RESEARCH ARTICLE

Mutational landscape of the human Y chromosome-linked genes and loci in patients with hypogonadism

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Abstract

Sex chromosome-related anomalies engender plethora of conditions leading to male infertility. Hypogonadotropic hypogonadism (HH) is a rare but well-known cause of male infertility. Present study was conducted to ascertain possible consensus on the alterations of the Y-linked genes and loci in males representing hypogonadism (H), which in turn culminate in reproductive dysfunction. A total of nineteen 46, XY males, clinically diagnosed with H (11 representative HH adults and eight prepubertal boys suspected of having HH) were included in the study. Sequence-tagged site screening, *SRY* gene sequencing, fluorescence *in situ* hybridization mapping (FISH), copy number and relative expression studies by real-time PCR were conducted to uncover the altered status of the Y chromosome in the patients. The result showed random microdeletions within the *AZFa* (73%)/*b* (78%) and *c*(26%) regions. Sequencing of the *SRY* gene showed nucleotide variations within and outside of the HMG box in four males (21%). FISH uncovered mosaicism for *SRY*, *AMELY*, *DAZ* genes and DYZ1 arrays, structural rearrangement for *AMELY* (31%) and duplication of *DAZ* (57%) genes. Copy number variation for seven Y-linked genes (2–8 rounds of duplication), DYZ1 arrays (495–6201copies) and differential expression of *SRY*, *UTY* and *VCY* in the patients' blood were observed. Present work demonstrates the organizational vulnerability of several Y-linked genes in H males. These results are envisaged to be useful during routine diagnosis of H patients.

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Introduction

Hypogonadism (H) refers to deficient or absent of male gonadal functions due to insufficient testosterone secretion and may arise from testicular disease (primary H) or dysfunction of the hypothalamic pituitary gonadal axis (secondary H) (Achermann and Jameson 1999). Secondary H, also known as hypogonadotropic hypogonadism (HH) is a genetic disorder that results in delayed/absent/incomplete puberty and/or infertility (Achermann and Jameson 1999; Fraietta *et al.* 2013). The occurrence of this form of H ranges from 1:10,000 to 1:86,000 in the population (Achermann and Jameson 1999; Fraietta *et al.* 2013). When associated with anosmia, it is known as Kallmann syndrome (KS) (Fraietta *et al.* 2013). The diagnosis of HH at prepubertal (PH) age is difficult and usually postponed until adulthood. HH phenotype is variable because several genes are associated with this disorder (Silveira *et al.* 2002; de Roux *et al.* 2003). The condition is reported to account for ∼1–2% of male factor infertility largely due to aberrant Y chromosome (Chudnovsky and Niederberger 2007).

Despite the presence of the palindromic sequences, innate safeguarding attributes and intrachromosomal recombination, the Y chromosome remains highly prone to genetical changes (Sun *et al.* 2000; Kuroda-Kawaguchi *et al.* 2001; Pathak *et al.* 2006; Premi *et al.* 2007, 2008, 2009, 2010; Kumari *et al.* 2012). Sporadic mutations and environmental factors both affect the Y chromosome causing alteration in genes and loci. These alterations predispose individuals to produce sperm with *de novo* mutations that are passed on to the progeny with defective Y chromosome. Of all the important genes implicated with normal male development, *SRY* located on chromosome Yp11.3 plays a pivotal role in male sex determination (Graves 2002). In addition to *SRY*, structural polymorphism of a single copy *AMELY* gene on Yp is yet another major cause of Y chromosome variation (Lattanzi *et al.* 2005; Jobling *et al.* 2007). A particular area of Yq spans the azoospermia factor regions (*AZF*) *a*, *b* and *c*, harbouring genes involved in spermatogenesis (Vogt *et al.* 1996). The *AZFc* region encompassing four copies of *DAZ* gene (*DAZ*1–4) have been reported to show deletions in the infertile males

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(Vogt *et al*. 1996; Reynolds and Cooke 2005; Choi *et al*. 2012; Kumari *et al*. 2012). Thus, a significant proportion of males across the population portray variations in *DAZ* copy number (Premi *et al.* 2007, 2008, 2009, 2010; Kumari *et al.* 2012). In addition, Y chromosome consists of 3.4-kb DYZ1 repeat arrays on the Yq12 region having ~3000–4500 copies in normal males (Premi *et al.* 2010). These copies are drastically altered in males exposed to natural background radiation (Pathak *et al.* 2006; Premi *et al.* 2009), groundwater arsenic pollution (Ali and Ali 2010), cases of prostate cancer (Pathak *et al*. 2006 Yadav *et al*. 2013), repeated abortion (Pathak *et al*. 2006; Yan *et al*. 2011) and other categories of disorders related to male infertility (Bashamboo *et al*. 2005; Tian *et al*. 2014; Yadav *et al*. 2014). Despite the advances made on Y chromosome genetics, our understanding on the affected genes and loci in males with clinical condition of H still remains inadequate. We evaluated Y chromosome instability among representative H males with HH/associated KS and also assessed PH boys suspected of having HH disorder. The study was conducted to ascertain possible consensus on the organization, expression and copy number variation of the genes among these patients. Indeed, we detected structural alterations in the Y chromosome-linked genes and loci. This work is envisaged to augment DNA-based diagnosis, lending support to genetic counselling.

Materials and methods

Ethical consent

Present study was reviewed and approved by the Ethical and Biosafety Committees (EBC) of the National Institute of Immunology (NII), New Delhi and Aligarh Muslim University (AMU), Aligarh, India. Patients in the present study visited Department of Endocrinology, Jawaharlal Nehru Medical College, AMU, Aligarh for reproductive disorders and infertility related problems. The peripheral blood samples were collected for diagnostic purpose from the patients with their informed consent. Majority of the patients were illiterate and thus after guiding and counselling about the whole study, their oral consents were obtained. In case of minors, caretakers gave their approval. This was reported to the committee and due approval was accorded. Once the approvals from both the EBC were obtained, the study was conducted.

