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Two new novel point mutations localized upstream and downstream of the HMG box region of the *SRY* gene in three Indian 46,XY females with sex reversal and gonadal tumour formation

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The Y chromosome-specific gene *SRY* is one of the key genes involved in human sex determination. The *SRY* gene encodes a testis-specific transcription factor that plays a key role in sexual differentiation and development in males and is located on the distal region of the short arm of the Y chromosome. Mutations in *SRY* gene result in XY sex reversal and pure gonadal dysgenesis. *SRY* expression initiates a network of gene activity that transforms the undifferentiated gonad, genital ridge into testis. Mutations in the *SRY* gene have been considered to account for only 10–15% of 46,XY gonadal dysgenesis cases, whereas the majority of the remaining cases may have mutation(s) in the *SRY* regulatory elements or other genes involved in the sex differentiation pathway. Patients both with gonadal dysgenesis and Y-chromosome presence are at high risk of developing gonadoblastoma. Using PCR, single strand conformational polymorphism (SSCP) and automated DNA sequencing, we analysed the mutations in the *SRY* gene in three 46,XY sex reversal patients. Two patients demonstrated nucleotide substitution (A → G) within the open reading frame just outside and upstream of the conserved DNA-binding motif called the high-mobility group (HMG) box, replacing glutamine at codon 57 with arginine. Altered SSCP patterns were also observed in these patients. Histological examination of gonads in patient 1 revealed the formation of gonadoblastoma. Patient 3 demonstrated A → T substitution which replaces serine at codon 143 with cysteine, just outside but downstream of the HMG box. Results suggest the involvement of *SRY* gene in sex reversal which further supports the relationship between *SRY* alterations, gonadal dysgenesis and/or primary infertility.

Key words: gonadoblastoma/*SRY* gene/46,XY sex reversal

Introduction

Sex determination is governed and regulated by a series of genetic switches that influence cellular differentiation during critical periods of gonadal development. Interestingly, the primordial fetal gonads are bipotential and gonadal development provides an excellent opportunity to identify genes involved in the differential organogenesis. During mammalian embryogenesis, the presence of the *SRY* gene determines whether the gonads develop as testes, which in turn determines whether the embryo will develop as a male. In the absence of the *SRY* gene, the fetal gonads develop as ovaries (Harley *et al.*, 1992). XY gonadal dysgenesis is the result of an embryogenic testicular regression event, and can occur in a pure or partial form. Pure gonadal dysgenesis is defined as 46,XY individuals with no testes but who have fully developed female-type external genitalia and normal Müllerian structures but only streak gonads. In contrast, patients with partial gonadal dysgenesis are characterized by partial testicular differentiation with genital ambiguity. Gonadal histology illustrates hypoplastic testicular tubules intermixed with ovarian stroma (Marcantonio *et al.*, 1994). These individuals may have unilateral or bilateral dysgenetic gonads and/or streak gonads.

Several genetic loci may play important roles in testis-determining pathways. Male-to-female sex reversal in patients with 46,XY karyotype results from the failure of testis development, which may be due to mutations in the *SRY* gene (Sinclair *et al.*, 1990). This gene is located on the short arm of the Y chromosome close to the pseudo-autosomal boundary. It consists of a single exon with a conserved central motif, termed high-mobility group (HMG) box, and has DNA-binding as well as DNA-bending activities, suggesting that it functions as a transcriptional regulator. This gene has been shown to be essential for initiating testis development and the differentiation of the indifferent and bi-potential gonads into the testicular

pathway (Sinclair *et al.*, 1990; Marx, 1995). In the presence of *SRY*, the supporting cells of the undifferentiated gonad become testicular Sertoli cells, and without *SRY* the supporting cells become ovarian follicular cells (Capel, 2000). This protein has been shown to possess sequence-specific DNA-binding activity and it is assumed to regulate other genes involved in the male determination pathways (Harley *et al.*, 1994). Normal *SRY* changes the architecture of DNA, thus allowing access of other factors needed for its expression (Behlke *et al.*, 1993). *SRY* gene product stimulates a cascade of regulatory events, allowing Leydig cell differentiation from steroid secretory cells, the arrest of mitosis in germ cells and proliferation and organization of connective tissue into a testicular pattern (Castineyra *et al.*, 2000). The HMG box is essential for *SRY* to bind and bend DNA, as well as for transporting the protein into the nucleus (Sinclair, 2001).

