Functional analysis of novel desert hedgehog gene variants improves the clinical interpretation of genomic data and provides a more accurate diagnosis for patients with 46,XY differences of sex development

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ABSTRACT
Background Desert hedgehog (DHH) gene variants are known to cause 46,XY differences/disorders of sex development (DSD). We have identified six patients with 46,XY DSD with seven novel DHH gene variants. Many of these variants were classified as variants of uncertain significance due to their heterozygosity or associated milder phenotype. To assess variant pathogenicity and to refine the spectrum of DSDs associated with this gene, we have carried out the first reported functional testing of DHH gene variant activity.

Methods A cell culture method was used to assess DHH variant induction of Hedgehog signalling in cultured Leydig cells. Protein expression and subcellular localisation were also assessed for DHH variants using western blot and immunofluorescence.

Results Our co-culture method provided a robust read-out of DHH gene variant activity, which correlated closely with patient phenotype severity. While biallelic DHH variants from patients with gonadal dysgenesis showed significant loss of activity, variants found as heterozygous in patients with milder phenotypes had no loss of activity when tested with a wild type allele. Taking these functional results into account improved clinical interpretation.

Conclusion Our findings suggest heterozygous DHH gene variants are unlikely to cause DSD, reaffirming that DHH is an autosomal recessive cause of 46,XY gonadal dysgenesis. Functional characterisation of novel DHH variants improves variant interpretation, leading to greater confidence in patient reporting and clinical management.

INTRODUÇÃO

The conserved hedgehog family of signalling molecules plays a central role in the development and differentiation of numerous organs during embryogenesis (Franco:2012iz). Desert hedgehog (DHH), a member of this family, is one of three hedgehog homologues present in mammals, and plays a key role in the development of the gonads. In the XY male, differentiation of the bipotential gonads into the testis involves specification of the supporting cells, the Sertoli cells. A subset of these cells produce and secrete DHH from early in development (embryonic day 11.5 in the mouse). This secreted DHH binds the PTCH1 receptor in the adjacent Leydig cells, activating the hedgehog signalling pathway and triggering differentiation of these steroidogenic cells. In the mouse, Dhh knockout female mice are viable and fertile but the majority of XY mice are externally female with a blind vagina. In these mice, the testes lacked spermatogonia and had a severe reduction in Leydig cell numbers, and hence reduced testosterone which resulted in typical female appearance. Work in mice has shown that Dhh and its signalling pathway are important for the upregulation of Nr5a1 (encoding steroidogenic factor 1), one of the key genes involved in gonad development and steroidogenesis.

Differences/disorders of sex development (DSD) in humans are defined as congenital conditions where the development of chromosomal, gonadal and anatomical sex is atypical (Chicago Consensus Meeting). DSDs are a heterogeneous group, including 46,XY disorders of testicular development. These patients have a 46,XY karyotype and external genitalia that range from mild undervirilisation phenotypes such as microphallus or hypospadias to normal female appearance (complete sex reversal). These conditions are most often caused by a breakdown in the genetic pathways required for testicular or ovarian differentiation and development. It is well established that pathogenic variants in DHH (OMIM 605423, chr12q13.12) are a rare cause of 46,XY DSD, in particular causing 46,XY partial or complete gonadal dysgenesis (OMIM 607080, OMIM 233420). To date, 12 different DHH pathogenic variants have been published in 17 cases of 46,XY DSD (figure 1). In some cases the patients present with gonadal cancers and/or minifascicular neuropathies. In 15 of the 17 reported cases the patients had DHH variants on both alleles (homozygous or compound heterozygous). Two patients reported to carry a heterozygous variant (p.Leu363Cysfs*4) also had
Previously reported and novel variants in desert hedgehog (DHH). A schematic figure showing the protein position of both previously reported variants in DHH (upperside), and the new ones reported in this study (underside). Publication references are in bold superscript after each variant. Yellow circles show missense variants, blue are in-frame deletions and red are deletions causing frameshifts. ** depicts those that were homozygous and * those that were heterozygous, and ? where the zygosity is unknown. Compound heterozygous variants are depicted by a dotted line joining the two variants.

sex chromosome mosaicism (45,X/46,XY). Based on these reports it could be concluded that when the sex chromosomes are normal, DHH variants act in a recessive manner to cause 46,XY complete or partial gonadal dysgenesis. Thus, it is still unclear whether carrying a variant in just one copy of DHH causes milder forms of DSD such as a 46,XY undervirilisation phenotype or hypospadias. In NCBI ClinVar there are 28 DHH variants reported associated with 46,XY DSD, 17 of which are listed as likely benign or of uncertain significance. Of those that are regarded as pathogenic and likely pathogenic (LP) just one is unpublished—c.T1027C.p.Cys343Arg (figure 1).

