Absence of inactivating mutations and deletions in the DMRT1 and FGF9 genes in a large cohort of 46,XY patients with gonadal dysgenesis
Absence of inactivating mutations and deletions in the DMRT1 and FGF9 genes in a large cohort of 46,XY patients with gonadal dysgenesis

Aline Zamboni Machado¹, Thatiana Evilen da Silva¹, Elaine Maria Frade Costa, Mariza Gerdulo dos Santos, Mirian Yumie Nishi, Vinicius Nahime Brito, Berenice Bilharinho Mendonca, Sorahia Domenice*

Unidade de Endocrinologia do Desenvolvimento, Laboratório de Hormônios e Genética Molecular (UM42) da Disciplina de Endocrinologia e Metabologia do Hospital das Clínicas da Faculdade de Medicina da Universidade de São Paulo, Brazil

1 These authors contributed equally to this article.

1 Corresponding author. Av. Dr. Enéas de Carvalho Aguiar, n°155, PAMB, 2° andar, bloco 6, São Paulo, SP; CEP 05403-900, Brazil. Tel.: +55 11 2661 7512; fax: +55 11 2661 7519.
E-mail address: sorahiad@gmail.br (S. Domenice).

© 2012 Published by Elsevier Masson SAS.

1769-7212/$ – see front matter © 2012 Published by Elsevier Masson SAS.
http://dx.doi.org/10.1016/j.ejmg.2012.07.012

A R T I C L E  I N F O

Article info
Received 8 December 2011
Accepted 31 July 2012
Available online 9 August 2012

Keywords:
46,XY gonadal dysgenesis
DMRT1
FGF9
Testicular development
3’ UTR microsatellite

A B S T R A C T

Despite advances in our understanding of the mechanisms involved in sex determination and differentiation, the specific roles of many genes in these processes are not completely understood in humans. Both DMRT1 and FGF9 are among this group of genes. DMRT1 controls germ cell differentiation, proliferation, migration and pluripotency and Sertoli cell proliferation and differentiation. FGF9 has been considered a critical factor in early testicular development and germ cell survival in mice. We screened for the presence of DMRT1 and FGF9 mutations in 33 patients with 46,XY gonadal dysgenesis. No deletions in either DMRT1 or FGF9 were identified using the MLPA technique. Eight allelic variants of DMRT1 were identified, and in silico analysis suggested that the novel c.968-15insTTCTCT variant and the c.774G>C (rs146975077) variant could have potentially deleterious effects on the DMRT1 protein. Nine previously described FGF9 allelic variants and six different alleles of the 3’ UTR microsatellite were identified. However, none of these DMRT1 or FGF9 variants were associated with increased 46,XY gonadal dysgenesis. In conclusion, our study suggests that neither DMRT1 nor FGF9 abnormalities are frequently involved in dysgenetic male gonad development in patients with non-syndromic 46,XY disorder of sex development.

1. Introduction

Sex determination in mammals is a dynamic process in which germ and somatic cells acquire sex-specific characteristics. In humans, the bipotential embryonic gonad begins to develop around the fifth week of foetal life [1]. In the 6th and 7th weeks, the differentiation of specific cell groups in the bipotential gonadal tissue occurs; this will determine the development of the ovaries or testes, two very different organs with specialised functions [2].

Despite advances in our knowledge of the mechanisms involved in sex determination and differentiation, the exact roles of many genes that participate in different stages of this complex process are not completely understood [2]. Both DMRT1 and FGF9 genes are among this group of genes. Several studies have shown evidence of their roles in testicular development in mice, but their exact functions in the human testis have not been established [3,4].

The DMRT1 (Doublesex and mab-3-related transcription factor 1) gene, a member of the DMRT family, plays diverse and essential roles in vertebrate testis development. In the mouse testis, Dmrt1 is suggested to control germ cell differentiation, proliferation, migration and pluripotency and Sertoli cell proliferation and differentiation [3,5]. In female gonads, Dmrt1 directly regulates Stra8 transcription and is involved in the postnatal formation of ovarian follicles [6].

