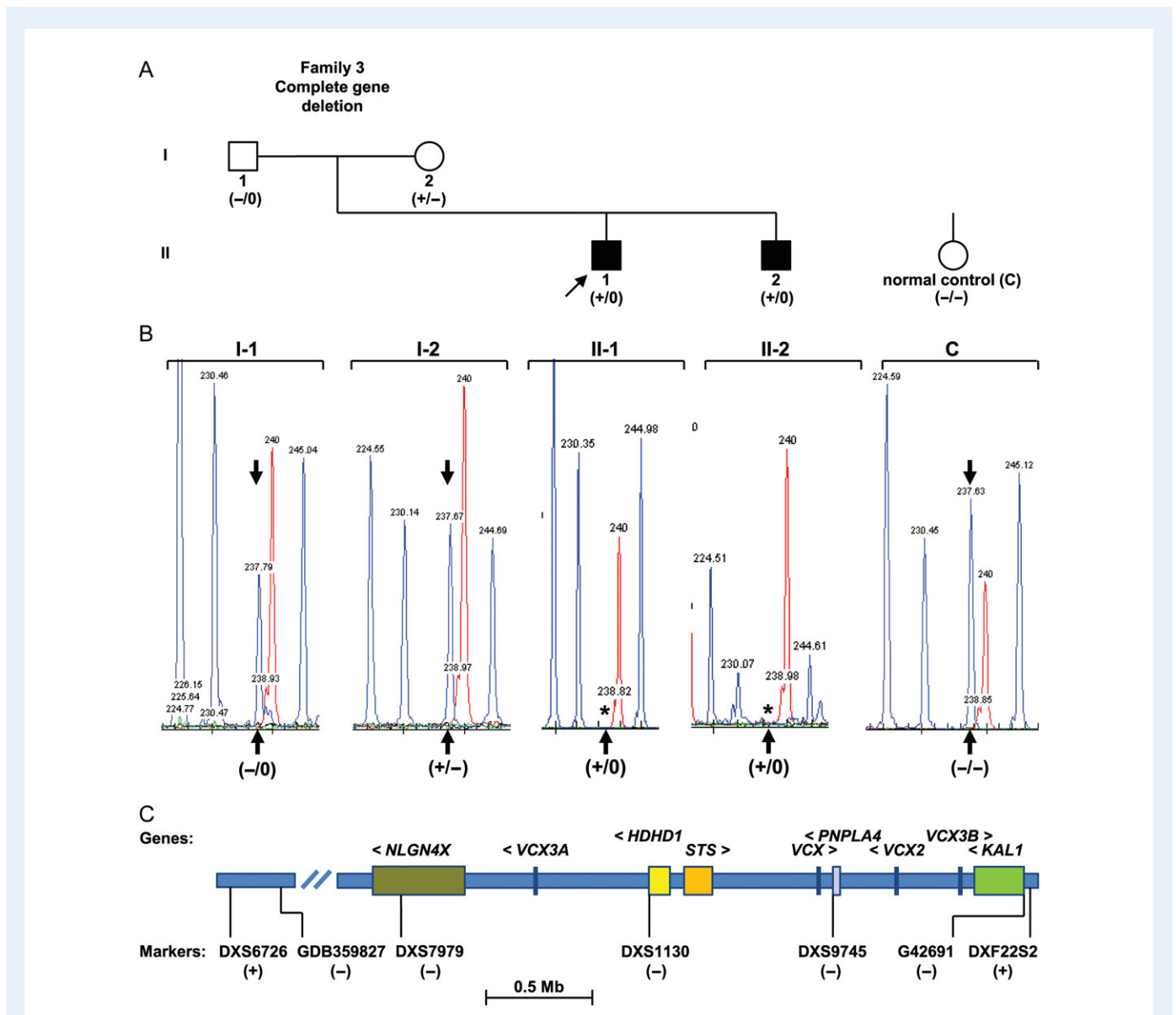


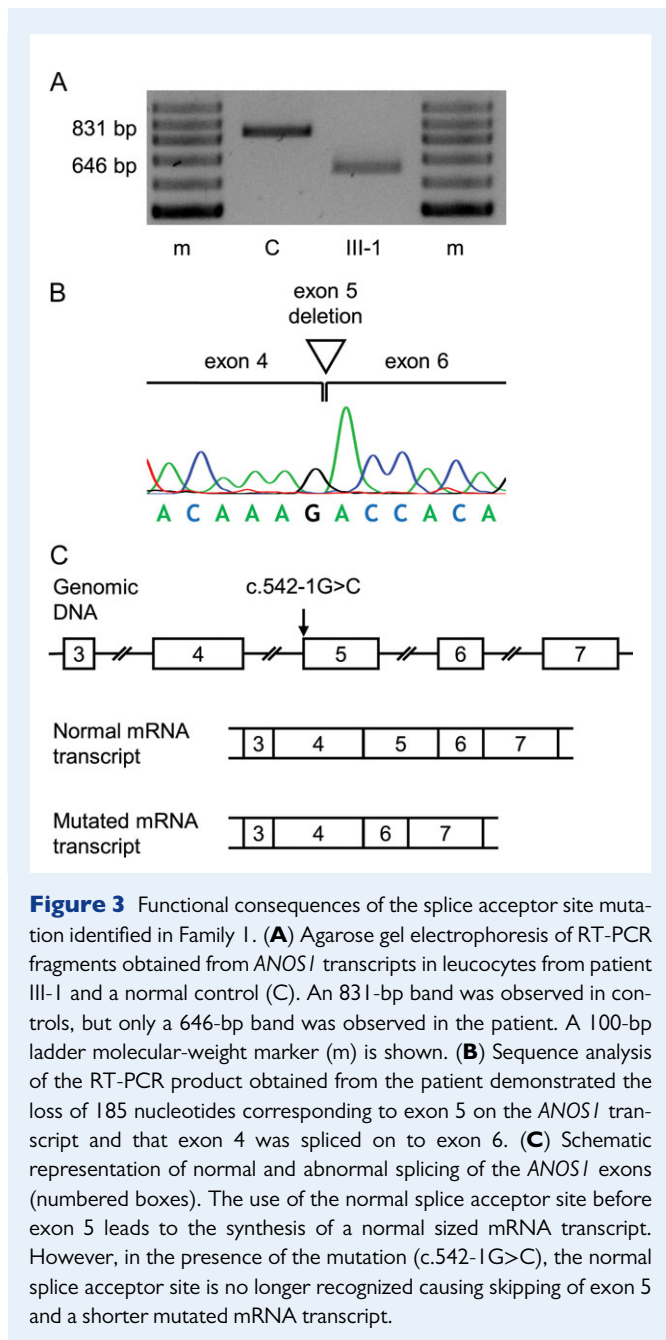
Figure 2 Identification of an *ANOS1* whole gene deletion. **(A)** Pedigree of the affected individuals. Squares denote males and circles denote females, filled symbols represent patients with CHH, open symbols represent unaffected individuals, arrow indicates the index case. The observed genotypes are represented under each individual, as hemizygous males with (+/0) and without (-/0) the mutation, and heterozygous (+/-) and normal homozygous (-/-) females. **(B)** Multiplex ligation-dependent probe amplification (MLPA) analysis of family members and a normal female control (C). Arrows indicate the location of the 237-bp probe signal corresponding to *ANOS1*, which is absent (asterisk) in the patients (II-1 and II-2) and present in the normal hemizygous father (I-1), normal heterozygous mother (I-2), and normal homozygous female control. A 240-bp DNA size marker is represented in red. **(C)** Schematic representation of the Xp22.32-p22.31 region (oriented from telomere (left) to centromere (right)) showing the genes that are deleted in the patients and the genetic markers used to assess the limits of the deletion. The presence (+) and absence (-) of the markers indicate that the deletion extends ~4.8 megabases (Mb). A 0.5-Mb scale bar is shown.

and this revealed that the deletion spanned a region of ~4.8 megabases (Mb). This deleted region affected several genes (ordered towards the telomere): *ANOS1*, *VCX3B*, *VCX2*, *PNPLA4*, *VCX*, *STS*, *HDHD1*, *VCX3A* and *NLGN4X* (Fig. 2C).

Additional family members were screened for the identified mutations and this showed segregation of the mutations with the disorder, in an X-linked recessive inheritance pattern (Figs 1A and 2A). The

clinical characteristics of patients with identified *ANOS1* mutations are summarized in Table 1.

The nonsense mutation and the complete deletion of *ANOS1* occurred in patients with anosmia (i.e. KS), whereas the splice site mutation occurred in two brothers with apparently normal olfaction. As *ANOS1* mutations occur only rarely in normosmic CHH, this prompted us to re-evaluate these patients for whom olfaction ability



had been self-reported as normal. Olfaction testing in these individuals revealed hyposmia in both. MRI of the olfactory bulbs and tracts in these patients was not performed because one of the brothers had fixed orthodontic braces and the other declined the exam.