Sample collection

Collection of samples spanned over a period of two years. Each patient's medical history was taken and physical examination was conducted. Semen analysis for sperm count, motility and viability were performed according to WHO (2010) criteria. In addition, total testosterone (T), follicle stimulating hormone (FSH), luteinizing hormone (LH), prolactin (PRL), and thyroid stimulating hormone (TSH) concentrations were measured by radioimmunoassay (Bashamboo *et al.* 2005; Premi *et al.* 2008). The patient group underwent magnetic resonance imaging (MRI) of the olfactory bulbs and tracts. Subjects with a decreased sense of smell or anosmia were assumed to have KS. A total of 19 H males (11 HH / 8 PH) were included in the present study. The diagnosis of HH was based on the absent/impaired puberty by the age $\geq$18 years and low serum Gn (Tian *et al*. 2014). Further, on the basis of unilateral or bilateral cryptorchidism and/or micropenis/anosmia, pubertal delay, low or absent gonadotropin response to a gonadotropin releasing hormone (GnRH) test, and eunuchoid body proportion (Marshall and Tanner 1970; Feldman and Smith 1975), eight prepubertal hypogonadism (PH) boys suspected of having HH were also included in this study. Twenty proven normal fertile males (NFM) were taken as positive controls. Details of the patients, karyotype status and diagnosis are given in table 1 in electronic supplementary material at http://www.ias.ac.in/jgenet/.

DNA/RNA isolation and cDNA synthesis

Genomic DNA was prepared from peripheral blood of H and NFM control samples using standard phenol : chloroform extraction procedure. RNA was isolated from blood using TRI reagent (MRC, Cat No.TB-126-200, Cincinnati, USA) following standard methods (Premi *et al.* 2009). Quality of DNA and RNA was checked on agarose gel. DNA contamination in RNA was checked using human β-actin primers. Using Verso cDNA synthesis kit (Thermo Fisher Scientific, Cat No. AB-1453/B, Waltham, USA), cDNA was synthesized following standard protocol. Amplification of DNA and cDNA was confirmed using β-actin and *GAPDH* primers, respectively (table 2 in electronic supplementary material).

Analysis of Y chromosome-specific genes and sequence-tagged site (STS)

A total of 87 STSs spanning the known regions of Y chromosome were selected from male-specific region of the Y chromosome (MSY) breakpoint mapper for detecting their presence or absence in genomic DNA of patients and controls using end point PCR. Additional 10 sets of Y-linked primers were used to confirm their intactness in the patients (table 2 in electronic supplementary material). The reaction of 35 cycles was carried out in 10 μL volume using Go Taq polymerase and 5$\times$ reaction buffer (Promega, Madison, USA), 200 μM dNTPs and 40 ng of template DNA. The annealing temperature of respective STSs was taken from the NCBI database. Primer for STS sY14 (*SRY*) was used to confirm the presence of Y chromosome and quantity of genomic DNA. The amplified products were resolved on agarose gel. In case of any ambiguity, the reactions were repeated twice before accepting the same to be negative.

Sequence analysis of SRY, ZFY and AMELY genes

Mutational status of *SRY*, *ZFY* and *AMELY* genes in H patients and controls was assessed by PCR amplification

of blood genomic DNA, followed by cloning and sequencing of the resultant amplicons (Premi *et al.* 2009; Kumari *et al.* 2012), see table 2 in electronic supplementary material. To screen Y chromosome polymorphism, STSs specific to *DUXY* and *KALPY* genes were sequenced. The sequences submitted to GenBank (Banklt) were then blast searched using NCBI BLASTN and aligned to reference sequence (*SRY*, NM_003140.2; *AMELY*, NC_000024.10; *ZFY*, XM_006724873.1; *DUXY*, XM_006726523; *KALY*, NC_000024.10) using ClustalW.

Single-nucleotide variant (SNV) screening

Identification of *DAZ* SNV in H patients was based on PCR amplification-restriction digestion method. Reaction was carried out for five SNVs (I–III, V (sY587) and VII (sY581) spanning from 5′ to the 3′ end of *DAZ* gene (table 3 in electronic supplementary material) following standard protocol (Premi *et al.* 2009; Kumari *et al.* 2012). The digests were resolved on 3% agarose gel. Two internal positive STSs, sY14 (*SRY*) and sY152 (marker for the *DAZ* 1+4 gene), were used along with NFM controls.

FISH mapping

For chromosome preparation, standard protocol was followed (Pathak *et al.* 2006). Patients were subjected to FISH analysis along with NFM controls. FISH was performed using clones of 3.4-kb DYZ1 fragment, *DAZ* (cos6B7), *AMELY* (cos9E) and LSI *SRY/CEPX* DNA (procured commercially from Abbott Molecular, Cat 32-191007, Illinois, Des Plaines, USA). DYZ1 clone was labelled with spectrum red/green (Pathak *et al.* 2006) and *DAZ/AMELY* clones with biotin/fluorescence UTP using Nick Translation Kit from Vysis (USA) following standard protocol (Premi *et al.* 2008, 2009). Images were captured with CCD camera attached with Olympus UCMAD-2 and analysed using Applied Imaging Systems CytoVision software ver. 3.93. At least 30–50 metaphases from each patient were screened for the presence/absence of respective probe signal(s). Experiments were repeated twice to avoid any artifacts.

Copy number estimation

Genomic DNA from H patients and that of controls (NFM and genomic DNA sample provided with the *RNase*P detection kit, ABI) were used for copy number estimation of seven different Y-linked genes using real-time PCR (ABI-7500) following standard protocol (Pathak *et al.* 2006; Premi *et al.* 2009). Taqman assays and probes were procured from Applied Biosystem. *RNaseP* gene (catalog number: 4316831) was taken as an endogenous control. Universal cyclic conditions recommended by ABI were used to amplify all the samples in triplicates. The copies were then calculated using $2^{-\Delta Ct}$ method (Kumari *et al.* 2012). The copy number of DYZ1 arrays was calculated based on absolute quantification assay using *SYBR* green dye. The standard curve had a slope of -3.4 and R2 value of >0.99. The details of the genes, their respective TaqMan assays, probes and primers are given in table 4 in electronic supplementary material.

Relative gene expression

The relative expression of *SRY*, *UTY*, *DAZ* and *VCY* transcripts in patients and control blood samples was estimated by conducting reactions in triplicate on real-time PCR (ABI-7500) (Premi *et al.* 2009; Kumari *et al.* 2012), see table 5 in electronic supplementary material. Two-fold dilution series of cDNA template was assayed using respective Taqman probe and universal cyclic condition as recommended by ABI. Due to logistic constrains, expression analysis for all the H patients could not be conducted. Testis cDNA was prepared from the total RNA procured commercially (catalog number: 636533, Clontech Takara, USA). The concentration of mRNA was checked using exon specific *GAPDH* primers. Further, Ct values were normalized twice, with the *HPRT* gene as an endogenous control and the sample showing highest Ct value of the respective gene. The expression levels were then calculated by $2^{-\Delta\Delta Ct}$ method (Premi *et al*. 2009). All experiments were repeated at least thrice.

Statistical analysis

Sigma Plot 11.0 (Systat Software, Erkrath, Germany) was used for statistical analysis. Differences among frequencies between NFM and H patients were calculated using Fisher's exact test. Probability (P) values ≤ 0.05 were regarded as statistically significant.

