A heterozygous mutation in the desert hedgehog gene in patients with mixed gonadal dysgenesis

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Aetiology of mixed gonadal dysgenesis (MGD) has not been completely elucidated. Molecular analyses have failed to demonstrate the presence of mutations in sex-determining region on Y chromosome (SRY); it has been suggested that these individuals may bear mutations in other genes involved in the testis-determining pathway. Desert hedgehog’s (DHH) importance regarding male sex differentiation has been demonstrated in various studies we describe here, for the first time, two cases of MGD in which a monoallelic single base deletion in DHH is associated with the disorder. Genomic DNA was isolated from paraffin-embedded gonad tissue from 10 unrelated patients with MGD and three controls; in addition to, DNA from peripheral blood leukocytes in 100 controls. Coding sequence abnormalities in DHH were assessed by exon-specific PCR, single-stranded conformation polymorphism (SSCP) and direct sequencing. In two patients, a heterozygous 1086delG in exon 3 was found. Comparing previously described mutations in DHH to the one observed in this study, we can affirm that the phenotypic spectrum of patients with gonadal dysgenesis due to mutations in DHH is variable. This study continues to demonstrate the importance that DHH has in mammalian male sexual differentiation, providing extended evidence that DHH constitutes a key gene in gonadal differentiation.

Key words: DHH gene/founder gene/male gonadal differentiation/MGD/mutation

Introduction

Male sex determination in mammals depends on the presence and action of the sex-determining region on Y chromosome (SRY) gene located on the Y chromosome and various other genes located on autosomal and X-linked loci. A main 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 vital for the establishment of testis morphology and hormone production (Yao et al., 2002).

Mixed gonadal dysgenesis (MGD) comprises an heterogeneous group of diverse chromosomal, gonadal and phenotypic abnormalities which are characterized by the presence of a testis on one side and a contralateral streak or an absent gonad. Most patients have a 45,X/46,XY chromosomal mosaicism and germ cell tumours, such as gonadoblastoma or dysgerminoma, which develop in about one third of patients with this syndrome (Robboy et al., 1982; Méndez et al., 1993). To date, the aetiology of MGD has not been completely elucidated; traditionally, it has been proposed that in MGD predominance of XY or X0 gonadal cells, would determine gonadal differentiation into a testis or a streak gonad. However, in various cases previously reported there has not been concordance between the gonadal karyotype and the differentiation of the gonad (Mailhes et al., 1979; Sugarman et al., 1994; Méndez et al., 1999). These reports have demonstrated that the theory which states that gonadal sex determination is due to the predominance of a specific cell type is not always confirmed. In patients with MGD, molecular analyses have failed to demonstrate the presence of mutations in the SRY gene; therefore, it has been suggested that these individuals may bear mutations in other genes involved in the testis-determining pathway (Álvarez-Nava et al., 2001; Canto et al., 2004a). One of these genes is desert hedgehog (DHH), a member of the hedgehog family of signalling proteins (Ingham, 1998) which in humans is localized in 12q12–q13.1 is constituted by three exons and encodes a protein of 396 amino acids (Tate et al., 2000).

The importance of DHH and its murine homologue Dhh regarding male sex differentiation has been demonstrated in various studies (Bitgood and McMahon, 1995; Bitgood et al., 1996). Clark et al. (2000) reported that the majority of Dhh null male mice developed into pseudohermaphrodites. Other studies have demonstrated that the differentiation of peritubular myoid cells and the consequent formation of testis cords is regulated by Dhh (Clark et al., 2000; Pierucci-Alves et al., 2001). Furthermore, it has been suggested that Dhh/Patched 1 signalling is a positive regulator of the differentiation of steroid-producing Leydig cells in the fetal testis (Yao et al., 2002). In studies in humans, Umehara et al. (2000) reported a homozygous missense mutation of the DHH gene, in one patient with 46,XY partial gonadal dysgenesis associated with minifascicular neuropathy; likewise, our group recently demonstrated a homozygous mutation in the DHH gene in three patients with 46,XY complete pure gonadal dysgenesis (PGD) (Canto et al., 2004b). All of these findings have demonstrated that Dhh and DHH are key molecules that intervene in male gonadal differentiation.

Here we describe, for the first time, two cases of MGD in which a monoallelic single-base deletion in the DHH gene is associated with the disorder.
Materials and methods

Patients
The study was approved by the Institute’s Human Research Committee. Ten unrelated patients with proven diagnosis of MGD were molecularly studied. All patients as well as all controls had a Mexican-mestizo ethnic origin, and in all cases family history was negative for consanguinity. Cytogenetic and histopathological findings in these patients are summarized in Table I. Gonads were obtained from control subjects from the department of pathology from three individuals, whom in years past were gonadectomized because of prostatic cancer and did not have any phenotypical abnormality. The 100 control DNA blood samples were obtained from a DNA bank of endocrinologically normal male individuals, with no phenotypic abnormalities.

