

Genomic Instability of the DYZ1 Repeat in Patients with Y Chromosome Anomalies and Males Exposed to Natural Background Radiation

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Abstract

We assessed genomic instability of 3.4 kb DYZ1 repeat arrays in patients encompassing prostate cancer (PC), cases of repeated abortion (RA) and males exposed to natural background radiation (NBR) using real-time PCR and fluorescence *in situ* hybridization (FISH). Normal males showed DYZ1 copies ranging from 3000 to 4300, RA, 0–2237; PC, 550; and males exposed to NBR, 1577–5700. FISH showed organizational variation of DYZ1 in these samples substantiating the data obtained from real-time PCR. Of the 10 RA samples, 7 were found to be affected of which, 5 showed deletion of 265 bp from nt 25 to 290 and 773 bp from 1347 to 2119 and 2 showed deletion of 275 bp from nt 3128 to 3402. Copy number variation of DYZ1 in these males correlated with genetic constraints/anomalies. Although precise mechanisms of genomic instability of DYZ1 remains unclear, we construe that this repeat plays a critical role in maintaining the structural integrity of the Y chromosome, possibly by absorbing the load of mutations. This may be used as a marker system to analyze genetic integrity of the DYZ1 repeat array(s) across the spectrum of patients.

Key words: copy number variation; DYZ1 repeat fraction; Y chromosome dysfunction; repeated abortion; prostate cancer

1. Introduction

The human Y chromosome has attracted a great deal of attention owing to its small size and the limited number of genes it carries.^{1,2} The recent genomic data showed presence of massive palindromic sequences having several isoforms and multiple copies of genes suggesting its unique organization and equally unique trail of evolution.^{3–5} Despite fewer functional genes located on the Y chromosome,^{6,7} its earlier image of genetic wasteland is totally changed. Complete sequencing of the euchromatic region revealed a total of 178 transcribed units,³ most of which are pseudogenes or their amplified copies. The Y encodes only 45 unique proteins and several of its genes are involved in sex determination and control and regulation of fertility.^{8,9}

The human Y chromosome has been divided into pseudoautosomal regions (PAR1 and PAR2), which represent

5% of its total sequences, whereas the remaining 95% NRY (Non-Recombining Y) contains euchromatic and heterochromatic regions. The genes in the PAR1 and PAR2 regions are inherited like autosomal ones.¹⁰ The distal region represents the heterochromatic portion corresponding to Yq12 with ~30 Mb sequences¹¹ and harbors two major repeat domains, DYZ1 and DYZ2.¹² The DYZ1 satellite, corresponding to ~40% of the total Y chromosome⁷ was first reported as 3.4 kb band from *Hae*III digested human male genomic DNA.¹³ Sequence analysis of a single array uncovered 229 copies of pentanucleotide repeat motifs 5'-TTCCA-3'.¹⁴ A normal human Y chromosome contains 2000–4000 copies of DYZ1¹³ in a sex-specific manner.^{15–17} However, it is not clear if copy number variation of DYZ1 affects its normal functioning, if any, and correlates with spermatogenesis, male fertility, sustenance to full-term pregnancy or overall reproductive potential of the males. DYZ1 has been found to show mosaicism in repeated abortion (RA),¹⁸ whereas other loci seem to be affected in prostate cancer (PC)^{19,20} and males exposed to natural background radiation

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(NBR).²¹ However, the changes that are brought about within the Y chromosome through the genomic instability of DYZ1 repeat fraction under these conditions remain unclear.

A reliable assay system for an accurate quantification of the copy number status of the DYZ1 array is not available. Fluorescence *in situ* hybridization (FISH) uncovered chromosomal translocation, mosaicism and aberrant Y chromosome^{17,18} but failed to detect copy number status of the DYZ1 since probe detected single signal on the Yq12 region. Here we describe assessment of copy number variation of the DYZ1 arrays in normal males, patients suffering from PC, cases of RA and males exposed to NBR employing real-time PCR and FISH. Our work shows copy number status of the DYZ1 arrays in the normal males and patients and establishes a genotype phenotype correlation. Prospects of this work in the area of Y chromosome genomics in general and clinical genetics in particular are highlighted.