Previously, using massively parallel sequencing (MPS) on DNA from 278 patients with 46,XY DSD we reported novel homozygous, compound heterozygous and heterozygous variants in DHH. Six patients carrying novel DHH variants presented with a wide spectrum of phenotypical severity. Here we detail patient phenotypes, and use the first published functional analysis to assess DHH variant activity. Our work highlights the importance of functional analysis for accurate variant curation and reporting in DSD, and refines the spectrum of disorders in which DHH likely plays a role.

MATERIALS AND METHODS

Study subjects and clinical evaluation

All patients were recruited by collaborating clinicians after written informed consent (as detailed in17).

Targeted gene capture, MPS and data analysis

Genomic analysis was carried out as previously described. Annotations are based on DHH NM_021044.2. Annotations were initially created by our custom pipeline and were also processed through Mutalyser. Sequencing covered the DHH exons without gaps and often extended into the intron or UTRs by up to 100 bp (online supplementary figure S1).

Variant curation

Novel variants were curated using a detailed clinically accredited scheme used at the Victorian Clinical Genetics Services. This is based on the American College of Medical Genetics (ACMG) guidelines, and allows classification of variants into one of five categories; pathogenic (P), likely pathogenic (LP), variant of uncertain significance (VUS), likely benign (LB) or benign (B). This classification assigns scores based on type of variant, minor...
Diagnostics

allele frequencies (MAFs), Grantham scores, protein domains, existing classifications of variants (ie, ClinVar), segregation or inheritance, phenotype match and functional evidence. For functional evidence, two categories are considered. The first, which is scored the strongest (either as pathogenic or benign strong) is a functional test of activity where the endogenous tissue/patient cells are used. The second category (pathogenic moderate or benign supporting) is reserved for functional evidence from an assay using an exogenous cell line. VUS are categorised as one of three—VUSa, VUSb, VUSc, based on whether conflicting evidence is sufficient to justify VUS classification. VUSa (potentially pathogenic) is regarded as a variant that is unambiguously classifiable with predominantly pathogenic evidence, VUSb is not unambiguously classifiable and VUSc is not unambiguously classifiable with predominantly benign evidence.

Mutant DHH expression vectors
A human DHH expression construct was from OriGene Technologies (True ORF clone RC206715). The mutant DHH expression vectors (p.Arg124Gln, p.Leu363Cysfs*4, p.Glu90del, p.Leu162Pro, p.Tryp173Cys, p.Ala227Val, p.Pro261Leu, p.Gly170Ser, p.Arg249His, p.Arg195Tryp, p.Arg27Gln, p.Arg164Pro) were created by site-directed mutagenesis (QuickChange II XL Site-directed Mutagenesis Kit, Agilent Technologies, Santa Clara, California, USA) according to the manufacturer's instructions (for primer sequences see online supplementary table 1). Sanger sequencing using vector primers was used to confirm the presence of the variant in the vectors.

DHH variant immunofluorescence
HEK293 cells were seeded on an eight-well chamber slide (Nunc), and transfected with DHH expression vectors (wild type [WT] and all mutants) or a PCMV (plasmid containing the cytomegalovirus constitutive promoter)-empty control using Lipofectamine 2000 (Invitrogen). Cells were processed as previously published 24 hours after transfection. Cells were incubated overnight with a goat polyclonal DHH (N-19) antibody (1:300, Santa Cruz sc-1193) and/or a polyclonal rabbit anti-FLAG antibody (1:10000, Sigma). Secondary antibodies used were donkey antigoat Alexa488 (1:1000, Invitrogen) and donkey antibovine Alexa594 (1:1500, Invitrogen). Nuclear counterstaining was performed with DAPI (4′,6-diamidino-2-phenylindole). Images were recorded using a Zeiss lazer scanning microscope (LSM) 780 confocal microscope. Images were taken at 20× and 40× magnification.