Different types of mutations in *SRY* gene have been found to account for ~15% of these cases with gonadal dysgenesis (Cameron and Sinclair, 1997;Margarit *et al.*, 1998; Assumpcao *et al.*, 2002).

Table I. Mutations in the *SRY* gene identified so far

Phenotype	SRY mutation	Familial history
Outside, upstream HMG box		
Partial ovarian function	Gln2CAA → StopTAA	−
Pure GD	Tyr4TAT → StopTAA	−
UTS	Tyr4TAT → 1base del StopTAG	−
Partial GD	Ser18AGT → AsnAAT	+
Pure GD	Arg30AGA → IleATA	+
Pure GD	Lys43AAG → 1 base insertion T Stop TAA	−
Pure GD	Glu57CAG → ArgCGG[a]	−
Within HMG box		
Pure GD	Arg59AGA → GlyGGA	−
Pure GD	Val60GTG → LeuCTG	+
TH	Val60GTG → LeuGCG	−
Pure GD	Arg62CGA → GlyGGA	−
Pure GD	Met64ATG → IleATA	−
Pure GD	Met64ATG → ArgAGG	−
Pure GD	Asn65AAC → HisCAC	−
Pure GD	Phe67TTC → LeuCTC	−
Pure GD	Phe67TTC → ValGTC	+
Pure GD	Ile68ATC → ThrACC	−
Pure GD	Trp70TGG → StopTAG	−
Pure GD	Arg72CGC → GlyGGC	−
Pure GD	Glu74GAG → StopTAG	+
Pure GD	Arg75AGG → AsnAAT	−
Pure GD	Arg76CGC → SerAGC	+
Pure GD	Met78ATG → ThrACG	−
TH	Ala79GCT → AlaGCC	−
Pure GD	Arg86CGA → StopTGA	−
Pure GD	Asn87AAC → TyrTAC	−
Pure GD	Ile90ATC → MetATG	+
Pure GD	Ser91AGC → GlyGGC	+
Pure GD	Lys92AAG → StopTAG	−
Pure GD	Gln93CAG → StopTAG	−
Pure GD	Gly95GGA → ArgCGA	−
Pure GD	Gly95GGA → GluGAA	−
Pure GD	Gly97CAG → StopTAG	+
TH	Leu101CTT → HisCAT	−
Pure GD	Lys106AAA → IleATA	−
Pure GD	Trp107TGG → StopTAG	−
Pure GD	Pro108CCA → 1 base deletion A ProCCT	−
Pure GD	Pro108CCA → ArgCGA	−
Pure GD	Phe109TTC → SerTCC	+
Pure GD	Ala113GCA → ThrACA	−
Pure GD	Arg121AGA → 4 base deltion; frameshift	−
Pure GD	Pro125CCG → leuCTG	+
Pure GD	Tyr127TAT → CysTGT	−
Pure GD	Tyr127TAT → CysTAA	−
Partial GD	Tyr129TAT → AsnAAT	−
Pure GD	Tyr129TAT → StopTAA	−
Pure GD	Pro131CCT → ArgCGT	−
Pure GD	Arg132CGT → GlyGGT	−
Pure GD	Arg133CGG → TrpTGG	−
Partial GD	Lys136AAG → SerAGT	−
Outside, downstream HMG box		
Pure GD	Ser143AGT → CysTGT[a]	−
Pure GD	Gln158CAA → 1 base deletion A; Frameshift	−
Pure GD	Leu163TTG → StopTAG	+

[a]Present cases.
GD = gonadal dysgenesis; TH = true hermaphroditism; UTS = Ullrich–Turner syndrome.

