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Association study of Yq11 AZFc subdeletion and recurrent pregnancy loss-A study in South Indian males

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Abstract

The aetiologies in recurrent pregnancy loss were focused only towards females with less consideration of male factors. This study was undertaken to analyze the association of AZF deletions in idiopathic recurrent pregnancy loss among South Indian couples. Eighty males whose female partners with the history of two or more idiopathic recurrent pregnancy loss were considered as a case group and fifty male volunteers with proven fertility as a control group. Semen and blood samples were collected from the entire study subject and examined for functionality of the sperm along with routine semen analysis. Standard PCR was performed for Yq microdeletion analysis in AZFa, b and c regions along with AZFc subdeletion analysis. RPL group showed statistically significant lower scores for total motility and sperm function test than the control. Abnormal semen parameters were reported in 18.8% among RPL males. AZFc subdeletions were reported in 8.75% of RPL males, none of the RPL individuals including control group showed no deletion for AZFa, AZFb and AZFc. We observed subnormal sperm function scores in AZFc sub deleted subjects. No significant association was observed between routine semen parameters and AZFc subdeletions. Our results revealed the potential association of abnormal sperm functioning and AZFc subdeletion in the idiopathic RPL cases in South Indian population.

Keywords: Recurrent pregnancy loss, Sperm function test, Y chromosome, AZF microdeletions, AZFc subdeletions

1. Introduction

The most common outcome of conception is embryonic or foetal death and only 30% of conceptions result in live birth (1). Approximately 15% of all clinically recognized pregnancies result in spontaneous loss and there are many more pregnancies that fail prior to being clinically recognized. Recurrent pregnancy loss (RPL) or habitual miscarriage is one of the common complications of fertility and is defined as the spontaneous loss of two or more consecutive abortions upto 20th week of gestation affecting 1-2% of couples trying to conceive (2, 3). RPL is a multi factorial condition with several etiologic factors including chromosomal abnormalities, alterations in hormonal levels, uterine abnormalities, thrombophilia disorders and maternal immune system dysfunction.

Despite of all these investigations about half of the cases remain unexplained.

RPL is usually approached from the women's perspective owing that the relation exist between the mother and the developing embryo. Though 50% of the genomic material is contributed by male gamete to the embryo potential male contributing factors has been poorly evaluated in RPL cases (4-6).

There are several evidences that male factors potentially affect fertilization, embryonic development, viability, placental proliferation and differentiation of trophoblast cells (7, 8).

Approximately 50% of all human reproductive failures appear to be associated with chromosome abnormalities and 1-4% of these anomalies have observed in RPL couples (9).

Chromosomal alterations such as minute deletions, substitutions, duplications, translocations or point mutations cannot be detected by cytogenetic analysis. These subtle abnormalities might account for increased rate of idiopathic miscarriages. Few recent studies have confirmed the association between microdeletion and RPL. Candidate genes

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which are essential for male fertility are located on the three AZF regions at Yq11 of Y chromosome (10).

The first region AZFa contains two single copy genes *USP9Y* and *DBY* whose deletion or mutation causes spermatogenic failure. The AZFb region contains several multi copy genes among which *RBMY* encodes for RNA-binding protein that helps in splicing (11, 12) and the third region AZFc harbors the *DAZ* gene family (13, 14).

Subdeletions within the AZFc region has been described and this results in the removal of small number of gene copies and its transcription units (15-17). The role of these deletions especially in RPL is still unknown and controversial. The association between Y chromosome microdeletion and RPL were studied by different researchers on their specific population groups.

Similar studies have never been documented in correlation with RPL among South Indian population. Hence, the aim of the present study is to investigate the Yq classical AZF deletions and also AZFc subdeletions to understand its association with RPL. To best of our knowledge, this study is the first of its kind to describe the association of Y chromosome AZF deletions on semen parameters and sperm function test among male partners of RPL females from South India.