More recently, it has been suggested that Dmrt1 is involved in maintaining male fate in mammals after birth, suppressing female gonad development pathways in the male gonad by antagonising Foxl2 [6,7]. This represents a new and important function for Dmrt1 in the male gonads after birth.

In humans, the deletion of DMRT1 has been rarely associated with the presence of isolated gonadal dysgenesis in 46,XY DSD patients [8,9].

The FGF9 (Fibroblast Growth Factor 9) gene, a member of the fibroblast growth factor family, has been considered a critical factor in early testicular development and germ cell survival in mice [4].
Recently, a new model of gene interaction was proposed to explain the activities of the Sox9/Fgf9 versus Wnt4/Rspo1 genes in gonadal development. In this model, Sox9 up-regulates Fgf9 expression, which maintains Fgf9 and Sox9 expression through a positive feedback mechanism. In this situation, the balance between Fgf9 and Wnt4 signals favours Fgf9, establishing the male pathway. Both Sox9 and Fgf9 knockout XY mice show sex reversal as a consequence of the inhibition of masculinizing events in the embryonic gonad [10]. Fgf9 mutant mice die soon after birth due to lung defects, but the XY embryos also had abnormalities in gonadal development similar to those observed in patients with 46,XY gonadal dysgenesis [11].

We screened for the presence of DMRT1 and FGF9 mutations in a large cohort of patients with 46,XY gonadal dysgenesis to evaluate the possible disruption of their function in these patients.

2. Materials and methods

2.1. Patients

This study was approved by the Ethics Committee of the Hospital das Clinicas da Faculdade de Medicina da Universidade de Sao Paulo, and written informed consent was obtained from all patients or their legal guardians.

Thirty-three patients with 46,XY disorder of sex development (DSD) due to abnormal gonadal development who were receiving treatment at the Unit of Endocrinology of the Development of Hospital das Clinicas were studied. Eleven patients presented complete gonadal dysgenesis (CGD), and 22 patients presented partial gonadal dysgenesis (PGD).

Subjects with mutations in the DAX1 or WNT4 genes were excluded from the study.

2.2. Molecular analysis

Genomic DNA was extracted from peripheral blood leukocytes using the proteinase K-SDS salting-out method [12].

2.3. MLPA (Multiplex ligation probe amplification)

MLPA analysis of DMRT1 was carried out using the commercial P334-A1 Gonadal Development Disorder Kit (MCR Holland, Amsterdam, The Netherlands). This kit contains 10 probes for the DMRT1 gene (2 probes for each of the 5 exons of DMRT1) in addition to probes for the CYP17A, SRD5A2 and HSD17b3 genes and control probes.

MLPA analysis of FGF9 was carried out using in-house FGF9 probes partially covering the coding region of the gene and four control synthetic probes (GABRA4, MPDZ, RB1 and UHRF2, located at chromosomes 4p12, 9p23, 13q14.2 and 9p24.1, respectively) [13–15]. The MLPA reactions were carried out using the in-house probe set and the reagents from P231 reagent kit. The MLPA probe sequences are described in Table 1. The results were analysed using Genescan 3.7 and further evaluated using Excel (Microsoft).

2.4. DMRT1 and FGF9 direct sequencing

The entire DMRT1 (NM_021951.2/ENSG00000137090) and FGF9 (NM_002010/ENST00000382353) coding region and the exon—intron boundary areas were PCR-amplified using specific primers. The primers used for DMRT1 were previously described by Vinci et al. [16], and FGF9 primers were designed using the Primer3 program (http://frodo.wi.mi.edu).

The PCR products were purified with “ExoSAP-IT” (GE Healthcare Life Sciences, Buckinghamshire, UK) and sequenced using the standard dideoxy chain termination method with the ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit 3.1 (Life Technologies, Foster City, CA, USA). Control DNA samples were obtained from 89 fertile males.

