Short Communication

Detection of Partial Deletions of Y-chromosome AZFc in Infertile Men Using the Multiplex Ligation-dependent Probe Amplification Assay

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Abstract

Background: In recent studies, partial deletions of the azoospermia factor c region (AZFc) on the Y-chromosome have been detected in males with infertility problems. However, there has been a lot of debate about their significance. In order to study such deletions, a simple but accurate method for their detection was applied in this study.

Methods: We present data obtained from the Multiplex Ligation-dependent Probe Amplification (MLPA) assay using a new Y-chromosome-specific MLPA probemix (P360) which allows the easy detection of partial AZFc deletions.

Results: Partial AZFc deletions were detected in 8% of our cohort of previously mutation-negative infertile males (and 0% of the fertile control cohort).

Conclusion: These results provide further evidence of the causality of partial AZFc deletions. None of the partial AZFc deletions were detectable by the standard multiplex PCR method, demonstrating the advantage of the MLPA method.

Keywords: Causality, Gene dosage, Infertility, Microdeletions, Molecular genetics, Y-Chromosome.
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Introduction

Large deletions of the AZF regions on the Y-chromosome (AZFa, AZFb and AZFc) were shown to result in fertility problems in males as long ago as 1976 (1). However, it is only recently that molecular techniques have advanced to the stage where partial AZF region microdeletions can be detected. The detection of partial AZFc deletions in infertile males has led to a lot of debate as to their causality (2). Various studies have described partial AZF deletions as either causative of infertility (3), not causative (4, 5) or potentially causative with variable expressivity and/or penetrance (6, 7). The variability in the conclusions of all of these reports was based not just on the differences between the types of microdeletions that were detected, but also on the high phenotypic variation between patients with the same microdeletion.

The standard Y-microdeletion assay recommended by the European (EAA/EMQN) best practice guidelines (8) does not detect the most commonly-reported partial deletion of AZFc. If further studies into the genotype/phenotype correlations of partial AZF deletions are to be carried out, an effective and easy method of detection is essential. The method must be able to accurately detect microdeletions of various sizes, preferably in a single reaction in order to minimize experimental time and cost.

We tested the Multiplex Ligation-dependent Probe Amplification (MLPA) assay (9) to ascertain if this would be a good method for the detection of partial AZF deletions.

The MLPA method lends itself to the simultaneous analysis of multiple chromosomal regions, offering an easy way to screen the three AZF

regions. The new P360 Y microdeletion MLPA probemix contains multiple MLPA probes for all three AZF regions–15 probes that bind within AZFa, 19 in AZFb and 23 in AZFc. The MLPA probe design for this probemix was complicated by the presence of an inverted tandem duplication in AZFc which contains many of the important spermatogenesis genes. As both of the repeat segments have identical DNA, all probes that hybridize within these repeat regions will bind to a minimum of two sites. If one copy of this repeat is lost, it will not be possible to determine which copy is missing. However, studies suggest that it is the reduction in copy number of these genes, rather than the loss of a particular copy, that is thought to be important (10); therefore, as long as the copy number can be accurately determined then the data will have diagnostic value.

Methods

Test populations: A cohort of 50 unrelated infertile men was collected at the Wessex Regional Genetics Laboratory (WRGL) following referrals from local and national clinicians. All patients had previously been tested for the presence of Y-chromosome microdeletions using the multiplex PCR method of Simoni et al. (8). None of the patients had been found to have a detectable deletion. Other common causes of infertility (CFTR gene mutations and karyotype abnormalities) had also been excluded.

In order to provide a matched control cohort, 50 healthy males (who were originally referred to the WRGL for linkage analysis) were chosen and anonymized. All individuals within this group had fathered at least two children and were, therefore, assumed not to have any significant fertility problems.

