Int J Biol Med Res. 2013; 4(2): 3121-3127

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International Journal of Biological & Medical Research

Journal homepage: www.biomedscidirect.com

Original Article

Specific genetic marker based molecular study of the AZFA & AZFD region microdeletion in infertile cases of northeast India.

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ARTICLE INFO

Keywords:
Y chromosome microdeletion
AZFa region
AZFd region
Markers

ABSTRACT

Introduction: The first cases of Y chromosome microdeletions and male infertility were reported in 1992 and many case series have subsequently been reported from various parts of the world. A very few studies have been done involving the patients of North-East Indian states, so this study aimed to detect frequency and incidence of AZF microdeletions in this population with special reference to AZFa and AZFd region of long arm of Y chromosome. Aims: The main objectives involve - a) Determination of microdeletion frequency for each type of infertility group included in the study. b) Determination and comparison of the microdeletion frequency of Y chromosomal AZFa and AZFd region in the cases using 2 gene-specific markers: USP9Y, DBY and 3 STS markers: sY145, sY152, sY153. c) Evaluation of the importance of using markers, not specified by European Academy of Andrology (EAA) to detect Y chromosome microdeletion, in Indian scenario. Methods: A total of 170 infertile males attending private infertility clinics of Guwahati, Assam were selected for the study. This includes 50 azoospermic, 82 oligozoospermic, 18 oligoasthenozoospermic and 20 asthenozoospermic cases. Both blood and semen samples were collected from 45 individuals. PCR amplification was carried out using specific primer sets and isolated genomic DNA. All genes and STS markers were amplified efficiently in samples from 50 fertile men tested, but failed to be amplified in samples from fertile women. Results: The frequency of Y chromosome microdeletion with respect to AZFa and AZFd region was found to be 25.3% (43/170). In azoospermic, oligozoospermic, oligoasthenozoospermic and asthenozoospermic cases it was found to be 28% (14/50), 26.8% (22/82), 11.1% (2/18) and 25% (5/20) respectively. In the cases with single marker microdeletion, microdeletion of only DBY gene was found to be associated with all the four infertility groups. In the 45 individuals from whom both blood and semen samples were available, frequency of Y chromosome microdeletion was found to be higher in semen samples (20%) than blood (17.8%) samples. Conclusion: It shows that markers not specified by EAA are also good enough in determining Y chromosome microdeletion in the present population. We also suggest that the use of DNA isolated from semen sample, for Y chromosome microdeletion screening can be a better option as it can detect the deletions not detected by blood sample analysis.

1. Introduction

Male infertility refers to the inability of a male to achieve a pregnancy in a fertile female. Globally about 15% couples are affected by this problem and abnormalities in male partner are estimated in about half of these cases [1]. This can occur due to various factors like, structural defects in the male reproductive organs, hormonal imbalance, varicocele, impotency, hypogonadotrophic hypogonadism, impaired sperm production due to genetic abnormalities etc. Since the path-breaking work of Tiepolo and Zuffardi (1976), it has been proposed that long arm of

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the human Y chromosome (Yq) hosts a number of genes and several types of recurrent Yq microdeletions are associated with spermatogenic failure [2, 3]. This region of Yq is referred to as AZF (Azoospermic factor) region and it is divided into four regions: AZFa, AZFb, AZFc and AZFd, AZFd being located in berween AZFb and AZFc [4]. Till date, 122 genes and 110 pseudogenes have been identified to be present in Y chromosome. Of the genes located in the AZF region, 31 genes are human testes-specific, 14 genes are protein-coding and 17 genes are non-protein-coding transcripts [5, 6, 7, 8]. The AZFa region, the most proximal portion of the long arm of human Y chromosome is located in the deletion interval 5C and its size ranges between 400-600 kb. This region contains three genes, namely USP9Y, DBY and UTY, and represents a high degree of homology with mouse Sxrb region. All these three genes have ubiquitously expressed X homologues that escape X-inactivation [9, 10]. The first gene identified to be located in the AZFa region is USP9Y (ubiquitin-specific protease 9, Y) previously known as DFFRY (Drosophila fat facets related Y). It transcribes an ubiquitin-specific protease/hydrolase, a member of the C19 cystein peptidase family. These enzymes play a role in the intracellular cleavage of ubiquitin molecules from ubiquitinated proteins. USP9Y protein shares 91% sequence similarity with its X chromosomal homologue USP9X, suggesting that they may also have functional similarity. USP9Y starts to express in the male germline only at the spermatid stage. Though, initially USP9Y deletions were thought to be exclusively associated with azoospermia, more recent studies indicate otherwise. DBY (DEAD box on the Y), previously known as DDX3Y, encodes a protein containing DEAD box motif, characteristics of ATP-dependent RNA helicases. It shares 91.7% sequence similarity with its X chromosomal homologue. Unlike USP9Y and DBX, DBY expression is testis-specific. DBY has been detected predominantly in the cytoplasm of spermatogonia. The precise role of these genes in spermatogenesis is still unclear. Interestingly, despite being ubiquitously expressed, the deletion of both these genes has been reported in male infertility cases suggesting their function to be tissue-specific. It is suggested that DBY could have a more important role in spermatogenesis than USP9Y [11, 12]. Unlike AZFa region, no candidate gene has been identified in the AZFd region till now. However, deletion of the sY153 STS marker located in the DYS237 locus has frequently been reported, indicating the possible role of the genes located in this region in spermatogenesis. The other two important STS markers identified in this region are, sY145 located in DYS51S1 locus and sY152 located in DYS236 locus. Microdeletions in AZFa region is believed to be associated with more severe forms of infertility like azoospermia and/or severe oligozoospermia representing SCO (Sertoli cell only) syndrome. But microdeletions of AZFd region has also been reported from patients with mild oligozoospermia and even in patients with normal sperm count associated with abnormal sperm morphology [9].