However, the majority of these patients may have mutations in other genes involved in the sex differentiation pathway or in the regulatory elements of *SRY* gene. To date, 51 mutations have been identified within the *SRY* gene open reading frame (Table I), and most of these are located in the HMG box, highlighting the crucial but critical role of this domain (Assumpcao *et al.*, 2002; Fernandez *et al.*, 2002; IMG; http://archive.uwcm.ac.uk/uwcm/mg/hgmd0.html; Zhou *et al.*, 2003). Only 10 mutations lie outside and upstream of the HMG box (eight of which are located in the 5′ region) and the remaining two lie downstream, 3′ of the HMG box (IMG, http://archive.uwcm.ac.uk/uwcm/mg/hgmd0.html; Baldazzi *et al.*, 2003). The analysis of *SRY* status in relation to female infertility has been done in most developed countries, yet to the best of our knowledge so far there are no reports (except one individual reported from India where the mutation lies within the HMG box: McElreavey *et al.*, 1992) of *SRY* gene mutations outside of the HMG box that have been documented in correlation with cytogenetic and biochemical anomalies in infertile female patients (46,XY) with gonadal dysgenesis from developing countries, particularly India where reproductive health problems are still considered as a social stigma.

Here we describe two novel point mutations in the *SRY* gene in three patients with 46,XY gonadal dysgenesis. An attempt has also been made to investigate the relationship between *SRY* mutations and gonadoblastoma formation in these patients. One mutation is found just outside, 5′ upstream of the HMG box and the other outside, 3′ downstream of the HMG box; both of these mutations are associated with variable phenotypes.

Materials and methods

Informed consent was obtained from all patients and control individuals who participated in the present study. This study was approved by the Ethics Committees both of the hospital and university.

Subjects

Patient 1, a 28 year old woman born to healthy unrelated parents, presented with primary amenorrhoea, but was otherwise healthy and had no history of illness. The patient had no dysmorphic features. The external genitalia and secondary sexual characteristics of this patient were like those of normal females. Other features included the presence of Müllerian structure, absence of Wolffian structures and formation of tubules. Pubic and sparse axillary hairs were also present. Pelvic ultrasound revealed streak gonads and presence of Müllerian ducts. Endocrine function studies showed elevated LH (28.4 mIU/ml) and FSH (69.2 mIU/ml). One atypical feature of the proband is the presence of secondary sexual characteristics including normal breast development. Histological examinations of the gonads also revealed proliferation of atypical germ cells admixed with granulomatous chronic inflammation consistent with gonadoblastoma formation.

Patients 2 and 3 were 22 and 20 year old females respectively born to healthy unrelated patients and were referred to us because of primary amenorrhoea. Both these patients had fully developed feminized phenotypes without any sexual ambiguity. Other features included presence of Müllerian structures, absence of Wolffian structures and formation of tubules with sparse axillary hairs. Pubic hair showed characteristic male-like distribution with clitoromegaly without any labial fusion. Pelvic ultrasound revealed the presence of streak gonads and presence of Müllerian ducts. The external genitalia and secondary sexual characteristics were like those of normal females. Endocrine function studies showed elevated LH (22.6 and 24.2 mIU/ml) and FSH (54.0 and 49.7 mIU/ml) respectively. Both patients had not developed any gonadoblastoma at the time of examination.

Three heparinized blood samples were collected from patients at the Department of Obstetrics and Gynaecology, Maulana Azad Medical College and Lok Nayak Hospital, New Delhi-110025 India and transported to the laboratory on ice. All patients and controls who participated in the present study were non-smokers and non-alcoholics.

Cytogenetic analysis

Chromosomal analyses were performed by conventional techniques on phytohaemagglutinin-stimulated peripheral blood culture from the infertile female patients (Husain and Bamezai, 1988). Conventional G-banding techniques were used for chromosome identification.

Hormonal analysis

The hormonal evaluation was conducted by the radioimmunoassay method using kits supplied by BARC, India (for LH) and Hormone Diagnostics, India (FSH).