Results

Patient diagnosis

Among the 19 patients, four H (HH10, HH14, PH*11 and PH*13) were diagnosed with anosmia/KS. The patients showed low serum levels of T, FSH and LH. None of the patient showed any characteristic features of ambiguous genitalia; Turner or Klinefelter syndrome. Chromosome analysis showed apparently normal 46, XY karyotype in all the males though the quality in most cases was inadequate for G-banding. Clinical status of the H patients is given in table 1 in electronic supplementary material.

AZF susceptibility

STSs screening of the Y chromosome showed random microdeletions in the *AZFa/AZFb/AZFc* regions in patients but not in controls. The deletion frequency was higher in *AZFb* region (78%) compared with that of *AZFa* (73%) and *AZFc* (26%) regions. Patient H (*1 and 17, cryptorchidism) showed maximum deletion of STSs. Remarkably, no microdeletions were observed in four H (*2, 10, *12 and 19) patients.

AZFa region

No provirus human endogenous retrovirus (HERV)-mediated recombination events were seen in *AZFa* region. However, prominent microdeletions in both (PH and HH) males were observed in proximal region of provirus A, STSs sY746, sY1179 and provirus B, STS sY1183 (see tables 1 & 2; figure 1a in electronic supplementary material). Further, STSs sY1182 H (15–17), sY1185 H (4 and 5), sY1186 H (9 and 16) and sY1066 H (15, 17 and 18) were found to be absent only in HH males. To uncover deletions between sY1064 and sY1065, we screened nine STSs using *USP9Y*, *DBY* and *UTY* genes-specific primers. Microdeletions clustered in *USP9Y* gene were observed in both subgroups of H males for STSs sY1317 and sY1316. The results suggest that possible deletion breakpoint in these patients lies within the *USP9Y* gene. *USP9Y* (1–3) gene region H (*11 and *13), STSs sY86 and sY87 (*H7) were absent in PH males, whereas sY88 was

Table 1. Details of Y chromosome STSs present in AZF region of H patients.

Patient ID	H male STS	*1	*3	4	5	6	*7	*8	9	*11	*13	14	15	16	17	18	No. of H males showing STS deletions
		Region															
										AZFa							
1	sY746	−	+ +	+	+	−	+	−	−	+	+	−	−	−	+	**7**	
2	sY1179	−	+	+	−	+	−	+	−	+	+	+	−	−	+	+	6
3	sY1180	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Nil
4	sY1181	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Nil
5	sY1064	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Nil
6	sY1065	+	+	+	+	+	+	+	+	+	+	+	+	+	−	+	1
7	sY1182	+	+	+	+	+	+	+	+	+	+	+	−	−	−	+	3
8	sY1183	−	+	+	+	+	+	+	−	+	+	+	−	−	−	+	5
9	sY1184	+	+	+	+	+	+	+	−	+	+	+	+	+	+	+	1
10	sY1185	+	+	−	−	+	+	+	+	+	+	+	+	+	+	+	2
11	sY1186	+	+	+	+	+	+	+	−	+	+	+	+	−	+	+	2
12	sY1066	+	+	+	+	+	+	+	+	+	+	+	−	+	−	−	3
13	sY83	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Nil
14	sY84	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Nil
15	sY86	+	+	+	+	+	−	+	+	+	+	+	+	+	+	+	1
16	sY1317	−	+	+	−	+	+	+	+	+	+	+	−	−	−	−	6
17	sY1316	−	−	+	+	+	−	−	+	+	+	−	−	−	−	−	9
18	sY87	+	+	+	+	+	−	+	+	+	+	+	+	+	+	+	1
19	sY1234	−	−	+	−	+	−	+	+	+	+	+	+	+	+	+	4
20	sY1231	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Nil
21	sY88	+	+	+	−	+	+	+	+	+	+	+	+	+	+	+	1
										AZFb							
22	sY113	−	+	−	−	−	+	+	+	+	+	+	+	+	+	+	4
23	sY117	−	+	−	−	+	+	+	+	+	+	+	+	+	−	−	5
24	sY124	+	+	+	+	+	−	−	−	−	−	−	−	+	+	+	7
25	sY125	−	+	+	+	+	+	+	+	+	+	+	+	+	−	−	3
26	sY127	+	+	+	+	+	−	+	+	+	+	+	+	+	+	−	2
27	sY131	−	+	+	+	+	+	+	+	+	+	+	+	−	−	−	4
28	sY129	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−	3
29	sY1233	−	−	−	+	+	+	−	+	+	+	+	+	+	−	−	6
30	sY627	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Nil
										AZFc							
31	sY142	−	−	−	+	+	−	−	+	+	+	+	+	+	+	+	5
Total no. of STSs affected in each patient		12	4	5	8	1	9	4	6	2	1	2	8	9	12	9	

*Prepubertal H males, 87 STSs screened, −, STS absent; +, STS present, see figure 1 and table 1 in electronic supplementary material.

Table 2. Relative prevalence of deletions in *AZFa*, *AZFb* and *AZFc* regions of H males.

(A) H patients subgroup/number	*AZF* region STSs deleted *AZFa*	*AZFb*	*AZFc*	Relative frequency (%) STSs deletion *AZF* (*a*, *b* and *c*)
PH/8	sY86, sY87,*USP9Y* (1–3)	–	–	5/87 (0.05)
HH/11	sY1065, sY1182, sY1185, sY1186, sY1066, sY88	sY129	–	7/87 (0.08)
PH+HH/19	sY746, sY1179, sY1183, sY1317, sY1316, sY1234	sY113, sY117, sY124, sY125, sY127, sY131, sY1233	sY142	14/87 (0.16)
Total no. of STSs affected	17/21 (80%)	8/9 (88%)	1/30 (3)	26/87 (30%)
No. of H males showing STS deletions	14/19 (73%)	15/19 (78%)	5/19 (26%)	15/19 (78%)

(B) H patients subgroup/number	No. of patients showing (%) SNVs *DAZ*	Sequence polymorphism *SRY*	*KALPY* (sY182)	FISH rearrangement *AMELY*	*DAZ*	Copy number polymorphism *No. of genes	DYZ1
PH/8	1 (0.12)/SNVII	4 (0.5)	4 (0.5)	2/duplication (0.25)	4/duplication (0.5)	8 (1) / 2–8 copies	8 (1) / 528–6201
HH/11	9 (0.81)/SNVs I–III and V	2 (0.18)	1 (0.09)	4/duplication (0.36)	7/duplication (0.63)	11 (1) / 2–16 copies	9 (0.81) / 495–6105
PH+HH/19	10 (0.52)/ SNVs I–III and V	6 (0.31)	5 (0.26)	6/duplication (0.31)	11/duplication (0.57)	19 (1) / 2–16 copies	17 (0.89) / 495–6201

PH, prepubertal hypogonadism; HH, hypogonadotropic hypogonadism; **SRY*, *AMELY*, *XRY*, *HSFY*, *PRY*, *DAZ* and *BPY2*.

absent in a single HH male H (5). STS sY1234 representing *DBY* gene was deleted in four H (*1,*3, 5 and *7) males. Interestingly, *UTY* gene remained intact in all the patients (table 1; figure 1b in electronic supplementary material).

