0021-972X/04/$15.00/0
Printed in U.S.A.

The Journal of Clinical Endocrinology & Metabolism 89(9):4480–4483

doi: 10.1210/jc.2004-0863

Mutations in the *Desert hedgehog* (*DHH*) Gene in Patients with 46,XY Complete Pure Gonadal Dysgenesis

P. CANTO, D. SÖDERLUND, E. REYES, AND J. P. MÉNDEZ

Research Unit in Developmental Biology (P.C., D.S., J.P.M.), Hospital de Pediatría, Centro Médico Nacional Siglo XXI, Instituto Mexicano del Seguro Social, México, Distrito Federal 06725, México; and Department of Pathology (E.R.), Instituto Nacional de Ciencias Médicas y Nutrición "Salvador Zubirán", México, Distrito Federal 14000, México

Mutations of *SRY* are the cause of complete pure gonadal dysgenesis (PGD) in 10–15% of patients. In the remaining individuals, it has been suggested that mutations in other genes involved in the testis-determining pathway could be causative. We describe the first report in which three cases of 46,XY complete PGD are attributed to mutations of the *Desert hedgehog* (*DHH*) gene.

***DHH* was sequenced using genomic DNA from paraffin-embedded gonadal tissue from six patients with complete 46,XY PGD. Mutations were found in three patients: a homozygous mutation in exon 2, responsible for a L162P, and a homozygous 1086delG in exon 3.**

Mutated individuals displayed 46,XY complete PGD, differentiating from the only previously described patient with a homozygous *DHH* mutation, who exhibited a partial form of PGD with polyneuropathy, suggesting that localization of mutations influence phenotypic expression.

This constitutes the first report where mutations of *DHH* are associated with the presence of 46,XY complete PGD, demonstrating that the genetic origin of this entity is heterogeneous and that disorders in other genes, different from *SRY*, involved in the testis-determining pathway are implicated in abnormal testicular differentiation in humans. These data extend previous reports demonstrating *DHH* is a key gene in gonadal differentiation. (*J Clin Endocrinol Metab* 89: 4480–4483, 2004)

MALE SEX DETERMINATION in mammals depends on the presence of the *SRY* gene located on the Y chromosome, as well as on the action of several other genes, which are involved in the testis-determining pathway, located on autosomal and X-linked loci. A vital event in testicular organogenesis is the specification of somatic cell lineages, which include Leydig cells, Sertoli cells, and peritubular myoid cells. Specification of these lineages is critical for establishing testis morphology and hormone production (1).

Gonadal dysgenesis encompasses a heterogeneous group of different chromosomal, gonadal and phenotypic abnormalities (2, 3). Sex reversal in XY females is a consequence of failure of testis determination or differentiation. Individuals with the complete form of 46,XY pure gonadal dysgenesis (PGD) present a 46,XY karyotype, bilateral streak gonads, normally developed Müllerian ducts, and female external genitalia (2). It has been estimated that the presence of the Y chromosome in 46,XY PGD patients increases (10–30%) the risk of developing gonadal tumors, *i.e.* gonadoblastoma or dysgerminoma (4, 5).

Mutations of the *SRY* gene are the cause of 46,XY sex reversal in approximately 10–15% of patients with PGD. In the remaining individuals, a precise cause has not been determined, and it has been suggested that they may bear mutations in the *SRY* regulatory elements or in other genes involved in the testis-determining pathway (6, 7). One of these genes is *Desert hedgehog* (*DHH*), a member of the hedgehog family of signaling proteins, which also includes *Sonic hedgehog* and *Indian hedgehog* (8). In humans, *DHH* is located in 12q12→q13.1, is composed of three exons, and encodes a protein of 396 amino acids (9).