Western blot
HEK293t cells were seeded at approximately 80% confluency on a 24-well plate (3×10⁴ cells), and transfected with 0.5 µg of pCMV-DHH expression vector (WT or mutant) using Lipofectamine 2000 (Invitrogen). After 24 hours cells were trypsinised using 0.025% trypsin PBS (phosphate buffered saline) and reseeded at 20% confluency (approx. 1.6×10⁵ cells). T33 cells (mouse Leydig-like cells; 91060526 Cell Bank Australia) were also added at a 1:1 ratio (1.6×10⁵) with the HEK293t cells. The co-culture was left for 48 hours, after which the cells were washed once with PBS and Trizol reagent (Invitrogen) was used for RNA extraction. DNAseq was carried out using the Promega Dnase free kit. RNA was converted to cDNA with the GoTaq reverse transcriptase kit and a mix of oligoDT and random primers (Promega). qPCR (GoTaq qPCR master mix, Promega) was carried out with the mouse Gli1, Ptc1 genes as pathway read-outs, and the Tbp gene as a housekeeper (see supplementary table 1 for primers). Human DHH primers were used to assess the level of expression with the hRPL32 housekeeper primers. The experiment was replicated three times. ΔΔCT (threshold cycle) values were calculated for each target and expressed as a ratio of the control (empty vector). Error bars are based on SEM and p values were calculated using one-way analysis of variance (ANOVA). Activity of each variant is calculated as a percentage of the WT, normalised to the empty, control.

RESULTS
DHH variants in a wide range of patients with DSD
We have previously published the use of a targeted MPS DSD gene panel to find causative variants in patients with DSD. Here we have further characterised seven novel DHH gene variants identified in six patients (table 1). All novel DHH variants were rare alleles, with only three of the seven variants found in the online databases ExAC, gnomAD or Exome Variant Server (EVS), and none were found as homozygous on these data bases. In gnomAD p.Ala227Val had a total MAF of 0.0000581, p.Arg249His MAF was 0.00003231 and p.Arg27Gln MAF was 0.0001402 (table 1). The patients had a wide range of phenotypes. Patient 1, previously the subject of a clinical report, has a similar clinical presentation to those previously associated with DHH variants. This patient has a homozygous p.Arg164Pro variant and is 46,XY with female genitalia and bilateral dysgenetic testes. Variant curation using a clinically accredited scheme based on the ACMG guidelines (materials and methods) classified this DHH variant as LP (table 1). We also present two patients with compound heterozygous DHH variants (patients 2 and 3; table 1). Patient 2, a 46,XY female patient with clitoral hypertrophy, a blind ending vagina and no Müllerian duct derivatives, had two palpable gonads present in the labia majora with immature testicular tissue (table 1). This patient had the variants p.Ala227Val and p.Pro261Leu, and visualisation of sequencing reads showed these were present on different alleles (online supplementary figure S2). Each DHH variant is curated separately and both were considered VUS3a (table 1). In the second compound heterozygous case (patient 3), a 46,XY female patient with ambiguous genitalia, blind ending vagina and inguinal testis with an unknown histology, the vari ants p.Arg249His and p.Gly170Ser were identified. We were unable to obtain parental samples, but compound heterozygosity
<table>
<thead>
<tr>
<th>Patient ID</th>
<th>Karyotype</th>
<th>Sex of rearing</th>
<th>External genitalia</th>
<th>Genital location and histology (WML)</th>
<th>Millieuven structures</th>
<th>Neuroupathy</th>
<th>DHH variants</th>
<th>ExAC</th>
<th>gnomAD</th>
<th>EVS</th>
<th>Graham score</th>
<th>SIFT</th>
<th>Polyphen2</th>
<th>Mutation taster</th>
<th>CADD</th>
<th>Clinvar</th>
<th>Curation before functional</th>
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<tr>
<td>1</td>
<td>46,XY</td>
<td>Female</td>
<td>Well-formed labia. Prominent clitoral structure. Normal vaginal opening with blind vagina.</td>
<td>L: intrabdominal R: intrabdominal. Histopathology: Bilateral dysgenetic testes. No evidence of gonadoblastoma or germ cell neoplasia</td>
<td>Left paraurethral biopsy showed Mullerian tissue resembling endocast. Right paraurethral biopsy showed vasopapillary tissue</td>