AZFb region

AZFb deletions were detected in both the subgroups of H males. STSs sY117, sY124 and sY1233 were absent in majority of the patients served as hotspot for *AZFb* region (see tables 1 & 2; figure 1c in electronic supplementary material). In addition, scattered microdeletions were observed for STSs sY113, sY125, sY127, sY131 and sY129. None of the patients showed *gr/gr* or *b1/b3* or *b2/b3* deletions.

AZFc region

STS sY142 was absent in five PH (*1, *3, 4, *7 and *8) males. No other prominent deletion was seen in *AZFc* region (tables 1 & 2).

DAZ SNV analysis

PCR analysis of STS sY152 (*DAZ*1+4) showed amplification in all the patients confirming presence of four *DAZ* copies. In six adult HH males, H (4, 6, 9, 14, 16 and 19), allelic variation for SNVs (I–III) was seen. SNVII digestion site was missing in patient H4 and SNVV, related to sY587 was missing in seven adult HH males, H (4, 6, 9, 15, 18 and 19) and one PH male H (*8) (see table 2; table 3 in electronic supplementary material). No *DAZ* deletions were detected in NFM controls.

Gene sequence plasticity

Sequence analysis of *SRY*, *AMELY* and *ZFY* showed nucleotide variations only in *SRY* gene in both the subgroups of H males. The sequences were deposited in GenBank and accession numbers were obtained (table 3). *SRY* sequence alignment of representative H patients, with that of NFM controls showed nucleotide variations among six H males (two adult HH, H (6 and 18) and four PH, H (*2, *3, *7 and *8)), see table 2; figure 2 in electronic supplementary material. Further, bioinformatic translation (Transeq tool) of *SRY* CDS region (nt 89–703) uncovered nucleotide polymorphism in four H (*3, 6, *7 and 18) patients (figure 3 in electronic supplementary material). In two of these patients H (*3 and 18), amino acid (aa) changes were detected in HMG region (59–128 aa). Functional effect of these nucleotide polymorphisms using PolyPhen-2 (genetics.bwh.harvard.edu/pph2/) predicted probably damaging mutations, with the score of >0.99. Sequencing of sY182 in 19H cases showed random nucleotide variations in five H (one adult HH, H17 and four PH, H (*1, *3, *7, *8)) males (table 2; figure 4 in electronic supplementary material). Most of the nucleotide variations in *SRY* and *KALPY* genes

Table 3. Details of nucleotide variations and their possible peptide changes in *SRY* gene.

	Patient ID	GenBank accession number	Nucleotide changes in CDS region	Corresponding amino acid (aa) change	HMG region affected	*SRY* copies
1.	*H2	KF413436	No change	None	No	2
2.	*H3	KF413437	c.A>G352; c.A>G436	p.H120R	Yes	1
3.	H6	KF413438	c.T>A96; c.T>C182; c.A>G594	p.Y4N;p.K170E	No	4
4.	*H7	KF413439	c.644 _645Cins	p.P185_p.L190insHQRSQ; subsequent multiple aa changes	No	4
5.	*H8	KF413440	c.A>G597	None	No	2
6.	H18	KF413441	c.G>A156; c.A>T435; c.C>T481; c.A>T494	p.R132C;p.K136M	Yes	1

H, hypogonadism males. For details regarding nucleotide and amino acid (aa) changes, see figures 2 & 3 in electronic supplementary material.

were present in PH cases. These changes were not detected in control samples. Sequences of the *DUXY* gene in these patients remained intact.

SRY, AMELY, DAZ and DYZ1 mosaicism

FISH conducted with *SRY, AMELY, DAZ* and DYZ1 probes on metaphases and interphase nuclei showed ∼1–2% mosaicism in both the subgroups of H patients (figure 5 in electronic supplementary material). A higher level of mosaicism ∼5–8% was recorded for *SRY* gene in H (*H1, 6,*H7, *11, 15 and 17); for *AMELY* in H (*11–13 and 17), for *DAZ* in H (*1–3, *11, 17–19) and for DYZ1 arrays in H (*11, 14 and 17) males. The *SRY/CEPX* probe showed signal on X chromosome in all the cells. In NFM, signal for the four probes were detected in all the cells screened.

Rearrangement of AMELY gene

AMELY gene sequencing revealed no nucleotide alternation in H patients. Double FISH conducted using texas red labelled *DAZ* and fluorescence labelled *AMELY* probes showed *AMELY* gene signal on Yp and *DAZ* on Yq arms of H patients and NFM, see figure 1, E(d). However, along with normal signals, in 31% (6/19 cases), 1–3% metaphases of H (*11,*12, 1417) patients showed *AMELY* signals on the Yq arm (figure 1, A–D, E (b–d); table 2). In *H11 patient, *AMELY* signal was present on both the arms (figure 1, A&E (a)).

Unilocus duplication of DAZ gene

DAZ SNV showed sequence variation in H patients. FISH showed two merged signals for four *DAZ* copies in NFM controls. Patients H (*1–3, 4, 5, *7, 10, 11, 17–19) showed three *DAZ* signals and an overlaps (5–10% cells) suggesting unilocus duplication, (figure 2; table 2). In patients H (*7 and 10), signals were far apart between the two loci compared with that of NFM, signifying possible gene rearrangement. Further, 1% cells in patient *H2 showed two signals for the *DAZ* probe. Thus, *DAZ* gene seems to be rearranged in both the subgroups of H males.