2. Materials and methods

2.1. Collection of human blood and germline samples and isolation of DNA

Ten RA samples were obtained from Nobel hospital, Faridabad, North India, and two PC from Kerala, South India. A total of 56 samples containing 40 blood and 16 semen samples from males and 15 blood samples from females, all exposed to NBR were collected from Chavra, (Kerala) in accordance with the Institute's Ethical and Biosafety Committee. In addition, 20 blood samples from normal males and females each from North India were also used. DNA isolation was performed following standard protocols.²²

2.2. PCR amplification of DYZ1 fraction

To assess organizational variation of DYZ1 repeats (accession no. X06228), both at its copy number level as well as within the single array amongst individuals suffering from RA, PC and exposed to NBR, internal

Table 1. Details of primers used for amplification of different regions of the DYZ1 repeat array

S. no.	Primer no.	Primer Sequence 5'-3'	Position	Amplicon size (bp)
1	DYZ1.1F	TTCCCTTCGCT TGCATTCCAT	25–47	266
2	DYZ1.6R	TGAAATGGACTGGAAAGGAATG	268–290	
3	DYZ1.2F	TTTGAGTCGTTCCATAACAC	1347–1367	773
4	DYZ1.8R	TGGAATGGACTCGAACAGAGTG	2097–2119	
5	DYZ1.3F	GAGTCCATTCACTTCCAGAAC	3128–3149	
6	DYZ1.5R	GACTGGAAAGGCTGGGTGTCGA	3380–3402	

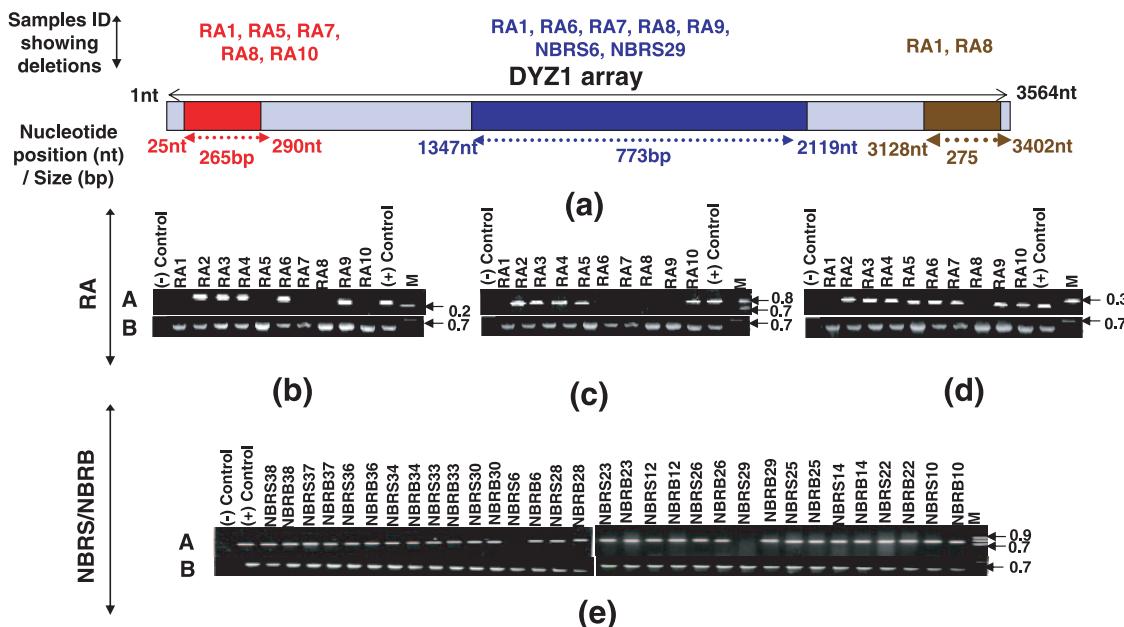


Figure 1. Schematic representation showing three deletion points in the DYZ1 arrays (a), their corresponding individual deletions in RA (b–d) and germline and blood samples of NBR (e). As control, amplification with β -actin primers is shown in panel B. Sample ID is given on top of each panel and molecular marker (M) is shown in base pairs.

