

# Mutations in the *SRY*, *DAX1*, *SF1* and *WNT4* genes in Brazilian sex-reversed patients

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## Abstract

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In most mammals, male development is triggered by the transient expression of the *SRY* gene, which initiates a cascade of gene interactions ultimately leading to the formation of a testis from the indifferent fetal gonad. Mutation studies have identified several genes essential for early gonadal development. We report here a molecular study of the *SRY*, *DAX1*, *SF1* and *WNT4* genes, mainly involved in sexual determination, in Brazilian 46,XX and 46,XY sex-reversed patients. The group of 46,XX sex-reversed patients consisted of thirteen 46,XX true hermaphrodites and four 46,XX males, and was examined for the presence of the *SRY* gene and for the loss of function (inactivating mutations and deletions) of *DAX1* and *WNT4* genes. In the second group consisting of thirty-three 46,XY sex-reversed patients we investigated the presence of inactivating mutations in the *SRY* and *SF1* genes as well as the overexpression (duplication) of the *DAX1* and *WNT4* genes. The *SRY* gene was present in two 46,XX male patients and in none of the true hermaphrodites. Only one mutation, located outside homeobox domain of the 5' region of the HMG box of *SRY* (S18N), was identified in a patient with 46,XY sex reversal. A novel 8-bp microdeletion of the *SF1* gene was identified in a 46,XY sex-reversed patient without adrenal insufficiency. The dosage of *DAX1* and *WNT4* was normal in the sex-reversed patients studied. We conclude that these genes are rarely involved in the etiology of male gonadal development in sex-reversed patients, a fact suggesting the presence of other genes in the sex determination cascade.

### Key words

- Sex reversal
- *SRY* gene
- *DAX1* gene
- *SF1* gene
- *WNT4* gene

## Introduction

The sex-determining gene region of the Y chromosome (*SRY*), located on the short arm of the Y chromosome, has been shown to be the testis-determining factor (1). Genetic evidence supporting this identification came from the observation of 46,XX sex-reversed patients who are carriers of *SRY* transloca-

tions from the Y to the X chromosome and of 46,XY sex-reversed patients who harbor mutations in the *SRY* gene (2,3). However, the occurrence of sex reversal cannot be explained by *SRY* alterations in most of the affected patients. These findings suggest that both "gain of function" and "loss of function" mutations in other genes in the sex determination cascade may cause sex rever-

sal. Another possibility is that the dosage of critical genes may also affect sex determination. Several autosomal genes (*WT1*, *SF1*, *SOX9*, *WNT4*, *DMRT1-DMRT2*) and sex-linked genes (*DAX1*, *ATRX*) interact in the process of sex determination, but their exact mechanisms of action are not completely understood (4,5).

The effect of gene dosage on the process of sex determination has been suggested in almost all of its steps. In humans, 46,XX sex reversal is determined by the duplication (overexpression) of *SOX9* and by the presence of the *SRY* gene (6,7). Duplication of the *DAX1* and *WNT4* genes, as well as haploinsufficiency of the *SOX9*, *SF1*, *WT1* and *DMRT1-DMRT2* genes, have been considered responsible for the development of 46,XY sex reversal (8-14).

We report here the results of the study of genes *SRY*, *DAX1*, *SF1* and *WNT4* in Brazilian 46,XX (N = 15) and 46,XY (N = 33) sex-reversed patients. Informed consent was obtained from all patients studied according to the criteria established by the Ethics Committee of the Hospital das Clínicas, Faculdade de Medicina, Universidade de São Paulo, São Paulo, SP, Brasil.

### ***SRY* gene**

The sex-determining region of the Y chromosome (*SRY*) is located in the distal region of the short arm of the Y chromosome and is responsible for initiating male sexual determination (15). It consists of a single exon with a central conserved motif, termed HMG box, and has DNA-binding and -bending activities suggesting that *SRY* functions as a transcriptional regulator. Mutations in *SRY* or its flanking regions were identified in several cases of 46,XY sex-reversed patients (complete gonadal dysgenesis, CGD, and partial gonadal dysgenesis, PGD). The frequency of *SRY* mutations in 46,XY sex-reversed patients was estimated to be 20% and almost of them induce a CGD (16). We

evaluated 7 cases of CGD, 17 of PGD and 9 of embryonic testicular regression syndrome. In a patient with PGD we identified a point mutation at codon 18 (S18N) upstream of the 5' border outside the HMG box of the *SRY* gene. This variant sequence was also found in his father and in his normal brother (17). Cases of familial mutations associated with normal fertile males and 46,XY females have been reported (18,19). Incomplete penetrance may be caused by the genetic background, a threshold effect due to altered biochemical properties of the *SRY* protein. Among these fathers the presence of undetected gonadal mosaicism was determined in rare cases.