MLPA analysis: MLPA was carried out according to the manufacturer's instructions with the P360 (version A1) Y microdeletion probemix (MRC-Holland, the Netherlands) on both the infertility patient cohort and the fertile control individuals. MLPA PCR products (0.5 *μl*) were added to 0.2 *μl* of Genescan^{TM} – 500 ROX^{TM} Size Standard (Applied Biosystems, USA) and 9 *μl* of Hi-Di Formamide (Applied Biosystems, USA) and separated on an ABI 3130xl Genetic Analyzer (Applied Biosystems, USA) using a 36 *cm* long array and 3130 POP-7^{TM} array polymer (Applied Bio-

Table 1. The AZFc MLPA probes in Y-chromosome order and the results of seven positive cases from the infertility (Inf) or control (Con) cohorts

AZFc probe	Y location	Probe Size (*bp*)	Inf +1	Inf +2	Inf +3	Inf +4	Inf +5	Con +1	Con +2
BPY2	23282390	418			Deleted			Duplicated	
BPY2	23282929	166			Deleted			Duplicated	
BPY2	23284612	178			Deleted			Duplicated	
BPY2	23327263	486 ***			Deleted			Duplicated	
BPY2	23376456	373 ***			Deleted				
DAZ2	23931533	283 **	Deleted	Deleted		Deleted	Duplicated		Duplicated
RBMY2CP	23979879	147 **	Deleted	Deleted		Deleted	Duplicated		Duplicated
DAZ2	24029525	301 **	Deleted	Deleted		Deleted	Duplicated		Duplicated
DAZ2	24271551	265 **	Deleted	Deleted		Deleted	Duplicated		Duplicated
CDY1B	24467621	361 **	Deleted	Deleted		Deleted	Duplicated		Duplicated
CDY2A	24660720	234 ***	Deleted	Deleted		Deleted	Duplicated		Duplicated
BPY2	24961006	486 ***	Deleted	Deleted		Deleted	Duplicated		Duplicated
BPY2	25010203	373 ***	Deleted	Deleted		Deleted	Duplicated		Duplicated
BPY2	25770903	373 ***			Deleted				
BPY2	25820108	486 ***			Deleted				
CDY2A	26120421	234 ***			Deleted				
CDY1B	24467621	361 **			Deleted				
DAZ2	26509515	265 **			Deleted				
DAZ2	26752077	301 **							
RBMY2CP	26801745	147 **							
DAZ2	26850094	283 **							
PPP1R12BP1	26891166	142							
RBMY2DP	28570146	389							

Some of the MLPA probes bind to more than one site on the Y-chromosome – those binding in two places are marked with **, those binding in three places are marked with ***. The tandem inverted repeat (DAZ2 to BPY2) is shown within the boxes. The Y chromosome location is given as the first base of the MLPA probe binding site, according to the HG19 human genome reference sequence

systems, USA). The run conditions were as follows: injection voltage=1.2 *kVolt*, injection time=5*s*, oven temperature=60^{o}C, run voltage=15 *kVolt*, and run time=20 *min*. Subsequent data was analyzed using the MLPA analysis function of the GeneMarker (version 1.85) software (SoftGenetics, USA).

The P360 MLPA kit was initially validated on a cohort of infertile patients with a known Y microdeletion, previously detected by multiplex PCR analysis. All of these deletions were detected by the MLPA kit (data not shown).

Any deletions identified at the WRGL in the test cohort were subsequently confirmed at the MRC-Holland testing laboratory. There was 100% concordance between the results from the two laboratories (data not shown).

Results

Of the 50 infertile men that were analyzed, four (8%) were found to have a microdeletion of some part of the AZFc region (see tables 1 and 2) whilst one individual (2%) had a microduplication of AZFc. No microdeletions were seen in the fertile control cohort, although two individuals within this group (4%) had an AZFc microduplication.

As some of the MLPA probes bind to multiple sites (due to the presence of a large inverted repeat on the Y-chromosome) the deletions shown in table 1 are just one of the several possibilities in each case. An example of the typical MLPA dosage ratios for one of the deletion-positive cases is shown in table 2. This table shows all of the possible deletions that can be interpreted from the data for that particular patient.

