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Tandem duplication and copy number polymorphism of the *SRY* gene in patients with sex chromosome anomalies and males exposed to natural background radiation

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Mutations in the *SRY* gene encompassing the HMG box have been well characterized in gonadal dysgenesis, male infertility and other types of sex chromosome related anomalies (SCRA). However, no information is available on copy number status of this gene under such abnormal conditions. Employing 'Taqman Probe Assay' specific to the *SRY* gene, we screened 16 DNA samples from patients with SCRA and 36 samples from males exposed to high levels of natural background radiation (HNBR). Patients with SCRA showed 2–16 copies of the *SRY* gene of which, one, Oxen (49, XYYYY) had eight copies with sequences different from one another. Of the 36 HNBR samples, 12 had one copy whereas 24 harboured 2–8 copies of the *SRY* gene. A HNBR male 33F had one normal and one mutated copy of this gene. Analysis of 25 DNA samples from blood and semen of normal males showed only one copy of this gene. Despite multiple copies in affected males, fluorescence *in-situ* hybridization (FISH) with *SRY* probe detected a single signal on the Y chromosome in HNBR males suggesting its possible localized tandem duplication. Copy number status of the other Y-linked loci is envisaged to augment DNA diagnostics facilitating genetic counselling to affected patients.

Key words: background radiations/copy number polymorphism (CNP)/endoreduplication/human Y chromosome/non-disjunction/tandem duplication

Introduction

SRY gene located on p11.3 region of the Y chromosome encodes a transcription factor that belongs to the HMG DNA-binding proteins and plays a dominant role in mammalian male sex determination (Gubbay *et al*., 1990). This gene triggers the differentiation of the Sertoli cells in a cell autonomous manner from one of the somatic cell lineages in the genital ridge (Jeske *et al*., 1995). In humans and mice, the onset of *SRY* mRNA expression defines testis determination. Expression of *SRY* gene in humans is not switched off and, unlike mice, continues until adulthood where it expresses transiently from d.p.c 10.5 to d.p.c 12.0 (Sekido *et al*., 2004).

Analysis of the *SRY* gene in gonadal dysgenesis, altered karyotypes (e.g. X/XYY), polysomy of the Y chromosome (Sirota *et al*., 1981) and XY females (Takagi *et al*., 1999) has shown mutations either within or up/down stream regions of the HMG box. Patients with XY gonadal dysgenesis carry point mutations in the *SRY* gene which affect its binding or bending properties (Hawkins *et al*., 1992). In humans, the *SRY* gene is reported to be single copy and haploid, whereas in some rodents it is present in one or more, monomorphic or polymorphic forms (Lundrigen and Tucker, 1997). Previously, owing to limitations in the power of detection, it was not feasible to assess copy number status of a gene and correlate the same with genetic anomalies. Real-time PCR enables detection of even a trace amount of DNA (low copy number) and minute differences between the two samples (copy number differences). Despite multiple copies of the Y chromosomes in patients, there is no report on copy number polymorphism (CNP) of the *SRY* gene and its biological consequence. Here, we report CNP of the *SRY* gene in patients suffering from sex chromosome related anomalies (SCRA) and males exposed to high levels of natural background radiation (HNBR) from Kerala, (South India) using real-time PCR assays.

The rationale to include HNBR samples is based on the fact that cancers, chromosomal lesions (Cheriyan *et al*., 1999) and mutations in the minisatellites and other regions of the genome are induced by the background radiation but its effect on the human Y chromosome has not yet been studied. Long term exposure to ionizing radiation leaves a permanent impression on the genome or on a given chromosome (Hande *et al*., 2003). In humans, long-term experimental irradiation and mutational analysis across the generations are impractical and unethical. In view of this, we used samples from a population living in the coastal region of Kerala that offers a natural setting of high background radiation.

In order to establish if the copy number status of the *SRY* gene is related to its organization on the Y chromosome, we conducted fluorescence *in-situ* hybridization (FISH) on the metaphase chromosomes of the HNBR males. FISH data showed a single signal of the *SRY* gene suggesting its localized tandem duplication. Our study demonstrates that human Y chromosome is indeed affected by HNBR showing CNP analogous to that of patients with SCRA.

This work is envisaged to augment DNA diagnostics and genetic counselling.

Materials and Methods

Collection of blood and semen samples and genomic DNA isolation

Samples were collected with informed consent from 25 normal males (15 blood and 10 semen samples), 16 SCRA patients and four normal females (negative control) from J N Medical College, Aligarh, India, strictly in accordance with the Institute's Ethical and Bio-safety Guidelines. In addition, blood and semen samples of 36 males belonging to 23 families exposed to HNBR (Chavara) and 16 samples (blood and semen each from eight males) living in non-radiation exposed area (Kochi) from Kerala were obtained with their informed consent. The coastal area of Kerala contains the world's highest levels of natural radioactivity which is due to local abundance of monazite, a mineral containing around 10% thorium phosphate. The radioactivity strip measures an area of only 10 km by 1 km and supports a population of several thousands, whose traditional occupation is fishing (Gruneberg *et al*., 1966). The biologically effective radiation dose received by the coastal population is 10 000–12 000 μSv per year which is 10 times greater than the worldwide average (Nair *et al*., 1999; Forster *et al*., 2002). DNA isolation from blood and semen samples was done following standard protocols (Ali *et al*., 1986). Genomic DNA from the cell lines of Oxen was kindly provided by Mark Jobling, Department of Genetics, University of Leicester, Leicester, England, UK.

Primers, Taqman probes and conditions for real-time PCR

For real-time PCR, Assay on Demand specific to *SRY* gene (ID: Hs00243216_s1, nucleotide location 453) and an endogenous control, *RNase* P gene (single copy per haploid genome; Catalog number: 4316831) having FAM/TAMRA and FAM/MGB probes, respectively, procured from Applied Biosystem, USA, were used. For the reaction, DNA dilution was adjusted to 5 ng/μl. The universal cyclic conditions for real-time PCR comprised 10 minutes of polymerase activation at 95°C followed by 40 cycles, each at 95°C for 15 seconds and 60°C for 1 minute. The reaction was conducted on 'Sequence Detection System' 7000 (ABI, USA). Taking triplicates of each sample, every assay was repeated at least five times to ensure error-free consistent Ct values (error rate ±0.05).