primers were designed (Table 1) using Primer Express Software V2.0 (ABI). PCRs were conducted in a 25 μ l volume containing *Taq* Polymerase, 10x PCR buffer (Promega, Madison, WI, USA), 200 μ M dNTPs and 100 ng of target DNA for a total of 35 cycles. Each cycle involved denaturation at 95°C for 1 min, annealing 60°C for 1.5 min and primer extension at 72°C for 1 min. The amplified products were resolved on 1.5% agarose gel. The quantity and quality of the above mentioned samples were confirmed by PCR amplification using a set of Human β -actin (F 5'-GTGGGCCGCTCTAGACACC-A-3' and R 5'-CGGTTGCCCTAGG G TTCAGGGGG-GG-3') primers.

2.3. Slot-blot hybridization

In order to confirm deletions shown by endpoint PCR, 500 ng of genomic DNA from different RA samples in 100 μ l of 2x SSC was slot blotted onto a nylon membrane (Manifold Apparatus, Schleicher & Schuell, Germany) and UV fixed. For control, genomic DNA from normal male and female was included in the blot. Independently amplified PCR products of 265, 773 and 275 bp from normal human male were used as probe. Hybridization and autoradiography was performed following standard procedure.²³

2.4. FISH

For FISH with human metaphase chromosomes, DYZ1 cloned probe was labeled using Nick Translation Kit from Vysis (IL, USA) following supplier's instructions. Hybridization, washing, counter staining and mounting of the slides were performed following established protocol.¹⁷ Slides were screened under the Olympus microscope (BX51) fitted with vertical fluorescence illuminator U-LH100HG UV, excitation and barrier filters. Images were captured with a CCD camera. For karyotyping, CytoVision 2.81 software from Applied Imaging was used.

2.5. Copy number calculation of DYZ1 repeat

The copy number of DYZ1 was calculated based on absolute quantification assay using SYBR green dye and Sequence Detection System-7000 (ABI, CA). A set of primers specific to DYZ1 (DYZ1F: 5'-TGGAAATGGAA-TCGAATGGAATGGAA-3' and DYZ1R: 5'-TGCCAA-ATCATTGCATTCCTTCC-3') was designed using Primer Express Software V2.0 (ABI). The efficiency of primers was assessed by using a 10-fold dilution series of the recombinant plasmid and standard male genomic DNA. The reactions were performed in triplicate using 96-well plates in a 25 μ l reaction volume employing conditions of 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 95°C for 10 s and 60°C for 1 min. Copies of the DYZ1 arrays were calculated by extrapolation of the standard curve obtained with known copies of the recombinant plasmid.

Table 2. Details of the DNA samples and primers used for the screening DYZ1 array(s)