Variant sequences of the FGF9 3' UTR, (GA)3(AG)2(GA)7 and (TG)nTA(TG)nTA(TG)n, which were previously described by Chen et al., were screened for polymorphic microsatellite alleles. The region from c.*127 to c.*395 (from 932 to 1199 bp relative to the transcription start site of FGF9), was amplified and sequenced as described by Chen et al. [17].

2.5. In silico analysis

The DMRT1 variants c.774G>c and c.968-15insTCTCTC were analysed in silico using the Human Splicing Finder prediction program (HSF; http://www.umd.be/HSF/).

2.6. Statistical analysis

The frequencies of allelic variants of DMRT1 and microsatellite motifs in the 3' UTR of FGF9 were compared between controls and the study group using the Chi-square test for categorical samples. A p value <0.05 was considered statistically significant. All statistical analyses were performed with SigmaStat for Windows (version 2.03; SPSS, Inc., San Rafael, CA, USA). Allele frequency analysis

Table 1

<table>
<thead>
<tr>
<th>Probes</th>
<th>Size (bp)</th>
<th>Probe 5'</th>
<th>Probe 3'</th>
<th>UCSC position</th>
<th>Chr Region</th>
</tr>
</thead>
<tbody>
<tr>
<td>GABRA</td>
<td>84</td>
<td>CACGCTTGTCATAAACCCTG</td>
<td>ACCAAGCTTCCAGGATCCGG</td>
<td>46994861–46994902</td>
<td>4p12</td>
</tr>
<tr>
<td>FGF9 – Exon2</td>
<td>94</td>
<td>CTCCCTTACGGAATGTTGAG</td>
<td>CAGGATCGCTGCTTGGGAT</td>
<td>22246056–22246114</td>
<td>13q11.2-q12</td>
</tr>
<tr>
<td>FGF9 – Exon 1</td>
<td>101</td>
<td>ACTGCACTCTACCCGCTGAGGAG</td>
<td>AGGCCGGCTGCTGCTACTAAG</td>
<td>22255237–22255288</td>
<td>13q11.2-q12</td>
</tr>
<tr>
<td>FGF9 – Exon 3</td>
<td>112</td>
<td>ACCATATAAAGGAGGTGAGCCGAGCGC</td>
<td>ATGTGCTTACTAAAATAGG</td>
<td>22273599–22273546</td>
<td>13q11.2-q12</td>
</tr>
<tr>
<td>MPDZ</td>
<td>123</td>
<td>GTGATGTCAGCTCTGTCGATAG</td>
<td>ATATACCCCGATCTCTCAGAAG</td>
<td>13176317–13176397</td>
<td>9p23</td>
</tr>
<tr>
<td>RB1</td>
<td>129</td>
<td>GTCCGCAATCTACCTCTGGCGCGAG</td>
<td>AGGCGAGATCTTATATGC</td>
<td>49039352–49039438</td>
<td>13q14.2</td>
</tr>
<tr>
<td>UHRF2</td>
<td>132</td>
<td>CAGGAGAGAGAGAGAGAGAGAGAGAGA</td>
<td>CTGTTGCAATGCGGCTGAC</td>
<td>6482011–6482100</td>
<td>9p24.1</td>
</tr>
</tbody>
</table>

* The 5' probe is always preceded by a universal primer attached to the sequence (GGGTCCTCAAGGGTTGGA).
* The 3' probe is always nested in a universal primer attached to the sequence (TCTAGATGCTTGGCAGC), and its 5' end is phosphorylated.
* UCSC assembly: February 2009. The numbers indicate the coordinate of the first base of the 5' probe and the last base of the 3' probe. Chr, chromosome.
showed that all polymorphisms of DMRT1 were in Hardy–Weinberg equilibrium.

3. Results

3.1. DMRT1 and FGF9 gene dosage imbalance

No DMRT1 and FGF9 deletions or amplifications were identified by the MLPA technique in the 33 patients studied.

3.2. DMRT1 and FGF9 allelic variants

Eight allelic variants of DMRT1 were identified, seven of them had been previously described (Ensembl and NCBI) (Table 2). The effects of the DMRT1 variants c.968-15insTCTCTCT and c.774G>C (rs146975077) on splice site selection were analysed in silico using the web-based program HSF.

