

Multiplex PCR based screening for microdeletions in azoospermia factor region of Y chromosome in azoospermic and severe oligozoospermic south Indian men

Ramaswamy Suganthi Ph.D., VV Vijesh M.Sc., Sanjay Jayachandran M.Sc., Jahangir Ali Fathima Benazir Ph.D.

Department of Biotechnology, Dr. G. R. Damodaran College of Sciences, Coimbatore, 641014, India.

Corresponding Author:
Ramaswamy Suganthi, School of Biotechnology, Dr. G. R. Damodaran College of science, Coimbatore-641014, India.
Email: sugantham2000@gmail.com
Tel/Fax: (+91) 9843134681

Received: 14 February 2012
Revised: 19 June 2012
Accepted: 19 June 2012

Abstract

Background: Y chromosomal microdeletion is an important genetic disorder, which may arise due to intrachromosomal recombination between homologous sequences in the male specific region of the human Y chromosome. It is frequently associated with the quantitative reduction of sperm. The screening for Y chromosomal microdeletions has a great clinical value.

Objective: To develop a sequence tagged site (STS) based multiplex PCR protocol, which could be specific for the rapid detection of AZF deletions and thereby estimating the frequency of AZF sub deletions in infertile South Indian men.

Materials and Methods: In the current study, PCR based Y chromosomal microdeletion screening analysis was performed in 75 men including 30 non-obstructive azoospermic men, 20 severe oligozoospermic, and 25 normozoospermic fertile men (controls) using 15 known STS primer pairs mapped within the AZF locus. Deletion frequency was estimated after successful PCR amplification.

Results: We designed and optimized a STS based multiplex PCR protocol, which could be helpful for the clinicians to detect the Y chromosomal deletions rapidly and specifically. In our study, we estimated an overall deletion frequency of 36%. Among these 12 (40%) were azoospermic and 6 (30%) were oligozoospermic. No microdeletions were observed in normozoospermic fertile men.

Conclusion: Our Study emphasizes the fact that Y chromosomal microdeletion screening tests are unavoidable in the workup of idiopathic male infertility. Mandatory screening for Y deletions should be done in all azoospermic and severe oligozoospermic patients before undergoing assisted reproductive technology.

Key words: *Y chromosomal microdeletions, AZF region, Male infertility, Multiplex PCR.*

Introduction

Infertility silently emerges as a major public problem related to reproductive health. It adversely affects the social, psychological and economic status of the affected individuals and couples. It is estimated that the global prevalence of infertility ranges from 3.5-16.7% in developed nations and from 6.9-9.3% in developing and under developed nations (1).

Astonishingly, a male factor can be observed in about half of the cases (2). Even though there were several proposed causes of male infertility, the causative factor of 60-75% cases are still unknown and termed as idiopathic male infertility (3). In these cases, it is speculated that the genetic factors may directly or with the interaction of

environmental factors act at some stages of testicular development and disrupt spermatogenesis (4, 5). Disruption in spermatogenesis will result in qualitative decline and quantitative reduction of sperm. Azoospermia and oligozoospermia are frequently observed in men with impaired spermatogenesis, and most of these patients seek for intracytoplasmic sperm injection (ICSI) (6).

ICSI is a revolutionized assisted reproductive technique (ART) which fulfills the dream of millions to have their own baby (7, 8). Since the ICSI, technique bypasses all the natural barriers related to natural fertilization, it raises serious concern about the transmission of known and unknown genetic defects including Y chromosomal microdeletions to the descendants (9-13). This

implies the necessity for testing genetic defects in infertile men before undergoing ICSI.

Y chromosomal microdeletion is defined as the missing up of gene or gene sequences from the functionally active part of the Y chromosome. It represents the most common structural chromosomal anomaly associated with non-obstructive azoospermia and severe oligozoospermia (14). To date, most of the studies on Y deletions are centred on a hotspot region known as the azoospermia factor (AZF), which is located at the distal portion of the long arm of the Y chromosome (Yq11.2) (15).