Copy number estimation of the SRY *gene*

Copies of the *SRY* gene were calculated using the formula: copy number = $(1 + E)^{-\Delta Ct}$, where E is the efficiency of the PCR, ΔCt difference in threshold cycle value between the test sample and endogenous control. To achieve the maximum efficiency (one) of the real-time PCR, the amplicon size was kept small (50–70 bp) so that copy number of the test gene remains $2^{-\Delta Ct}$.

End point PCR of the SRY *gene, agarose gel electrophoresis, cloning and sequencing*

SRY gene fragment was amplified from genomic DNA using a set of forward 5′GACAATGCAATCATATGCTTCTGC3′ and reverse 5′CTGTAGCG GTCCCGTTGCTGCGGT3′ primers (Bashamboo *et al*., 2005) in a 25 μl reaction volume containing Taq Polymerase, 10 X PCR buffer (Promega, Madison, WI, USA), 200 μM dNTP's and 100 ng of target DNA. The reaction was conducted for a total of 30 cycles, each involving denaturation of the template at 95°C for 1 minute, annealing of the primers at 65°C for 1 minute and extension of the same at 72°C for 1 minute. The amplified products resolved on 1.5% agarose gel were purified (QIAGEN Gel Extraction Kit) and cloned in *pGEM*-T easy vector (Promega, Madison, WI, USA). Twenty recombinant plasmids containing *SRY* insert from Oxen and ten from HNBR male 33F were sequenced. Distinctly different sequences of the eight independent clones (pSOx1-pSOx8) from Oxen (Accession number: AY998484-AY998491), two (pS33F3 and pS33F13) from 33F male (Accession no. DQ062157 and DQ062158), respectively, were deposited in the GenBank. For confirmation of the point mutations, direct sequencing of PCR product was also done.

FISH

Blood from HNBR-exposed males, unexposed males and females was used for chromosome preparation using the standard protocol (Bashamboo *et al*., 2005). FISH was conducted using dual colour LSI *SRY* probe from VYSIS (Part # 32–191007). This probe hybridizes simultaneously to band Yp11.3 of the human Y chromosome and Xp11.1-q11.1 locus of the human X chromosome. Hybridization, washing, counterstaining, and mounting of the slides were done using the standard protocol (Rahman *et al*., 2004). Slides were screened under the Olympus Fluorescence microscope (BX51) fitted with a vertical fluorescence illuminator U-LH100HG UV, excitation and barrier filters. Metaphase images were captured with a CCD camera and karyotyping was done using Cytovision 2.81 software from Applied Imaging Systems.

Results

CNP of the SRY *gene in patients with SCRA and HNBR males*

Copy number status of the *SRY* gene studied by real-time PCR was found to be abnormal. Owing to haploid status of the Y chromosome, Ct value for *RNase P* gene was −1 as compared to that of *SRY* gene [ΔCt = (Ct of *SRY*-Ct of *RNaseP*) = 1] in blood (diploid) DNA. The Ct values for *RNase*P and *SRY* genes from the semen DNA (haploid) of the normal males were found to be the same (ΔCt = 0) corresponding to single copy status of the *SRY* gene in normal human males (Figure 1). This was substantiated by subsequent analysis of 15 blood and 10 semen DNA samples used as controls (data not shown).

Of all the DNA samples analysed from the patients with SCRA, *SRY* gene showed CNP in the range of 2–16 (Table I). This gene was absent in a Turner patient p6697 but present in two others, p2C and p4 showed one and four copies, respectively. An unusually tall Turner patient p65971 was found to have 16 copies, whereas the father of this patient (F-p65971) had two copies of the *SRY* gene (Table I). A Swyer syndrome patient, p20 and Oxen showed four and eight copies, respectively (Figure 2). DNA analysis of hypogonadism/Cryptorchidism patients p65974 and p65973 showed four copies in each. Clinical details and hormonal status of the patients with SCRA used in the present study have been reported previously (Bashamboo *et al*., 2005).

For the copy number status of the *SRY* genes in males exposed to HNBR, 36 samples analysed included 23 families. Fathers and sons (F, father; B, boy for son) in several families showed two or more copies of this gene (Table II). Of all the samples analysed, twelve showed single copy of the *SRY* gene, fifteen showed two, six showed 1–2 (owing to Y chromosome mosaicism), two showed eight and one sample showed four copies affecting about 66% of the males. A HNBR-exposed male (33F) and his son (33B), both carried two copies of the *SRY* gene (Figure 3). Yet another HNBR male 7F, and his son 7B had eight and four copies, respectively (Table II). Copy number and sequence of the *SRY* gene in 16 samples (comprising semen and blood samples from eight males) from Kochi City, a non-radiation area of Kerala was found to be normal upon the analysis (data not shown). Interestingly, end point PCR showed stronger band intensity of the *SRY* gene in case of males with additional copies compared to that of the normal males (Figure 4).

Uniqueness of the copies of the SRY *gene in oxen and 33f*

Oxen, which represents a rare instance of Y chromosome polysomy (49, XYYYY) showed eight copies of the *SRY* gene with unique nucleotide changes at several positions (Table III). *In silico* peptide analysis (http://www.ncbi.nlm.nih.gov/gorf/gorf.html) of these sequences showed several known and novel mutations (Figure 5A). Two of the recombinant clone's pSOx1 and pSOx2, showed silent nucleotide changes at several places. The pSOx3, representing another

