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Y chromosome microdeletions are not associated with spontaneous recurrent pregnancy loss in a Sinhalese population in Sri Lanka

Tithila Kalum Wettasinghe, Rohan W. Jayasekara, and Vajira H.W. Dissanayake*

Human Genetics Unit, Faculty of Medicine, University of Colombo, 271 Kynsey Road, Colombo 8, Sri Lanka

*Correspondence address. E-mail: vajirahwd@hotmail.com

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BACKGROUND: Many advances have been made in reproductive medicine, yet the spontaneous loss of a pregnancy remains the most common complication of pregnancy. The aetiology of spontaneous recurrent pregnancy loss (RPL) is multifactorial. Y chromosome microdeletions are found in ~7% of men with low sperm counts and, compared with the general population, a higher frequency of spontaneous pregnancy loss occurs in infertile couples. The current study was designed to examine whether Y chromosome microdeletions were associated with RPL in a Sinhalese population in Sri Lanka.

METHODS: The subjects were 76 male partners of couples where the female partner had experienced three or more RPLs. One hundred and twenty random males from the general population were also analysed as a control group. DNA extracted from peripheral blood was tested for Y chromosome microdeletions in the azoospermic factor (AZF), AZFa, AZFb, AZFc regions using a multiplex PCR amplification system. Partial deletions within the AZFc region were also tested.

RESULTS: None of the men (76 with RPL, and the 120 controls) had any microdeletions in the AZF*a*, AZF*b*, AZF*c* regions or partial deletions in the AZFc region.

CONCLUSIONS: Y chromosome microdeletions do not appear to be important in the aetiology of RPL in this population in Sri Lanka.

Key words: Y chromosome microdeletions / recurrent pregnancy loss / AZFa / AZFb / AZFc

Introduction

Recurrent pregnancy loss (RPL), defined as three or more consecutive pregnancy losses, is estimated to affect ~1% of all couples trying to conceive (Rai and Regan, 2006). This is a higher frequency than expected by chance. It is estimated that more pregnancies are lost spontaneously than are actually carried to term (Rai and Regan, 2006; Stephenson and Kutteh, 2007). Despite much research and the many advances that have been made in reproductive medicine during the past decades, spontaneous loss of a pregnancy remains the most common complication of pregnancy (Pandey *et al.*, 2004; Stephenson and Kutteh, 2007).

The aetiology of spontaneous RPL is multifactorial (Christiansen, 2006). The male factor, i.e. sperm quality, chromosomal anomalies and paternal age, is rarely discussed and has been poorly evaluated in RPL. The repetitive pregnancy losses in some couples and the high incidence of unexplained RPL indicate that there are specific underlying causes that have not yet been found.

At least 50% of spontaneous RPLs in the first trimester are associated with genetic abnormalities in the foetus (Daniely *et al.*, 1998). In spite of this, cytogenetic abnormalities are detected only in a minority of the couples experiencing RPL (Stirrat, 1990). Embryos bearing the 45, X karyotype have a higher risk of spontaneous miscarriage (Hassold *et al.*, 1988; Daniely *et al.*, 1998). It has been hypothesized that the loss of the second X chromosome occurs in men who have microdeletions in their Y chromosome (Oates *et al.*, 2002).

Y chromosome microdeletions are found in ~7% of men with very low sperm counts (Ferlin *et al.*, 2007). Compared with the general population, a higher frequency of spontaneous pregnancy losses has been observed in infertile couples, and the prevalence of infertility among repeated spontaneous aborters is high (Coulam, 1992). The current study was designed to examine whether Y chromosome

microdeletions were associated with RPL in a Sinhalese population in Sri Lanka.

Materials and Methods

Subjects

Two hundred couples with three or more spontaneous RPLs were recruited for a case–control genetic association study of genetic thrombophilic polymorphisms with RPL between January 2006 and January 2008 at the Human Genetics Unit, Faculty of Medicine, University of Colombo according to a protocol approved by the Ethics Review Committee of the Faculty. These women were selected using strict criteria that included a detailed medical, clinical, reproductive and family history. Details from recent laboratory reports of autoimmune disorders (lupus anticoagulant antibodies, antiphospholipid, anticardiolipin antibodies), hormonal evaluations (progesterone, thyroid panel and diabetes mellitus), obstetric radiography and ultrasonography were taken from each woman to rule out other possible causes of RPL. These women and their partners had also undergone karyotyping prior to recruitment and those with chromosomal abnormalities had been excluded. In addition, the women had undergone screening for genetic thrombophilias (i.e. the methylenetetrahydrofolate 677C > T; factor II 20210G > A and factor V 1691G > A polymorphisms). We re-contacted the couples where both male and female partners were karyotypically normal, and the female partner had tested negative for genetic thrombophilic polymorphisms and invited the male partner to participate in the current study.