Later on, Vogt *et al* reclassified the concept of the azoospermia factors into three non-overlapping regions, AZFa, AZFb and AZFc (16). Recent evidence directs to the existence of new pattern of AZF deletions in which distal part of AZFb overlaps with the proximal region of AZFc (17). Each region contains candidate genes, which are essential for normal development of germ cells. So far, at least fourteen proteins encoding Y genes were identified in AZF locus (18).

The homologous recombination between identical Y-specific repeats generates deletions that cause the removal of functional AZF genes which in turn leads to spermatogenic failure (19). In the current study, we made an effort to develop a STS based multiplex PCR protocol to determine the frequency of Y chromosomal microdeletions in azoospermic and severe oligozoospermic infertile South Indian men.

Materials and methods

Patient selection

The study was carried out on non-obstructive azoospermic and severe oligozoospermic infertile men referred to leading ART centres in South India. Patient selection was done based on the alterations detected in sperm count according to WHO guidelines (20). We excluded the patients with known causes of infertility like, karyotype abnormalities, obstructive azoospermia, varicocele and testicular tumors.

A total of 75 men, including 30 non-obstructive azoospermic men, 20 severe oligozoospermic and 25 normozoospermic fertile men (Controls) were enrolled in the

study. Written informed consent was obtained from all the patients enrolled in the study. One drop of blood embedded in Whatmann filter paper in sterile eppendorf were collected and transported to the laboratory in airtight cool packs and stored in the refrigerator for screening.

DNA Isolation

Genomic DNA was extracted from a single drop of peripheral blood samples using a rapid DNA extraction protocol (21). The concentrated DNA was diluted using RNase free water (1:3 ratios) before being analysed by multiplex PCR.

Multiplex PCR screening analysis

The processed DNA of patients was subjected to multiplex PCR using 15 sets (Five multiplex set) of STS (Table I). Each primer pair amplifies a specific region of the AZF locus located in the long arm of Y chromosome. The AZF specific STS primers were selected according to the guidelines jointly recommended by the European Academy of Andrology and the European Molecular Genetics Quality Network and also from previous literatures (22-24).

The multiplex PCR reaction comprised a total volume of 16 μ l of which 8 μ l was the master mix (Qiagen, Germany), 5 μ l of multiplex STS primer mix and 3 μ l of diluted DNA. Amplifications were carried out on eppendorf thermocycler. 40 cycles were performed as follows: 94°C for 5 min for denaturation, 55°C for 45 sec for primer annealing and 67°C for 4 min for extension.

The programs were followed by the final extension step at 67°C for 4 min. The PCR products were then analyzed by electrophoresis at 50 V on 1.5% agarose gels (Himedia, Mumbai) and visualized by staining the gel with ethidium bromide (10.5 μ g/ml). Failure of amplification of any STS markers indicated microdeletion by comparing it with the control. Sample showing deletions were tested at least three times with single primer to confirm the results.

Statistical analysis

The frequency distribution of Y chromosomal microdeletions was calculated using Microsoft Excel 2010, Windows Installer version.

Results

Thirty non-obstructive azoospermic, twenty severe oligozoospermic infertile men and twenty five normozoospermic fertile men (Controls) were screened for Y chromosomal microdeletions using multiplex PCR method. DNA was separated from the peripheral blood leucocytes of all patients, and PCR amplification was successfully performed

using AZF specific STS markers. PCR amplification produced bands of expected size for all the fifteen AZF loci investigated in all fertile controls (Figure 1).

Eighteen patients, including twelve azoospermic and six oligozoospermic patients showed deletion of one or more STS markers corresponding to AZF locus. The details of the results are shown in Figure 2 and summarized in Table II.