Copy number polymorphism (CNP)

PCR analyses for *SRY*, *AMELY*, *XKRY*, *HSFY*, *PRY*, *DAZ* and *BPY2* genes showed amplification in all the patients (figure 6 and table 6 in electronic supplementary material), suggesting their intactness. Taqman assay uncovered CNP for seven Y-linked genes, showing 2–4 rounds of duplication in H males ($P < 0.001$, compared with those of NFM) (table 4). *PRY* present in two copies followed by *SRY* and *AMELY*, present in single copy in NFM was among the most frequently duplicated genes detected in H males. *PRY* showed 4–16 rounds of duplications in both HH and PH males. *SRY* showed 2–8 rounds of duplication in HH and 2–4 rounds in PH males. Similarly, *AMELY* showed 2–4 rounds of duplication in both the subgroups. Apparently, *HSFY* was least affected with respect to its copies and only four H (4, *11, *13 and 17) patients showed CNP. Despite repeated reactions, in some patients copy number remained undetermined. In these patients *RNaseP* gene Ct value was consistently found to be −1 (haploid Y chromosome), however, ΔCt values for the seven genes mentioned earlier was >1 (not shown). Significantly, copies for DYZ1 arrays ranged from 495–6201 in H males (528–6201 copies in PH and 495–6105 in HH) (tables 2 and 4), compared to NFM, 3000–4500 ($P < 0.001$).

Differential expression of SRY, UTY and VCY in blood lymphocytes

Owing to logistic constrains, it was not possible to conduct expression analysis of Y-linked genes in patient's tissues

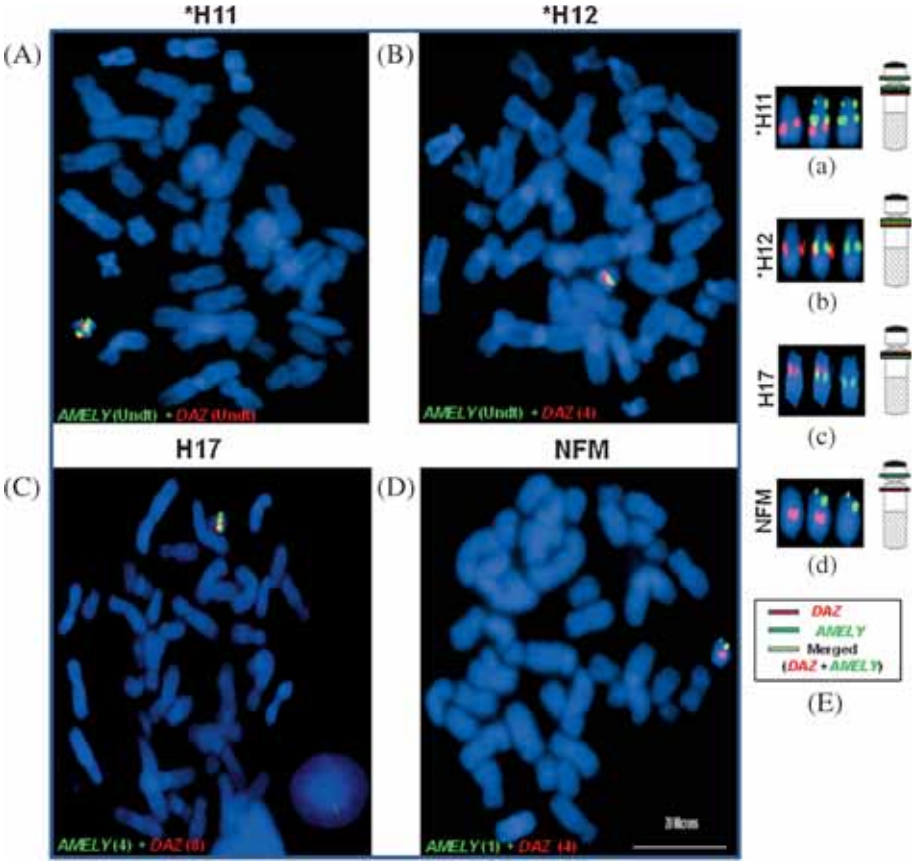

Figure 1. *AMELY* gene rearrangement in H males. Representative panels (A–D) showing structural reorganization of *AMELY* gene on metaphase chromosome of H patients. Signals in green represent *AMELY* and red represent *DAZ* gene. Panel E (a–d) shows different arrangement of *AMELY* gene on Y chromosome. Location of each gene is shown on the ideogram to the right side of the panel (E). Yellow signal results from overlapping of red and green signals. Patient IDs are provided on top and copy number on bottom left of the panels. Undt, undetermined copy number; NFM, normal fertile males used as control. Scale is given at the bottom of the panel (D).

or gonads. Control NFM testis cDNA showed expression of all the genes confirming fidelity of the probes. *DAZ* gene showed no expression either in blood lymphocytes of NFM controls or that of H patients. Respective average ΔCt values (within the same range) were taken as normal male calibrator. In H patients, differential expression of *SRY, UTY* and *VCY* genes was observed (figure 7 and table 5 in electronic supplementary material) compared with that in NFM controls, where *SRY* showed highest expression followed by *UTY* and *VCY* genes in both the subgroups of H males.

Discussion

Disorders affecting the temporospatial regulation of hypothalamic – pituitary – gonadal (HPG) axis are susceptible to genetic defects, resulting in impaired male sexual development and fertility. Similarly, endocrine disruptors may also bring alteration to the HPG axis, leading to reproductive disorders (Svechnikov *et al.* 2014). Known endocrine causes of male disorder comprise hypogonadotrophic and hypergonadotrophic H. HH is a rare disorder and mutations in autosomal and X-chromosome-linked genes have been identified in patients with HH (Gorlov *et al.* 2002; Qin *et al.* 2014). Yet these genetic defects account for only 30% of all cases. Some genetic mutations are sufficient to cause H while others results from the combination of more than one genetic abnormality. Thus, to understand the mechanism of reproductive failure in H males, need for identification of more number of genes is prerequisite. We analysed group of H (HH adult and PH boys suspected of having HH) male patients with clinical subfeatures of cryptorchidism, micropenis, gynecomastia and Kallmann syndrome. In majority of adult males, incomplete or absent pubertal development is linked with HH condition. However, for boys in the pubertal age, distinguishing HH from constitutional delay of growth and puberty may be difficult. H cases showed low hormone levels of testosterone suggesting disruption of spermatogenesis. PH cases mainly showed phenotypic features of cryptorchidism/anosmia/micropenis whereas adult HH cases showed mixed phenotype of cryptorchidism/anosmia/micropenis/aspermia. In few of the H cases, these syndromes were also associated with low levels or no sperm count. Despite endocrine imbalance and frequent gene alteration in patients with HH/PH/KS syndrome,