<td>No sign at 14yo.</td>
<td>NC_000012.11: g.49484188C&gt;G, NM_021044.2:c.491G&gt;C:p.Arg164Pro</td>
<td>Homozygous</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>103</td>
<td>Damaging</td>
<td>Probably damaging</td>
<td>Disease causing</td>
<td>32</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>46,XY</td>
<td>Female</td>
<td>Clitoral hypertrophy (7.5 cm). Otherwise female external appearance. Short-blind ending vagina (2.5 cm).</td>
<td>L: Painless in labia majora. R: Painless in labia majora. Histopathology: Immature testicular tissue with reduced spermatogenesis and tubal without lumen.</td>
<td>Compound heterozygote</td>
<td>No sign at 20yo.</td>
<td>NC_000012.11: g.49484153G&gt;A, NM_021044.2:c.680C&gt;T:p.Ala227Val, NM_021044.2:c.782C&gt;T:p.Pro261Leu</td>
<td>Compound heterozygote</td>
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<td>0.0000646</td>
<td>1</td>
<td>64</td>
<td>98</td>
<td>Tolerated</td>
<td>Tolerated</td>
<td>Disease causing</td>
<td>Disease causing</td>
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<tr>
<td>3</td>
<td>46,XY</td>
<td>Male</td>
<td>Proximal hypospadias and disorder</td>
<td>L: scrotal R: scrotal. Histology unknown</td>
<td>Not present</td>
<td>Not present</td>
<td>NC_000012.11: g.49484027C&gt;T, NM_021044.2:c.508G&gt;A:p.Gly170Ser</td>
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<td>0.0000323</td>
<td>1</td>
<td>29</td>
<td>56</td>
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<td>Tolerated</td>
<td>Disease causing</td>
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<td>Severe hypospadias</td>
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<td>Not reported</td>
<td>NC_000012.11: g.49484027C&gt;T, NM_021044.2:c.508G&gt;A:p.Gly170Ser</td>
<td>Compound heterozygote</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>29</td>
<td>56</td>
<td>Tolerated</td>
<td>Probably damaging</td>
<td>Disease causing</td>
<td>32</td>
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<td>0</td>
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<td>Probably damaging</td>
<td>Disease causing</td>
<td>32</td>
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<td>Severe hypospadias</td>
<td>L: scrotal R: scrotal. Histology unknown</td>
<td>Not present</td>
<td>Not reported</td>
<td>NC_000012.11: g.49484221C&gt;T, NM_021044.2:c.508G&gt;A:p.Arg170Gln</td>
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<td>0.0041482</td>
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<td>Probably damaging</td>
<td>Disease causing</td>
<td>29.9</td>
<td>VUS (invitae)</td>
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Patient phenotypes are described along with the DHH variants found. Minor allele frequencies of each variant are shown from the Exome Aggregation Consortium (ExAC), the Genome Aggregation Database (gnomAD) and Excess Variant Server (EVS) databases. Grantham Score and CADD (Combined Annotation-Dependent Depletion) score are shown for likely pathogenic variants. Note three programmes (SIFT, Polyphen2 and Mutation Taster) are shown. Variant curation following ACMG guidelines was performed using functional analysis. ACMG, American College of Medical Genetics; EHH, ethicopathology; EHS, exome/whole genome sequencing; VUS, variant of uncertain significance.
was confirmed by cloning the genomic fragment containing these two variants (online supplementary figure S3). Based on the existing data, both variants were classified as VUS3a, due to conflicting in silico predictions and low Grantham Scores as well as a lack of functional evidence to support pathogenicity (table 1). We also found three patients with heterozygous variants (table 1). Patient 4 was a 46,XY male patient with severe hypospadias, testes in the scrotum (p.Gly170Ser). Patient 5 (p.Arg193Trp) was a 46,XY male patient with one testis in the scrotal region and one inguinal gonad of unknown histology (table 1). Finally, a p.Arg27Gln change was found in patient 6, a 46,XY male patient with severe hypospadias (table 1). None of the patients had a reported peripheral neuropathy. The heterozygous variants found in patients 4, 5 and 6 were also classified as VUS3a (possibly pathogenic), in part due to the lack of evidence that DHH variants can cause DSD in an autosomal dominant manner (table 1).