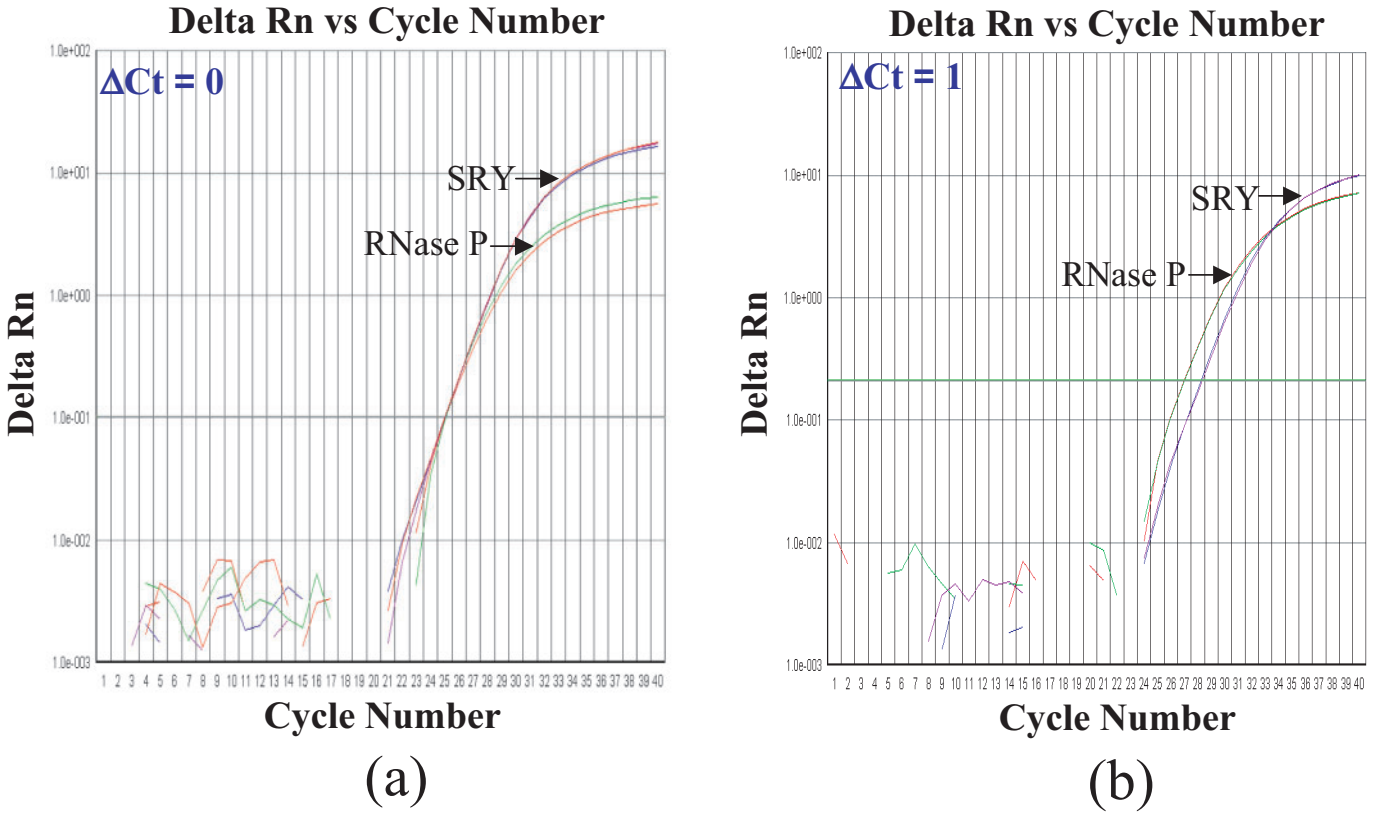

Figure 1. Real-time PCR amplification plot showing single copy of the *SRY* gene (ΔCt = 0) in germline (**a**) and blood (ΔCt = 1) DNA (**b**) from normal human males.

Table I. Copy number polymorphism of the *SRY* gene amongst patients with sex chromosome related anomalies

Number	Patients	Karyotype	Phenotype	Ct RNase *P* (±0.05)	Ct SRY (±0.05)	ΔCt (approximate)	*SRY* copies
1	p2B	45,XO 46,XX 46,XY (T)	Female	29.51	29.56	0	2
2	p2C	NA (T)	Female	31.50	32.58	1	1
3	p4	45,XO 46,XX 46,XY (T)	Female	30.00	29.20	–1	4
4	p65971	46,XX 46,XY 46,XXXp (T)	Female	31.60	28.67	–3	16
5	F-p65971	NA	Male	30.01	30.11	0	2
6	p20	46,XY (S)	Female	32.00	31.93	0	2
7	p65972	NA (T)	Female	35.77	32.74	–3	16
8	p65973	NA (C & H)	Male	32.59	31.63	–1	4
9	p65974	NA (C & H)	Male	31.93	30.89	–1	4
10	p65975	NA (T)	Female	32.87	30.80	–2	8
11	Oxen	49,XYYYY	Male	28.96	27.12	–2	8
12	p21	47,XXY (K)	Female	28.27	28.21	0	2
13	p6697	NA (T)	Female	26.00	Undetermined	Undetermined	0
14	p17698	NA (T)	Female	28.82	29.79	1	1
15	PC	46,XY	Male	24.00	25.05	1	1
16	Normal male	46,XY	Male	28.07	29.05	1	1

copy of the *SRY* gene showed a known F110S mutation, a characteristic feature of the gonadal dysgenesis and a novel Y163F mutation downstream of the HMG box. In pSOx4, a mutation R59S, upstream to HMG box known to be responsible for poor binding of the *SRY* protein to target DNA was detected (Harley *et al*., 2003). This clone was devoid of an amino acid at position 198. In pSOx5, another change K170E, not reported earlier, was detected whereas in pSOx6, P108A mutation responsible for gonadal dysgenesis and four novel mutations Y198D, A7V, F34S and D58E, also not reported previously, were detected. We observed Y127N change in pSOx7. In an earlier study, the Y127C mutation detected at the same position was found to cause a reduction in the binding of the HMG box with target DNA (Harley *et al*., 2003). In pSOx7 and pSOx8, seven amino acid residues DNRLYRD from 160–166 were altered into GQQVVQG. In pSOx8, four mutations (A6V, P26S, D73G and Q97R) known to be implicated with gonadal dysgenesis were detected (Table III). Analysis of the