In mice, *Dhh* has a sexually dimorphic expression. Analysis of gene transcripts demonstrated that expression of *Dhh* is observed in fetal testes at 11.5 d postcoitum, whereas no transcripts are detected in fetal ovaries (10). The product of the *Dhh* gene is specifically expressed in Sertoli cells and Schwann cells along peripheral nerves (11, 12). The importance *Dhh* has in testicular morphology was originally described by Clark *et al.* (13) in a study in which the majority of *Dhh* null male mice developed into pseudohermaphrodites. Likewise, it was demonstrated that the differentiation of peritubular myoid cells and the consequent formation of testis cords is regulated by Dhh (13, 14). Furthermore, Hung-Chang *et al.* (1) suggested that *Dhh/Ptch1* signaling is a positive regulator of the differentiation of steroid-producing Leydig cells in the fetal testis. *Dhh* is expressed in Sertoli cells, being the only mammalian hedgehog protein expressed in the gonad between 11.5 and 13.5 d postcoitum. *Ptch1*, one of the hedgehog receptors, is expressed in interstitial cells. In conclusion, *Dhh/Ptch1* signaling triggers Leydig cell differentiation by up-regulating Steroidogenic Factor 1 and *P450* Side Chain Cleavage enzyme expression in *Ptch1*-expressing precursor cells, which are located outside the testis cords.

In 2000, Umehara *et al.* (15) reported a homozygous missense mutation of the *DHH* gene in one patient with 46,XY partial gonadal dysgenesis associated with minifascicular neuropathy. The authors suggested that *DHH* is a key molecule that intervenes in both male gonadal differentiation and perineural formation in peripheral nerves.

Here we describe the first report in which in three cases of

Abbreviation: PGD, Pure gonadal dysgenesis.

JCEM is published monthly by The Endocrine Society (http://www.endo-society.org), the foremost professional society serving the endocrine community.

46,XY complete PGD, homozygous mutations of the *DHH* gene are associated with the disorder.

Patients and Methods

Patients

The study was approved by the Institute's Human Research Committee. Gonadal tissue obtained from gonadectomies between 1983 and 1998, from six unrelated phenotypic females with 46,XY complete PGD, was molecularly studied. All patients had a Mexican-mestizo ethnic origin, and in all cases family history was negative for consanguinity. All individuals had a nonmosaic 46,XY karyotype in at least 50 cells (peripheral blood leukocytes). Clinical and histopathological findings in these patients are shown in Table 1.

Methods

Genomic DNA was isolated from peripheral blood leukocytes in control subjects by standard techniques (16) and from paraffin-embedded gonadal tissue from the patients and three controls using the MagneSil genomic fixed tissue system (Promega Corp., Madison, WI), following the conditions recommended by the manufacturer. DNA was amplified by PCR in 50 μl of reaction mixture containing 0.3 μg genomic DNA, 0.1 mM dNTPs, 2.0 U of thermostable DNA polymerase (AmpliTaq, Applied Biosystems, Foster City, CA), and 250 nM of each specific set of *DHH* primers. The sequences of the *DHH* primers for all three exons and the PCR conditions were described by Umehara *et al.* (15) (GenBank accession number AB010581 for exon 1, AB010993 for exon 2, and AB010994 for exon 3).

After amplification, PCR products were electrophoresed in a 1.2% agarose gel and afterward purified using the QIAEX II gel extraction kit (QIAGEN GmbH, Hilden, Germany). These products were then sequenced (0.1 μg DNA template reaction) on an ABI 377 automated DNA sequencer (Perkin-Elmer, Applied Biosystems Division, Foster City, CA) using the BigDye terminator cycle sequencing ready reaction kit (Perkin-Elmer). For all exons, both strands were sequenced and compared. Each mutation was confirmed in three independent PCR amplifications and sequencings.

TABLE 1. Clinical and histopathological findings in six patients with 46,XY complete PGD

Patient	Age (yr)	Gonads	External genitalia
1	17	Bilateral streaks	Female
2	19	Bilateral streaks with bilateral gonadoblastoma	Female
3	25	Bilateral streaks	Female
4	16	Bilateral streaks	Female
5	13	Bilateral streaks with bilateral gonadoblastoma	Female
6	26	Bilateral streaks with bilateral dysgerminoma	Female

All patients presented infantile uterus and bilateral fallopian tubes. Patient 2 presented a left epididymis, and patient 5 presented bilateral epididymis.

Results

In all patients, the sequence of the open reading frame of the *SRY* gene was analyzed in gonadal tissue and no mutations were found (data not shown).