S. no	Patients ID	Primer sets		
		DYZ1.1 & 1.6 (265 bp)	DYZ1.2 & 1.8 (773 bp)	DYZ1.3 & 1.5 (275 bp)
1	RA1	—	—	—
2	RA2	+	+	+
3	RA3	+	+	+
4	RA4	+	+	+
5	RA5	—	+	+
6	RA6	+	—	+
7	RA7	—	—	+
8	RA8	—	—	—
9	RA9	+	—	+
10	RA10	—	+	+
11	NBRS38	+	+	+
12	NBRB38	+	+	+
13	NBRS37	+	+	+
14	NBRB37	+	+	+
15	NBRS36	+	+	+
16	NBRB36	+	+	+
17	NBRS34	+	+	+
18	NBRB34	+	+	+
19	NBRS33	+	+	+
20	NBRB33	+	+	+
21	NBRS30	+	+	+
22	NBRB30	+	+	+
23	NBRS6	+	—	+
24	NBRB6	+	+	+
25	NBRS28	+	+	+
26	NBRB28	+	+	+
27	NBRS23	+	+	+
28	NBRB23	+	+	+
29	NBRS12	+	+	+
30	NBRB12	+	+	+
31	NBRS26	+	+	+
32	NBRB26	+	+	+
33	NBRS29	+	—	+
34	NBRB29	+	+	+
35	NBRS25	+	+	+
36	NBRB25	+	+	+
37	NBRS14	+	+	+
38	NBRB14	+	+	+
39	NBRS22	+	+	+
40	NBRB22	+	+	+
41	NBRS10	+	+	+
42	NBRB10	+	+	+
43	PC1	+	+	+
44	PC2	+	+	+

RA denotes DNA samples from Repeated Abortion, NBRB and NBRS from blood and semen, respectively, of the males exposed to Natural Background Radiations. PC denotes Prostate Cancer males. Figures in parenthesis denote size of the amplicons.

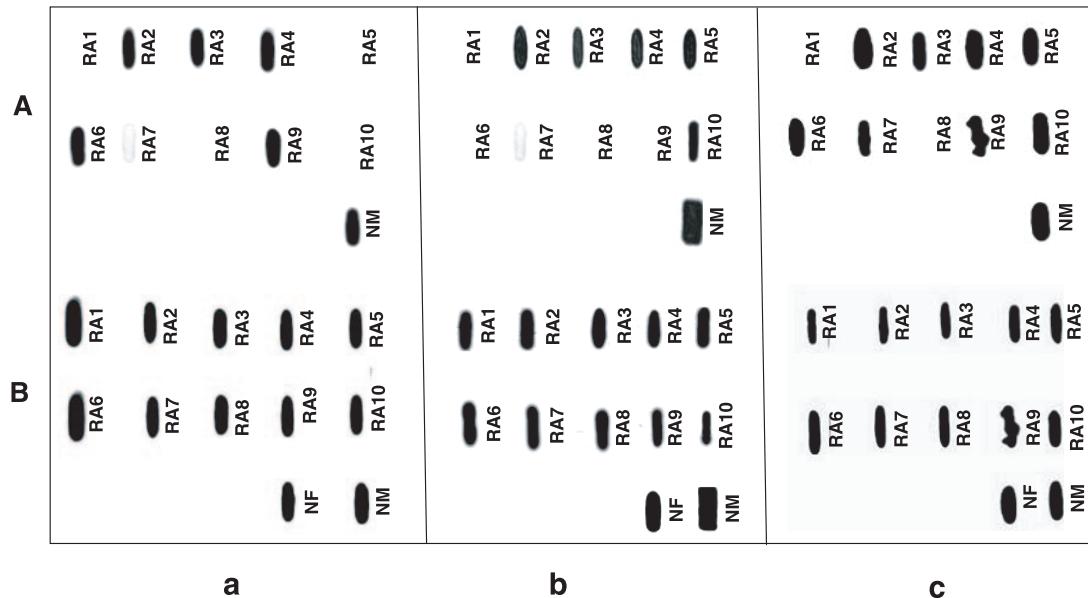


Figure 2. Slot-blot hybridization of genomic DNA from RA with PCR amplified fragments of the DYZ1 arrays of: 265 bp (a); 773 bp (b) and 275 bp (c) from normal male (panel A) and β -actin (panel B). Note the lack of signals in seven RA samples. NM and NF denote normal human male and female genomic DNA, respectively.