2. Materials and Methods

2.1. Study design

The study was conducted after obtaining Institutional ethical committee clearance (IHEC-UOM No.52/2011-12). Eighty male partners (aged 25-40) from the couples with the history of two or more consecutive pregnancy loss in their early first or second trimester were recruited as subjects for the present study. All RPL women were examined through ultrasonography and hysterosalpingography to rule out anatomical defects, immunological, endocrinological and infectious problems. The exclusion criteria consisted of endocrine disorders and uterine structural abnormalities. Husbands of women fulfilling the above said criteria and those who approved to provide blood and semen samples voluntarily were enrolled. Detailed familial, clinical, occupational and reproductive information were obtained using pre-designed proforma and clinical work-up was obtained from respective clinics. Fifty volunteers who had fathered live normal child/children without the history of miscarriage and unassisted pregnancies were recruited as control group for the present study. Written informed consent was obtained from all the study subjects.

2.2. Semen analysis

Semen samples were obtained from both subjects and control group by means of masturbation and collected into sterile wide mouth non-toxic vials after recommending three days of sexual abstinence. Collected samples were allowed to liquefy at 37^0C for 30 minutes and analyzed within an hour of collection by performing physical and microscopic examinations as per the WHO guidelines (18).

The quality of the spermatozoa was assessed through function tests. Functional integrity of plasma membrane was performed using hypo-osmotic swelling (HOS) test. Fructose and sodium citrate were mixed in equal ratio to prepare Hypo-osmotic solution. This solution was incubated at 37^0C for 10 minutes and semen sample was mixed and incubated for 30 minutes at 37^0C. This mixture was dropped on glass slide, covered with cover slip and observed under microscope. Percentage of coiled (curled) tail was recorded. If more than 60% of spermatozoa, shows coiled tail then it is considered as normal (19).

Nuclear chromatin decondensation (NCD) test was carried out to check the ability of the spermatozoa nuclear material to decondense in vitro. Semen sample was centrifuged to separate plasma from pellet. The pellets were washed with 0.05M borate buffer. Sample was mixed with EDTA-SDS mixture and incubated at 37^0C for an hour. An equal volume of glutaraldehyde borate buffer was added. A drop of this mixture was transferred on to clean glass slide and covered with cover slip and observed under microscope. The number of condensed and decondensed heads were counted. If more than 70% of spermatozoa show decondensed nuclear chromatin then it is considered as normal (20).

2.3. Analysis of Y chromosome micro deletion

Two milliliter of intra venous blood was anti-coagulated with EDTA and stored at 4^0C until DNA isolation was performed. The whole genomic DNA was isolated using QIAamp DNA blood mini kit (Qiagen, Netherlands). The purity of DNA was assessed using UV-Vis Spectrophotometer and the concentration of DNA was diluted to 50ng concentration using Tris-EDTA buffer to perform standard PCR. All the DNA samples were subjected for Yq microdeletion analysis using STS markers sY84 and sY86 for AZFa region, sY127 and sY134 for AZFb and sY254 and sY255 for AZFc region using PCR according to the EAA/EMQN guideline. Subsequently AZFc subdeletion analysis was performed using sY1291 (gr/gr deletion) and sY1191 (b2/b3 deletion) sY1197 (b1/b3 deletion) STS markers (17, 21).

The PCR primers were obtained from Integrated DNA Technologies (USA). The primer details, target region and PCR product size are provided in Table 1. PCR was carried out in a 25µl reaction mixture. Amplification conditions were started with an initial activation step at 94^0C for 4 minutes followed by 30 cycles of denaturation at 94^0C for 30s, annealing at 58^0C (sY86, sY127), 58.2^0C for (sY254, sY255 and sY1197), 57 ^{0}C for (sY84 and sY134) and 60.4^0C (sY1291 and sY1191) for 30 seconds, extension at 72 ^{0}C for 30 seconds and final elongation for 5 minutes at 72^0C. The PCR was carried out using Corbett thermal cycler. PCR products were separated using electrophoresis on 2% agarose gel with ethidium bromide and visualized under UV trans-illuminator. Samples in which a deletion of a particular STS marker was detected were further confirmed by two repeats of the particular sample. The deletion of complete AZFa region was observed by the absence for the amplification of markers sY84 and sY86, absence of markers sY127 and sY134 indicates the deletion of AZFb region and the AZFc region deletion was observed by the absence of marker sY254 and sY255. The gr/gr deletion was screened by the absence of marker sY1291. The b2/b3 deletion was detected by absence of sY1191 and the absence of sY1197 indicates only b1/b3 deletion in the subjects.