With the advent of assisted reproductive techniques (ART), there always lies a huge possibility of transmission of any genetic abnormality related to male factor infertility like Y chromosome microdeletion, from father to son, as these techniques bypass the physiological mechanisms related to fertilization. Therefore, in today's medical scenario it is of great importance to know the exact reason of the male factor infertility for proper counseling of the patients before they opt for any of the ART techniques for conception. In case, the patient is diagnosed with any genetic abnormality it is also possible to minimize the risk of transmission by using preimplantation genetic diagnosis (PGD). We, for the first time made an attempt to assess the extent of association between Y chromosome microdeletion and different infertile patient groups amongst men of seven North-East Indian states, with special reference to AZFa and AZFd regions.

2. Materials and Methods

Patient selection

A total of 170 infertile men attending private infertility clinics of Guwahati, Assam were selected for the study. The semen analysis for the categorization of cases was carried out by the clinic using World Health Organization (WHO) guidelines [13]. Thus the present study includes 50 azoospermic, 82 oligozoospermic, 18 oligoasthenozoospermic and 20 asthenozoospermic cases (details given in table-1). The cases were aged between 24 – 52 years. The positive control samples were collected from 50 normozoospermic men, while blood samples collected from females had been used as negative control.

Sample Collection

Two ml of venous blood was collected in a tube containing ethylenediamine tetraacetate (EDTA) as an anticoagulant. Semen samples produced by masturbation were collected in wide mouthed collection vials. All samples were stored at -20°C prior to DNA extraction.

Detection of Y chromosome microdeletion

The genomic DNA was extracted from both blood and semen samples using commercially available genomic DNA extraction kits (GeneiPure™.) and PCR amplification were carried out using specific primer sets to detect microdeletion in Y chromosome. All genes and STS markers included in the study were located on the long arm of the Y chromosome. The markers used in the study were: USP9Y and DBY genes from AZFa region and STS markers sY145, sY152 and sY153 from AZFd region. None of these markers are EAA approved [14]. The primer sequences are given in table-2.

Each marker was amplified separately for 35 cycles in 0.2ml microfuge tubes using a TC-512 gradient thermocycler (ABI Biosystems™). The 25µl of reaction mixture contained 100ng of genomic DNA, 10X PCR buffer with 15mM Mgcl2, 2.5 mM of each dNTP and 3U/µl Taq DNA polymerase enzyme. The PCR conditions used were as followed: initial denaturation at 94°C for 3 min was common for all markers. Subsequent denaturation condition (94°C for 30 sec) was also same for USP9Y, DBY, sY145 and sY153, but for sY152 it was done at 94°C for 45 sec. The annealing conditions followed for different markers were: 57°C for 30sec for USP9Y, 59°C for 30sec for DBY, 55°C for 1 min for sY152, 57°C for 45 sec for sY145 and sY153. Subsequent extension was done at 72°C for 45 sec for USP9Y and DBY. For sY152, sY145 and sY153 it was done at 72°C for 1 min. Final extension for all the markers was done at 72°C for 10 min. In each PCR reaction, markers were amplified with a normozoospermic positive control sample, a female negative

control sample and a water control to determine chemical contamination. The PCR amplified products were submitted to electrophoresis on 1.5% agarose gel stained with 0.5μg/mL ethidium bromide and visualized by gel documentation system. A 100 bp DNA ladder was loaded alongside the PCR products to estimate the band size. Microdeletion of any genetic marker had been confirmed after conducting three PCR reactions with same result to rule out amplification failure.