3. Results

3.1. Organizational variation within the DYZ1 region

Of all the samples used in the present study, nine showed distinct deletions within the DYZ1 arrays (Fig. 1a). Of these, a 265 bp deletion from nt 25 to 290 was detected in five (RA1, RA5, RA7, RA8 and RA10) individuals (Fig. 1b, panel A). A 775 bp deletion from nt 1347 to 2119 was detected in seven (RA1, RA6, RA7, RA8, RA9, NBRS6 and NBRS29) individuals (Fig. 1c and e, panel A). Another 275 bp deletion, spanning from nt 3128 to 3402 was detected in two (RA1 and RA8) individuals (Fig. 1d, panel A). Significantly, samples RA1 and RA8 showed all the three (265, 773 and 275 bp) deletions. However, all the samples showed amplification with β -actin primer giving rise to expected 650 bp fragment indicating intactness of the target DNA (Fig. 1b–e, panels B). Screening of the DNA samples from normal men showed no deletion. Table 2 represents details of DNA samples and primers used for screening of the DYZ1 arrays.

3.2. Slot blot hybridization substantiates deletions in RA samples

Slot-blot analysis of the DNA from RA samples probed independently with 265, 773 and 275 bp fragments showed absence of signals in several individuals. RA1, RA5, RA7, RA8 and RA10 showed absence of signals when hybridized with labeled probe of 265 bp (Fig. 2a, panel A). Similarly, RA1, RA6, RA7, RA8 and RA9 showed no signal with labeled probe of 773 bp (Fig. 2b,

panel A), and RA1 and RA8 with 275 bp (Fig. 2c, panel A). Hybridization of genomic DNA with β -actin showed signals in all the samples (Fig. 2a–c, panel B).

3.3. FISH uncovers DYZ1 (in)stability on the Y chromosome

FISH of DYZ1 with metaphase chromosomes and interphase nuclei of one RA, 24 individuals exposed to NBR and two PC, showed varying levels of signals. Of the two PCs, PC1 showed DYZ1 signal on the autosome 10 along with Y chromosome in ~80% metaphases, which was substantiated by three signals in the interphase nuclei (Fig. 3a and b). In addition, single DYZ1 signal was also detected in ~20% cells in PC1 indicating DYZ1 mosaicism. PC2 showed DYZ1 signal in ~40% cells and total absence of the same in the remaining 60% cells (Fig. 3c and d, arrows), indicating a gross level of DYZ1 mosaicism. The FISH analysis on sample RA10 showed overall reduced but varying signals of DYZ1 on the Y chromosome and interphase nuclei (Fig. 3e). Similar results showing variations in the DYZ1 signal intensity were obtained from the screening of 13 families exposed to NBR, where five males 1b, 3b, 8f, 9f and 13f showed relatively reduced signals (Fig. 3f). In addition, the 8b male showed positional change and 2f, 4f and 10f males showed enhanced signals of the DYZ1 arrays suggesting their organizational variations (Fig. 3f). Present study establishes a correlation between the specific genotype (PC, NBR and RA) and varying level of DYZ1 instability, reflected as presence/absence or reduced level of signals.