### Novel DSD-associated DHH gene variants are found throughout the protein

In humans, the DHH gene is autosomal (12q12.13) and comprises three exons encoding a 396 amino acid protein. DHH, like all hedgehog proteins is produced as a 45 kDa precursor and the 25 kDa C-terminal drives autolytic cleavage assisted by the covalent addition of cholesterol moiety to the N terminus. This releases the 19 kDa N-terminal fragment, which acts as the active molecule. All of these features are thought to ensure proper secretion and efficient extracellular movement of HH proteins, allowing them to act as secreted morphogens (reviewed in 24). All previously described DHH variants in patients with normal sex chromosome complement are homozygous or compound heterozygous (figure 1). While our biallelic variants fall in both the N-terminal and C-terminal regions, our heterozygous variants are all found within the N-terminal (figure 1). The DHH gene is highly conserved and shows constraint against both missense variants and loss of function (LOF) variants in ExAC (z=3.96 and probability of loss of function intolerance/PLI=0.26). All of our variants affect highly conserved amino acids, in some cases conserved back to the single hedgehog protein in fruit flies (online supplementary figure S4).

### Novel DHH variants do not alter DHH cellular localisation

To assess cellular localisation, the WT and mutant DHH alleles were transfected into HEK239t cells with an expression vector carrying a C-terminalFLAG tag. This allowed detection of the DHH protein with both an anti-DHH antibody (raised against an N-terminal peptide, detecting both cleaved N-terminal and full-length DHH) and an anti-FLAG antibody which will detect full-length DHH and C-terminal DHH. In WT DHH-FLAG overexpression we found that DHH was mostly expressed in the cytoplasm and at the cell membrane (figure 2A and online supplementary figure S3), with no nuclear staining. In general, good overlap was seen between the Flag and DHH antibodies (online supplementary figure S5), the former of which was more sensitive and stained some cells more strongly (online supplementary figure S5). This may be an indication that the majority of the DHH detected in the cells is uncleaved, consistent with previous reports.26 The only DHH variant that had significantly reduced DHH staining intensity was the previously reported p.Leu363CysFs*4 variant, with no nuclear staining. In general, this assay shows no effect on protein function, a BS3 (benign strong) score is assigned. We therefore sort a reliable in vitro test to DHH variant activity. Previous studies have shown that DHH is not effectively cleaved or secreted from cells, preferentially activating the hedgehog signalling pathway in adjacent cells via paracrine activity.25 To test the paracrine activity of each DHH mutant compared with WT, we used a previously reported co-culture assay25 (figure 4A,B). In this system, HEK293t cells are transfected with the DHH expression plasmids for 24 hours, after which these DHH-expressing cells are cultured with mouse Leydig-like cells (TM3), which are responsive to the DHH signal (figure 4A,B). After 48 hours of co-culture, RNA is extracted and qPCR is used to assess the levels of expression of hedgehog pathway target genes Ptc1 and Gli1 in the TM3
cells, using mouse-specific primers. In this assay, we found that overexpressed WT human DHH induced mGli1 expression 18-fold compared with an empty plasmid control. We found that mPtch1 levels were less sensitive where WT DHH induced mPtch1 expression twofold to threefold compared with the control. To validate the method, we tested four of the previously reported pathogenic variants and found that all of these had a significant reduction in activity compared with WT, observed for both mGli1 (figure 4C) and mPtch1 (figure 4D) expression levels. Of the four known DHH variants tested, three had almost complete loss of activity, (<10% of WT activity; p.Glu90del, p.Leu363Cysfs*4, p.Leu162Pro) whereas p.Arg124Glu retained around 30% of WT function (figure 4C,D). The three alleles with a complete LOF were found in 46,XY female patients with complete gonadal dysgenesis, whereas the p.Arg124Glu variant was reported in two 46,XY female sisters with the slightly milder phenotype of partial gonadal dysgenesis. Thus, DHH variant activity measured in this assay correlates with phenotype severity.

We next tested our seven novel variants. Using the mGli1 read-out all variants but one (p.Arg27Gln) had reduced activity when tested individually (figure 4E). Those found in the most severely affected patients (46,XY female patients; patient 1 and patient 2) had the most reduced activity, with less than 2% of WT activity (figure 4E, table 2). Even when combined, the variants found in patient 2 had an activity of around 2% of the WT allele (figure 4E and table 2). The other patient with biallelic DHH variants (patient 3) who was less severely affected (46,XY male patient with hypospadias) had variants p.Gly170Ser and p.Arg249His, which showed a 26% and 47% activity, respectively, in this assay (figure 4E and table 2). Combined, these variants had an activity of 46% of the WT allele (table 2). Finally, two of the three heterozygous variants found in patients had a reduced activity (p.Gly170Ser, 47% and p.Arg195Trp, 16%, table 2). However, when these variants were tested in combination with the WT allele, to model the in vivo environment, they appeared to lose this inactivity, presenting activities close to WT (90% and 98%) (figure 4E and table 2). Finally, the heterozygous variant p.Arg27Gln found in patient 6 had no significant loss of activity compared with WT, either alone or in combination with WT DHH (figure 4E and table 2). mPtch1 expression, although a less sensitive read-out, showed similar results (figure 4F and table 2), with variants present as homozygous or compound heterozygous tending towards LOF, whereas those present as heterozygotes show activity closer to WT DHH (figure 4F and table 2). Importantly, qPCR analysis found that DHH expression from all constructs was similar (online supplementary figure S7). Together, these data show that the co-culture assay is a sensitive read-out of DHH activity and variant pathogenicity.