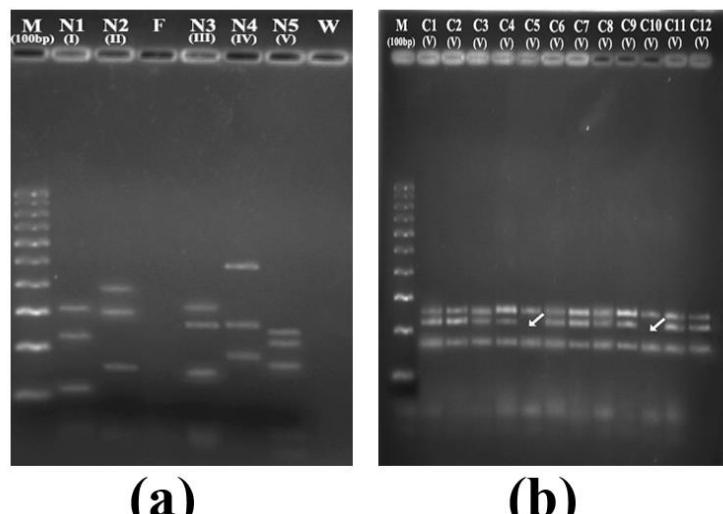
Table I. Sequence-Tagged Sites (STS) primer used for Y chromosomal microdeletion analysis

Multiplex primer sets	STS	Locus	Primer sequence	Base pairs (bp)
Multiplex primer set I	DFFRY	AZFa	F: 5'GAGCCCATTTGTCAGTTA C 3' R: 5'CTGCCATTTCACATCAAC C 3'	111
	sY143	AZFb	F: 5'GCAGGATGAGAAGCAGGTAG 3' R: 5'CCGTGTGCTGGAGACTAATC 3'	311
	sY158	AZFc	F: 5'CTCAGAAGTCCTCCTAATAGTT C 3' R: 5'ACAGTG GTT TGT AGC GGG TA 3'	231
Multiplex primer set II	DBY	AZFa	F: 5'AGTTTATTCTAACCTAGG CAAACG 3' R: 5'TCCAACCAGGCCTGTAGTGAGGCC 3'	169
	sY134	AZFb	F: 5'GTCTGCCTCACCATAAAACG 3' R: 5'ACCACTGCCAAAACCTTCAA 3'	301
	sY254	AZFc	F: 5'GGGTGTTACCAGAAG GCAAAA 3' R: 5' GAACCGTATCTACCAAAG CAG C 3'	400
Multiplex primer set III	sY84	AZFa	F: 5'AGAAGGGTCTGAAAGCAG GT 3' R: 5'GCCTACTACCTGGAGGCTTC 3'	320
	sY127	AZFb	F: 5'GGCTCACAAACGAAAAGAAAA 3' R: 5'CTGCAGGCAGTAATAAGGGA 3'	274
	CDY	AZFc	F: 5'TCATAATTCCAATTGTACTG G 3' R: 5'TTCTATCCCTCGGGCTGAGCTC 3'	132
Multiplex primer set IV	sY83	AZFa	F: 5'CTTGAATCAAAGAAGGCCCT 3' R: 5'CAATTGGT TTGGCTGACAT 3'	275
	sY143	AZFb	F: 5'AGCTTCTAT TCGAGGGCT TC 3' R: 5'CTCTCTGCAATCCCTGAC AT 3'	196
	sY283	AZFc	F: 5'CAGTGATAACACTCGGACTTGTGTA 3' R: 5'GTTATTGAAAAGCTACACGG G 3'	497
Multiplex primer set V	sY90	AZFa	F: 5'CAGTGCCCCATAACACTTTC 3' R: 5'ATGGTAATACAGCAGCTCGC 3'	176
	sY117	AZFb	F: 5'GTTGGTCCATGCTCCATAC 3' R: 5'CAGGGAGAGAGC CTTTACC 3'	260
	sY158	AZFc	F: 5' CTCAGAAGT CCTCTAATAGTT 3' R: 5'ACAGTGGTTGTAGCGGGTA 3'	231