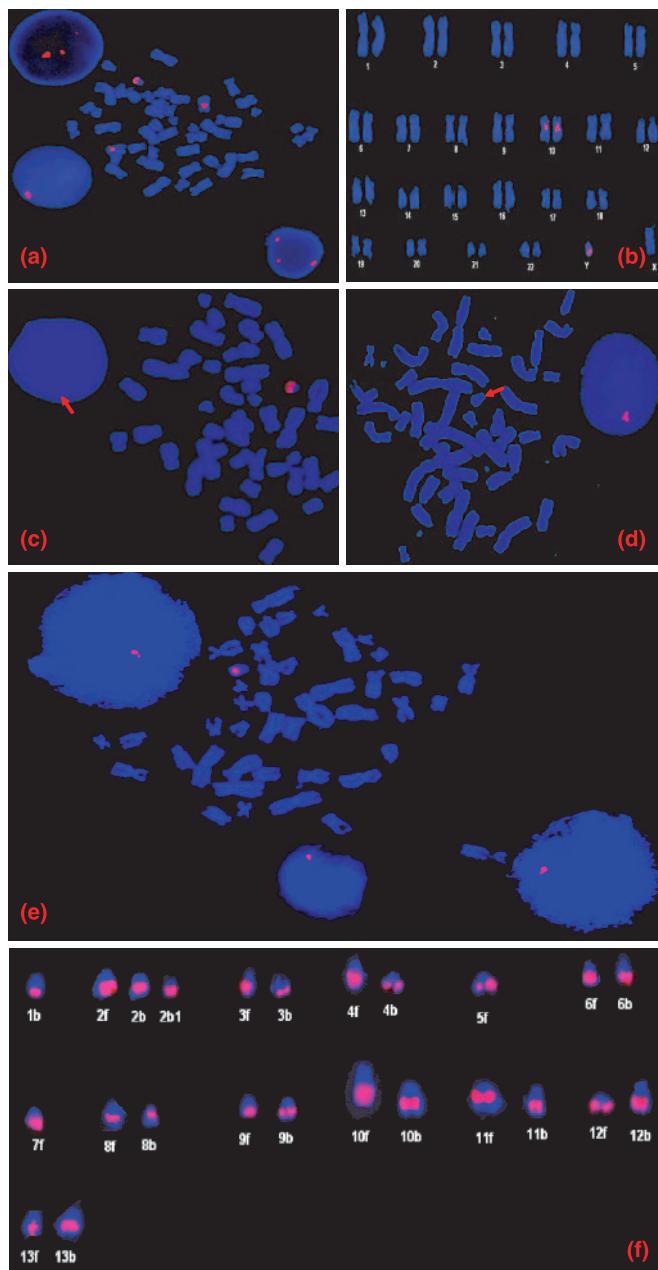


Figure 3. Fluorescent *in situ* hybridization of DYZ1 arrays with human Y chromosome in males representing PC-1 (a–b) and PC-2 (c–d), RA10 (e) and 13 families of males exposed to NBR (f). Note the DYZ1 signal on autosome 10 and Y chromosome (a–b), absence of the same on the Y chromosome in panel d (arrow) and its unequal distribution (e–f).

3.4. Copy number status of DYZ1 in normal males, RA, PC and NBR

High quality intact DNA samples of all the categories were subjected to real-time PCR to assess copy number status of the DYZ1 arrays. The standard curve in real-time PCR with a slope of -3.4 and single dissociation peak reflected 100% efficiency of the assay system (data not shown). The primers were highly specific as evident from the females showing no amplification. In normal males, DYZ1 copies ranged from 3000 to 4300, in RA,

0 to 2237 and in NBR, 1577 to 5700. PC2 showed 550 copies of the DYZ1 arrays. The real-time PCR results were consistently reproducible as evident from the samples subjected to reactions at least for three times. Copy number status of the DYZ1 arrays amongst group of different samples is shown in a representative bar diagram (Fig. 4). Compared with DNA used as control from normal males, all the other samples, showed CNV of DYZ1 either below or above the normal range. Genomic instability of DYZ1 seems to be more common in RA samples compared with that in NBR exposed males.

4. Discussion

4.1. Organizational variation of the DYZ1 arrays

In the present study, our aim was to assess copy number status of the DYZ1 arrays in normal males and compare the same with that in patients representing PC, RA and NBR samples. DYZ1 represents a major satellite fraction of ~ 3.4 kb from the long arm of the human Y chromosome with overabundant STR motif 5'-TTCCA-3'.¹⁴ STRs have been known to expand and shrink possibly owing to unequal cross-over during meiosis²⁴ giving rise to hypervariable regions²⁵ or variable number tandem repeat (VNTR) loci.²⁶ Sequences on the Y chromosome show rapid rate of evolution as opposed to that on the autosomes and X chromosomes and move equally rapidly in an intra-chromosomal and inter-chromosomal environment.²⁷ This is well corroborated with translocation of DYZ1 on chromosome 10 in PC1. Compared with the normal males, DYZ1 seems to be affected in all the abnormal genomes. It does not transcribe but is propagated from generation to generation across the cell lineages. This seems to play a critical role, possibly in chromatin folding and maintenance of the structural integrity of the Y chromosome. This inference is based on the fact that DYZ1 arrays showed three discernible deletions in seven different RA samples but not in any of the normal males. Deletions detected in the present study by PCR and supported by FISH data seem to have contributed towards the vulnerability of the Y chromosome, obstructing the sustenance of the pregnancy. This is because all the RA samples were of the male origin. In this context, female aborted fetus may be analyzed to include/exclude such possibility. Owing to small sample size statistical analysis was not conducted. This issue may be resolved unequivocally with the analysis of more number of samples.