Methods
Genomic DNA was isolated from peripheral blood leukocytes in 100 control subjects by standard techniques (Sambrook and Russell, 2001) and from paraffin-embedded gonadal tissue from the patients and three controls, using the MagneSil Genomic Fixed Tissue System (Promega, Madison, WI, USA), following the conditions recommended by the manufacturer.

Coding sequence abnormalities in the DHH gene were assessed by exon-specific PCR, single-stranded conformation polymorphism (SSCP), analysis and direct sequencing. Exon DNA amplifications were carried out with primers and PCR conditions described elsewhere (Umehara et al., 2000; Canto et al., 2004b). After amplification, PCR products were electrophoresed in a 1.2% agarose gel following the conditions recommended by the manufacturer. Coding sequence abnormalities in the DHH gene were assessed by exon-specific PCR, single-stranded conformation polymorphism (SSCP), analysis and direct sequencing. Exon DNA amplifications were carried out with primers and PCR conditions described elsewhere (Umehara et al., 2000; Canto et al., 2004b). After amplification, PCR products were electrophoresed in a 1.2% agarose gel and afterwards purified using the Qiaex II, Gel extraction kit (Qiagen GmbH, Hilden, Germany). Analysis of conformation-depend polymorphism of single-stranded DNA was performed according to the method of Orita et al. (1989), with minor modifications as previously described (Canto et al., 1997). Direct sequencing of the PCR fragments was carried out with the Thermosequenase (α-33P)dNTP terminator cycle sequencing kit (USB, Cleveland, OH, USA), using the forward primer DHH3 (5′-TACGCCGTTTTCGAGATCACCAGTTGG-3′). Mutant and normal PCR products were also sequenced bidirectionally using the primers DHH3 and DHH51 as described by Umehara et al. (2000) and the BigDye™ terminator cycle sequencing ready reaction kit (PE Biosystems, Foster City, CA, USA) with the ABI 377 automated DNA sequencer (Perkin-Elmer, Applied Biosystems Division, Foster City, CA, USA). Each mutation was confirmed in three independent PCR amplifications and sequencings.

Results
In all patients, the sequence of the open-reading frame of the SRY gene was analysed, and no mutations were found (data not shown).

Abnormalities in the migration pattern were detected by SSCP analysis in exon 3 of the DHH gene in patients 1 and 9 (Figure 1). Direct sequencing of the DHH gene demonstrated that both patients presented a heterozygous nucleotide deletion 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 (Figure 2). In both patients, no sequence variations were observed in the first two exons of the gene.

In the remaining eight patients, direct sequencing of exons 1–3 of the DHH gene did not reveal any molecular abnormality. One hundred normal male individuals (200 alleles) did not harbour the mutation, being homozygous wild type.

Discussion
Immediately after the initiation of SRY expression, activation of Dhh transcription is observed, and it constitutes one of the first indications of male-specific development, before overt sexual dimorphism of the gonads. Pre-Sertoli expression of Dhh makes it a plausible candidate for direct regulation by the sex-determining gene (Bitgood et al., 1996). The Dhh-null mouse presents an altered phenotype, owing to the absence of the protein, characterized by undescended and extremely small testis which lack adult-type Leydig cells and numerous undifferentiated fibroblastic cells in the interstitium that produce abundant collagen. The basal lamina, normally present between the myoid cells and Sertoli cells, is focally absent (Clark et al., 2000; Pierucci-Alves et al., 2001). In heterozygous males, the external phenotype and the testicular size, as well as microtubules of Sertoli cells are similar to that of wild-type males (Clark et al., 2000). Umehara et al. (2000) studied the DHH gene in peripheral blood from a 46,XY patient describing the presence of a missense mutation at the initiation codon of exon 1. Recently, our group described homozygous mutations in the DHH gene in three cases of 46,XY-complete PGD (Canto et al., 2004b). Taking into consideration all studies regarding Dhh and DHH, it has been proposed that DHH is a key gene in mammalian gonadal differentiation.

In this study, we analysed all three exons of the DHH gene in 10 patients with MGD, finding the same heterozygous mutation in two of them who presented a 45,X/46,XY chromosomal complement. Unfortunately, in these cases, we could not investigate the possibility of polynuropathy because the molecular studies were carried out with DNA obtained from fixed gonadal tissues processed between 1983 and 1989. Likewise, parents of both subjects were not available for DNA analysis and, therefore, we could not determine whether these constitute de novo mutations.