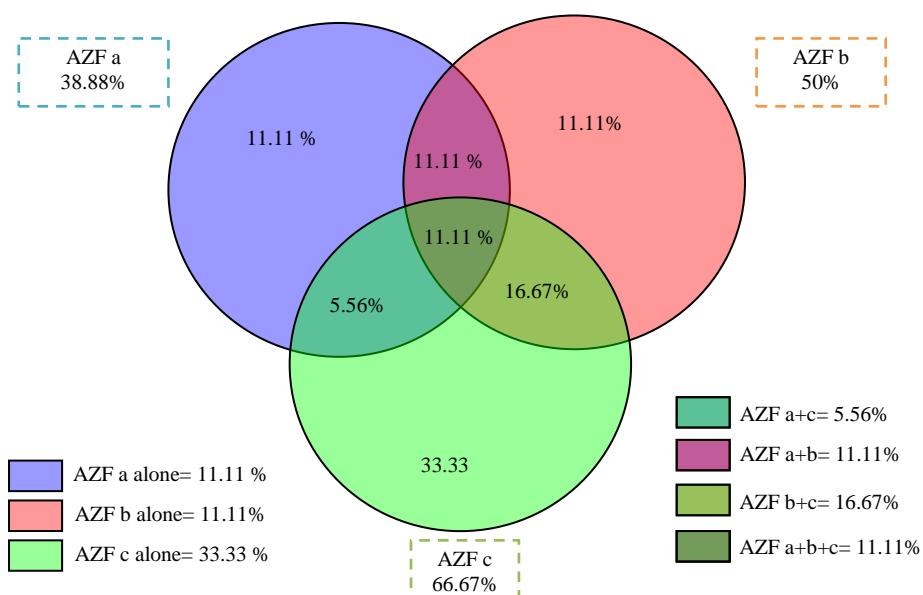
Table II. Frequencies of AZF microdeletions

Spermatogenetic defect (Spermatozoa in ejaculate)	Total patients screened	Sample studied	No. of STS markers used	Karyotype	Patients with microdeletions	Frequency of microdeletions (%)
Non-obstructive Azoospermia (None)	30	Blood	15	normal*	12	40%
Oligozoospermia (<5 mln/ml)	20	Blood	15	“	6	30%
Normozoospermia (>20 mln/ml)	25	Blood	15	“	0	-
Total	75	-	15	-	18	36%

* All patients have normal karyotype

**Figure 1.** Gel images showing the multiplex PCR products.

(a) Amplified multiplex PCR bands of normozoospermic fertile men (control) using various multiplex primer set. Lane 1, 100bp molecular weight marker , Lane 2,3,5,6,7 showing PCR amplified products using multiplex primer set I, II,III,IV & V respectively, Lane 4, female DNA, Lane 8, water. (b) Amplified multiplex PCR bands of infertile clinical samples using multiplex primer set V. The arrow marks indicates deletion in corresponding STS marker.

**Figure 2.** Vendiagram showing AZF deletions in the human Y chromosome.

Discussion

A gene cluster which is essential for the normal spermatogenesis is located on interval 6 of the long arm of the Y chromosome (i.e., Azoospermia factor) (15, 16). Deletions in AZF locus are the most common event seen in azoospermic and severe oligozoospermic patients (14). Worldwide the incidence of AZF deletions reported in infertile samples is about 1-55.5% and none of the deletions were observed in a large number of fertile controls (25-27).

The average frequency of Y chromosomal microdeletions in Indian studies comes around 7% (28). The lack of stringent patient selection criteria, the difference in experimental design and ethnic variations among different populations accounts for the variation in frequency (29, 30). In the present study, we observed deletions in 18 patients with a frequency range of 36%. Among these 12 (40%) were azoospermic and 6 (30%) were oligozoospermic, and our result are in accordance with the previous literatures. The small sample size (75 patients) and stringent patient inclusion criteria may accounts for the slight increase in the deletion frequency.

Furthermore, Y chromosome microdeletions in azoospermic patients were found to be at a much higher rate than the oligozoospermic patients (31). Our study also reflect this finding and strengthen the speculation that Y chromosome microdeletions are associated with the quantitative reduction than the qualitative decline of sperm (30).

The presence of the large number of repetitive sequence and massive palindromes in the male specific region of Y chromosomes accelerates the intrachromosomal recombination between homologous sequences, which in turn remove the AZF genes partially or completely (19). The removal of candidate genes from the AZF locus has variable clinical consequences.