The HSF analysis suggested that the novel variant c.968-15insTCTCTCT, located in intron 4, may be able to cause the insertion of a mutant silencer motif sequence, which could result in a premature termination codon and a truncated protein (Fig. 1). This DMRT1 variant was found in 6% of the patient alleles and in 12% of the control alleles (Table 3).

A single copy of the c.774G>C (rs146975077) DMRT1 variant was identified in one patient with 46,XY partial GD and was not found in control subjects. The analysis of the c.774G>C variant using HSF suggested that this change might create a novel site in the Exonic Splicing Enhancer (ESE) region, with a potentially deleterious effect on the DMRT1 protein. This DMRT1 variant was reported to have a prevalence of 2.8% in the African population in the 1000Genomes database.

The c.*11_12insT (rs11290071) variant was identified in 9 alleles of 33 patients with 46,XY GD (13.5%) and in 12 alleles of the 89 controls 6.7% (Table 3).

Nine allelic variants of FGF9, all previously described (Ensembl and NCBI), were identified in this group of patients with 46,XY gonadal dysgenesis (Table 2).

The microsatellite motif in the 3' UTR is composed of two repeat sequences, (GA)3(AG)2(GA)7 and (TG)3TA(TG)13, in the 3'-untranslated region of the DMRT1 mRNA. The first repeat sequence was not polymorphic in our cohort, while six different alleles (TG)10−18 of the second repeat sequence were found (c.*275TG). We analysed the distribution of the polymorphic alleles between patients with 46,XY gonadal dysgenesis and control males (Table 4).

Recent insights into the mechanisms involved in the transformation of undifferentiated gonadal tissue to organs with specialised functions have revealed several essential genes in this process: Sry/Dmrt1, Sox9, Fgf9/Pdgf2 and Wnt4/Rspo1.

The Dmrt1 gene is a very important factor in inducing male sex determination in invertebrates and vertebrates. Dmrt1 might function as the first signal of the male gonadal cascade in animals whose sex determination is dependent on environmental factors. Moreover, in male mice, the involvement of Dmrt1 in early male gonadogenesis has been well demonstrated in knockout animals [18,19].

However, the role of DMRT1 in testicular development in humans has not yet been completely elucidated. Although comparative genomic hybridization (CGH) and array CGH techniques, powerful tools for the detection of minor chromosomal imbalances, have been used to investigate 46,XY GD patients, DMRT1 deletion has rarely been identified in patients without syndromic features [9,20].

In this cohort of 46,XY patients with abnormal gonadal development, the absence of DMRT1 dosage imbalances was identified by MLPA analysis, confirming the previous reports that submicroscopic deletions of DMRT1 are uncommon in non-syndromic 46,XY GD patients.

Several DMRT1 polymorphisms have been described, and some of these polymorphisms have been associated with an increased risk of testicular germ cell tumour development in humans [21,22].

Although the HSF analysis had suggested that the novel c.968-15insTCTCTCT variant and the c.774G>C (rs146975077) variant had potentially deleterious effects on the DMRT1 protein, the distribution of the c.774G>C (rs146975077) allelic variant did not show significant differences between 46,XY GD patients and controls (Table 3).