SRY analysis

Genomic DNA from the blood samples was extracted by digestion with Proteinase K (Boehringer) followed by routine phenol chloroform isolation and precipitation with ethanol or isopropanol and chilled 3 M sodium acetate (pH 5.2). Vacuum-dried DNA samples were dissolved in TE (Tris–EDTA) buffer and DNA concentration was determined by gel electrophoresis. Two sets of oligonucleotide primers (F1 5′-CATGAACGCATTCATCGTGTGGTC-3′; R1 5′-CTGCGGGAAGCAACTGCAATTCTT-3′, and F2 5′-CAGTGTGAAACGGGAGAAAACAGT-3′; R2 5′-GTTGTCCAGTTGCACTTCGCTGCA-3′) were used in the PCR reactions to amplify fragments of 254 and 351 bp respectively from the open reading frame of the *SRY* gene. Exon 5 of the *p53* gene was amplified as an internal control using oligonucleotide primers F5′-TACTCCCCTGCCCTCAACAA-3′ and R5′-CATCGCTATCTGAGCAGCGC-3′ yielding a PCR product of 184 bp. PCR amplification was performed in 25 ml reaction volume containing 10 mmol/l Tris–HCl (pH 8.4), 50 mmol/l KCl, 1.5 mmol/l $MgCl_2$, 200 μmol/l each of dNTP (dATP, dCTP, dGTP, dTTP), 5 pmol of oligonucleotide primers, 100–500 ng of DNA and 0.5 IU Taq DNA polymerase (Perkin–Elmer Cetus, USA). PCR thermal cycling conditions were; a 4 min denaturation period at 95°C and 35 cycles of the following: 95°C for 30 s, 55°C for 30 s and 72°C for 30 s, and a final extension of 7 min at 72°C. Every PCR included negative (normal XX female) and positive (normal XY male) controls. PCR products (7.0 μl) were visualized on 2% agarose gel with ethidium bromide.

Single-strand conformational polymorphism (SSCP)

PCR products were labelled with [α-P^{32}]dCTP by performing an additional 15 cycles of PCR and loaded on 6% non-denaturing polyacrylamide gel containing 5% glycerol. The electrophoresis was carried out overnight at 200 V at 17 ± 1°C. The dried gel was exposed to X-ray film for 48 h at − 70°C (Orita *et al.*, 1989).

Automated DNA sequencing

PCR products showing altered band mobility and/or shift in SSCP patterns were sequenced using an ABI prism 310 automated sequencer. Before sequencing, PCR products were purified using ammonium acetate–ethanol precipitation method. The cycle sequencing of the purified PCR products was performed using BIG Dye terminator sequencing ready reaction mix with AmpliTaq DNA polymerase FS, on GeneAmp PCR 9700. PCR conditions were set as: 96°C × 10 s, 56°C × 5 s and 60°C × 4 min for 25 cycles. After cycle sequencing, extension products were purified to remove any unincorporated dye-labelled terminators using ethanol–sodium acetate precipitation method. Template suppressor reagent was added and samples were heat-denatured, chilled on ice and loaded on the 310 sequencer. The sequences were analysed using sequencing analysis software 3.4.1 on a Mac OS 9.1.

Results

Chromosome analysis from peripheral blood lymphocyte cultures showed a 46,XY karyotype in patients with primary infertility. Alterations of the investigated biochemical parameters were sufficient to exclude the diagnosis of androgen insensitivity. Based on these findings, we set up PCR to amplify the *SRY* gene. PCR products were also scanned for mutations using single strand conformational polymorphism (SSCP) analysis. All three samples showed