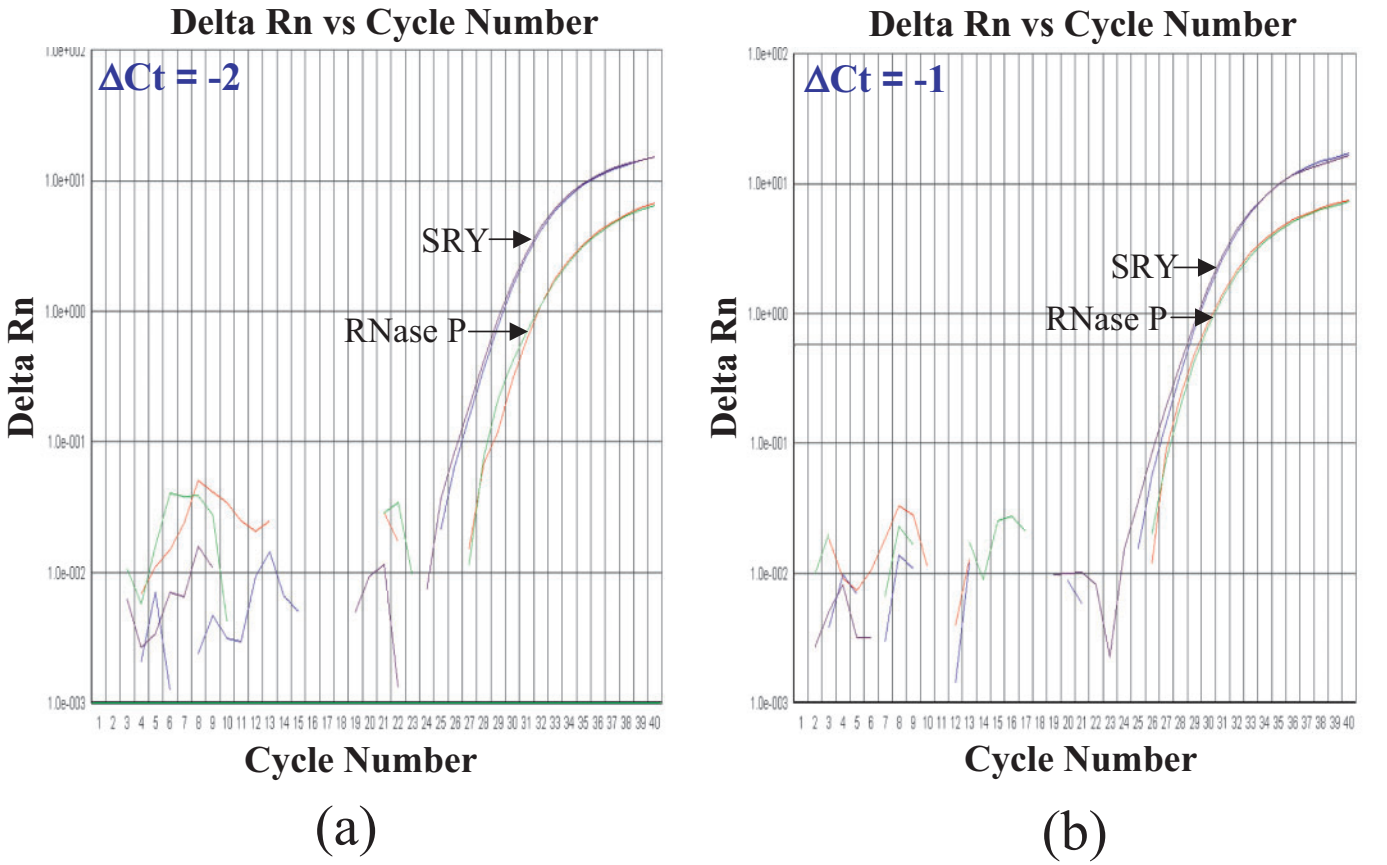

Figure 2. Real-time PCR amplification plot showing eight copies of the *SRY* gene (ΔCt = –2) in Oxen (**a**) and four copies of the same (ΔCt = –1) in Swyer syndrome p20 (**b**).

tertiary structure of the HMG box (www.searchlauncher.bcm.tmc.edu/seq-search/struc-predict.html) showed a number of alterations in all the eight copies of the SRY peptide (data not shown).

Similarly, analysis of the two copies of the *SRY* gene from the HNBR male 33F showed abnormal sequences (Table IV). One clone, pS33F13, showed a massive c-terminal deletion involving half of the HMG box and the other one, pS33F3, showed a point mutation towards the amino terminal and 20 'aa' deletion in the c-terminal region from residues 179–198 (Figure 5B). Details of the nucleotide changes in 33F male are given in Table IV.

Possible tandem duplication of the SRY *gene*

The FISH conducted on the metaphase chromosomes and interphase nuclei of the normal males showed a single signal of the *SRY* and *DXZ1* probes in the interphase nuclei and on the chromosomes (Figures 6a–c). A single signal was detected in the centromeric region of each X chromosome in the normal female metaphases taken as negative control (data not shown). Males exposed to HNBR also showed a single signal of the *SRY* gene even with CNP. The HNBR male 7F carrying eight copies of the *SRY* gene showed a relatively stronger signal on the Y chromosome compared to that of a normal male (Figures 6d–f). Depending upon the condensation status of the sister chromatids, two signals of *SRY* were also detected (Figures 6g–k). However, in several cases with 2–4 copies of the *SRY* gene, the differences in the signal intensity were not discernible (Figure 7).

Discussion

The human genome contains blocks of duplicated sequences resulting in genomic variation (Sebat *et al*., 2004; Sharp *et al*., 2005). Thus, CNP of the *SRY* gene is well within the realm of genetic diversity and process of genome evolution. Multiple copies of the Y chromosome, possibly caused due to non-disjunction, are expected to harbour proportionate numbers of their genes, maintaining a correlation between the two. In the present study, no such correlation between the Y chromosome and *SRY* gene was seen in the patients with SCRA and the males exposed to HNBR.