Functional studies alter DHH variant curation and reporting

Having established that we can test DHH variant activity in vitro, the results of which are highly consistent with patient phenotypes and additional variant data, we now sought to assess whether functional outcomes would change variant curation. We performed a second curation taking into account the overall outcomes of the functional studies. For patient 1, inclusion of the functional data, showing LOF, did not give enough additional evidence to change the variant curation, which remains at LP (table 2). Patient 2 had two variants in trans, which were each considered VUS3a initially. When the LOF observed in the co-culture assay was taken into account, this additional evidence was sufficient to elevate these variants to LP. Patient 3 also had biallelic DHH variants, p.Arg249His and p.Gly170Ser, which were initially classified as VUS3a. Following our findings that each of these had a loss of activity, separately and combined, the p.Arg249His variant was elevated to LP, while the p.Gly170Ser variant remained at VUS3a (table 2), largely due to its conflicting in silico predictions (table 1). Interestingly, this variant was also found as a heterozygous variant in patient 4, where it was initially classified as VUS3a. Our functional findings suggested that when combined with the WT allele, as it would be in vivo, no significant loss of activity was observed—providing evidence for this variant being benign. Therefore this variant in this patient was downgraded to VUS3b (not unambiguously classifiable) (table 2). Patient 5 had a heterozygous p.Arg195Trp allele initially classified as VUS3a. Our data suggest that when alone, this allele shows a severe LOF (between 2% and 16% of WT activity), whereas when combined with the WT allele, no significant loss of activity is observed. This, combined with other data including the heterozygous state not previously shown to cause DSD, lead to a curation of VUS3b (not unambiguously classifiable). Finally, patient 6, also carrying a heterozygous DHH allele, p.Arg27Gln classified as VUS3a, was downgraded to VUS3c, due to the overwhelming evidence...
that alone or with combination with the WT allele, this variant exhibits no LOF (table 2).

**DISCUSSION**

DSDs represent a major paediatric concern as they are estimated to affect 1.7% of live births.26 We have previously shown that MPS can deliver a diagnosis rate of up to 43% in 46,XY DSD.17 In the 52 patients with a disorder of testicular development, biallelic DHH variants were observed in four patients (concluding to 7.7%) and three patients carried a single DHH variant (heterozygous).17

The ACMG variant curation guidelines20 use functional evidence from ‘well-established functional assays’ as strong evidence for or against variant pathogenicity (PS3 or BS3). The authors state that ‘one must consider how closely the functional assay reflects the biological environment’ and they give more weight to assays that use biopsied tissues. Unfortunately, access to biopsied gonadal tissue from 46,XY female patients is not often possible, and it is not clear that this dysgenic gonadal tissue would allow direct testing of DHH activity, which is important earlier in embryonic and fetal development. Thus a robust test of DHH variant activity using in vitro or cell culture methods is required. A recent review discussing the nuances of the ACMG guidelines suggests that any model for variant activity should be closely related to the disease aetiology and that a ‘well-validated functional assay should provide variant level evidence of the effect on the gene or gene product’.27 Here, we have used a co-culture cell method to functionally test DHH variants, specifically assessing their ability to activate the HH signalling pathway in Leydig cells, recapitulating the main function of DHH in the gonad. This is the first report of a functional test to assess the activity of DHH variants in vitro. Strande et al state that functional assays ‘require benchmarking against multiple variants with definitive clinical interpretations as determined by genetics or other evidence’.27 Testing of four previously reported pathogenic DHH variants in our co-culture method revealed that the functional activity of each variant in our assay correlated closely with gonadal phenotypical severity. Three of these variants; p.Leu363cysFS*, p.Arg124Glu and p.Leu162Pro, are reported in ClinVar as pathogenic, consistent with our functional studies which showed loss of activity, consistent with phenotype severity. Furthermore, one of patients had a homozygous missense variant—patient 1, who was a 46,XY female patient with complete gonadal dysgenesis. Consistent with this clinical phenotype, the variant found in this patient (p.Arg164Pro) demonstrated a complete lack of function in our assay. Our data suggest that our functional assay is a robust and reliable test for DHH variant gonadal activity. There are currently 11 non-synonymous DHH variants classified as VUS for 46,XY DSD in ClinVar, and we believe that our functional assay could be used to validate these variants. While it is well established that biallelic DHH variants (in a homozygous state) cause DSD, this is just the second report of patients carrying two different DHH alleles (compound heterozygous), giving us the rare opportunity to functionally characterise each variant separately and in combination. Patient 2 was a 46,XY female patient carrying the variants p.Ala227Val and p.Pro269Leu which had an almost complete LOF, leading to a change in curation of the variants from VUS3a to LP. Patient 3 was also biallelic, carrying the p.Arg249His and p.Gly170Ser variants. This patient had the milder phenotype of 46,XY male with proximal hypospadias and chordee. Interestingly, both of these alleles had a milder reduction in function—between 13% and 50%. When tested in combination they had an activity around 45% of the WT DHH. These data confirm that DHH variants can cause DSD in a compound heterozygous inheritance pattern.