Deletions within the DYZ1 region in the semen samples of NBR exposed males indicated that despite being a slow process, NBR causes genome instability. This is faithfully recorded by the DYZ1 arrays as substantiated by FISH results. CNV of DYZ1 may also be caused due to non-disjunction, large duplications and deletions.^{28–30} Besides duplication, reverse transcriptase activity may also contribute to copy number variation.³¹ Irrespective of the

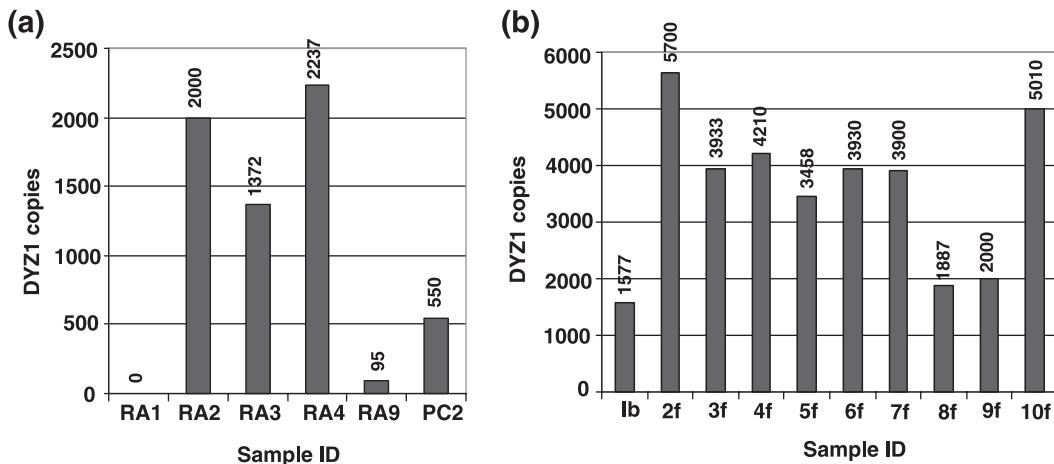


Figure 4. A bar diagram showing copy number status of the DYZ1 arrays in representative RA, PC (a) and NBR samples (b). Note the variations amongst different RA and NBR samples.

mechanisms involved, environment seems to contribute to genomic instability of DYZ1, thereby, supporting the concept of rapid rate of evolution of the Y chromosome.³² Despite evidence (*albeit in direct*) that genomic instability of the DYZ1 is correlated with RA, we wish to refrain from any such conclusion, since work is underway on this using more number of samples to prove this point or otherwise. The abnormal copies of the DYZ1 in patients with PC have never been reported earlier. Once again, it is difficult to draw a major conclusion owing to study based on two samples. This warrants analysis of more number of PC samples to resolve this issue.

5. Conclusion

DYZ1 seems to be affected in RA, PC and NBR samples though its diagnostic potential has neither been explored nor acknowledged. The most intriguing observation was well-defined deletions in three different regions of DYZ1 repeat in RA samples. In addition, its copy number fluctuation below or above the normal range correlated with abnormal genotype. Present study indicates its diagnostic and prognostic potentials suggesting that this approach may be used as a marker system for ascertaining its copy number status and establishing genotype/phenotype correlation in routine clinical cases.

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