Genetic defects in the *DHH* gene were found in three of six patients. In patient 4, a homozygous mutation in exon 2 consisting of a T-to-C substitution at position 485 was observed. This mutation was responsible for a leucine (CTG) into proline (CCG) substitution at codon 162 (Fig. 1). Exons 1 and 3 showed no sequence variations. In patients 2 and 6, a nucleotide deletion was found in exon 3, at position 1086, which comprises the third nucleotide (guanine) of codon 362. This deletion caused a stop codon (TAG), four codons after the deletion was located (Fig. 2). No sequence variations were observed in the first two exons of the gene.

Direct sequencing of exons 1–3 of the *DHH* gene in the

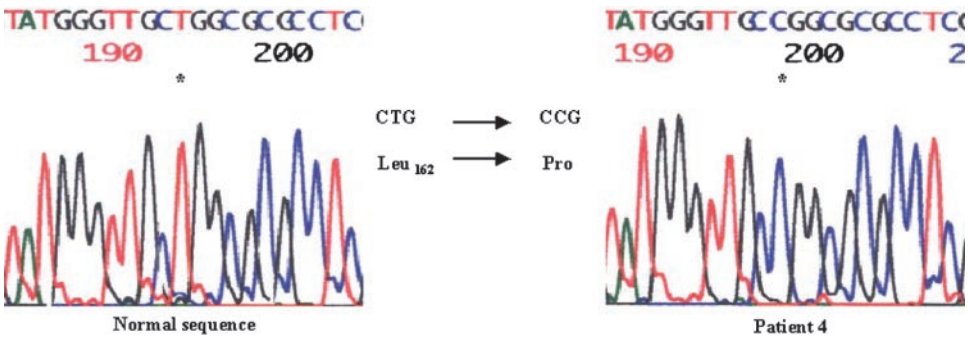

FIG. 1. Partial sequence of exon 2 of the *DHH* gene from patient 4 and from a normal male control. A homozygous thymine-to-cytosine (T→C) mutation that results in the substitution of leucine into proline at codon 162 is observed in the patient.

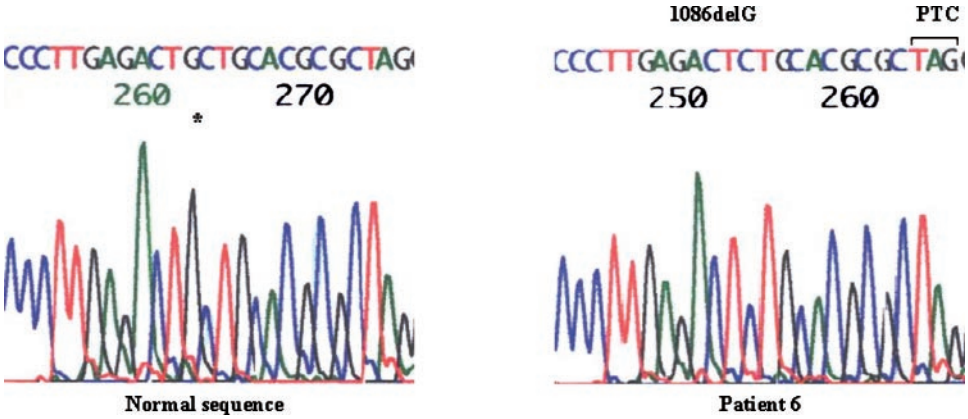

FIG. 2. Partial sequence of exon 3 of the *DHH* gene from patient 6 and from a normal male control. A nucleotide deletion (guanine) at position 1086 is observed. The deletion causes a frameshift and a premature termination codon (PTC), four codons after the deletion was located.

remaining three patients did not reveal any molecular abnormality.

One hundred normal male individuals (200 alleles) did not harbor any of the mutations, being homozygous wild type.

Discussion

Gonadal dysgenesis comprises a variety of clinical conditions characterized by abnormal development of the fetal gonad, including 45,X Ullrich Turner syndrome and its variants, mixed gonadal dysgenesis, as well as 46,XX and 46,XY PGD. The latter includes a complete and a partial form; in 46,XY complete PGD, bilateral streak gonads are always formed (2). In the majority of cases, the precise origin of this entity has not been determined; the *SRY* gene is mutated in only 10–15% of patients with 46,XY PGD. In the remaining individuals, the cause of the disorder has not been established (6, 7), although some autosomal and X-linked loci have been linked with failure to develop a testis (17).