2.4. Statistical analysis

Statistical analysis was performed using Independent- samples t test to analyze the significant difference using statistical program SPSS (version 14.0). Results are reported as mean ± standard deviation. Chi square test was performed to ascertain any significant deviation in the deletion frequency between RPL and control group. Differences were considered significant when $p<0.05$.

Table 1. Sequence Tagged Sites (STS) primer sequences, target region and size of PCR product, employed for Y chromosome microdeletion analysis

STS	Region/Subregions	PCR product size (bp)	Primer's (5'-3')	Reference
sY84-F	AZFa	326	AGAAGGGTCTGAAAGCAGGT	Simoni et al (2004)
sY84-R			GCCTACTACCTGGAGGCTTC	
sY86-F	AZFa	320	GTGACACACAGACTATGCTTC	Simoni et al (2004)
sY86-R			ACACACAGAGGGACAACCCT	
sY127-F	AZFb	274	GGCTCACAAACGAAAAGAAA	Simoni et al (2004)
sY127-R			CTGCAGGCAGTAATAAGGGA	
sY134-F	AZFb	301	GTCTGCCTCACCATAAAACG	Simoni et al (2004)
sY134-R			ACCACTGCCAAAACTTTCAA	
sY254-F	AZFc	380	GGGTGTTACCAGAAGGCAAA	Simoni et al (2004)
sY254-R			GAACCGTATCTACCAAAGCAGC	
sY255-F	AZFc	120	GTTACAGGATTCGGCGTGAT	Simoni et al (2004)
sY255-R			CTCGTCATGTGCAGCCAC	
sY1191-F	b2/b3	385	CCAGACGTTCTACCCTTTCG	Repping et al (2002)
sY1191-R			GAGCCGAGATCCAGTTACCA	
sY1197-F	b1/b3	453	TCATTTGTGTCCTTCTCTTGGA	Repping et al (2002)
sY1197-R			CTAAGCCAGGAACTTGCCAC	
sY1291-F	gr/gr	527	TAAAAGGCAGAACTGCCAGG	Repping et al (2002)
sY1291-R			GGGAGAAAAGTTCTGCAACG	

3. Results

The mean age of men in RPL group was 33.5 (SD=6.3) and in control was 32.3 (SD=5.9). The mean number of abortion in female partner was 2.6 (SD=0.87). As depicted in Table 2 we did not observe any significant difference in the pH, ejaculate volume and sperm concentration between the groups, where as we recorded significant difference in total motility and sperm function tests between RPL and control group. Based on semen analysis according to WHO criteria 81.25% RPL males were normozoospermic, however 5% of RPL individual exhibited less sperm count, 6.25% of less sperm motility and 7.5% scored lesser value for sperm count and motility. AZFc subdeletions were reported in 7 males of 80 RPL couples (Figure 1 C-E), no deletions were observed for AZFa, b and c regions (Figure 1A

and 1B).

The frequency of b2/b3 is higher than gr/gr deletion. gr/gr deletion alone was identified among two individuals and in another two cases it was associated with b1/b3 and b2/b3;b1/b3. Similarly isolated b2/b3 deletion was demonstrated among three subjects and in another case, it was associated with gr/gr; b1/b3. Isolated b1/b3 deletion was not observed but it was associated with b2/b3 and gr/gr. Details of the semen profile of the individuals observed with deletions are depicted in Table 4.