Eighty-nine couples were excluded because of genetic thrombophilia in the female partner. A total of 111 couples were re-contacted. Seventy-six male partners gave written informed consent and participated in the study (Table I). Among the men recruited for the study, a seminal fluid analysis report was available for 50, of which 5 (10%) men were oligozoospermic, 2 (4%) men were asthenozoospermic, 2 (4%) men were asthenozoospermia/oligozoospermic and 41 (82%) men were normospermic.

In addition, 120 random DNA samples of males from the general population were also subjected to Y chromosome microdeletion testing to determine the prevalence of Y chromosome microdeletions in the general population. These samples came from a population-based DNA collection maintained in our Unit for studies of this nature with the approval of the Ethics Review Committee of the Faculty of Medicine, University of Colombo. Ethics clearance was obtained to use the samples for the current study.

Table I Clinical characteristics of the 76 couples experiencing spontaneous RPL.

No. of pregnancy losses	Mean age (years)		No. of couples	Couples (%)
	Female	Male		
3	31	35	32	42.1
4	32	37	22	29.0
5	31	34	8	10.5
6	33	37	5	6.6
7	29	34	4	5.3
9	25	30	2	2.6
11	35	36	1	1.3
12	36	36	1	1.3
13	27	32	1	1.3
Total	31	35	76	100%

Y chromosome microdeletion analysis

Peripheral blood (3 ml) was collected from each male in the study group and genomic DNA was extracted using the blood DNA mini extraction kit (Qiagen, Germany) according to the manufacturer's protocol.

All the azoospermic factor (AZF) regions, that is, AZF*a*, AZF*b* and AZF*c*, were tested for Y chromosome microdeletions in the Yq *AZF* region in two steps. In the first step, aimed at detecting *AZFa*, *AZFb* and *AZFc* microdeletions, we used the multiplex PCR amplification system suggested in the state of the art recommendations made by the European Academy of Andrology/European Molecular Genetics Quality Network (EAA/EMQN) guidelines (Simoni *et al.*, 2004). Two multiplex reactions (A and B) were used for the analysis of the three AZF deletion regions on the Y chromosome. Both multiplexes contain five fragments, i.e. the three AZF loci and the two control fragments sex-determining region of the Y (SRY) and Zinc Finger Protein of Y Chromosome/Zinc Finger Protein of X Chromosome (*ZFY/ZFX*). The 25 μl PCR reaction mix contains: 12.5 μl 2× Quiagen Multiplex PCR MasterMix [containing HotStarTaq DNA Polymerase, Qiagen Multiplex PCR Buffer (containing 6 mM $MgCl_2$) and dNTP Mix], 2 μl 10× Primer mix, ~1 μl template DNA and 25 μl of sterile distilled water.

Amplification conditions start with an initial activation step of 15 min at 95°C, followed by 35 cycles of 30 s denaturation (94°C), 90 s annealing (57°C) and 60 s elongation (72°C), plus a final elongation step of 10 min and cooling to 4°C.

The PCR products were separated by electrophoresis on a 3% agarose gel impregnated with ethidium bromide and visualized under UV light (Fig. 1). Genomic DNA from a man with an already confirmed deletion (AZFb) was used as a positive control whereas the negative control DNA was obtained from a woman. *ZFY/ZFX* (zinc finger protein genes) were used as an internal controls. The *SRY* gene (sex-determining region of the Y) was examined to confirm the sex of the donor.

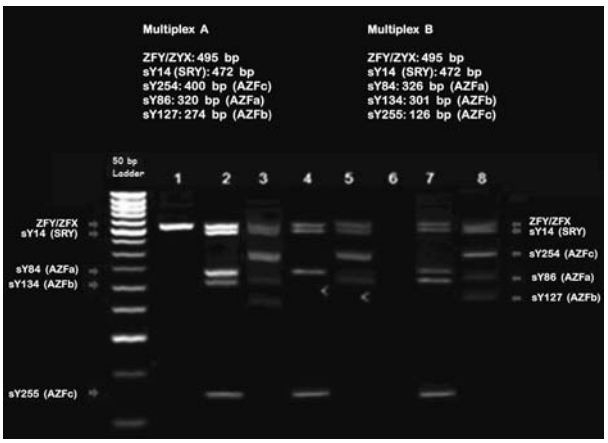

Figure 1 Electrophoresis results of multiplex PCR products amplified on a 3% agarose gel. A female sample as the negative control in the first lane, normal male in the second and third lanes, men with deletions in lanes 4 and 5 where the deleted bands are marked with an arrow, blank in the sixth lane and study population sample in the seventh and eighth lanes.