Gene duplication increases the complexity of a genome, particularly when it is not clear if all the copies follow normal levels of expression (He and Zhang, 2005). Incidentally, it cannot be resolved by Southern blot hybridization (Murthy *et al*., 2005). In the present study, this limitation was circumvented by the real-time PCR assay system. The results showed as many as 16 copies of the *SRY* gene in two cases (p65971 and p65972) (see also Table I) suggesting multiple rounds of tandem duplication. However, presence of two copies of this gene in the father (F-p65971) of a patient (p65971) suggests that the patient did not inherit 16 copies, instead it was due to multiple rounds of tandem duplications. Although no direct evidence is available, these observations suggest that non-disjunction of the Y chromosome and duplication of the *SRY* gene are independent events. This was also corroborated by the HNBR data where fathers and sons often showed uneven copy number of the *SRY* gene (Table II). These observations are important because in normal human males, *SRY* has always remained single copy as substantiated by the analysis of two

Table II. Copy number polymorphism of the *SRY* gene in males exposed to high level of natural background radiation

Number	Patient ID	Ct RNase *P* (±0.05)	Ct SRY (±0.05)	ΔCt (approximate)	*SRY* copies
1	1F	26.00 (28.67)	27.01 (28.70)	1 (0)	1–2
2	1B	26.15	26.20	0	2
3	3F	26.34	27.31	1	1
4	3B	34.42	34.38	0	2
5	4F	26.62	26.67	0	2
6	5F	34.33 (36.01)	35.28 (35.99)	1 (0)	1–2
7	5B	26.38	26.41	0	2
8	6F	26.00	25.98	0	2
9	7F	30.98	29.00	–2	8
10	7B	29.33	29.35	0	2
11	8F	25.79 (30.23)	26.74 (30.15)	0	1–2
12	8B	30.46 (27.04)	31.39 (27.10)	1 (0)	1–2
13	9F	27.30	27.40	0	2
14	10F	26.95	27.01	0	2
15	10B	27.77	27.81	0	2
16	11B	26.01	26.99	1	1
17	12F	25.98	26.84	1	1
18	13F	25.84	26.99	1	1
19	13B	26.11	27.01	1	1
20	14F	28.99	29.95	1	1
21	14B	29.80	30.88	1	1
22	15F	26.11 (29.99)	27.01 (29.97)	1 (0)	1–2
23	15B	28.70 (31.69)	29.69 (31.78)	1 (0)	1–2
24	22F	28.55	29.55	1	1
25	26F	28.46	26.50	–2	8
26	26B	30.79	30.59	0	2
27	27F	26.40	27.39	1	1
28	27B	29.99	29.98	0	2
29	28F	26.55	27.50	1	1
30	29F	31.37	30.50	–1	4
31	30F	21.56	21.49	0	2
32	30B	31.41	31.38	0	2
33	31B	24.44	24.35	0	2
34	32F	24.74	25.74	1	1
35	33F	24.67	24.60	0	2
36	33B	28.19	29.15	1	1
37	Normal male	27.12	28.29	1	1

sets of DNA including that from blood and semen samples from non-exposed areas (see materials and methods).

CNP and Y chromosome mosaicism

Our quest to demonstrate if a single Y chromosome could harbour more than one copy of the *SRY* gene became clear from the analysis of a patient (p4) with mosaic chromosomes (46,XX/46,XY/45,XO). This patient with a single Y chromosome had four copies of the *SRY* gene, suggesting two rounds of tandem duplication. Yet another Turner patient (p6697) who had undetectable Y chromosome and *SRY* gene represented a case of pure XO conceptus (not a mosaic of the Y chromosome) though majority of the Turner cases show varying degrees of Y chromosome mosaicism (Ali and Hasnain, 2003; Bashamboo *et al*., 2005). The instance(s) of pure XO conceptus cannot be demonstrated unequivocally because chromosome analysis does not uncover the lowest level of Y chromosome mosaicism. Thus, employing real-time PCR Assay System, all the ambiguous cases may be resolved. CNP seems to be more common (>80%) in patients suffering from SCRA. However, the *SRY* gene never remained normal (single copy) in about 66% of the males exposed to HNBR.

Possible mechanism of CNP

A single copy gene gives rise to a number of mRNA transcripts, some of which may be reverse transcribed and transposed into the DNA resulting in its multiple copies (Moran *et al*., 1996). Thus, besides putative tandem duplication, reverse transcriptase activity on its mRNA may also contribute to the CNP. However, this seems to be a less likely mechanism because all the *SRY* copies were in the multiples of the two, and thus far a maximum of only 16 were found. Owing to the availability of a large number of mRNA transcripts, reverse transcriptase like activities would have generated far more numbers of *SRY* copies.

A classical model suggests that after gene duplication, one copy preserves the ancestral function while the other one is free to conform to a new function (Van Hoof, 2005; Ohno, 1999). In an alternate duplication, divergence and complementation model, duplicated genes are preserved because each copy loses some but not all of its functions through degenerating mutations (Hughes, 1994; Force *et al*., 1999). The second model seems to be a more likely explanation for eight copies of the *SRY* gene in Oxen and two copies in the HNBR male 33F, all having different sequences. It is well established that gene duplication and alternate splicing are inversely correlated evolutionary mechanisms fuelling the process of new biological functions (Kopleman *et al*., 2005). In the present study, the results suggest that HNBR also contributes to CNP, perhaps associated with the (initiation) process of duplication. Large-scale CNPs in humans have been described (Sebat *et al*., 2004; Sharp *et al*., 2005) but the CNP of the *SRY* gene reported herein seems to be the first such study.