AZFc types of deletions are most frequently observed in a majority of the Y chromosomal microdeletion screening studies. This may be due to the presence of a large number of repetitive sequences compared to another locus (32).

It is also speculated that there is a positive correlation between the size of the targets available for homologous recombination and incidence of each deletion (33). AZFc (66.67%) was the most frequently deleted region found in our study followed by AZFb (50%) and AZFa (38.88%). The relative frequency of AZFa, b and c region in infertile men in this study is AZFa alone in (11.11%); AZFb alone in (11.11%); AZFc alone in (33.33%); AZFa+c (5.56%), AZFa+b (11.11%), AZFb+c(16.67%) and AZFa+b+c deletions detected in two people (11.11%) (Figure 2). Hence the significant frequency rate of AZF deletions indicates the growing need of Y chromosomal screening analysis in infertile patients.

AZFa deletions are responsible for the spermatogenic disruption, which leads to Sertoli cell-only (SCO) syndrome. A complete deletion of AZFa locus removes 792kb, including the two candidate genes *USP9Y* and *DBY* (34). AZFb deletions are associated with complete absence of elongating spermatids and spermatozoa or SCOS (29, 35, 36). AZFc deletions quantitatively reduce sperm density and show capricious phenotypical features ranges from azoospermia to mild/severe oligozoospermia (32).

Furthermore, the retrieval of mature sperm upon testicular sperm extraction (TESE) for the use in IVF/ICSI is practically impossible in AZFa and AZFb deleted patients whereas sperm retrieval was possible in AZFc deleted men. At the same time risk associated with transmission of AZFc deletions was also reported (36-39) Hence the Y chromosomal microdeletion screening itself has a diagnostic, prognostic and a preventive value. The Y chromosomal microdeletion tests are

now a reality in most of the andrology labs. However, the major hurdle faced by the Y chromosomal microdeletion screening is the lack of well standardized protocols. PCR amplification using STSs has most commonly used to identify microdeletions in the Y-chromosome. The variation in the number of STS markers used in various studies also made confusion. Now it is clear that there is no correlation between the number of STS markers used and the deletion frequency.

The usage of a minimum number of highly specific, non-polymorphic STS markers will protect against inaccuracy (9). The simultaneous amplification of several loci in one reaction is achieved with the help of multiplex PCR. The optimization of multiplex PCR often poses difficulties in making different primer pairs work together. In the present study, we successfully develop a multiplex PCR protocol with 15 sets of AZF specific STS markers. The multiplex PCR protocol we developed is convenient, cost effective, fewer time-consuming and specific for the rapid detection of AZF deletions in infertile patients.

Conclusion

Bypassing the barriers of natural fertilization with the help of ART without proper screening analysis may increase the number of infertile population. New innovations and researches are essential for identifying known, and unknown molecular genetic defects associated with ART. Here we presented a simple multiplex protocol for detecting AZF deletions in azoospermic and severe oligozoospermic South Indian men before undergoing ART.

Acknowledgments

We are thankful to the management of Krishna Assisted Reproduction and Endoscopy Centre (KARE) and Center for

Research in Assisted Reproduction and Fetal Therapy (CRAFT), Kerala for providing clinical samples to conduct the present study. The volunteers who donated blood samples for this study are gratefully acknowledged.

Conflict of interest

All authors have seen and agreed with the contents of the manuscript and there is no conflict of interest to report.