The phenotype of case 17 with deletion in all sub regions sY1191 (b2/b3), sY1291 (gr/gr) and sY1197(b1/b3) was oligospermic, while the case 65 and 78 with deletion for sY1291(gr/gr) had lesser score for both the sperm function tests NCD and HOS, where as the case 9, 54 and 56 with deletion in sY1191(b3/b3) had lesser scores for HOS test, sperm count and motility respectively, case 13 with deletion for sY1291 (gr/gr) and sY1197 (b1/b3) has abnormal score for NCD test. No significant association was observed between sub normal scores for semen parameters and AZFc subdeletions.

Table 2. Semen parameters and sperm function test in control and RPL groups

Study group	Control	RPL	*p* value
Number of subjects	50	80	
Age (years)	32.3±5.9	33.5±6.3	0.32
Semen parameters			
pH	7.7±0.3	7.8±0.4	0.171
Ejaculate volume	2.3±1	2.2±1.2	0.752
Total motility	63.4±11.8	58.4±19.6	0.001*
Sperm concentration	61.3±28.3	47.5±30.6	0.494
Function test			
HOS	75.5±17.7	65.9±13.9	0.001*
NCD	75.9±7.5	59±17.7	0.001*

p value <0.05 was considered to be significant HOS=hypo osmotic swelling test, NCD=nuclear chromatin decondensation test.

Table 3. Frequency of different types of deletions in control and RPL groups

STS	Control	RPL
AZFa	-	-
AZFb	-	-
AZFc	-	-
sY1291 alone	-	2
sY1191 alone	-	3
sY1197 alone	-	-
sY1291 & sY 1191	-	-
sY1291 & sY1197	-	1
sY1191 & sY 1197	-	-
sY1291, sY1191 & sY1197	-	1
Total	-	7 (8.75%) (p>0.05)

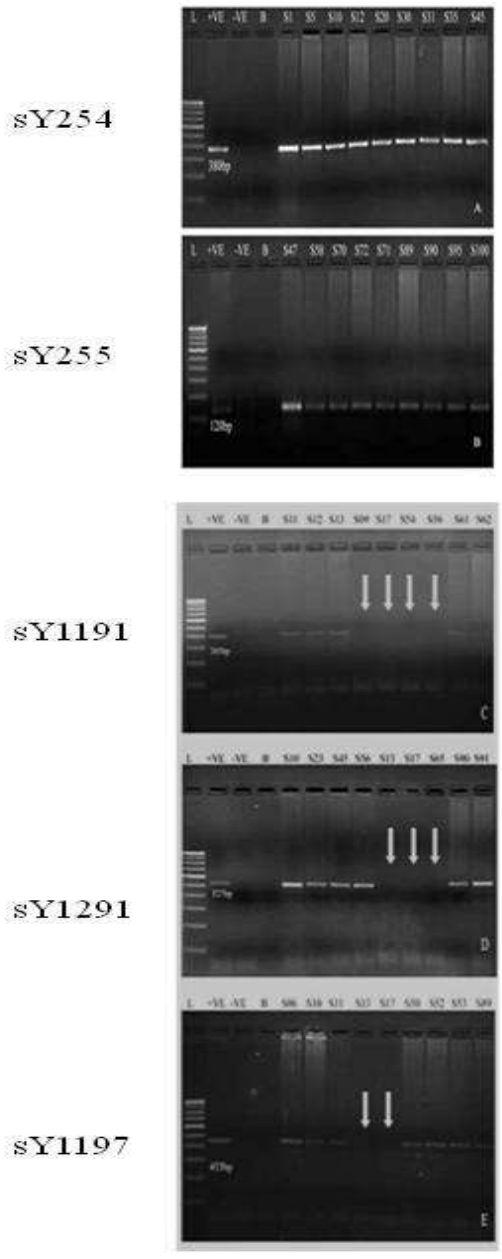

Figure 1. A-E gel image showing PCR product of sequence tagged sites (STS) markers used for screening Y chromosome (Yq) AZFc region in RPL males. White arrow (C-E) indicates the deletion in the markers in the given sample number. Lane 1 represents the marker 100bp, +ve as control DNA sample, -ve as female DNA sample and B corresponds to water used as blank.