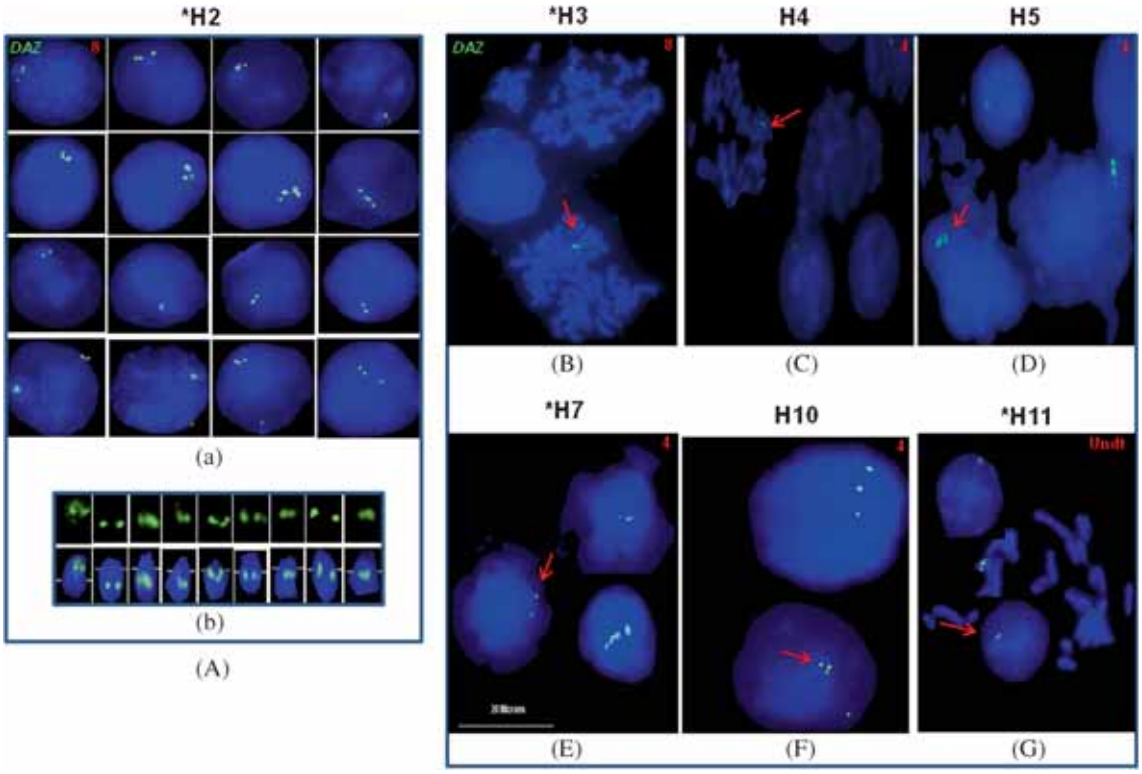

Figure 2. *DAZ* gene organization in H males. FISH panels (A–G) shows differential arrangement of *DAZ* on the interphase nuclei and metaphase chromosomes (red arrow) of representative H males. Panel (A) displays altered arrangements of the *DAZ* on interphase nuclei (a) and Y chromosome (b) of representative patient *H2. Patients IDs are mentioned on top of the panels and copy number on right. Undt, undetermined copy number. Scale is provided at the bottom of the panel (E).

we failed to find a correlation between spermatogenic failure and a specific Y-chromosome-linked gene. Thus, Y chromosome vulnerability was observed albeit without having a consensus on the altered genes and loci.

Table 4. Copy number analysis of the Y-linked, genes and arrays in patients with H.

	Patient ID	*SRY*	*AMELY*	*XKRY*	*HSFY*		*DAZ*	*BPY2*	*DYZ1*
		Copy number							
NFM		1	1	2	2	2	4	3	3000–4500
H	*H1	4	2	2	2	16	8	3	2783
	*H2	2	2	2	2	8	8	3	1853
	*H3	1	2	2	2	4	8	3	1815
	H4	2	2	2	4	16	4	3	4709
	H5	2	2	2	2	16	4	3	2310
	H6	4	1	2	2	8	4	3	5445
	*H7	4	2	2	2	4	4	3	4977
	*H8	2	1	2	2	8	4	3	1386
	H9	1	1	4	2	4	4	3	2640
	H10	1	1	2	2	2	4	3	5280
	*H11	Undt	Undt	Undt	Undt	Undt	Undt	Undt	528
	*H12	1	Undt	2	2	2	4	3	1584
	*H13	2	4	2	4	2	4	3	6201
	H14	1	2	2	2	2	6	3	495
	H15	8	2	8	2	16	4	6	4290
	H16	1	1	2	2	4	4	3	3330
	H17	4	4	2	1	8	8	3	820
	H18	1	1	2	2	8	8	3	5115
	H19	1	1	2	2	8	8	3	6105

Undt, undetermined copies, H, hypogonadism; NFM, normal fertile males.

Y chromosome microdeletions are frequently associated with the quantitative decrease in the sperm production (Kumari *et al.* 2012; Rives 2014) along with other infertility disorder (Krausz *et al.* 2006; Kumari *et al.* 2012; Khabour *et al.* 2014). Although, there was no definitive genotype/phenotype correlation, H patients showed higher frequency of microdeletions in *AZFb* region followed by *AZFa*. Our study showed several random noncontiguous deletions in H males. *AZFa* region, STS sY1316 of *USP9Y* gene deleted in nine males was taken to be the hotspot for microdeletion in H (both PH and HH) males. This is an important observation, as the gene is known to act as a fine-tuner of spermatogenesis (Krausz *et al.* 2006). Interestingly, sY1066, 1185 and 1186 were found to be deleted only in HH patients, making it a HH marker.

Genes on *AZFb* region have important roles in spermatogenesis (Elliott 2000). *AZFb* region, STS sY117, deleted in five males, sY124 deleted in seven males and sY1233 in six males were taken to be as hotspots in H (both PH and HH) males. Deletions in *AZFc* region are the most commonly reported among AZF microdeletions (Ferlin *et al.* 2005). In our study, however *AZFc* (sY142) deletion accounted for only 26% (5/19) of the total deletion detected in the patients. Men with complete *AZFa* or *AZFb* deletions or with larger deletions are reported to be azoospermic (Krausz *et al.* 2014). We speculate that deletions in *AZFa* and *AZFb* regions are disrupting spermatogenesis in H males. Study on large number of H males with different ethnic group will resolve this issue.

Conventional karyotyping missed Y chromosome mosaicism in H males. However, FISH of interphase nuclei and metaphases showed Y chromosome mosaicism in both HH and PH cases. In few patients, higher percentage of mosaicism (∼5–8%) for *SRY*, *AMELY*, *DAZ* genes and DYZ1 arrays was observed. Association between Y microdeletions and 46, XY mosaicism has been reported in individuals with sex-chromosome-related anomalies (SCRA) and males exposed to background radiation (Lenz *et al.* 2005; Premi *et al.* 2009). We propose that genome instability during cell division results in Y chromosome mosaicism in the patients. Thus, chromosomal studies in cases of H patients with atypical clinical features are relevant. Accordingly, FISH may be conducted in case of H patients to uncover mosaic and or nonmosaic conditions before resorting to prognosis and genetic counselling.