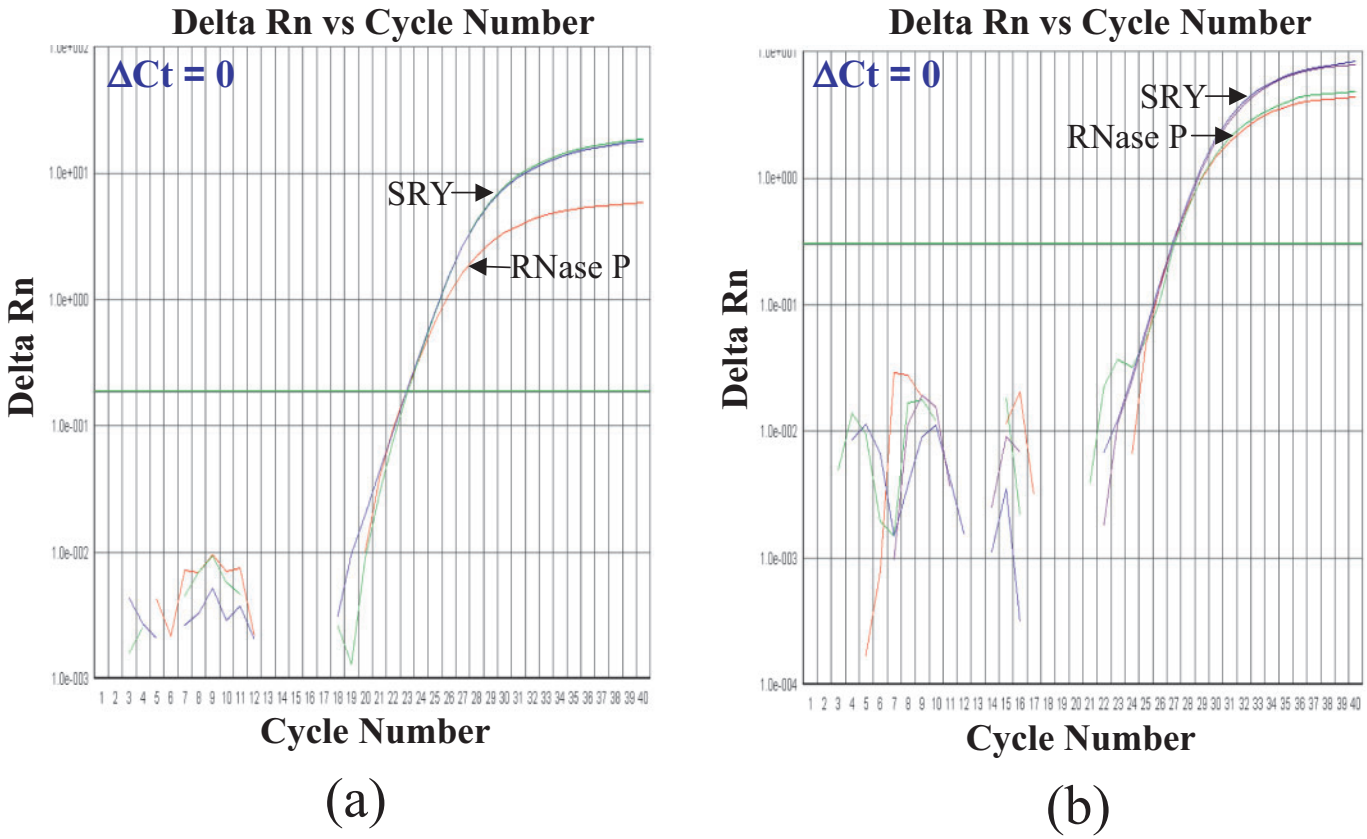

Figure 3. Real-time PCR amplification plot showing two copies (ΔCt = 0) each of the *SRY* gene in HNBR male 33F (**a**) and his son 33B (**b**).

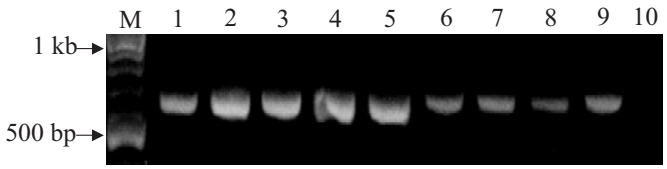

Figure 4. End point PCR amplification of the *SRY* gene with DNA from different sources. Lanes representing 1, 33F; 2 and 3, Oxen; 4 and 5, p65971; 6 and 7, father of patient p65971; 8 and 9, normal males as positive control; and 10, female DNA used as negative control. Barring 33F, all the samples used were in duplicate. 'M' denotes molecular marker given in base pairs. The difference in the signal intensity of PCR product is correlated with the copy number status of the *SRY* gene.

Table III. Details of nucleotide changes in the eight copies of the *SRY* sequences of oxen

Number	Sequence ID	Nucleotide length	Mutation at nucleotide position
1	Normal	600	—
2	pSOx1	599	13 (A-C), 208 (C-T), 486 (deletion of A)
3	pSOx2	596	486 (deletion of A), 555 (C-G), 559 (deletion of C), 564 (deletion of A), 579 (deletion of C)
4	pSOx3	600	333 (T-C), 495 (A-T), 526 (A-C), 547 (C-G), 587 (G-C)
5	pSOx4	600	181 (A-T), 598 (C-A), 600 (G-T)
6	pSOx5	597	1 (deletion of G, A, C), 512 (A-G)
7	pSOx6	597	1 (deletion of G, A, C), 24 (C-T), 95 (A-G), 105 (T-C), 178 (T-A), 186 (C-G), 596 (T-G)
8	pSOx7	600	1 (deletion of G, A), 383 (T-A), 483 (Insertion of G)552 (T-C), 586 (deletion of A)
9	pSOx8	600	1 (deletion of G, A), 24 (C-T), 80 (C-T), 222 (A-G), 483 (Insertion of G), 493 (T-C), 519 (deletion of A), 553 (A-T)

Conclusion

Irrespective of the mechanisms involved, CNP of the *SRY* gene uncovered in the present study provides ample opportunity to establish a correlation between the abnormal genotype, number of Y chromosome(s) and copies of the *SRY* gene(s).

Screening of the additional Y chromosome(s) and assessment of CNP of more genes together with the chromosome analysis and hormonal assays would enhance our understanding of the biological consequences of such phenomenon, enabling more focused genotype phenotype correlation. Similarly, DNA screening at the prenatal level using real-time PCR assay may be of immense help to monitor high-risk pregnancies, facilitating more accurate prognosis of the genetic anomalies. In turn, this would uplift the accuracy of the DNA diagnostics and enable better genetic counselling to affected patients.