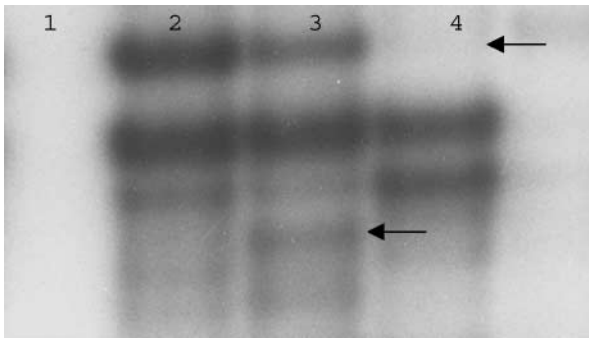

Figure 1. PCR-SSCP analysis of single exon of *SRY* gene in patients with 46,XY, Lanes 1: *SRY* negative control DNA; 2: *SRY* positive control DNA and *arrows* indiacate 3: *SRY* with additional band (patients 1 and 2) and 4: *SRY* with missing band (patient 3) indicating that these patients carry mutations in *SRY* gene.

aberrant migration in the SSCP assay (Figure 1). By direct sequencing of PCR products, we have identified a point mutation in the *SRY* gene in patients 1 and 2. This mutation is an A → G transversion mutation that results in the replacement of glutamine with an arginine residue at amino acid 57 in the open reading frame, just outside and upstream of conserved DNA-binding motif called HMG box (Figures 2A, B and 3). Gonadal examination revealed the presence of gonadoblastoma formation in patient 1. However, the third patient demonstrated A → T transversion mutation which leads to the replacement of the serine residue at codon 143 with a cysteine residue, just outside but downstream of the HMG box sequence (Figures 2C, D and 3). Neither of the other mutations nor nucleotide mosaicisms were found in the amplified products of the *SRY* gene. No nucleotide substitution mutations were found in our preliminary *SRY* sequencing from 25 normal males excluding the possibility of a polymorphism. It cannot be ascertained whether these mutations are *de novo* as paternal DNA was not available for analysis and therefore a parental germ cell mosaicism cannot be excluded.

Discussion

All three patients presented with a 46,XY karyotype and had no evidence of mosaicism. They had elevated FSH and LH levels, well-formed Müllerian structures, no Wolffian structures, streak gonads and fully formed feminized female genital structures. Patient 1 also had gonadoblastoma formation. There is evidence that the *SRY* gene is essential for sex determination (Sinclair *et al.*, 1990). The assignment of *SRY* as the testis-determining factor (TDF) is supported by many studies of human intersex abnormalities (Harley *et al.*, 1992; McElreavey *et al.*, 1992; Affara *et al.*, 1993; Zeng *et al.*, 1993; Bilbao *et al.*, 1996; Brown *et al.*, 1998). All these studies indicate that mutations in the *SRY* gene are associated with gonadal dysgenesis. The *SRY* protein belongs to the *SOX* family of transcription factors, which are further characterized by a HMG domain with DNA-binding and -bending properties, the ability to mediate protein–protein interactions, and containing signals for nuclear import (Südbeck and Scherer, 1997; Wilson and Koopman, 2002). Here, we describe three females with gonadal dysgenesis and mutations in the *SRY* gene at codon Q57R in patients 1 and 2 and S143C in patient 3. Both these mutations lie just outside the highly conserved HMG box. The polar neutral amino acid, glutamine, is replaced with a polar charged arginine (with pK_a of 12.5) in patients 1 and 2. It is possible that with this mutation in place, mutated *SRY* protein may not be able to enter the nucleus to elicit the male gene expression or it may have disrupted the nuclear localization signal necessary to