The homologous recombination between MSY palindromes could generate rearrangements (Lange *et al.* 2009). This was noticed for *AMELY* gene known to be located on the short arm of the Y chromosome. However, in six H patients (one PH and five HH), the gene was present on long arm of Y chromosome. We speculate that intrachromatid crossingover between inverted repeats might have led to inversion/duplication of *AMELY* gene. This error in crossingover might lead to aberrant spermatozoa and consequently becoming yet another cause of infertility. Occasionally, duplication and rearrangement of *AMELY* gene have been described (Murphy *et al.* 2007; Lange *et al.* 2013). However, no such rearrangements in males with clinical diagnosis of H have been reported so far. Interstitial Y deletions, duplications and isodicentric Y chromosomes are associated with a wide range of sex disorders, including male infertility. We presume that such complex rearrangement with respect to *AMELY* is relatively a rare event, brought about by error in crossingover. Study on larger sample size of such patients is warranted to establish a correlation between *AMELY* gene alteration and their fertility status.

FISH showed unilocus *DAZ* duplication in the interphase nuclei and metaphase Y chromosome in ∼5–10% of representative H patients. We are not sure if similar arrangement is maintained in germ cells as we could not ascertain this due to unavailability of semen samples. However, *DAZ* rearrangement in the blood lymphocytes in number of both HH and PH cases provides compelling evidence on Y chromosome susceptibility. Similar susceptibility was evident for *SRY* gene showing 2–8 copies among H patients in blood DNA.

Sequence analysis of *SRY* gene in H patients showed nucleotide variations. Two of the H patients with a single copy *SRY* gene showed mutation(s) in HMG box region. Since *SRY* is also involved in testis development, we infer that mutations observed here are not '*de novo*', rather represent somatic variations. Further, lack of nucleotide variations in representative H patients showing CNP by real-time PCR, suggests that more number of clones need to be sequenced to uncover their polymorphic nature. We observed significantly higher expression of *SRY* gene in the blood DNA of H males and construed this to be its derailed regulation. Besides testes, *SRY* is also expressed in other tissues including heart, liver, kidney and brain (Mayer *et al.* 1998).

Reportedly, *SRY* gene may influence brain and behaviour directly via its expression in neural tissue, or indirectly by exerting its effect on testis development, thereby modulating hormonal secretion (Kopsida *et al.* 2009). Overexpression of autosomal genes has been associated with molecular pathogenesis of various syndromes (Letourneau *et al.* 2014). In the present study, higher expression of *SRY* in H males corroborates this hypothesis. It would be significant to uncover autosomal genes involved in regulation of Y-linked genes. Analysis of larger sample size of such patients is required to obtain a clear picture on the genomics of the human Y chromosome and its overall function both in normal and abnormal genomes.

Conclusions

Present study demonstrates that genomic architecture of the Y chromosome in HH/PH/associated KS patients is prone to alteration. H and associated disorders can be corrected by hormonal treatment and or surgical intervention. However, the damages leading to genetic abnormalities may be transmitted to the offspring if an abnormal sperm happens to fertilize the egg and implantation takes place. In all such

cases, genetic screening of the patients would be useful. This would minimize the undue mutational load in the population contributing towards healthy demography.

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References

Achermann J. C. and Jameson J. L. 1999 Fertility and infertility: genetic contributions from the hypothalamic-pituitary-gonadal axis. *Mol. Endocrinol.* **13**, 812–818.

Ali S. and Ali S. 2010 Genetic integrity of the human Y chromosome exposed to groundwater arsenic. *BMC Med. Genomics* **3**, 35.

Bashamboo A., Rahman M. M., Prasad A., Chandy S. P., Ahmad J. and Ali S. 2005 Fate of SRY, PABY, DYS1, DYZ3 and DYZ1 loci in Indian patients harbouring sex chromosomal anomalies. *Mol. Hum. Reprod.* **11**, 117–127.

Choi J., Song S. H., Bak C. W., Sung S. R., Yoon T. K., Lee D. R. *et al.* 2012 Impaired spermatogenesis and gr/gr deletions related to Y chromosome haplogroups in Korean men. *PLoS One* **7**, e43550.

Chudnovsky A. and Niederberger C. S. 2007 Gonadotropin therapy for infertile men with hypogonadotropic hypogonadism. *J. Androl.* **28**, 644–646.

de Roux N., Genin E., Carel J. C., Matsuda F., Chaussain J. L. and Milgrom E. 2003 Hypogonadotropic hypogonadism due to loss of function of the KiSS1-derived peptide receptor GPR54. *Proc. Natl. Acad. Sci. USA* **100**, 10972–10976.

Elliott D. J. 2000 RBMY genes and AZFb deletions. *J. Endocrinol. Invest.* **23**, 652–658.

Feldman K. W. and Smith D. W. 1975 Fetal phallic growth and penile standards for newborn males. *J. Pediatr.* **86**, 395–398.

Ferlin A., Tessari A., Ganz F., Marchina E., Barlati S., Garolla A. *et al.* 2005 Association of partial AZFc region deletions with spermatogenic impairment and male infertility. *J. Med. Genet.* **42**, 209–213.

Fraietta R., Zylberstejn D. S. and Esteves S. C. 2013 Hypogonadotropic hypogonadism revisited. *Clinics* **68**, 81–88.

Graves J. 2002 The rise and fall of SRY. *Trends Genet.* **18**, 259–264.

Gorlov I. P., Kamat A., Bogatcheva N. V., Jones E., Lamb D. J., Truong A. *et al.* 2002 Mutations of the GREAT gene cause cryptorchidism. *Hum. Mol. Genet.* **11**, 2309–2318.

Jobling M. A., Lo I. C., Turner D. J., Bowden G. R., Lee A. C., Xue Y. *et al.* 2007 Structural variation on the short arm of the human Y chromosome: recurrent multigene deletions encompassing Amelogenin Y. *Hum. Mol. Genet.* **16**, 307–316.

Khabour O. F., Fararjeh A. S. and Alfaouri A. A. 2014 Genetic screening for *AZF* Y chromosome microdeletions in Jordanian azoospermic infertile men. *Int. J. Mol. Epidemiol. Genet.* **5**, 47–50.

Krausz C., Degl'Innocenti S., Nuti F., Morelli A., Felici F., Sansone M. *et al.* 2006 Natural transmission of USP9Y gene mutations: a new perspective on the role of *AZFa* genes in male fertility. *Hum. Mol. Genet.* **15**, 2673–2681.