The observation of 46,XX sex-reversed patients revealed that some of them carried *SRY* translocations from the Y to the X chromosome (3,20). XX males can be classified as Y-positive and Y-negative depending on the presence or absence of Y-derived specific sequences. Molecular analyses have demonstrated that approximately 90% of these patients carry a variable amount of Y material, including *SRY*, due to a Y-to-X interchange originated by an illegitimate recombination during paternal meiosis. In XX males with Y-to-X translocation preferential inactivation of the Y-bearing X chromosome could be the major mechanism causing a sexually ambiguous phenotype (21). Approximately 10% of XX males have no detectable *SRY* or other Y-sequences. The majority of these patients have ambiguous external genitalia of variable degrees, although a complete male phenotype could be observed. To explain testicular development in the absence of *SRY*, it has been proposed that a mutation at a normally inactive locus downstream of the *SRY*, autosomal or X-linked, may confer a gain of function activity to this gene for initiating the testis development pathway. In our experience, the *SRY* gene was present in two of four 46,XX males studied. These two patients presented normal male external genitalia. Only one patient

presented ambiguous external genitalia and was *SRY* negative (22).

The majority of XX true hermaphrodites have no cytogenetic evidence for mosaicism, chimerism or translocated Y chromosome sequences and molecular studies have shown that *SRY* and other Y-specific sequences are absent in the great majority of them (20). *SRY* was not identified in any of the eleven 46,XX true hermaphrodites of our group (22).

Thus, the *SRY* gene is an important factor in the cascade of genetics events necessary for male sex determination, but alone it cannot explain the majority of the sex-reversed cases.

### **Dosage sensitive sex reversal locus *DAX1* gene**

It has been suggested that *SRY* represses a negative regulator that normally inhibits testicular development (23). When duplicated, this negative regulator gene may escape *SRY* inactivation resulting in absent or incomplete testis development. Supporting this hypothesis, the duplication of the dosage sensitive sex (DSS) reversal locus, a 160-kb region of Xp21.3, was identified in XY sex-reversed patients (10). The analysis of this locus identified the gene named *DAX1* (DSS reversal, adrenal hypoplasia congenita on the X chromosome, gene 1) that encodes a transcriptional repressor factor (24). It has been speculated that the duplication of the *DAX1* gene is the cause of gonadal dysgenesis in XY sex-reversed patients in whom the DDS duplication was identified. On the other hand, the presence of loss of function of this gene may prevent the repressor effect on the masculinizing genes and thus determine testicular development in XX individuals.

*DAX1* is expressed in adrenal cortex, gonads, hypothalamus and pituitary (25). It has been speculated that *DAX1* might be such a gene (26). To investigate the role of *DAX1*

dosage in the induction of abnormal gonadal development in patients with sex reversal, we searched for the presence of inactivating mutations in *DAX1*, as well as deletions or extra copies of this gene in patients in whom the *SRY* gene was not implicated in the etiology of the disease.

We screened eleven 46,XX true hermaphrodites and two 46,XX males for the presence of inactivating mutations of the *DAX1* gene and detected no mutations in any of them.

*DAX1* dosage was determined by the Southern blotting technique with a *DAX1*-specific probe obtained from a PCR product compared to a P450c1alpha probe as an internal control. We analyzed the ratio between the signals of the two probes and compared the values obtained between normal controls and patients. Eleven 46,XX sex-reversed patients were studied to determine the presence of deletions of the *DAX1* gene.

The results obtained for the patients were similar to those for normal female controls, suggesting the absence of *DAX1* deletion in this group. Since no inactivating mutations or deletions of *DAX1* were identified in these patients, loss of *DAX1* function seems not to be involved in the molecular etiology of male gonadal development in these *SRY*-negative 46,XX sex-reversed patients. In addition, twenty-one 46,XY sex-reversed patients were studied to determine the presence of duplications of the *DAX1* gene and the results obtained demonstrated the absence of *DAX1* duplication in this group of patients, ruling out the presence of extra *DAX1* copies as the cause of dysgenetic gonads in these patients. Although there is evidence in the literature suggesting that excess expression of *DAX1* determines the abnormal development of male gonads, no patient with restricted *DAX1* duplication has been described thus far (27). These data indicate that genetic mechanisms related to the development of dysgenetic gonadal tis-

sues in patients with DSS locus duplication may be more complex than a simple dosage effect of *DAX1*.