In mammals, testis development is initiated in response to the expression of *Sry* in Sertoli cell precursors. In Sertoli cells, the activation of *Dhh* transcription occurs immediately after the initiation of *Sry* expression (11). Bitgood *et al.* (10) generated knockout mice genetically null for the *Dhh* gene, observing male sterility. Likewise, Clark *et al.* (13) studied the testes from adult and prepubertal mice lacking *Dhh*, demonstrating peritubular cell defects that may be indicative of the role these cells have in the development of tubular morphology, Leydig cell differentiation, and spermatogenesis. Umehara *et al.* (15) analyzed the *DHH* gene in peripheral blood from one subject with 46,XY partial PGD and polyneuropathy who presented premature female genitalia, a blinded vagina and immature uterus, as well as a testis in one side and a streak gonad on the other. These authors found a missense mutation at the initiation codon of exon 1, which abolished initiation of translation at the normal start site, suggesting that failure of translation of the *DHH* gene may disturb the differentiation of male gonads and may result in 46,XY partial PGD.

In the present study, we analyzed all three exons of the *DHH* gene in six patients with 46,XY complete PGD, finding homozygous mutations in three of them. Mutated individuals displayed a 46,XY complete PGD (female external genitalia, bilateral fallopian tubes with infantile uterus, and streak gonads). To date, the only *DHH* mutation described in 46,XY PGD is the one by Umehara *et al.* (15); comparing this mutation with our cases, we can affirm that the phenotypic spectrum of 46,XY PGD patients with mutations in the *DHH* gene is variable, ranging from a partial form of PGD with polyneuropathy (15) to complete PGD without polyneuropathy. All of our patients had normal motor functions of both extremities and superficial and deep sensations; reflexes were also normal, there was no presence of symptoms or signs that suggested polyneuropathy, and mental function was not impaired. The phenotypic differences observed between our patients and the one reported by Umehara *et al.* (15) suggest that the localization of the mutations as well as a variety of other factors influence the expression of the phenotype. The presence of *DHH* mutations in patients with 46,XY partial PGD, as well as in 46,XY complete PGD, is similar to what has been observed regarding the *SRY* gene, where mutations have been shown to induce 46,XY complete PGD or partial PGD (18).

The L162P mutation exhibited by patient 4 is located in the mature amino-terminal domain of the DHH protein, constituting the first spontaneous mutation described in this domain. This mutation led to a nonconservative amino acid substitution, changing a highly conserved residue (Table 2A). We assume that the L162P mutation affected DHH function considering that the mature amino-terminal domain of Hh proteins has been shown to be essential for all the known long- and short-range activities of this protein (19, 20), and perhaps this mutation could alter the ability to bind with the transmembrane protein Ptc, which is required for cellular responsiveness to DHH (21).

The second mutation was found in patients 2 and 6, who presented a nucleotide 1086delG in exon 3. This mutation led to a nonconservative amino acid substitution, changing a highly conserved residue (Table 2B). Taking into consideration that two of six patients exhibited this mutation and although there is no known relationship between them, the mutation could derive from a common ancestor by a founder gene effect. An alternative explanation is that the identical deletion observed might indicate the presence of a hot spot in this region of the *DHH* gene. This deletion is located in the carboxyl-terminal domain of the DHH protein. Lee *et al.* (19) examined the effects of several distinct types of mutations in the carboxyl-terminal domain of *Drosophila melanogaster*, demonstrating that deletion or alteration of residues within this domain is associated with absence or reduced efficiency of autoproteolysis of the hedgehog. Furthermore, Porter *et al.* (22) observed that the autoprocessing activity of the carboxy-terminal domain influences the cellular localization of the amino-terminal domain.