Krausz C., Hoefsloot L., Simoni M. and Tüttelmann F. 2014 European academy of andrology; European molecular genetics quality network: EAA/EMQN best practice guidelines for molecular diagnosis of Y-chromosomal microdeletions: state-of-the-art 2013. *Andrology* **2**, 5–19.

Kopsida E., Stergiakouli E., Lynn P. M., Wilkinson L. S. and Davies W. 2009 The Role of the Y chromosome in brain function. *Open Neuroendocrinol. J.* **2**, 20–30.

Kumari A., Yadav S. K. and Ali S. 2012 Organizational and functional status of the Y-linked genes and loci in the infertile patients having normal spermiogram. *PLoS One* **7**, e41488.

Kuroda-Kawaguchi T., Skaletsky H., Brown L. G., Minx P. J., Cordum H. S., Waterston R. H. *et al.* 2001 The AZFc region of the Y chromosome features massive palindromes and uniform recurrent deletions in infertile men. *Nat. Genet.* **29**, 279–286.

Lange J., Noordam M. J., Van Daalen S. K., Skaletsky H., Clark B. A., Macville M. V. *et al.* 2013 Intrachromosomal homologous recombination between inverted amplicons on opposing Y-chromosome arms. *Genomics* **102**, 257–264.

Lange J., Skaletsky H., Van Daalen S. K., Embry S. L., Korver C. M., Brown L. G. *et al.* 2009 Isodicentric Y chromosomes and sex disorders as byproducts of homologous recombination that maintains palindromes. *Cell* **138**, 855–869.

Lattanzi W., Di Giacomo M. C., Lenato G. M., Chimienti G., Voglino G., Resta N. *et al.* 2005 A large interstitial deletion encompassing the amelogenin gene on the short arm of the Y chromosome. *Hum. Genet.* **116**, 395–401.

Lenz P., Luetjens C. M., Kamischke A., Kühnert B., Kennerknecht I. and Nieschlag E. 2005 Mosaic status in lymphocytes of infertile men with or without Klinefelter syndrome. *Hum. Reprod.* **20**, 1248–1255.

Letourneau A., Santoni F. A., Bonilla X., Sailani M. R., Gonzalez D., Kind J. *et al.* 2014 Domains of genome-wide gene expression dysregulation in Down's syndrome. *Nature* **508**, 345–350.

Marshall W. A. and Tanner J. M. 1970 Variations in the pattern of pubertal changes in boys. *Arch. Dis. Child.* **45**, 13–23.

Mayer A., Lahr G., Swaab D. F., Pilgrim C. and Reisert I. 1998 The Y-chromosomal genes *SRY* and ZFY are transcribed in adult human brain. *Neurogenetics* **1**, 281–288.

Murphy K. M., Cohen J. S., Goodrich A., Long P. P. and Griffin C. A. 2007 Constitutional duplication of a region of chromosome Yp encoding AMELY, PRKY, and TBL1Y: implications for sex chromosome analysis and bone marrow engraftment analysis. *J. Mol. Diagn.* **9**, 408–413.

Pathak D., Premi S., Srivastava J., Chandy S. P. and Ali S. 2006 Genomic instability of the DYZ1 repeat in patients with Y chromosome anomalies and males exposed to natural background radiation. *DNA Res.* **13**, 103–109.

Premi S., Srivastava J., Chandy S. P. and Ali S. 2007 *AZFc* somatic microdeletions and copy number polymorphism of the DAZ genes in human males exposed to natural background radiation. *Hum. Genet.* **121**, 337–346.

Premi S., Srivastava J., Chandy S. P. and Ali S. 2009 Unique signatures of natural background radiation on human Y chromosomes from Kerala, India. *PLoS One* **4**, e4541.

Premi S., Srivastava J., Epplen J. T. and Ali S. 2010 *AZFc* region of the Y chromosome shows singular structural organization. *Chromosome Res.* **18**, 419–430.

Premi S., Srivastava J., Panneer G. and Ali S. 2008 Startling mosaicism of the Y chromosome and tandem duplication of the *SRY* and *DAZ* genes in patients with turner syndrome. *PLoS One* **3**, e3796.

Qin M., Gong C., Qi Z., Wu D., Liu M., Gu Y. *et al.* 2014 Children with idiopathic hypogonadotropic hypogonadism: clinical data analysis and mutations analysis of KAL1 and FGFR1 gene. *Zhonghua Er Ke Za Zhi* **52**, 942–947.

Reynolds N. and Cooke H. J. 2005 Role of the *DAZ* genes in male fertility. *Reprod. Biomed. Online* **10**, 72–80.

Rives N. 2014 Y chromosome microdeletions and alterations of spermatogenesis, patient approach and genetic counseling. *Ann. Endocrinol.* **75**, 112–114.

Silveira L. F., MacColl G. S. and Bouloux P. M. 2002 Hypogonadotropic hypogonadism. *Semin. Reprod. Med.* **20**, 327–338.

Sun C., Skaletsky H., Rozen S., Gromoll J., Nieschlag E., Oates R. *et al.* 2000 Deletion of Azoospermia factor a (AZFa) region of human Y chromosome caused by recombination between HERV15 proviruses. *Hum. Mol. Genet.* **9**, 2291–2296.

Svechnikov K., Stukenborg J. B., Savchuck I. and Söder O. 2014 Similar causes of various reproductive disorders in early life. *Asian J. Androl.* **16**, 50–59.

Tian L., Chen M., Peng J. H., Zhang J. W. and Li L. 2014 Clinical characteristics, cytogenetic and amolecular findings in patients with disorders of sex development. *J. Huazhong Univ. Sci. Technolog. Med. Sci.* **34**, 81–86.

Vogt P. H., Edelmann A., Kirsch S., Henegariu O., Hirschmann P., Kiesewetter F. *et al.* 1996 Human Y chromosome azoospermia factors (*AZF*) mapped to different subregions in Yq11. *Hum. Mol. Genet.* **5**, 933–943.

Yadav S. K., Kumari A. and Ali S. 2013 Fate of chromosome linked genes and loci in prostate cancer cell lines DU145 and LNCaP. *BMC Genomics* **14**, 323.

Yadav S. K., Kumari A., Javed S. and Ali S. 2014 DYZ1 arrays show sequence variation between the monozygotic males. *BMC Genet.* **15**, 19.

Yan J., Fan L., Zhao Y., You L., Wang L., Zhao H. *et al.* 2011 DYZ1 copy number variation, Y chromosome polymorphism and early recurrent spontaneous abortion/early embryo growth arrest. *Eur. J. Obstet. Gynecol. Reprod. Biol.* **159**, 371–374.

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