Table II Sequences of the primer sets used to investigate Y chromosome microdeletions in partners of women with spontaneous RPL.

STS	Primer sequence 5′ to 3′	PCR product size (bp)	Reference
ZFY-F	ACCRCTGTACTGACTGTG	495	Simoni et al. (2004)
ZFY-R	GCACYTCTTTGGTATCYGAGAAAGT		
SRY-F	GAATATTCCCGCTCTCCGGA	472	Simoni et al. (2004)
SRY-R	GCTGGTGCTCCATTCTTGAG		
sY84-F	AGAAGGGTCTGAAAGCAGGT	326	Simoni et al. (2004)
sY84-R	GCCTACTACCTGGAGGCTTC		
sY86-F	GTGACACAGACTATGCTTC	320	Simoni et al. (2004)
sY86-R	ACACACAGAGGGACAACCCT		
sY127-F	GGCTCACAAACGAAAAGAAA	274	Simoni et al. (2004)
sY127-R	CTGCAGGCAGTAATAAGGGA		
sY134-F	GTCTGCCTCACCATAAAACG	301	Simoni et al. (2004)
sY134-R	ACCACTGCCAAAACTTTCAA		
sY254-F	GGGTGTTACCAGAAGGCAAA	380	Simoni et al. (2004)
sY254-R	GAACCGTATCTACCAAAGCAGC		
sY255-F	GTTACAGGATTCGGCGTGAT	123	Simoni et al. (2004)
sY255-R	CTCGTCATGTGCAGCCAC		
sY1191-F	CCAGACGTTCTACCCTTTCG	385	Repping et al. (2002)
sY1191-R	GAACCGTATCTACCAAAGCAGC		
sY1291-F	TAAAAGGCAGAACTGCCAGG	527	Repping et al. (2002)
sY1291-R	GGGAGAAAAGTTCTGCAACG		
SY1201-F	CCGACTTCCACAATGGCT	677	Repping et al. (2002)
SY1201-R	GGGAGAAAAGTTCTGCAACG		
sY1206-F	CTGGGCTTCTGTGGCTTT	412	Lynch et al. (2005)
sY1206-F	GCCAATTTGACCAGTGACTTC		
sY1161-F	CGACACTTTTGGGAAGTTTCA	330	Repping et al. (2002)
sY116-R	TTGTGTCCAGTGGTGGCTTA		

Patients without the classical AZF*a*, AZF*b* and AZF*c* deletions and all 120 control subjects underwent assessment for AZFc subdeletions. In the second step, we used a multiplex system to determine the presence or absence of the AZF*c* subdeletions by using sequence tagged sites (STSs) markers as described by Repping *et al.* (2003). Primer sequences were as described (Repping *et al.*, 2003 and GenBank) except for sY1206, which used the primers sY1206* F (5′-ctgggctttctgtggcattt-3′) and sY1206 R (5′-gccaatttgaccagtgacttc-3′) from within the GenBank sY1206 sequence to allow better size separation of multiplex PCR products on agarose gels (Lynch *et al.*, 2005). The sequences of all primer pairs and the expected size of the PCR products are shown in Table II. A 2 μl aliquot of the genomic DNA was amplified by a multiplex PCR in a total volume of 25 μl containing 12.5 μl ×2 Quiagen Multiplex PCR MasterMix, 2 μl ×10 Primer mix and 25 μl sterile distilled water. The PCR result was visualized on a 2% agarose gel with ethidium bromide including a 100 bp DNA ladder as a marker. In all PCRs, a female DNA and a water sample (no template) were included as negative controls (Fig. 1).

Results

None of the men (76 with RPL, and the 120 controls) had any microdeletions in the AZF*a*, AZF*b*, AZF*c* regions or partial deletions in the AZF*c* region.

Discussion

We set out to determine the association of Y chromosome microdeletions with RPL, studying male partners of women experiencing RPL, and found that none of the men in our population had Y chromosome microdeletions. In addition, the seminal fluid analysis reports indicated the majority of them to be normospermic.