Table 4. Semen details of the subjects with deletions

Sample ID	Age	No. of pregnancy losses	Deleted markers	Volume (ml)	Sperm concentration (10^6/ml)	Total motility (%)	NCD (%)	HOS (%)
9	40	2	sY1191	3.3	34	65	70	50*
13	31	2	sY1291, sY1197	1*	20	65	60*	80
17	32	2	sY1291,sY1191, sY1197	3	13*	50	70	70
54	24	2	sY1191	0.7*	14*	32*	80	80
56	32	2	sY1191	2	31	55	70	60
65	31	2	sY1291	3	19	45	30*	50*
78	39	3	sY1291	1.5	36	50	50*	40*

*Indicates lower reference value than the WHO guidelines (2010)

4. Discussion

Evaluation of male factor in RPL involves chromosomal analysis alone and the role of sperm factors has totally been ignored. Conventional semen parameters provides valuable information pertaining to sperm production, motility and viability but these are scanty to predict the exact fertility outcome of a couple trying to achieve parenthood as even men with normal semen parameters are not able to have children. Hence the relation between standard semen parameters and RPL has been a controversial theme. Considering the ambiguity in the conventional semen analysis, the sperm function tests have been developed to uncover the biological capacity of sperm to perform the functional characteristic which is essential to reach and fertilize ova resulting in successful pregnancy delivering healthy offspring. Association of functional status in early embryogenesis among RPL male partners has not been thoroughly intensively investigated. Few studies have revealed the association between increased abnormal sperm morphology and RPL (22, 23). Sperm membrane integrity is fundamentally important in the fertilization process and is evaluated through HOS test (24). The alteration in sperm nuclei might result in defective decondensation of the nucleus during fertilization, leading to early embryonic transience or poor embryonic development. Chromatin integrity was analyzed in vitro using NCD test, where Saxena and co-workers have reported abnormal sperm morphology in 8% of RPL subjects and significant lower sperm motility scores and sperm function test in RPL group than control (25). In our study, we observed abnormal values for count and motility in 18.75% of the RPL males and 2% of the males with severe abnormality in sperm morphology. Sperm function test (HOS and NCD) scores show significantly lower in RPL than control group wherein, 21.25% and 66.25% of the RPL individuals showed sub normal score for HOS and NCD respectively. Some of the previous studies have reported in spite of apparently normal sperm count showing subtle membrane defect in the spermatozoa and sub-optimal hypo osmotic swelling scores have been implicated as factor in the RPL (24, 26).

Y chromosome microdeletion is the most frequently observed structural abnormalities in the male specific region of Y chromosome. Individual with Y-chromosome micro-deletion have no vital symptoms but a significant percentage of them end up with fertility complications having negative impact on the sperm quality and abnormal spermatozoa that may be associated with RPL (27-29). Geoffroy- siraudin et al., reported that AZFc genes may have direct effect on early prophase by decreasing the rate of normal paring in pachytene stage of spermatocytes (30). This could be one of the major mechanism for the increased chromosomal anomalies of sperms in infertile by increasing the chance pregnancy loss (29). Few studies have reported the positive association of Y chromosome microdeletion in AZF region and RPL. Dewan *et al.* have reported 82% of male partners in couples with RPL had at least one and 65% with three or more microdeletions in AZF region of the Y-chromosome (31). This study has been criticized because of several pitfalls in their experimental design and result interpretation (32). Karaer et al. reported 7/46 had microdeletion in sY129 for AZFb 2/43 in sY153for AZFd region, but these are not true microdeletions because individuals STS absence should be considered as polymorphisms or methodological mistakes (33). A very recent study on Egyptian population using real time PCR SYBR green assay

revealed 10% of the single microdeletion, of these two were in AZFb region (*RBMY* gene), one in AZFa (*DBY* gene) and another in AZFc (*DAZ* gene) (34). According to previous study *DBY* gene is frequently deleted in infertile males and results in sever spermatogenic impairment leading to significant decrease in germ cells or its complete absence (35). Deletion in AZFb region which harbours *RBMY* gene leads to spermatogenetic failure which are predominantly observed in infertile (35, 36). However, this study fails to reveal the semen details to support the results.