Statistical analysis

The t-test has been used to compare the results between the groups. $p<0.05$ has been considered as significant.

3. Results

The frequency of Y chromosome microdeletion in the infertile cases of Northeast India, using only the markers for AZFa and AZFd regions was found to be 25.3% (43/170), with 15 cases (34.9%) showing microdeletion involving only AZFd region, 14 (32.6%) cases of only AZFa region and another 14 (32.6%) cases showing microdeletions of markers of both AZFa and AZFd regions [table-3]. Amongst the four groups of infertility cases, highest percentage of Y chromosome microdeletion was observed in azoospermic cases (28%; 14/50) followed by oligozoospermic (26.8%; 22/82), asthenozoospermic (25%; 5/20) and oligoasthenozoospermic (11.1%; 2/18) males. None of the control men showed deletion for any marker. In azoospermic and asthenozoospermic groups 10% cases showed microdeletion of AZFa region while, 7.3% oligozoospermic cases and 5.6% oligoasthenozoospermic men showed microdeletion of AZFa region. The frequency of microdeletion of markers of AZFd region only was found to be highest in azoospermic group (12%) followed by oligozoospermic (9.8%) and asthenozoospermic (5%) group. No oligoastherozoospermic case showed microdeletion of AZFD region only. Microdeletion involving markers of both regions was found in 6% azoospermic, 9.8% oligozoospermic, 5.6% oligoastherozoospermic and 10% asthenozoospermic males.

Simultaneous screening of both blood and semen samples from same individual was possible for 45 cases. Among these cases frequency of microdeletion was found to be 17.8% (8/45) in blood sample and 20% (9/45) in semen sample analysis. The microdeletion profile also varies considerably between blood and semen samples for these cases. One oligozoospermic man (P210*) showed absence of only sY145 marker in blood sample analysis but showed microdeletion of entire AZFD region in semen sample analysis. Another oligozoospermic man (P133*) was detected with absence of only USP9Y gene in blood sample but showed absence of sY145 marker also in semen sample analysis. Oligozoospermic case P209* did not show any microdeletion in blood sample analysis, but found to have microdeletion of DBY gene in semen DNA analysis. An asthenozoospermic man (P112*) showed microdeletion of only sY145 marker in blood sample analysis, but absence of markers sY145 and DBY was detected in semen sample analysis.

Most of the azoospermic individuals had microdeletion of at least one STS marker of AZFd region, with sY153 (3/14) having highest microdeletion frequency. But none of the oligoasthenozoospermic males had microdeletions in this region.

Of the 43 individuals with microdeletion, single marker microdeletion was detected in 26 cases but none of them showed microdeletion of only sY145 marker. In these cases of single marker microdeletion, only DBY gene microdeletion was found to be associated with all the four infertility groups.

Table-1: The spermiogram of infertile cases included in the study

Type of Infertility	Sample type			Total
	Blood	Semen	Both Blood and Semen	
Azoospermic	50	0	0	50
Oligozoospermic	48	22	12	82
Oligoasthenozoospermic	0	0	18	18
Asthenozoospermic	2	3	15	20
Total	100	25	45	170

Table-2: Primer sequences of the primers included in the present study

Gene/STS marker	Primer sequence	Product size (bp)
USP9Y	Forward 5'- GGGCTCAGAGGTGAAACTGACCCT-3' Reverse 5'- ACACATACTCCACACAGCCACCA-3'	716
DBY	Forward 5'- ACCTGGGCCTTGCCACCTCA-3' Forward 5'- ACCACTGCGGCTGCTGCTTC-3'	463
sY153	Forward 5□-GCATCCTCATTTTATGTCCA-3□ Reverse 5□ATGAGTCACGAAAACCCAAC - 3□	135
sY152	Forward 5□-AAGACAGTCTGCCATGTTTCA-3□ Reverse 5□-ACAGGAGGGTACTTAGCAGT-3□	125
sY145	Forward5'-CAACACAAAAACACTCATATACTCG-3' Reverse 5'-GGGCATTGTATGTTAATAAGAGTT-3'	125