References

1. Boivin J, Bunting L, Collins JA, Nygren KG. International estimates of infertility prevalence and treatment seeking: potential need and demand for infertility medical care. *Hum Reprod* 2007; 22:1506-1512.
2. World Health Organization .Towards more objectivity in diagnosis and management of male fertility. *Int J Androl* 1997; 7: 1-53.
3. Dohle GR, Colpi GM, Papp GK, Hargrave TB, Jungwirth A, Weidner W. EAU guidelines on male infertility. *Eur Urol* 2005; 48: 703-711.
4. Sharpe RM, Irvine DS. How strong is the evidence of a link between environmental chemicals and adverse effects on human reproductive health. *BMJ* 2004; 328:447-451.
5. Krausz C. Genetic Aspects of Male Infertility. *Eur Urol Rev* 2008; 3: 93-96.
6. Martínez-Garza SG, Gallegos-Rivas MC, Vargas-Macié M, Rubio-Rubio JM, de Los Monteros-Rodríguez ME, González-Ortega C, et al. Genetic screening in infertile Mexican men: chromosomal abnormalities, Y chromosome deletions, and androgen receptor CAG repeat length. *J Androl* 2008; 29: 654-660.
7. Palermo G, Joris H, Devroey P, Van Steirteghem AC. Pregnancies after intracytoplasmic injection of a single spermatozoon into an oocyte. *Lancet* 1992; 340: 17-18.
8. Nudel DM, Lipshultz LI. Is Intracytoplasmic Sperm Injection Safe? Current Status and Future Concerns. *Curr Uro Rep* 2001; 2: 423-431.
9. Kent-First M, Muallem A, Shultz J, Pryor J, Roberts K, Nolten W, et al. Defining regions of the Y-chromosome responsible for male infertility and identification of a fourth AZF region (AZFd) by Y-chromosome microdeletion detection. *Mol Reprod Dev* 1999; 53: 27-41.
10. Bonduelle M, Van Assche E, Joris H, Keymolen K, Devroey P, Van Steirteghem A, et al. Prenatal testing in ICSI pregnancies: incidence of chromosomal