While it is well established that DHH variants lead to autosomal recessive DSD, it was unclear whether heterozygous DHH variants contribute to milder undervirilisation phenotypes such as proximal hypospadias or micropenis. Here we report three patients with normal sex chromosome complements and heterozygous DHH variants. These patients were all 46,XY male patients with severe hypospadias, and two patients had tests in the scrotum while the third had one undescended testis. All variants were initially curated as VUS. Our functional testing
revealed that p.Gly170Ser, present in patient 4, had a partial LOF when tested alone meaning that it may be functionally pathogenic, but when in combination with WT DHH (as it would be in the patient), it had no significant LOF. This leads us to conclude that this variant will only cause DSD when in combination with a second allele, as is seen in patient 3, and is therefore unlikely to be the definitive or sole cause of DSD in patient 4. Thus clinically, this variant remains a VUS3a. Similarly, the p.Arg195Trp allele (patient 5) had an almost complete LOF functionally, but when combined with the WT allele, no LOF was observed—again meaning that clinically it is unlikely to be considered pathogenic. Finally, the p.Arg27Gln allele found in patient 6, had no significant LOF either alone or with WT DHH, suggesting that unlike the others, this is a benign Single Nucleotide Variant (SNV) both functionally and clinically. This led us to reduce the curation from VUS3a to VUS3c, meaning this variant is unambiguously classifiable with predominantly benign evidence. Thus, taken together our data lead us to conclude that heterozygous DHH variants are unlikely to cause DSD alone. It must be pointed out that our genetic sequencing only covers the coding regions of the DHH gene, extending into the introns by about 100bp. Therefore, we cannot discount the small possibility that we have missed a second variant in these patients that lies deep within the introns or the five or three prime regions. In addition, it is possible that a second hit in another gene within the DHH signalling pathway or testicular pathway could be contributing.

The molecular mechanism underlying the loss of activity in our variants is not known. Neither qPCR nor western blot suggested a significant loss of mRNA or protein for any of our novel DHH variants. No change in localisation was observed in immunofluorescence (IF), although p.Arg164Pro did show reduced number of cells with staining, HOPE analysis\(^ {28} \) suggests that this change (from a positive to a smaller neutral amino acid) will have severe effects on the structure of the protein as it lies within an α-helix, and forms a hydrogen bond with Val41 and E168. However, there did not appear to be reduced protein stability in western blot analysis. Thus, while our co-culture analysis provides an excellent read-out for DHH activity, which appears to correlate with gonadal phenotypical severity, further studies are required to pinpoint the exact disease mechanisms of each variant.

We had variants that fell in both the N-terminal and C-terminal domains of DHH. While it is well established that the N-terminal of DHH is the active molecule, the exact function of the C-terminus is unclear. A recent study from the Werner Lab has found that, given the right conditions (addition of Dithiothreitol or β-mercaptoethanol), a cell-free in vitro transcribed and translated human fusion DHH protein does undergo cleavage.\(^ {16} \)

In addition, they have shown that two variants found in patients within the C-terminus lead to reduced or absent cleavage efficiency. Nevertheless, there is currently little evidence for DHH cleavage in testis cells, and studies have found that DHH C-terminal is less efficient in cleaving the Sonic Hedgehog (SHH) N-terminal, and that unlike SHH, DHH proteins function in cell–cellcontact-mediated juxtacrine signalling.\(^ {23} \) Indeed, like us, Pettigrew \textit{et al} found that DHH expressed in HEK 293t cells was mostly uncleaved and yet it was still able to function in juxtacrine signalling. Tajouri \textit{et al} suggest that DHH autoprocessing is necessary to mediate signalling from Sertoli cells to promote differentiation of the Leydig cells.\(^ {16} \) While this may be the case in vivo, our cell culture experiments show that while DHH expressed in this system is mostly uncleaved, variants that lie within the C-terminal domain can still have a significant effect on signalling activation in TM3 mouse Leydig cells. Interestingly, one of our C-terminal variants, p.Ala227Val, affects a residue important for autoprocessing in SHH.\(^ {25} \) Thus, it appears our assay is equally able to assess the pathogenicity of both N-terminal and C-terminal variants. As it is possible that a small amount of cleavage is occurring (undetectable in western blot), it would be interesting to test the cleavage efficiency of these C-terminal variants in the future.

Receiving a genetic diagnosis in DSD is important for many reasons—it can reduce stigma and blame, and increase acceptance. An early genetic finding can also reduce the ‘diagnostic odyssey’, including uninformative clinical tests patients may be subject to. As DHH gene variants are most often autosomal recessive, a genetic finding also informs family planning. Of the six patients reported here, four carried two affected DHH alleles raising the possibility of familial inheritance and importance of cascade/carryer testing. A DHH-associated DSD genetic diagnosis can also guide clinical management and patient monitoring, in particular, in relation to gonadal malignancies and neopathies. Five patients with DHH pathogenic variants and gonadal cancers (including seminoma in situ, dysgerminoma and gonadoblastoma) have been reported.\(^ {7} \)\(^ {10} \)\(^ {11} \) Both homozygous missense variants and nonsense variants appear to cause these.