Table-3: Details of men with different AZF deletions

Case No.	Type of Infertility	Gene/STS Marker					AZF deleted
		USP9Y	DBY	sY145	sY153	sY152	
B8	Azoospermic	+	-	+	+	+	AZFa
B15	Azoospermic	+	+	-	-	+	AZFd
B22	Azoospermic	+	+	-	-	-	AZFd
B33	Azoospermic	-	+	+	+	-	AZFa+AZFd
B44	Azoospermic	-	+	+	+	-	AZFa+AZFd
B78	Azoospermic	+	+	-	-	+	AZFd
B96	Azoospermic	+	+	-	-	+	AZFd
B118	Azoospermic	+	+	+	+	-	AZFd
B122	Azoospermic	-	+	+	+	+	AZFa
B158	Azoospermic	+	-	+	+	+	AZFa
B240	Azoospermic	+	+	-	-	-	AZFd
B325	Azoospermic	-	+	+	+	+	AZFa
B342	Azoospermic	+	-	+	+	+	AZFa
B353	Azoospermic	+	-	+	+	-	AZFa+AZFd
S17	Oligozoospermic	+	+	-	-	+	AZFd
S76	Oligozoospermic	+	+	-	-	+	AZFd
S90	Oligozoospermic	-	+	-	-	+	AZFa+AZFd
S122	Oligozoospermic	+	+	-	-	+	AZFd
S123	Oligozoospermic	+	-	+	+	+	AZFa
S133	Oligozoospermic	-	+	+	+	-	AZFa+AZFd
S142	Oligozoospermic	+	+	-	-	+	AZFd
S147	Oligozoospermic	+	-	+	+	+	AZFa
B376	Oligozoospermic	+	-	+	+	+	AZFa
B328	Oligozoospermic	+	+	+	+	-	AZFd
B264	Oligozoospermic	+	+	-	-	+	AZFd
B241	Oligozoospermic	+	-	+	+	-	AZFa+AZFd
B235	Oligozoospermic	-	+	-	-	+	AZFa+AZFd
B224	Oligozoospermic	-	+	-	-	+	AZFa+AZFd
B220	Oligozoospermic	-	+	+	+	+	AZFa
B46	Oligozoospermic	+	+	-	-	+	AZFd
B45	Oligozoospermic	+	-	+	+	+	AZFa
B41	Oligozoospermic	+	-	+	+	-	AZFa+AZFd
P133*	Oligozoospermic	-	+	+	+	+	AZFa+AZFd
P201*	Oligozoospermic	+	-	+	+	+	AZFa
P209*	Oligozoospermic	-	+	-	-	-	AZFa+AZFd
P210*	Oligozoospermic	+	+	-	-	-	AZFd
P116*	OAZ	-	+	+	+	+	AZFa+AZFd
S154	OAZ	+	-	+	+	+	AZFa
B339	Asthenozoospermic	-	+	+	+	-	AZFa+AZFd
P102*	Asthenozoospermic	+	+	+	+	-	AZFd
P112*	Asthenozoospermic	+	-	-	+	+	AZFa+AZFd
P131*	Asthenozoospermic	-	+	+	+	+	AZFa
P142*	Asthenozoospermic	+	-	+	+	+	AZFa

Footnote: a) OAZ= Oligoasthenozoospermic cases; b) B= only blood sample available
c) S= only semen sample available; d) P*= both type of samples available, but semen DNA deletion profile given

4. Discussion

The prognostic value of Y chromosome microdeletion has been well documented by many researchers around the world [15, 16, 17, 18]. In our study population the frequency of Y chromosome microdeletion, using only the markers for AZFa and AZFd regions was found to be 25.3% (43/170). This is consistent with the findings of other studies in which the reported range is between 0.9% and 55.56% [1, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28], but is quite high in comparison to other Indian studies including our own studies [29, 30, 31, 32, 33, 34, 35, 36, 37]. Studies in European populations show that Y chromosome microdeletion could affect about 15% of azoospermic and 5%-10% of oligozoospermic men [38]. The incidence of microdeletion in azoospermic men varies greatly in Indian population with the range between 5.29% [34] and 29.63% [30]. In the present study, the proportion of azoospermic men (28%) with microdeletion of Y chromosome falls in this range. The reported range of Y chromosome microdeletion in oligozoospermic men of Indian origin is between 0% - 21.1% [29, 30, 32, 34, 37]. But we found a higher incidence of microdeletion in oligozoospermic (26.8%) men in our study. Sachdeva and co-workers [29] reported to have higher proportion of oligozoospermic men with microdeletion than azoospermic males, but in our case highest incidence of microdeletion was observed in azoospermic cases. We also detected 25% asthenozoospermic and 11.1% oligoasthenozoospermic men with Y chromosome microdeletion, but unfortunately no Indian data is available to validate our findings. A study on Chinese population reported 11.6% oligoasthenozoospemic men with Y chromosome microdeletion [39] which is in accordance to our finding.

