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# The novel p.E89K mutation in the *SRY* gene inhibits DNA binding and causes the 46,XY disorder of sex development

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## Abstract

Male sex determination in humans is controlled by the *SRY* gene, which encodes a transcriptional regulator containing a conserved high mobility group box domain (HMG-box) required for DNA binding. Mutations in the *SRY* HMG-box affect protein function, causing sex reversal phenotypes. In the present study, we describe a 19-year-old female presenting 46,XY karyotype with hypogonadism and primary amenorrhea that led to the diagnosis of 46,XY complete gonadal dysgenesis. The novel p.E89K missense mutation in the *SRY* HMG-box was identified as a *de novo* mutation. Electrophoretic mobility shift assays showed that p.E89K almost completely abolished *SRY* DNA-binding activity, suggesting that it is the cause of *SRY* function impairment. In addition, we report the occurrence of the p.G95R mutation in a 46,XY female with complete gonadal dysgenesis. According to the three-dimensional structure of the human *SRY* HMG-box, the substitution of the conserved glutamic acid residue by the basic lysine at position 89 introduces an extra positive charge adjacent to and between the positively charged residues R86 and K92, important for stabilizing the HMG-box helix 2 with DNA. Thus, we propose that an electrostatic repulsion caused by the proximity of these positive charges could destabilize the tip of helix 2, abrogating DNA interaction.

Key words: Gonadal dysgenesis; HMG-box; Missense mutation; Sex reversal *SRY*; Streak gonads; Testis determination

## Introduction

In humans, as in other mammals, sex determination is controlled by a dominant switch located on the Y-chromosome, the *SRY* gene, also referred to as the sex-determining region on the Y (1,2). The human single copy monoexonic *SRY* gene encodes a 204-amino acid protein that contains a conserved domain formed by 79 residues known as the high mobility group box (HMG-box), which is flanked by two nuclear localization signals (3). The HMG-box characterizes the *SRY* as a transcription regulator with DNA-binding and bending abilities (4-6). Mutations within the *SRY* gene sequence cause male-to-female sex reversal in XY individuals as a result of failure of indifferent gonads to develop into testes, leading to complete XY gonadal dysgenesis (46,XY CGD) (7), included among the 46,XY disorders of sex development (8). Patients with 46,XY CGD present

a normal female phenotype and streak gonads at birth, whereas they will present primary amenorrhea and failure to develop female secondary characteristics at puberty.

The importance of *SRY* function has been indicated by a number of studies describing amino acid changes within this protein in patients with 46,XY CGD (9). To date, up to 68 mutations have been identified within the open reading frame of the *SRY* gene (10). The majority of these mutations are located within the HMG-box, highlighting the critical role of this domain in DNA binding and transcriptional activation (11). Although mutations outside the *SRY* HMG-box domain are rare, they can also cause 46,XY CGD (12,13). Furthermore, a small subset of reports describe familial mutations (13,14), whereas the majority are *de novo* mutations affecting only one individual in a family. In summary,

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a large spectrum of phenotypic variation is observed among patients carrying different *SRY* gene mutations; therefore, phenotype and genotype correlations in patients with XY gonadal dysgenesis are not straightforward.

In the present study, the novel p.E89K and p.G95R mutations within the *SRY* HMG-box were identified in patients with 46,XY CGD. The E89 residue is conserved among the *SRY* proteins and the p.E89K mutation significantly affects the protein DNA-binding activity *in vitro*. Interestingly, both mutations introduce an extra positive charge adjacent to and between the positively charged residues R86 and K92 important for stabilizing the HMG-box helix 2 and DNA complex. The role of the electrostatic repulsion caused by the proximity of those positive charges on DNA interaction is discussed.

## Patients and Methods

### Patients

**Patient 1.** A 19-year-old female was referred to us due to hypogonadism and primary amenorrhea. She was born at term by normal delivery, but her birth weight is unknown. She was the first child of non-consanguineous parents in a sibship of 8 (6 sisters and 1 brother) with unremarkable family history. Upon physical examination, her weight was 42.4 kg and height 158 cm. She had female external genitalia with pubertal development in Tanner stage M1P2, and there was no dysmorphic picture. Cytogenetic analysis revealed a 46,XY karyotype. There were high levels of FSH (164 mIU/mL; normal range (NR): 1.5-12.4 mIU/mL) and LH (31.1 mIU/mL; NR: 1.7-8.6 mIU/mL), and ultrasonography revealed infantile uterus, but no female gonads were visualized. Both dysgenetic gonads were removed and a microscopic gonadoblastoma (2 x 2 mm) was found in the right gonad.