### **SF1 gene**

The orphan nuclear receptor steroidogenic factor 1 (*SF1* or *NR5A1*) is a key regulator of endocrine function within the hypothalamus, pituitary and gonadal axis and adrenal cortex (28). Disruption of the mouse *Nr5a1* gene encoding *Sf1* causes adrenal and gonadal agenesis, XY sex reversal, structural abnormalities of the ventromedial hypothalamic nucleus and altered gonadotropin expression by the pituitary gonadotrope. Only three mutations in *SF1* have been described in humans (12,29,30). In all of these patients, the *SF1* mutation caused adrenal insufficiency, and 46,XY sex reversal was detected in two of them. We searched for inactivating mutations in the *SF1* gene in thirty-two 46,XY sex-reversed patients without adrenal insufficiency. We identified a novel 8-bp microdeletion of the *SF1* gene in a 46,XY Brazilian patient with ambiguous genitalia, embryonic testicular regression syndrome and unexpected normal adrenal function. This microdeletion resulted in a premature stop codon at the 378 position determining a loss of function of the SF1 protein.

### **WNT4 gene**

*WNT4* is a member of a large family of WNT signaling glycoprotein molecules. It is involved in gonadal development and hence is also expressed in the developing mesonephros (31). Mouse knockout models have illustrated the primary role of *Wnt4* in female development (14). Mutants of both sexes fail to develop Müllerian ducts, and Wolffian ducts are stabilized in the female null mutant because of differentiation of Leydig-like interstitial cells in the ovary. *Wnt4* appears to prevent Leydig cell differentiation in the ovary. The expression of *Wnt4* is similar to

*Dax1*, suggesting that *Wnt4* may function in a similar manner as an anti-testis gene. A 46,XY sex-reversed patient with duplication of 1p31-p35, in whom *WNT4* was overexpressed (32) was reported, suggesting an effect of *WNT4* gene dosage on the steps of human sex determination. It was proposed that the mechanism of sex reversal was via *WNT4* induced up-regulation of *DAX1*.

To investigate the role of *WNT4* dosage in the induction of abnormal gonadal development in patients with sex reversal, we searched for the presence of inactivating mutations in *WNT4* as well as deletions or extra copies of this gene in sex-reversed patients. We screened for the presence of inactivating mutations of the *WNT4* gene in eleven 46,XX true hermaphrodites and two 46,XX males and detected no mutations in any of these patients.

The search for *WNT4* dosage was performed using the Southern blotting technique with a *WNT4*-specific probe obtained from a PCR product compared with a *DAX1* probe, as an internal control. We analyzed the ratio between the signals of the two probes and compared the values obtained for the normal controls and patients. We studied thirteen 46,XX sex-reversed patients to determine the presence of deletions of the *WNT4* gene and twenty-one 46,XY sex-reversed patients to determine the presence of duplications of this gene. The results obtained for the patients were similar to those obtained for normal controls, suggesting the absence of abnormal *WNT4* dosage in both groups of patients (Domenice S, Corrêa RV and Mendonca BB, unpublished data). No inactivating mutations of *WNT4* were identified in 46,XX patients (Domenice S, Corrêa RV and Mendonca BB, unpublished data). These results demonstrate the absence of abnormalities of the *WNT4* gene in these sex-reversed patients.

Over the past few years, considerable progress has been made in the molecular characterization of sex reversal disorders by

using a combination of strategies such as cell biology and animal models and especially by studying patients with these disorders. The majority of the key regulatory steps in fetal sex development relevant to clinical investigation were studied here and they may be responsible for a minority of the sex-reversed cases. Although genetic analyses have suggested that gene dosage appears to be important in human sexual development, we did not identify any abnormality in two important genes, *DAX1* and *WNT4*, related to the development of dysgenetic gonads.

We conclude that the *SRY*, *DAX1*, *SF1* and *WNT4* genes are rarely involved in the etiology of male gonadal development in sex-reversed patients (Table 1), a fact suggesting the involvement of other genes in the sexual determination cascade.

Table 1. Molecular findings of *SRY*, *DAX1*, *SF1* and *WNT4* genes in Brazilian sex-reversed patients.

Sex reversed	Gene	Molecular finding	Patients*	Diagnosis
46,XX	<i>SRY</i>	<i>SRY</i> +	2/15	XX male
	<i>DAX1</i>	No mutations	0/13	-
		Normal gene dosage		
	<i>SF1</i>	Not studied	-	-
	<i>WNT4</i>	No mutations	0/13	-
		Normal gene dosage		
46,XY	<i>SRY</i>	S18N	1/33	PGD
	<i>DAX1</i>	Not studied	-	-
	<i>SF1</i>	Del 8nt 1058-1065	1/32	ETRS
	<i>WNT4</i>	Not studied	-	-

ETRS = embryonic testicular regression syndrome; PGD = partial gonadal dysgenesis.

\*Number of patients with molecular alterations/total number of studied patients.

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