As it has been proposed for the *SRY* gene (18), we assume that a given mutation in *DHH* may produce sufficient DHH activity to reach the threshold required for testis formation. However, the same mutation on a different genetic background may reduce DHH activity, preventing testis development. Bitgood *et al.* (10) observed that the severity of the phenotype presented in their colony of *Dhh*-null mice varied depending upon the genetic background. Those on an inbred

TABLE 2. Alignment of the human DHH sequences with other mammalian Dhh and *D. melanogaster* Hh, demonstrating the highly conserved leucine 162 (substituted in patient 4 by a proline) and leucine 362 (nucleotide 1086delG in patients 2 and 6)

	Amino acids
A	
Patient 4	NKYGL **P** ARLAVEAGFDWVYYESRN
H. sapiens (wild type)	NKYGL **L** ARLAVEAGFDWVYYESRN
M. musculus	NKYGL **L** ARLAVEAGFDWVYYESRN
R. norvergic	NKYGL **L** ARLAVEAGFDWVYYESRN
D. melanogaster	SKYGM **L** ARLAVEAGFDWVSYVSRR
B	
Patients 2 and 6	FAPLR **1086delG**
H. sapiens (wild type)	FAPLR **L** LHALGALLPG
M. musculus	FAPLR **L** LHALGALLPG
R. norvergic	FAPLR **L** LHALGALLPG
D. melanogaster	LAPMR **L** LSTLEAWLPA

Amino acids 162 (A) and 362 (B) are depicted in *bold*.

background of the *129/Sv* strain developed germ cells up to primary spermatocytes, whereas germ cells in some of the *129/Sv-C57BL/6JF*$_1$ hybrids developed through meiosis to become step-15 spermatids. Furthermore, Clark *et al.* (13) reported that in their colony of *Dhh*-null mice bred on a mixed genetic background, the phenotypic outcome of the *Dhh*-null condition was more severe than the ones previously described.

We identified mutations of the *DHH* gene in only three of the six patients studied. The true prevalence of such mutations is difficult to assess because of the rarity of this entity. Larger samples of patients will need to be studied to determine the true prevalence of *DHH* mutations in humans. The absence of mutations in the other patients studied indicates that molecular defects in such patients could be present in the untranslated regulatory regions of the *DHH* gene or within introns; besides, defects in other gene(s) could explain the disorder.

In conclusion, to our knowledge, this constitutes the first report where mutations of the *DHH* gene are associated with the presence of 46,XY complete PGD. These data demonstrate that the genetic origin of 46,XY complete PGD is heterogeneous and that disorders in other genes, different from *SRY*, that are involved in the testis-determining pathway are directly implicated in abnormal testicular differentiation in humans. Likewise, these data extend previous reports in humans and other species demonstrating that *DHH* constitutes a key gene in gonadal differentiation.

Acknowledgments

We thank Leonor Enciso from the Unidad de Instrumentos, Coordinación de Investigación en Salud, and Irak León-O'Farrill from Unidad de Investigación Médica en Biología del Desarrollo, Instituto Mexicano del Seguro Social, for their technical assistance.

Received May 11, 2004. Accepted June 16, 2004.

Address all correspondence and requests for reprints to: Juan Pablo Méndez, M.D., Unidad de Investigación Médica en Biología del Desarrollo, Coordinación de Investigación en Salud, Coahuila 5, Apartado Postal A-047, Colonia Roma, C.P. 06703, México, D.F., México. E-mail: jpmb@servidor.unam.mx.

This work was supported by the Consejo Nacional de Ciencia y Tecnología (CONACYT), México (Grant G29790M).