**Patient 2.** A 17-year-old female was referred to us due to hypogonadism and primary amenorrhea. She was born at term by normal delivery to nonconsanguineous parents, with 2.6 kg and 46 cm of birth weight and length, respectively. Her mother had a stillborn girl and a first-trimester miscarriage, followed by an 18-year-old girl and the proposita; family history was unremarkable. On physical examination, her weight was 67.8 kg and height 174.3 cm. She had female external genitalia with pubertal development in Tanner stage M1P3, and no dysmorphic characteristics. Cytogenetic analysis revealed a 46,XY karyotype. There were high levels of FSH (80.1 mIU/mL; NR: 1.5-12.4 mIU/mL) and LH (21.4 mIU/mL; NR: 1.7-8.6 mIU/mL), and ultrasonography revealed infantile uterus, but no female gonads were visualized. The patient was informed about the risk of malignant development of gonadal tissue and was referred to other services in order to perform prophylactic bilateral gonadectomy.

The study was approved by the Ethics Committee of the Universidade Estadual de Campinas, and informed consent was obtained from the patients and controls.

### Methods

Genomic DNA was extracted from peripheral blood by standard techniques (15). PCR was performed with 0.5 µg genomic DNA, 10% DMSO, 200 µM each deoxyribonucleoside triphosphates, 0.5 µM each primer, 1X Taq DNA polymerase buffer solution (Amersham-GE, USA), and 1.5 U Taq DNA polymerase (Invitrogen, USA). Each PCR contained normal female and male control DNA. The *SRY* open reading frame was amplified as described before (13) and sequenced twice with products from different PCR assays. Both sense and antisense primers were used to confirm sequence results. Amplified *SRY* fragments were purified with Wizard SV Gel and PCR Clean-UP System (Promega, USA) and directly sequenced with the Big Dye Terminator Cycle Sequencing Kit V3.1 Ready Reaction (Applied Biosystems, USA). Wild-type and mutant *SRY* gene samples were amplified by PCR to be cloned into pET28A bacterial expression vector (Novagen, USA) to produce N-terminal His-tagged fusion proteins as described elsewhere (13). Resulting constructs were used to transform BL21 (DE3) competent *Escherichia coli* cells, and the expression of wild-type and mutant *SRY* proteins was induced with 0.1 mM isopropyl-1-thio-beta-D-galactopyranoside (IPTG). Cells were collected and lysed and protein was purified with an Ni-NTA agarose column (Qiagen, Germany). *SRY* proteins were eluted and stored at -70°C. Specific oligonucleotides containing the *SRY*-target DNA sequence were synthesized and subsequently annealed to generate a double-stranded probe (oligoA) as previously described (13,16). The ds-oligonucleotide was labeled with ( $\alpha$ -<sup>32</sup>P) dCTP (Amersham-GE) using the standard Klenow fill-in reaction. The mixtures were incubated in the presence of increasing concentrations of either normal or mutant *SRY* proteins. Electrophoretic mobility shift assays (EMSAs) were carried out on non-denaturing 6% polyacrylamide gels and the shifted bands were visualized on Hiperfilm MP autoradiographies (Amersham-GE).