Interestingly, none of the markers used in the present study are EAA recommended. EAA claims that by using 6 markers - sY84, sY86 (for AZFa), sY127, sY134 (for AZFb), sY254 and sY255 (for AZFc) for microdeletion analysis about 90% of Y chromosome microdeletion can be detected. It also disapproves the presence of AZFd region [14]. But many recent studies found contradictory report regarding efficacy of these markers in Indian population. One Indian study, detected only 6 of 200 males with microdeletion with EAA prescribed marker, but additional 15 men were found to have microdeletion with the help of another 27 markers expanding to AZFa, AZFb and AZFc region. Of 21 men with microdeletion, 15 (71%) showed microdeletion of sY153, in combination with other markers. No deletion of sY84 and sY86 was detected, but they found 2 cases with deletion of sY742 marker of AZFa region [29].

Another Indian research group, used sY746, sY84, sY86, USP9Y for detection of deletion in AZFa region. Unlike the previous study, both the cases with AZFa deletion showed absence of sY86. They found no case with microdeletion of USP9Y, but we found 14 cases with microdeletion of this marker either singularly or in combination with other markers. They reported 6 cases with microdeletion of sY153 in combination with other markers [32]. However, in both these studies, unlike us, sY153 has been used as a marker of AZFc region. But that can not disapproves the fact that a large number of Indian males with idiopathic infertility show microdeletion of this STS marker and it is therefore an important marker to be included in the Y chromosome microdeletion screening of Indian men. Thangaraj and his group also reported majority of the men with AZFa deletion had microdeletion of sY746 marker and not of sY84 and sY86. They reported 2 cases with microdeletion of USP9Y gene of AZFa region and 7 cases with microdeletion of sY746 of AZFa region. We found 2 azoospermic men showing deletion of only 1 marker (USP9Y), thus showing deletion of AZFa region [33]. This is in accordance with the finding of former study group. Previous studies conducted by our group in the same study population also showed low frequency of microdeletion with sY84 marker [36]. Recently, a Chinese study group used sY145 and sY152 as marker of AZFc region along with sY254, sY255 and sY157. They found, that all the 194 cases with deletion of sY254 and sY255 had the presence of sY145 and sY152. Thus they suggested that sY145 and sY152 can be omitted in AZFc screening [40].

Kent-First and co-workers reported (Kent-First et al., 1999) single STS microdeletions of the SY152 marker in 6 individuals and SY153 marker in 8 individuals. They also reported microdeletions of multiple STSs, in 2 individuals that involved the markers of AZFd region but excluded the AZFc or the DAZ regions. Thus they suggested that there is a correlation between microdeletions involving AZFd region and male factor infertility represented by mild oligozoospermia, oligoasthenozoospermia and asthenozoospermia [4]. In the present study, we have found deletion of AZFd region markers in all the 4 infertility groups, either as single marker microdeletion or in association with other markers. DBY gene microdeletion has also been reported by many Indian studies where it has been included in screening regime [41]. Many other study groups of Middle-East Asia and Africa have also reported lower frequency of Y chromosome microdeletion in their study population using the markers prescribed by EAA [42, 43]. Thus, it can be suggested that the EAA recommended markers alone are not sufficient enough to screen the Y chromosome microdeletion in Indian population. Indian population consists of people of many ethnic groups and their also lies a need to consider this fact while choosing the markers for this purpose. The 5 markers included in this study had showed relative efficacy in detecting microdeletion pattern in our study population. Other factors that should also be considered are,- the sample size, biological sample used for microdeletion study and the inclusion and exclusion criteria, as by restricting patient selection criteria only to those with azoospermia or severe oligozoospermia, pathological microdeletions causing less severe phenotypes will be missed

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In the present study, simultaneous screening of both blood and semen samples for 45 cases showed higher frequency of microdeletion in semen sample, than blood sample analysed. This fact has also been established by many other studies. Thus supporting the previous findings the present report can suggest that use of DNA isolated from semen sample, for Y chromosome microdeletion screening can be a better option as it can detect the deletions not detected by blood sample analysis.

5. Conclusion

Microdeletion analysis of human Y chromosome AZFa and AZFd region with 5 markers showed that they are efficient enough to detect microdeletion pattern in our study population though not specified by EAA. We also found higher frequency of microdeletion in semen samples, compared to blood samples.

Acknowledgement

This work was supported by Government of India, Department of Science and Technology (DST) Grant under Women Scientist-A (WOS-A) scheme to P. N. Barbhuiya.

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