References

1. **Hung-Chang Yao H, Whoriskey W, Capel B** 2002 Desert hedgehog/Patched 1 signaling specifies fetal Leydig cell fate in testis organogenesis. Genes Dev 16:1433–1440
2. **Berkovitz GD, Fechner PY, Zacur HW, Rock JA, Snyder HM, Migeon CJ, Perlman EJ** 1991 Clinical and pathologic spectrum of 46,XY gonadal dysgenesis: its relevance to the understanding of sex differentiation. Medicine 70: 375–383
3. **Méndez JP, Ulloa-Aguirre A, Kofman-Alfaro S, Mutchinick O, Fernández del Castillo C, Reyes E, Pérez-Palacios G** 1993 Mixed gonadal dysgenesis: clinical, cytogenetic, endocrinological, and histopathological findings in 16 patients. Am J Med Genet 46:263–267
4. **Scully RE** 1970 Gonadoblastoma: a review of 74 cases. Cancer 25:1340–1356
5. **Verp MS, Simpson JL** 1987 Abnormal sexual differentiation and neoplasia. Cancer Genet Cytogenet 25:191–218
6. **Schäffler A, Barth N, Winkler K, Zietz B, Rümmele P, Knüchel R, Schölmerich J, Palitzsch K-D** 2000 Identification of a new missense mutation (Gly95Glu) in a highly conserved codon within the high-mobility group box of the sex-determining region Y gene: report on a 46,XY female with gonadal dysgenesis and yolk-sac tumor. J Clin Endocrinol Metab 85:2287–2292
7. **Assumpcao JG, Benedetti CE, Maciel-Guerra AT, Guerra Jr G, Baptista MT, Scolfaro MR, de Mello MP** 2002 Novel mutations affecting SRY DNA-binding activity: the HMG box N65H associated with 46,XY pure gonadal dysgenesis and the familial non-HMG box R30I associated with variable phenotypes. J Mol Med 80:782–790
8. **Ingham PW** 1998 Transducing hedgehog: the history so far. EMBO J 17:3505–3511
9. **Tate G, Satoh H, Endo Y, Mitsuya T** 2000 Assignment of desert hedgehog (DHH) to human chromosome bands 12q12 → q13.1 by *in situ* hybridization. Cytogenet Cell Genet 88:93–94
10. **Bitgood MJ, Shen L, McMahon AP** 1996 Sertoli cell signaling by Desert hedgehog regulates the male germline. Curr Biol 6:298–304
11. **Bitgood MJ, McMahon AP** 1995 *Hedgehog* and *Bmp* genes are coexpressed at many diverse sites of cell-cell interaction in the mouse embryo. Dev Biol 172:126–138
12. **Parmantier E, Lynn B, Lawson D, Turmaine M, Sharghi Namini S, Chakrabarti L, McMahon AP, Jessen KR, Mirsky R** 1999 Schwann cell-derived Desert hedgehog controls the development of peripheral nerve sheaths. Neuron 23:713–724
13. **Clark AM, Garland KK, Russell LD** 2000 Desert hedgehog (Dhh) gene is required in the mouse testis for formation of adult-type Leydig cells and normal development of peritubular cells and seminiferous tubules. Biol Reprod 63:1825–1838
14. **Pierucci-Alves F, Clark AM, Russell LD** 2001 A developmental study of the *Desert hedgehog*-null mouse testis. Biol Reprod 65:1392–1402
15. **Umehara F, Tate G, Itoh K, Yamaguchi N, Douchi T, Mitsuya T, Osame M** 2000 A novel mutation of *desert hedgehog* in a patient with 46,XY partial gonadal dysgenesis accompanied by minifascicular neuropathy. Am J Hum Genet 67:1302–1305
16. **Sambrook J, Russell D** 2001 Preparation and analysis of eukaryotic genomic DNA. In: Nolan C, ed. Molecular cloning: a laboratory manual. 3rd ed. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press; 6.1–6.31
17. **Veitia RA, Salas-Cortes L, Ottolenghi Ch, Pailhoux E, Cotinot C, Fellous M** 2001 Testis determination in mammals: more questions than answers. Mol Cell Endocrinol 179:3–16
18. **Cameron FJ, Sinclair AH** 1997 Mutations in *SRY* and *SOX9*: testis-determining genes. Hum Mutat 9:388–395
19. **Lee JJ, Ekker SC, von Kessler DP, Porter JA, Sun BI, Beachy PA** 1994 Autoproteolysis in hedgehog protein biogenesis. Science 266:1528–1537
20. **Bumcrot DA, Takada R, McMahon AP** 1995 Proteolytic processing yields two secreted forms of Sonic hedgehog. Mol Cell Biol 15:2294–2303
21. **Pathi S, Pagan-Westphal S, Baker DP, Garber EA, Rayhorn P, Bumcrot D, Tabin CJ, Blake Pepinsky RB, Williams KP** 2001 Comparative biological responses to human Sonic, Indian and Desert hedgehog. Mech Dev 106:107–117
22. **Porter JA, von Kessler DP, Ekker SC, Young KE, Lee JJ, Moses K, Beachy PA** 1995 The product of hedgehog autoproteolytic cleavage active in local and long-range signalling. Nature 374:363–366