## Results

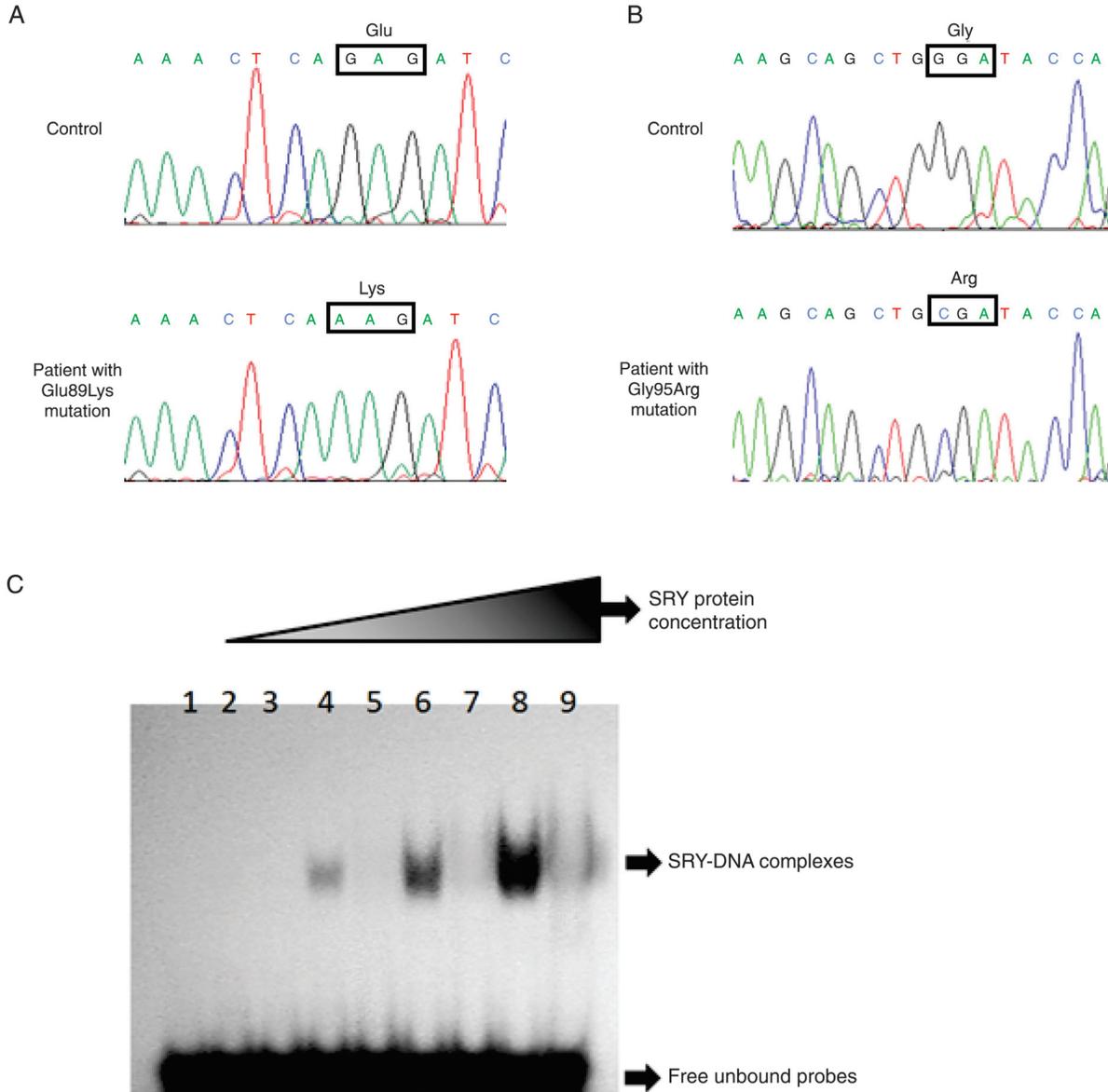
The 380-bp amplified fragment encoding the *SRY* conserved domain was sequenced with 46,XY CGD in both female patients. A hemizygous transition of a guanine (GAG) to an adenine (AAG) in codon 89 was observed in patient 1 (Figure 1A). This novel mutation causes the substitution of a glutamic acid by a lysine (p.E89K) within the HMG-box of the *SRY* gene. Sequencing the whole *SRY* coding region did not show any other nucleotide variation. The patient's father and brother did not present the nucleotide change, suggesting that this mutation had occurred as a *de novo* event. The p.G95R mutation was identified in patient 2 as a result of the GGA>CGA nucleotide change (Figure 1B).

To investigate the effect of the p.E89K mutation on the *SRY* DNA-binding activity, EMSAs were performed with the purified wild-type and mutant proteins. The assay with

increasing protein concentrations showed that the mutant protein was unable to bind to the DNA probe even at high concentrations, as compared to the wild-type protein, indicating that the mutation drastically decreased the SRY DNA-binding activity (Figure 1C).