Discussion

We identified three different *ANOS1* mutations in 42 unrelated male patients with CHH. These consisted of a novel splice acceptor site mutation (c.542-1G>C), a previously reported nonsense mutation (c.571C>T; p.Arg191*) (Oliveira et al., 2001), and a novel 4.8-Mb contiguous gene deletion in the Xp22.31-p22.32 region that included the *ANOS1* locus. These mutations are predicted to be highly deleterious as

the splice site mutation was found to result in exon skipping and the production of an abnormal mRNA, nonsense mutations result in premature stop codons that lead to the production of truncated proteins or to nonsense-mediated mRNA decay (Baker and Parker, 2004), and whole gene deletions result in complete absence of gene expression. Further evidence for the pathogenicity of the mutations came from the demonstration of their presence in additional family members with the same phenotype as the patients and their absence in normal individuals.

The prevalence of *ANOS1* mutations in the literature varies between 10 and 20% in male patients with KS (Semple and Topaloglu, 2010; Hu and Bouloux, 2011), but is exceptionally rare in patients with normal olfaction. Sato et al. (2004) reported a case of normosmic CHH with an *ANOS1* mutation, however, even this case had hypoplastic, rather than normal, olfactory bulbs on MRI. Another patient reported in the same study was allegedly normosmic until his early twenties and had hyposmia from his late twenties. More recently, Li et al. (2016) reported two children with *ANOS1* mutations, and with normal olfaction and normal MRIs.

Given that *ANOS1* mutations occur almost exclusively in patients with olfactory defects, our finding of two brothers with an *ANOS1* mutation and self-reported normal olfaction led us to re-evaluate these patients through olfaction testing and this did indeed reveal hyposmia in both. Thus, this confirms previous observations that self-reported olfactory assessment may underestimate the true olfactory deficit in some cases of CHH (Lewkowitz-Shpuntoff et al., 2012; Jagtap et al., 2013) and suggests that male patients with self-reported normal olfactory function should not be excluded from *ANOS1* genetic testing.

Our study identified a submicroscopic chromosomal deletion in a patient with KS. Although whole or partial *ANOS1* gene deletions are common in KS (Pedersen-White et al., 2008; Montenegro et al., 2013), the deletion identified in this patient extended along a 4.8-Mb region that contains several genes (i.e. *ANOS1*, *VCX3B*, *VCX2*, *PNPLA4*, *VCX*, *STS*, *HDHD1*, *VCX3A* and *NLGN4X*). Thus, this represents a contiguous gene deletion and explains why the patient has additional phenotypic features, such as ichthyosis and cognitive delay, which are not directly related to KS. In particular, mutations in *STS* (Steroid Sulphatase; MIM 300747) are responsible for X-linked ichthyosis, a genetic disorder of keratinization, characterized by generalized desquamation of the skin (Hernandez-Martin et al., 1999); and mutations in *NLGN4X* (Neurologin 4; MIM 300427) and *VCX3A* (Variably Charged, X chromosome, 3 A; MIM 300533) have been implicated in autism and mental retardation (Fukami et al., 2000; Jamain et al., 2003). Two similar deletions of 4.5 Mb (Macarov et al., 2007) and 3.7 Mb (Mochel et al., 2008), affecting the same set of genes, have been reported previously, and were associated with KS, ichthyosis and normal to mildly affected intellectual ability.

Our study also highlights the clinical heterogeneity of phenotypes that often challenge clinicians and hinder targeted genetic diagnostic testing. The variability of clinical manifestations, such as the severity of olfactory deficits, renal abnormalities or neurological signs (such as synkinesis or mirror movements) found among families and even within the same family, suggests a possible role of modifier genes, and/or epigenetic factors, in determining the variable expressivity of the disease. Although we searched for oligogenicity by sequencing other commonly affected CHH genes, other rarer loci were not analysed and it remains to be determined if a more comprehensive genetic analysis (e.g. through whole exome sequencing) would help elucidate the phenotypic variability.

Table 1 Clinical characteristics of patients with ANOS1 mutations.