Table 2

<table>
<thead>
<tr>
<th>Allelic variants in DMRT1 and FGF9 found in a cohort of 33 patients with 46,XY DSD.</th>
<th>Frequency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMRT1</td>
<td></td>
</tr>
<tr>
<td>c.14C&gt;T</td>
<td>12.1</td>
</tr>
<tr>
<td>c.133A&gt;T</td>
<td>12.1</td>
</tr>
<tr>
<td>c.663T&gt;C</td>
<td>4.5</td>
</tr>
<tr>
<td>c.774G&gt;C</td>
<td>1.5</td>
</tr>
<tr>
<td>c.783C&gt;T</td>
<td>1.5</td>
</tr>
<tr>
<td>c.968-15insTCTCT</td>
<td>6.06</td>
</tr>
<tr>
<td>c.1037A&gt;C</td>
<td>1.5</td>
</tr>
<tr>
<td>c.*11_12insT</td>
<td>13.6</td>
</tr>
<tr>
<td>FGF9</td>
<td></td>
</tr>
<tr>
<td>c.125_124 ins TT</td>
<td>27.3</td>
</tr>
<tr>
<td>c.124 ins T</td>
<td>39.4</td>
</tr>
<tr>
<td>c.278-28 T</td>
<td>18.2</td>
</tr>
<tr>
<td>c.278-14 C-T</td>
<td>45.4</td>
</tr>
<tr>
<td>c.327C&gt;T-G109C</td>
<td>27.3</td>
</tr>
<tr>
<td>c.309C&gt;T-G103G</td>
<td>3.0</td>
</tr>
<tr>
<td>c.381+17C&gt;T</td>
<td>2.2</td>
</tr>
<tr>
<td>c.447A&gt;G-S149S</td>
<td>75.7</td>
</tr>
<tr>
<td>c.*9G&gt;A</td>
<td>96.9</td>
</tr>
</tbody>
</table>

nv, Novel variant.

Table 3

<table>
<thead>
<tr>
<th>Allele</th>
<th>Patients (n = 33)</th>
<th>Control group (n = 89)</th>
<th>Chi-square</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>c.14C&gt;T</td>
<td>8</td>
<td>36</td>
<td>1.626</td>
<td>0.202</td>
</tr>
<tr>
<td>c.133A&gt;T</td>
<td>8</td>
<td>36</td>
<td>1.626</td>
<td>0.202</td>
</tr>
<tr>
<td>c.663T&gt;C</td>
<td>3</td>
<td>2</td>
<td>1.363</td>
<td>0.241</td>
</tr>
<tr>
<td>c.774G&gt;C</td>
<td>1</td>
<td>0</td>
<td>0.271</td>
<td>0.602</td>
</tr>
<tr>
<td>c.783C&gt;T</td>
<td>1</td>
<td>2</td>
<td>0.166</td>
<td>0.684</td>
</tr>
<tr>
<td>c.968-15insTCTCTCT</td>
<td>4</td>
<td>22</td>
<td>1.400</td>
<td>0.237</td>
</tr>
<tr>
<td>c.1037A&gt;G</td>
<td>1</td>
<td>2</td>
<td>0.166</td>
<td>0.684</td>
</tr>
<tr>
<td>(c.*11_12insT)T</td>
<td>9</td>
<td>12</td>
<td>2.099</td>
<td>0.147</td>
</tr>
</tbody>
</table>

Fig. 1. Chromatogram of the novel variant c.968-15insTCTCTCT in the DMRT1 gene. The beginning of 5' exon is indicated by black arrow.
The c.11L→12InsT variant, previously identified in a 46,XY partial GD patient by Mello et al. [23], was found in this cohort of patients (9 of 33 patients) but also in the control subjects (12 of 89 individuals). Statistical analysis indicated that this variant was not significantly more prevalent in the patient group (Table 3).

Although the role of Fgf9 in male gonadal development has been well established in animal models, few studies in humans are available. Until now, the only mutation identified in Fgf9 was p.Ser99Asn, which was identified in several members of a five-generation Chinese family with multiple synostosis syndrome. However, neither the genetic characteristics nor the gonadal function of the 15 affected members of that family (six 46,XX and nine 46,XY) were described [24].

In addition, several FGF9 polymorphisms are described on the Ensembl database (www.ensembl.org). In our cohort of 33 patients with non-syndromic 46,XY GD, we identified nine allelic variants with frequencies similar to those found in previous reports. In 2007, Chen et al. studied the coding and untranslated regions of FGF9 and identified microsatellites in the 3′ UTR of FGF9, the (TG)n motif. They identified four different alleles, (TG13–16) (c.275TG)n. The (TG)n motif was identified in their patients with 46,XY DSD of different aetiologies and rarely in controls (P = 0.01). Functional studies demonstrated that the (TG)14 allele had the greatest promotor activity in luciferase expression assays and also longer mRNA stability than the other alleles [17].