### Discussion

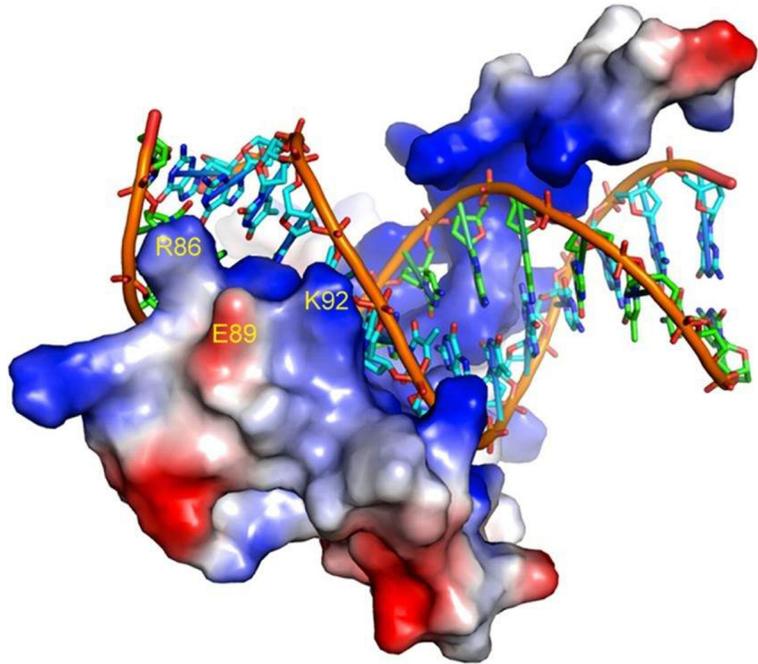
In the present study, we report a case of p.G95R substitution associated with sex reversal and describe a novel mutation (p.E89K) within the SRY HMG-box in patients with



**Figure 1.** Partial electropherograms of SRY gene DNA sequences of normal control and patients with 46,XY complete gonadal dysgenesis are shown. *A*, Patient 1 showing the G>A substitution that leads to a change in the amino acid E89 to K, within the highly conserved DNA-binding motif (HMG-box) of the SRY protein. *B*, Patient 2 showing the G>C substitution that leads to a change in the amino acid G95 to R, within the highly conserved DNA-binding motif (HMG-box) of the SRY protein. *C*, Effect of the p.E89K mutation on the DNA-binding activity of the SRY protein. Electrophoretic mobility shift assays (EMSA) were performed using the purified wild-type and mutant SRY proteins. *Lane 1* corresponds to the <sup>32</sup>P-labeled free probe. *Lanes 2, 4, 6, and 8* correspond to reactions comprising the probe + wild-type SRY protein at increasing concentrations (0.5, 1, 2.5, and 5 µg, respectively). The arrow indicates the SRY-DNA complex. *Lanes 3, 5, 7, and 9* correspond to reactions containing the probe + mutant SRY<sup>E89K</sup> protein at increasing concentrations (0.5, 1, 2.5, and 5 µg, respectively).

46,XY pure gonadal dysgenesis. The p.G95R mutation has already been described in cases of complete XY gonadal dysgenesis and has been shown to affect the DNA-binding activity of the protein (17-19).

The majority of the SRY mutations reported in patients with diseases of sex differentiation occur within the HMG-box domain (20). Not surprisingly, we found that the p.E89K mutation also impaired significantly the *in vitro* DNA-binding activity of the SRY protein. Although the E89 residue does not make contacts with DNA (21), its replacement by a positively charged residue is likely to have caused alterations in the electrostatic net charge of the protein surface, since E89 is located outside the HMG-box helix 2 (21). In addition, it is worth noting that in the 3-D structure of SRY (Protein Data Bank - PDB code 1J46), the side chain of E89 is located adjacent to and between the side chains of residues R86 and K92, both of which stabilize helix 2 through hydrogen bonds with the phosphate groups of DNA (see Ref. 21 and Figure 2). Since in the protein-DNA complex the atomic distances between E89 and the adjacent R86 and K92 side chains are outside the normal range for a strong electrostatic interaction (>4 Å), E89 may play a critical role to hold both R86 and K92 side chains in position to interact with DNA when the protein is not bound to its target sequence. Therefore, it is reasonable to suggest that the insertion of an extra positive charge between R86 and K92 would cause strong electrostatic repulsion among the side chains leading to the destabilization of the tip of helix 2, precluding DNA interaction. The fact that E89 is conserved in SRY protein in various mammalian species (22) is a strong indication that an acidic side chain in this position is important to maintain the protein function. Mutations in the same region of the SRY protein involving residues 90, 91, 94, and 95 also produced proteins with reduced binding activity (18). Accordingly, the G to R change at position 95 reported here also places a strong positive charge in the



**Figure 2.** Representation of the SRY-DNA complex depicting the tip of helix 2 involved in the DNA interaction. The electrostatic surface of SRY shows that the side chain of E89 sits between R86 and K92. The picture was created with the program PyMOL using the 3-D coordinates of the SRY protein (PDB code 1J46) published by Murphy et al. (21).

region adjacently to the K92 side chain. However, in this case, the insertion of an R residue might favor an inadvertent contact with the phosphate group, breaking hydrogen bonds formed by the R86 and K92 side chains with the DNA.

In conclusion, there is a good correlation between *in vitro* and *in vivo* effects involving mutant SRY proteins identified in the present report.

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