The heterozygous mutation described is identical (in a homozygous trait) to the one described recently by our group in two patients with 46,XY-complete PGD; this mutation lead to a non-conservative amino-acid substitution, changing a highly conserved residue located in the carboxyl-terminal domain of the DHH protein (Canto et al., 2004b). Lee et al. (1994) studied the effects of several distinct types of mutations in the carboxy-terminus domain of Drosophila melanogaster demonstrating that deletion or alteration of residues within this domain is associated with reduced or even absence of efficiency of autoproteolysis of the hedgehog. Taking into consideration that four individuals from the same ethnic group have exhibited this

Table 1. Cytogenetic and histopathological findings in patients with mixed gonadal dysgenesis (MGD)

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age (year)</th>
<th>Karyotype (peripheral blood)</th>
<th>Gonads and internal genitalia (right side/left side)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>19</td>
<td>45,X/46,XY</td>
<td>Testis with gonadoblastoma/streak, bilateral Fallopian tubes and epididymis</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>45,X/46,XY</td>
<td>Testis/streak, bilateral Fallopian tubes, right epididymis</td>
</tr>
<tr>
<td>3</td>
<td>1/12</td>
<td>45,X/46,XY</td>
<td>Streak/testis, bilateral epididymis, right Fallopian tube</td>
</tr>
<tr>
<td>4</td>
<td>20</td>
<td>45,X/46,XY</td>
<td>Testis/streak, right Fallopian tube and epididymis</td>
</tr>
<tr>
<td>5</td>
<td>14</td>
<td>45,X/46,XY</td>
<td>Testis with gonadoblastoma/streak, bilateral Fallopian tubes</td>
</tr>
<tr>
<td>6</td>
<td>17</td>
<td>45,X/46,XY</td>
<td>Testis with gonadoblastoma/streak, bilateral Fallopian tubes</td>
</tr>
<tr>
<td>7</td>
<td>11/12</td>
<td>45,X/46,XY,idic(Y)</td>
<td>Streak/testis, right Fallopian tube/ left epididymis</td>
</tr>
<tr>
<td>8</td>
<td>1</td>
<td>45,X/46,XY</td>
<td>Testis/streak, right epididymis/ left Fallopian tube</td>
</tr>
<tr>
<td>9</td>
<td>19</td>
<td>45,X/46,XY</td>
<td>Testis/streak, right epididymis/ left Fallopian tube</td>
</tr>
<tr>
<td>10</td>
<td>2</td>
<td>45,X/46,XY, dic(Y)</td>
<td>Streak/testis, bilateral Fallopian tube, left epididymis</td>
</tr>
</tbody>
</table>

*All patients presented either infantile or rudimentary uterus.
heterozygous mutations in MGD and although they are from different parts of the nation, the mutation could derive from a common ancestor by a founder gene effect. An alternative explanation is that the same deletion might indicate the presence of a hot spot in this region of the DHH gene. We consider that in both patients, the presence of the mosaicism, besides the existing mutation, prevented the development of testicular tissue. The magnitude in which the 45,X cell line or the DHH mutation affected the existing phenotype can not be ascertained.

Comparing the mutation described by Umehara et al. (2000) and the one previously described by our group in patients with 46,XY-complete PGD (Canto et al., 2004b), with the one observed in this study, we can affirm that the phenotypic spectrum of patients with gonadal dysgenesis because of mutations in the DHH gene is variable, ranging from MGD to complete PGD (Figure 3). Phenotypic diversity of patients with DHH mutations is similar to what has been described regarding the SRY gene, where mutations have been shown to induce 46,XY-complete PGD or partial PGD (Cameron and Sinclair, 1997). In their report, Umehara et al. (2000) also studied the father of the affected patient who presented a homozygous mutation at the initiation codon of exon 1 of the DHH gene. The father had the same mutation but in a heterozygous trait. The authors proposed that this heterozygous mutation was insufficient to cause PGD in the parent. However, in our patients, the heterozygous mutation apparently contributed to the phenotypic disorder. As it has been proposed for the SRY gene, 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 (Cameron and Sinclair, 1997).

The absence of mutations in the other patients studied indicates that molecular defects in those individuals could be present in the untranslated regulatory regions of the DHH gene or within introns; besides, defects in other gene(s) different from SRY and DHH could explain the existence of the disorder, i.e. SF1, WT1 and SOX9. Mutations in these genes have been implicated in gonadal dysgenesis (Canto et al., 2004a).

We identified mutations of the DHH gene in only two of the 10 patients studied. The true prevalence of such mutations is difficult to assess because of the rarity of this entity. The limited sample size in this investigation precludes any conclusions from being drawn about a direct role of DHH in the aetiology of MGD. Larger samples of patients will need to be studied to determine the true prevalence of DHH mutations in humans with this disorder of sex differentiation. This study continues to demonstrate the importance that DHH has in mammalian male sexual differentiation providing extended evidence that DHH constitutes a key gene in gonadal differentiation.

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