- anomalies in 1586 karyotypes and relation to sperm parameters. *Hum Reprod* 2002; 17: 2600-2614.
11. Kallen B, Finnstrom O, Nygren KG, Olausson PO. In vitro fertilisation (IVF) in Sweden: risk for congenital malformations after different IVF methods. *Birth Defects. Res A Clin Mol Teratol* 2005; 73: 162-169.
 12. Feng C, Wang LQ, Dong MY, Huang HF. Assisted reproductive technology may increase clinical mutation detection in male offspring. *Fertil Steril* 2008; 90: 92-96.
 13. Kai CM, Juul A, McElreavey K, Ottesen AM, Garn ID, Main KM, et al. Sons conceived by assisted reproduction techniques inherit deletions in the azoospermia factor (AZF) region of the Y chromosome and the DAZ gene copy number. *Hum Reprod* 2008; 23: 1669-1678.
 14. Ferlin A, Arredi B, Speltra E, Cazzadore C, Selice R, Garolla A, et al. Molecular and clinical characterization of Y chromosome microdeletions in infertile men: a 10-year experience in Italy. *J Clin Endocrinol Metab* 2007; 92: 762-770.
 15. Tiepolo L, Zuffardi, O. Localization of factors controlling spermatogenesis in the nonfluorescent position of the human Y chromosome long arm. *Hum Genet* 1976; 34: 119-134.
 16. Vogt PH, Edelmann A, Kirsch S, Henegariu O, Hirschmann P, Kiesewetter F, et al. Human Y chromosome azoospermia factors (AZF) mapped to different subregions in Yq11. *Hum Mol Genet* 1996; 5: 933-943.
 17. Repping S, Skaletsky H, Lange J, Silber S, Van Der Veen F, Oates RD, et al. Recombination between palindromes P5 and P1 on the human Y chromosome causes massive deletions and spermatogenic failure. *Am J Hum Genet* 2002; 71: 906-922.
 18. Vogt PH. Azoospermia factor (AZF) in Yq11: towards a molecular understanding of its function for human male fertility and spermatogenesis. *Reprod Biomed Online* 2005; 10: 81-93.
 19. Skaletsky H, Kuroda-Kawaguchi T, Minx PJ, Cordum HS, Hillier L, Brown LG, et al. The male-specific region of the human Y chromosome is a mosaic of discrete sequence classes. *Nature* 2003; 423: 825-37.
 20. World Health Organization. WHO Laboratory Manual for the Examination of Human Semen and Sperm-Cervical Mucus Interaction. 3rd Ed. 1999.
 21. Suganthi R, Manonayaki S, Benazir JF. Molecular analysis of Y-chromosome microdeletions in infertile men. *Int J Med Sci* 2009; 2: 54-60.
 22. Simoni M, Bakker E, Krausz C. EAA/EMQN best practice guidelines for molecular diagnosis of y-chromosomal microdeletions. *Int J Androl* 2004; 27: 240-249.
 23. Lahn BT, Page DC. Functional coherence of the human Y chromosome. *Sci* 1999; 278: 675-680.
 24. Kostiner DR, Turek PJ, Reijo RA. Male infertility: analysis of the markers and genes on the human Y chromosome. *Hum Reprod* 1998; 13: 3032-3038.
 25. Van derVenK, Montag M, PeschkaB, Leygraaf J, SchwanitzG, Haidl G, et al. Combined cytogenetic and Y chromosome microdeletion screening in males undergoing intracytoplasmic sperm injection. *Mol Hum Reprod* 1997; 3: 699-704.
 26. Foresta C, Ferlin A, Garolla A, Moro E, Pistorello M, Barbaux S, et al. High frequency of well-defined Y-chromosome deletions in idiopathic Sertoli cell-only syndrome. *Hum Reprod* 1998; 13: 302-307.
 27. Krausz C, Forti G, Mc Elreavey K. The Y chromosome and male fertility and infertility. *Intl J Androl* 2003; 26: 70-75.
 28. Simoni M. Clinical consequences of microdeletions of the Y chromosome: the extended Münster experience. *Reprod Biomed Online* 2008; 16: 289-303
 29. Girardi SK, Mielnik A, Schlegel PN. Submicroscopic deletions in the Y chromosome of infertile men. *Hum Reprod* 1997; 12:1635-1641.
 30. Calogero AE, Garofalo MR, D'Agata R. Current status of the molecular diagnosis of Y chromosome microdeletions in the work-up of male infertility. Factors influencing the variable incidence of Y chromosome microdeletions in infertile patients. *Hum Reprod* 1999; 14: 272-275.
 31. Krausz C, McElreavey K. Y chromosome and male infertility. *Frontiers in Bioscience* 1999; 4: 1-8.
 32. Kuroda-Kawaguchi T, Skaletsky H, Brown LG, Minx PJ, Cordum HS, Waterston RH, et al. The AZFc region of the Y chromosome features massive palindromes and uniform recurrent deletions in infertile men. *Nat Genet* 2001; 29: 279-286.
 33. Noordam MJ, Repping S. The human Y chromosome: a masculine chromosome. *Curr Opin Genet Develop* 2006; 16: 225-232.
 34. Kamp C, Hirschmann P, Voss H, Huellen K, Vogt PH. Two long homologous retroviral sequence blocks in proximal Yq11 cause AZFa microdeletions as a result of intrachromosomal recombination events. *Hum Mol Genet* 2000; 9: 2563-2572.
 35. Krausz C, Quintana-Murci L, McElreavey K. Prognostic value of Y deletion analysis: what is the clinical prognostic value of Y chromosome microdeletion analysis. *Hum Reprod* 2000; 15: 1431-1434.
 36. Ferlin A, Moro E, Rossi A, Dallapiccola B, Foresta C. The human Y chromosome azoospermia factor b (AZFb) region: sequence, structure, and deletion analysis in infertile men. *J Med Genet* 2003; 40: 18-24.
 37. Oates RD, Silber S, Brown LG, Page DC. Clinical characterization of 42 oligospermic or azoospermic men with microdeletion of the AZFc region of the Y chromosome, and of 18 children conceived via ICSI. *Hum Reprod* 2002; 17: 2813-2824.

38. Hopps CV, Mielnik A, Goldstein M, Palermo GD, Rosenwaks Z. Detection of sperm in men with Y-chromosome microdeletions of the AZFa, AZFb and AZFc regions. *Hum Reprod* 2003; 18:1660-1665.
39. Kuehnert B, Gromoll J, Kostova E, Tschanter P, Luetjens CM, Simoni M, et al. Case Report: Natural transmission of an AZFc Y-chromosomal microdeletion from father to his sons. *Hum Reprod* 2004; 19: 886-888.