This study was designed to overcome some deficiencies in the original study reporting an association between Y chromosome microdeletions and RPL (Dewan *et al.*, 2006). In their pilot study (Dewan *et al.*, 2006), reported that a significant proportion (82%) of 17 male partners of women with RPL had Y chromosomal microdeletions, primarily in the AZFc region, which may have been the reason for the RPL experienced by their partners. It is also known that deletions involving the AZFc region account for up to 90% of all Yq deletions with phenotypic variations (Reijo *et al.*, 1995; Vogt *et al.*, 1996). A detailed analysis of the AZFc region of the Y chromosome using new molecular markers has confirmed the existence of three such deletions; namely, gr/gr, b1/b3 and b2/b3 (also known as g1/g3) (Repping *et al.*, 2003; Fernandes *et al.*, 2004; Repping *et al.*, 2004).

The study by Dewan *et al.* (2006) on deletions in the human Y chromosome has been criticized because several pitfalls were

identified in the experimental design and interpretation of their results (Noordam *et al*., 2006). A similar study (Karaer *et al*., 2008) reported 7 of the 43 infertile men (16%) from couples with RPL had microdeletions in the AZF region of the Y chromosome, after studying four-specific regions of the Y chromosome [DYS220 (AZFb), DYS235, DYS236 and DYS237 (AZFd) subregions]. The STSs labelled with DYS 220 yielded the most deletions.

The existence of an AZF*d* region has not been confirmed in other studies, and it is believed that this deletion does not exist, while the partial deletions in the AZF*c* region supports the non-existence of an AZF*d* region (Simoni *et al*., 2004). The six primer sets suggested by the EAA/EMQN, which are highly robust and reliable in detecting Y chromosome microdeletions (Simoni *et al*., 2004) were not used in studies carried out by Dewan *et al*. (2006) and Karaer *et al*. (2008). This primer set can also be expanded with five additional primer pairs to study partial deletions in the AZFc region (Repping *et al*., 2003; Lynch *et al*., 2005). The marker sY67/DYS262 (used by Dewan *et al*. which is in fact located on the short arm of the Y chromosome), is nowhere near the AZF regions on Yq (Noordam *et al*., 2006). According to The National Center for Biotechnology Information UniSTS Database, the STS marker DYS262 is located on the short arm of the Y chromosome, and the markers DYS220, SY152 and SY150 on the long arm in the AZFc region. Dewan *et al*. (2006) report that they found patients with deletions of both DYS262, which is nowhere near the AZF regions on Yq, and an additional marker. This result indicates that these patients would have multiple deleted regions of the Y chromosome, which is highly unlikely in a normal male.

The absence of sY152 could be explained by a partial AZFc deletion that removes two DAZ copies. However, Dewan *et al*. (2006) did not screen their samples for these partial AZFc deletions, although the STSs that are necessary to do so are publicly available (Noordam *et al*., 2006).

According to guidelines provided by the EAA/EMQN for diagnostic laboratories, with the use of six STSs it should be possible to detect up to 95% of all reported Y microdeletions in the AZF regions (Simoni *et al*., 2004). In addition to the STSs recommended in the guidelines, we screened for the AZFc subdeletions using the five Yq STS markers described by Repping *et al*. (2003), therefore, it is highly unlikely that a known deletion would have been missed.

Kaare *et al*. (2008) conducted a similar study using 37 STS loci spanning the whole Y chromosome on 40 male partners of women with RPL and concluded that Y chromosome microdeletions were not associated with RPL in the Finnish population.

High rates of chromosomal abnormalities in sperm have been observed in RPL patients with normal lymphocyte karyotypes since the highly repetitive structure of the Y chromosome sequence predisposes to *de novo* deletions (Al-Hassan *et al*., 2005). Although in our study, we did not detect any microdeletions, it is important to keep in mind that a normal lymphocyte karyotype does not exclude sperm abnormalities. DNA extracted from peripheral blood was used in this study and therefore, there is a possibility that the abnormalities leading to RPL could arise in spermatocytes during spermatogenesis through *de novo* mutations.

The contribution of those events to RPL may be small as the vast majority of men in our series were normospermic. Investigations into Y chromosome microdeletions in sperm of karyotypically normal men with abnormal seminal fluid analysis reports are ongoing in our laboratory.

In conclusion, therefore, constitutional Y chromosome microdeletions do not appear to be important in the aetiology of RPL and routine Y chromosome microdeletion testing cannot be recommended for male partners of women experiencing RPL.

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