In our study all RPL males and control group were analyzed for AZF microdeletion using 6 STS markers recommended by EAA/EMQN guideline (21) and also AZFc subdeletions was tested using specific STS markers sY1191, sY1291 and sY1197 specified by Repping *et al*. (17). Standard PCR analysis revealed no classical deletions in control. However, 8.75% of RPL males showed subdeletion in the AZFc region of which 1.25% of the RPL males exhibit subdeletions for all the three markers used (sY1291, sY1191 and sY1197), 1.25% for two makers (sY1291 and sY1197)and 6.25% RPL males exhibit subdeletions for single marker (sY1291 and sY1191 separately). We did not observe any significant association between abnormal semen parameters with AZFc subdeletion pattern. Individuals with abnormal semen condition did not harbor any AZF deletion. Among AZFc subdeletion 57.1% individuals showed sub normal values for NCD and HOS where, 14.2% sub normal total motility scores and 28.5% of lower sperm concentration was observed.

A study on Mexican population employed eight STSs according to EAA/EMQN and along with they also utilized additional STSs reported by Dewan et al., (31) and Karaer et al., (33) and they observed no deletions among controls and RPL (37). Two independent studies have confirmed the frequency of AZF deletion in RPL males studying on Iranian population. Ghorbian and colleagues employed the marker recommended by EAA/EMQN guideline for AZFa, b and c, addition this group also utilized sY150 and sY152 reported by Dewan et al., (38). The study revealed no microdeletion in any of the regions in the case and control group. However, Soleimanaian et al., reported 13.3% of microdeletions in one of four different STR (DYS220, DYS262, DYF85S1 and DYF86S1) in RPL males (39). Another study on Sri Lankan population by Wettasinghe et al., revealed no classical AZF deletions and partial deletions in AZFc region (40). The study on Turkey population did not show any significant association between RPL and Y chromosomal microdeletions (41). The utilization of the six primer set provided by the EAA/EMQN guidelines will enable the detection of appropriate deletions and 95% of the deletions reported in the literature in the three AZF regions and is sufficient for routine analysis (21). In addition to these STSs we also screened for sY1291, sY1191 and sY1197 revealed for AZFc subdeletion in normozoospermic RPL males which accords with the study carried by Shahid et al. (42). This group have observed gr/gr (sY1291) deletion in 2.91% normozoospermic men used as control group. It is known that the mechanism for the formation of various Yq deletions is homologous recombination between specific palindromic sequences (12, 43, 44). The gr/gr (sY1291) deletion is caused by recombination between repeat sequences g and r, resulting in the loss of two of the four copies of the *DAZ* gene and one of the three copies of the *BPY2* gene. The result of the b2/b3 (sY1191) and b1/b3 (sY1197) deletion leads to the retention of the two *DAZ* gene copies and one or two *BPY2* gene copies. All AZFc subdeletions reported so far resulted in the reduction of their specific copy number; however, a clear dosage effect on sperm production was not been studied.

The different observation by different study groups may be due to the limitation in the studies of association of RPL and Y chromosome microdeletion, variation in the selection of the RPL subjects, sample size, the STSs employes for the study and their possible methodological error during experimental approach. One of the other possibilities reason might due to change in different geographic location and ethnicity which cannot be ruled out. Several population based studies are available for infertile cases such similar studies also required to conduct RPL males to draw a specific conclusion.

5. Conclusion

Our data suggest potential connection of RPL with sperm function and AZFc subdeletions and these may be one of the etiology which results in RPL. But we cannot predict the answer for the effect of subdeletions on RPL. So screening the both the partners of RPL couples is recommended simultaneously for the better outcome.

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