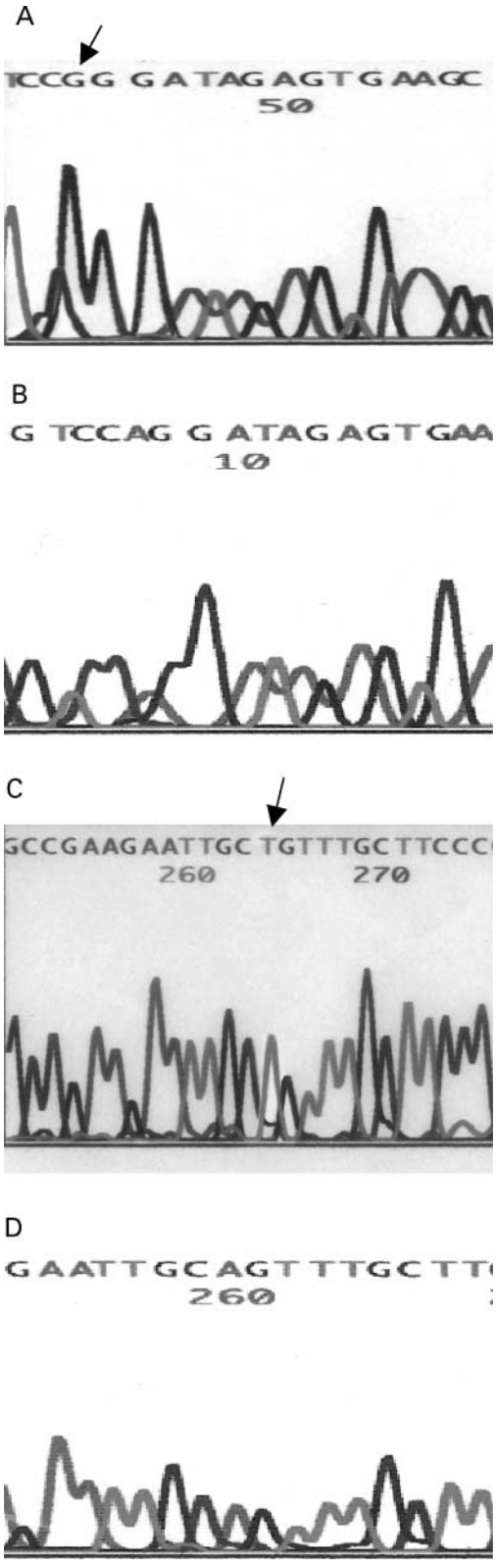

Figure 2. Partial electropherograms of the *SRY* gene in patients 1 and 2 (A: showing mutation Q57R and B: normal or wild type sequence) and patient 3 (C: showing a mutation S143C and D: normal or wild type sequence); *Arrows* indicate the substituted nucleotides.