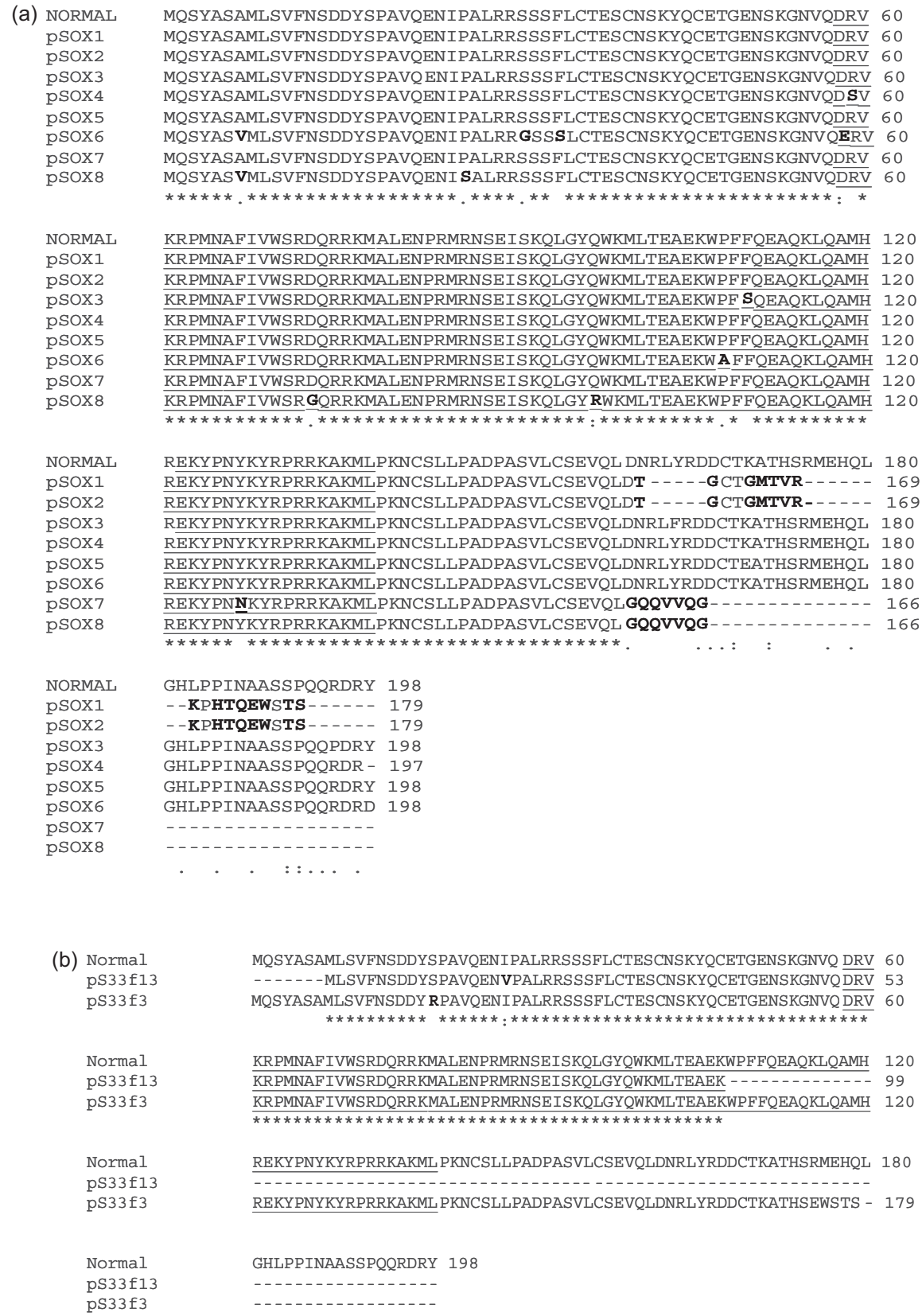

Figure 5. (**a**) ClustalW alignment of the eight copies of SRY peptides from Oxen showing distinct change in the amino acid sequences is highlighted in bold. HMG box is underlined. Note the deletions of the amino acids at the c-terminus of most of the clones. (**b**) ClustalW alignment of the two copies of the SRY peptide in HNBR male 33F showing deletion of half of the HMG box in one copy (pS33F13) and 20 amino acids in the c-terminus in the other one (pS33F3). The HMG box is underlined.

Table IV. Nucleotide changes in the two copies of the *SRY* gene in 33F male exposed to high level of natural background radiation

Number	Sequence ID	Nucleotide length	Mutation at nucleotide position
1	Normal male	600	Normal sequences
2	pS33F3	599	58 (T-A), 145 (A-G), 527 (deletion of A), 571 (A-G)
3	pS33F13	600	77 (A-G), 324 (G-A), 486 (A-G), 569 (G-A)

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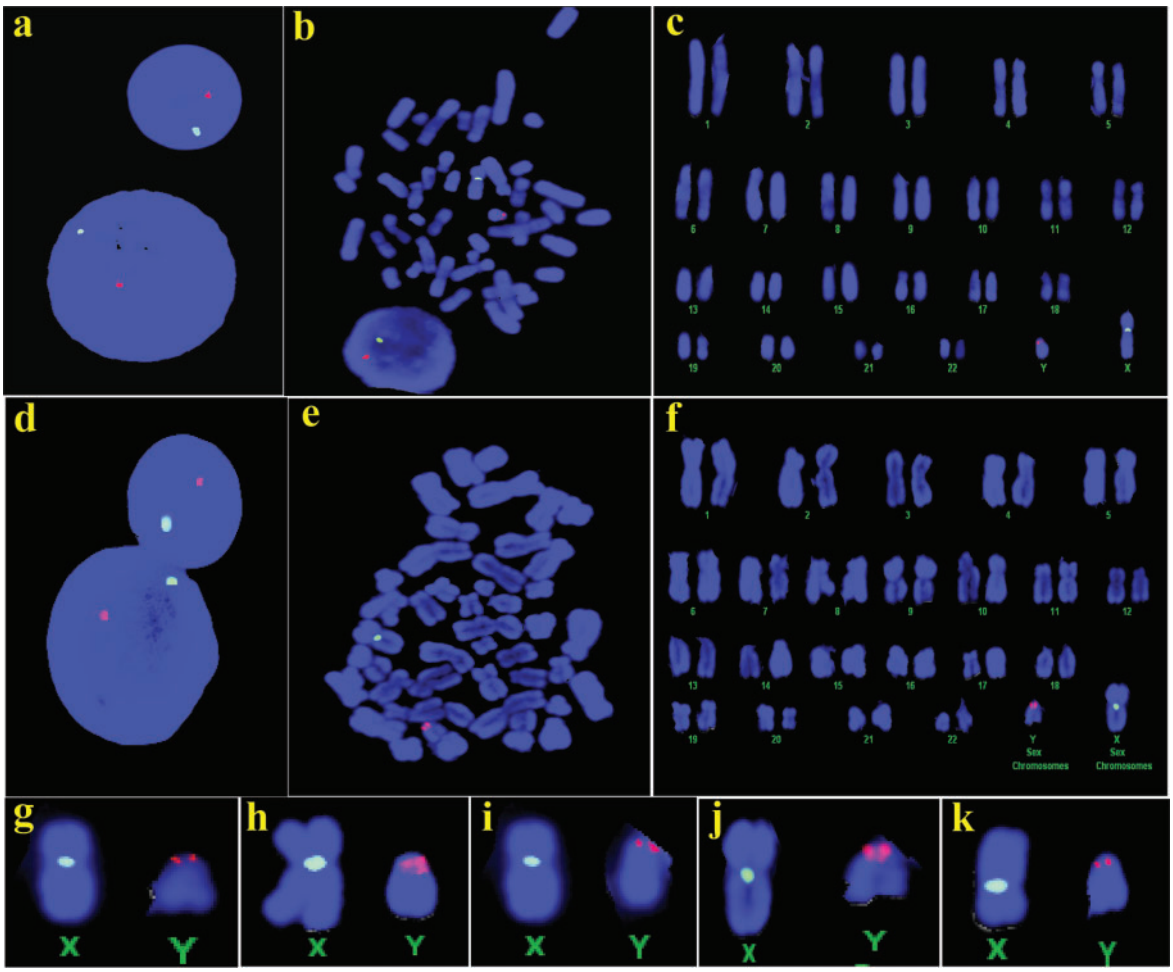