Table 2  
\textbf{DHH gene variant functional activity and second curation}

<table>
<thead>
<tr>
<th>Patient ID</th>
<th>DHH variants</th>
<th>Curation before functional</th>
<th>% of WT activity (mGIF)</th>
<th>Adjusted p value (mGIF)</th>
<th>% of WT activity (mPatch1)</th>
<th>Adjusted p value (mPatch1)</th>
<th>Final curation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>NM_021044.2:c.491G&gt;C:p.Arg164Pro</td>
<td>Homozygous</td>
<td>LP</td>
<td>1%</td>
<td>&lt;0.0001</td>
<td>4%</td>
<td>0.0398</td>
</tr>
<tr>
<td>2</td>
<td>NM_021044.2:c.680C&gt;T:p.Ala227Val</td>
<td>Compound</td>
<td>VUS-3a</td>
<td>~1%</td>
<td>&lt;0.0001</td>
<td>~20%</td>
<td>0.0059</td>
</tr>
<tr>
<td>3</td>
<td>NM_021044.2:c.728C&gt;T:p.Pro261Leu</td>
<td>heterozygote</td>
<td>VUS-3a</td>
<td>2%</td>
<td>&lt;0.0001</td>
<td>4%</td>
<td>0.0377</td>
</tr>
<tr>
<td>4</td>
<td>NM_021044.2:c.746G&gt;A:p.Arg249His</td>
<td>Compound</td>
<td>VUS-3a</td>
<td>26%</td>
<td>0.0002</td>
<td>13%</td>
<td>0.0448</td>
</tr>
<tr>
<td>5</td>
<td>NM_021044.2:c.680C&gt;T:p.Ala227Val</td>
<td>Heterozygous</td>
<td>VUS-3a</td>
<td>47%</td>
<td>0.0096</td>
<td>31%</td>
<td>0.2342</td>
</tr>
<tr>
<td>6</td>
<td>NM_021044.2:c.80G&gt;C:p.Arg27Gln</td>
<td>Heterozygous</td>
<td>VUS-3a</td>
<td>67%</td>
<td>0.9956</td>
<td>78%</td>
<td>0.9957</td>
</tr>
</tbody>
</table>

\(^ {3} \)Results from co-culture functional assay are shown, including percentage of WT activity and p-values for both mGIF and mPatch1 read-outs. Where variants were found as compound heterozygous or heterozygous, functional read-outs for combined alleles are also shown. Cureation outcomes for each variant taking these functional outcomes into account are also shown. 

DHH, desert hedgehog; LP, likely pathogenic; VUS, variant of uncertain significance; WT, wild type.
and our functional studies did not reveal any trend between the variants that caused malignancies and those that didn’t. Importantly, a DHH genetic diagnosis alerts the clinicians to increase monitoring of these patients.

DHH is also expressed in the Schwann cells of the peripheral nervous system where it signals the formation of the connective tissue sheath around peripheral nerves and is key for their survival. In Dhh null mice, peripheral nerve defects are observed.90 Seven of the 17 patients previously reported with DHH-associated DSD also had neuropathies, usually presenting between the ages of 20 years and 30 years.9 9 13 14 While none of our patients reported any signs of peripheral neuropathy, in several cases the genetic diagnosis prompted clinicians to test for this, and continued monitoring will now be carried out. Tajouri et al hypothesised that C-terminal variants affecting autoprocessing are more likely to lead to gonadal dysgenesis without neuropathies, as even unprocessed DHH protein may induce peripheral nerve development.90 Interestingly though, our patient with a homozygous variant in the N-terminal (patient 1; p.Arg164Pro) which showed a complete LOF has not reported neuropathies. We suggest that until larger cohorts of patients with DHH-associated DSD are followed longitudinally, all patients with DHH variants be monitored.

In conclusion, we report a functional assay that can assess DHH variant activity. Taking the results of this assay into account during variant curation improves the clinical interpretation of genomic data and provides a more accurate diagnosis for patients with 46,XY DSD.

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Contributors KA and JvdB carried out sequencing analysis and functional work. KA designed the study and wrote the manuscript with aS. gr carried out sequencing analysis and compiled patient data. nl carried out cloning/allelic segregation. Jr, Ka and JvdB carried out sequencing analysis and functional work.

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