Family	Member (a)	Sex	Age of diagnosis (years)	Clinical presentation	Olfaction (b)	Associated features	Basal hormone levels (normal reference range)	Brain MRI	ANOS1 mutation
1	III-1	M	15.0	Delayed puberty. Tanner stage 2. Testicular volume 2 ml	Normal (c)	Right renal agenesis	FSH 0.4 mIU/ml (0.8–8.2); LH 0.1 mIU/ml (0.7–7.2); total testosterone 0.20 ng/ml (2.20–8.00)	n/a	c.542 –1G>C
	III-2	M	15.8	Delayed puberty. Tanner stage 2. Testicular volume 2 ml	Normal (c)	Right renal agenesis. Attention deficit hyperactivity disorder	FSH 0.1 mIU/ml (0.8–8.2); LH 0.3 mIU/ml (0.7–7.2); total testosterone 0.10 ng/ml (2.20–8.00)	n/a	c.542 –1G>C
2	II-5	M	43	Delayed puberty. Tanner stage I. Bilateral cryptorchidism (2/3 ml testes in inguinal canals). Micropenis. Synkinesis (very mild)	Anosmia	Normal kidneys. Low bone mineral density (lumbar spine T/Z-score –2.1/–2.9; femoral neck T/Z-score 0.8/0.6)	FSH 0.7 mIU/ml (1.5–12.4); LH 0.1 mIU/ml (1.7–8.6); total testosterone 0.35 ng/ml (2.80–8.00)	Bilateral agenesis of the olfactory bulbs and tracts	c.571C>T (p.Arg191*)
	II-9	M	34	Delayed puberty. Tanner stage I. Bilateral cryptorchidism (3 ml testes in inguinal canals). Micropenis	Anosmia	Normal kidneys. Low bone mineral density (lumbar spine T/Z-score –2.5/–2.8; femoral neck T/Z-score –1.0/–1.1)	FSH 0.8 mIU/ml (1.5–12.4); LH 0.3 mIU/ml (1.7–8.6); total testosterone 0.29 ng/ml (2.80–8.00)	Bilateral agenesis of the olfactory bulbs and tracts	c.571C>T (p.Arg191*)
	III-1	M	21	Delayed puberty. Tanner stage I. Bilateral cryptorchidism (1 ml testes in inguinal canals). Micropenis	Anosmia	Low bone mineral density (lumbar spine T/Z-score –2.4/–2.3; femoral neck T/Z-score –1.3/–1.4). Renal imaging n/a	FSH 0.4 mIU/ml (1.5–12.4); LH < 0.4 mIU/ml (1.7–8.6); total testosterone 0.15 (2.80–8.00)	n/a	c.571C>T (p.Arg191*)
3	II-1	M	17.1	Delayed puberty. Tanner stage I. Testicular volume 2 ml	Anosmia	Right renal agenesis. Ichthyosis. Cognitive delay. Short stature (SDS –2.81). Delayed bone age (13.0 yr). Morbid obesity (BMI 43.6 kg/m ²)	FSH 0.4 mIU/ml (0.8–8.2); LH 0.1 mIU/ml (0.7–7.2); total testosterone 0.25 ng/ml (2.60–10.00)	Bilateral hypoplastic olfactory bulbs tracts and sulci	contiguous gene deletion
	II-2	M	10.8	Bilateral cryptorchidism	n/a	Normal kidneys. Ichthyosis. Cognitive delay. Normal stature (SDS 0.53). Obesity (BMI 28.8 Kg/m ² , SDS 3.04)	n/a	Bilateral hypoplastic olfactory bulbs and tracts, and hypoplasia of right sulcus	contiguous gene deletion

(a) As indicated in Figs 1 and 2; (b) self-reported; (c) Reclassified as hyposmia after undergoing an in-house psychophysical olfactory test, by asking the patient to identify the odour of ground coffee beans, cocoa, pepper, cinnamon and vinegar in an opaque container placed beneath each nostril (patients were able to detect the odours, but were unable to discriminate any of the substances, except vinegar); M, male; n/a, not available; SDS, standard deviation score. Hormone levels were measured by chemiluminescence immunoassays. Bone mineral density was measured by dual-energy X-ray absorptiometry. Kidneys were assessed by renal ultrasonography. All patients (except II-2 from Family 3, who was pre-pubertal) were treated with regular intramuscular injections of testosterone enanthate resulting in improvement of secondary sexual characteristics, muscle strength and subjective well-being. None of the patients has so far undergone fertility induction.

In conclusion, this study identified novel ANOS1 mutations in patients with CHH, thus expanding the known spectrum of ANOS1 mutations, and suggests that patients with self-reported normal olfactory function should not be excluded from ANOS1 genetic testing.

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Authors' roles

C.G. and M.C.L. conceived and designed the study. C.G. performed the genetic studies of the patients. F.F., T.B. and F.C. collected samples and acquired clinical data of the patients with mutations. C.G. and M.C.L. drafted the article and all authors revised it critically for important intellectual content and approved the final manuscript.

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Conflict of interest

The authors have no conflicts of interest to declare.

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