Figure 6. Fluorescence *in-situ* hybridization of the dual *SRY* probe with interphase nuclei (**a**) and metaphase chromosomes (**b,c**) from the normal male and HNBR male 7F (**d–f**). Green signal on the X chromosome corresponds to *DXZ1* and orange one on the Y chromosome to the *SRY* gene. The cut-out of the X and Y chromosomes are from 7F male representing different metaphases (**g–k**). Note the two *SRY* signals on the opposed sister chromatids occupying the same locus.

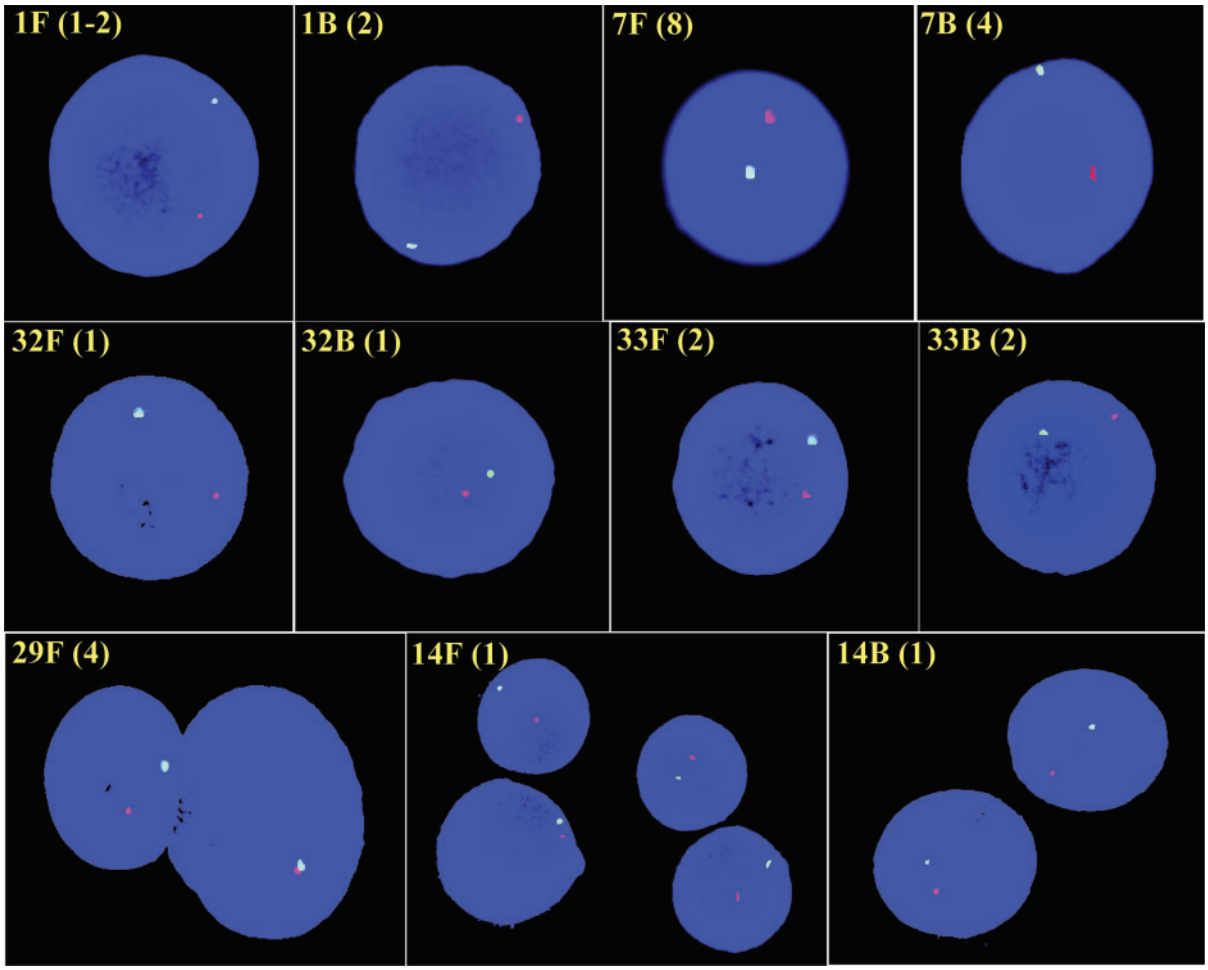

Figure 7. Fluorescence *in-situ* hybridization of the dual *SRY* probe with interphase nuclei of the HNBR males. The sample ID of each male is given on the top left of the panel. Figures in parenthesis denote number of copies of the *SRY* gene. Note the distinct green signal of *DXZ1* on X chromosome and orange of the *SRY* on the Y chromosome. The signal intensity of *SRY* is correlated with its copy number (see text for details).

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