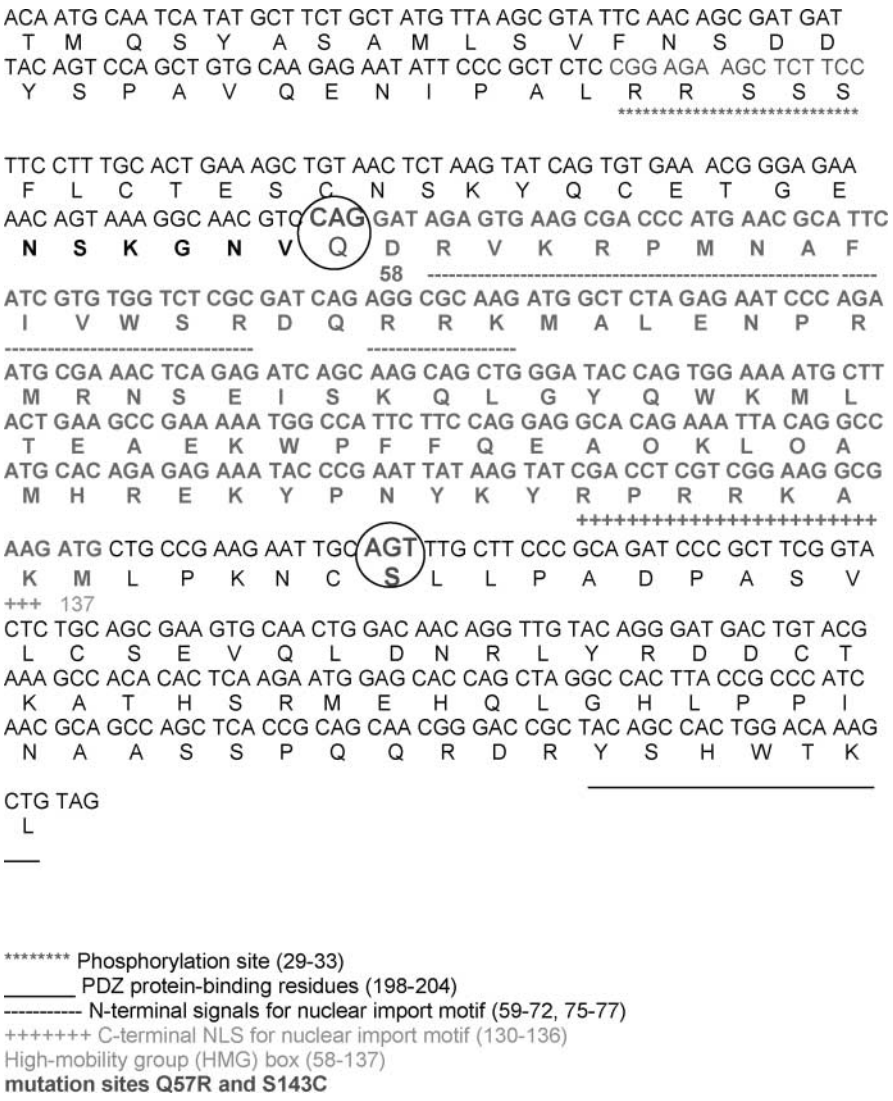

Figure 3. Partial upstream and downstream sequence of HMG box showing the position of mutations (circled amino acids, affected residues).

perform male gene expression. This change may form an electrostatic and hydrophobic interaction with phosphate and sugars respectively of DNA and could play an important role in altering the specific orientation and binding to DNA bases in the major groove. This may have totally or at least partially inhibited the interactions of *SRY* with DNA (Werner *et al.*, 1995). Patient 1 has also got normal breast development which is an atypical feature of the phenotype. The older patient (no. 1) developed gonadal tumours where as the younger patient (no. 2) did not, which is further supported by previous studies (Manuel *et al.*, 1976; Schmitt-Ney *et al.*, 1993; Tajima *et al.*, 1994; Uehara *et al.*, 1999, 2002). It is widely recognized that dysgenetic gonads possibly due to the *SRY* mutations frequently develop gonadoblastoma. However, the molecular cause of gonadoblastoma formation remains elusive. The *GBY* (gonadoblastoma locus, Y chromosome) locus on Yq region is thought to contain a proto-oncogene involved in the origin of these tumours (Page, 1987). This is further supported in that mutations in oncogenes, tumour suppressor genes and DNA repair genes accumulate with age and then cause tumour development in the streak gonads (Knudson, 1971). Although many patients with mutations in the *SRY* gene fail to develop secondary sexual characteristics at puberty, a small subset, similar to patient 1, do develop these to varying degrees (Scully, 1970; Warner *et al.*, 1985). Polar neutral amino acid serine is found replaced with a neutral and non-polar cysteine (with pK_a 8.4) at codon 143 in patient 3. This change may result in an altered protein which may have lost some of its stabilizing potential.

The majority of the mutations detected so far in the *SRY* gene lie within the conserved motif, i.e. HMG box, causing alterations in DNA binding/bending activity, and possibly contributing to the origin of 46,XY females. To date, only 10 mutations that lie outside the HMG box have been detected and all have different effects on the patient phenotype. It is hypothesized that the regions outside the HMG box might be required to stabilize protein binding and to generate specificity by helping to discriminate between the protein–protein interactions (Wilson and Koopman, 2002). Proteins such as *SRY* possessing just one HMG box show sequence specificity for DNA binding by recognizing AT-rich sequences, thus inducing a bend in the DNA helix. DNA binding and bending capacities by *SRY* protein have been demonstrated to be essential in testis development (Harley *et al.*, 1992). The strong bending of DNA together with the lack of a potential transregulation domain in human *SRY* has led to the suggestion that protein may modulate transcription by acting architecturally in the assembly of a nucleoprotein complex

(Pontiggia *et al.*, 1994). However, despite the critical role of *SRY* in the cascade of gene regulation leading to maleness, the direct targets of *SRY* remain to be positively identified.

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