Based on these findings, the authors suggested that the 3′ UTR FGF9 microsatellite (TG)14 allele is a functional polymorphism that may influence the risk of human 46,XY DSD by altering the regulation of FGF9 expression both pre- and post-transcriptionally.

Our study of the 3′ UTR microsatellite region of FGF9 in a cohort consisting exclusively of patients with 46,XY GD did not confirm the previously reported results. Six different alleles (TG)13–18 (c.275TG)n in the 3′ UTR of FGF9 were identified in our patients, but none of them, including the (TG)14 allele, was statistically associated with the presence of gonadal dysgenesis (Table 4). The heterogeneous 46,XY DSD aetiologies of the patients described by Chen et al. may have contributed to the difference in results.

The mechanisms responsible for the regulation of FGF9 expression are poorly understood. An adenylate/uridylate-rich element (ARE) in the FGF9 3′ UTR was recently shown to be crucial in the control of FGF9 expression [25]. Mutations in these regulatory elements might cause a reduction in FGF9 expression and secondarily might affect normal male gonadal development. The effects of specific regulatory elements on FGF9 expression and their possible consequences for testicular development must be better evaluated.

In conclusion, this study suggests that complete or partial deletions and inactivating mutations of DMRT1 and FGF9 are not frequently found in 46,XY DSD patients with abnormal gonadal development. Moreover, the DMRT1 and FGF9 allelic variants identified in this cohort of patients had apparently no association with gonadal dysgenesis. Whole genome, exome sequencing and array comparative genomic hybridization analysis could be useful tools for the molecular diagnosis of XY GD by the identification of potential candidate genes, and elucidation of the real role of DMRT1 and FGF9 in male sexual development.

Conflict of interest

All authors declare that there is no conflict of interest in relation to this article.

Acknowledgments

This work was supported by grants from Fundacao de Amparo a Pesquisa do Estado de Sao Paulo – FAPESP (08/55952-8) and FAPESP (09/03872-3), Centro Nacional de Pesquisa – CNPq (483416/2009-6) and Pq/CNPQ (305743/2011-2). We would like to thank Gustavo Vieira Rubim and Prof. Dr. Alexander Augusto Lima Jorge for their help in the statistical analysis of this work.

References


Table 4

Allelic distribution of FGF9 3′ UTR polymorphic microsatellites.

<table>
<thead>
<tr>
<th>Allele</th>
<th>Patients (n = 33)</th>
<th>Control group (n = 89)</th>
<th>Chi-square</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>TG13</td>
<td>0</td>
<td>4</td>
<td>0.436</td>
<td>0.509</td>
</tr>
<tr>
<td>TG14</td>
<td>28</td>
<td>83</td>
<td>0.195</td>
<td>0.659</td>
</tr>
<tr>
<td>TG15</td>
<td>30</td>
<td>86</td>
<td>0.064</td>
<td>0.800</td>
</tr>
<tr>
<td>TG16</td>
<td>4</td>
<td>4</td>
<td>1.169</td>
<td>0.280</td>
</tr>
<tr>
<td>TG17</td>
<td>3</td>
<td>3</td>
<td>2.590</td>
<td>0.108</td>
</tr>
<tr>
<td>TG18</td>
<td>1</td>
<td>0</td>
<td>0.268</td>
<td>0.605</td>
</tr>
</tbody>
</table>

Variants near DMRT1, TERT and ATF7IP are associated with testicular germ cell cancer, Nature Genetics 42 (2010) 604–607.

A second independent locus within DMRT1 is associated with testicular germ cell tumor susceptibility, Human Molecular Genetics 20 (2011) 3109–3117.


Multiple synostoses syndrome is due to a missense mutation in exon 2 of FGF9 gene, American Journal of Human Genetics 85 (2009) 53–63.

AUF1 p42 isoform selectively controls both steady-state and PGE2-induced FGF9 mRNA decay, Nucleic Acids Research (2010).