Discussion

The review by Navarro-Costa et al. (2) gives a thorough summary of the recent studies of partial AZFc deletions and their possible clinical effects. Most of the early publications were contradictory or equivocal, a fact highlighted by simultaneous studies of the Han Chinese population by two research groups who came to opposing conclusions

Table 2. An example of the typical MLPA ratios seen (in deletion-positive infertility patient 3) and the possible deletion combinations (Del 1-4)

AZFc probe	Y location	Probe size (*bp*)	Number of binding sites	MLPA ratio	Copies remaining	Del 1	Del 2	Del 3	Del 4
BPY2	23282390	418	1	0	0/1	Deleted	Deleted	Deleted	Deleted
BPY2	23282929	166	1	0	0/1	Deleted	Deleted	Deleted	Deleted
BPY2	23284612	178	1	0	0/1	Deleted	Deleted	Deleted	Deleted
BPY2	23327263	486	3	0.34	1/3	Deleted	Deleted	Deleted	Deleted
BPY2	23376456	373	3	0.35	1/3	Deleted			Deleted
DAZ2	23931533	283	2	0.99	2/2				
RBMY2CP	23979879	147	2	0.92	2/2				
DAZ2	24029525	301	2	1	2/2				
DAZ2	24271551	265	2	0.49	1/2			Deleted	Deleted
CDY1B	24467621	361	2	0.5	1/2			Deleted	Deleted
CDY2A	24660720	234	3	0.7	2/3			Deleted	Deleted
BPY2	24961006	486	3	0.34	1/3			Deleted	Deleted
BPY2	25010203	373	3	0.35	1/3		Deleted	Deleted	Deleted
BPY2	25770903	373	3	0.35	1/3	Deleted	Deleted	Deleted	
BPY2	25820108	486	3	0.34	1/3	Deleted	Deleted		
CDY2A	26120421	234	3	0.7	2/3	Deleted	Deleted		
CDY1B	24467621	361	2	0.5	1/2	Deleted	Deleted		
DAZ2	26509515	265	2	0.49	1/2	Deleted	Deleted		
DAZ2	26752077	301	2	1	2/2				
RBMY2CP	26801745	147	2	0.92	2/2				
DAZ2	26850094	283	2	0.99	2/2				
PPP1R12BP1	26891166	142	1	1.04	1/1				
RBMY2DP	28570146	389	1	1.01	1/1				

The tandem inverted repeat (DAZ2 to BPY2) is shown within the boxes. The Y-chromosome location is given as the first base of the MLPA probe binding site, according to the HG19 human genome reference sequence

(11, 12). However, other studies since then have provided more evidence that such deletions do affect the total motile sperm count (10). Although our study cohort was small in comparison to other published results, the presence of partial AZFc deletions in 4/50 infertile men (8%) compared to 0/50 fertile controls adds further weight to this argument. Our figure of 8% for partial AZFc deletions is similar to other larger studies, with most published microdeletion rates being approximately 5% (10, 13). Partial duplications were seen in both test groups (1/50 infertile males and 2/50 fertile controls) which agrees with the conclusion of Giachini et al. (13); namely, these small duplications do not have a significant effect on spermatogenesis.

The main problem with comparing data from different published studies is the variability in the size of the AZFc microdeletions in individual patients. In some cases the size is known, but in other individuals the deletion size has not been fully characterized. The selection criteria for the study populations is also likely to influence the outcome of the results.

In order to accurately ascertain genotype/phenotype correlations, it is essential to know which genes are missing. PCR-based deletion detection methods often need a separate test for each of the common Y microdeletions, such as the gr/gr deletion (14) or the b2/b3 deletion (15), and very few of these studies look at all possible deletions.

The MLPA technique has many advantages over PCR-based methods as it allows the detection of almost all of the potential Y-chromosome microdeletions or microduplications in a single reaction. The MLPA technique has previously been shown to be an ideal dosage detection technique in a large number of publications, ranging from the detection of single copy deletions or duplications of autosomal or sex chromosome genes (16–25) to the dosage analysis of genomic regions with variable copy number (26–28). The MLPA results obtained in this study were highly reproducible, the data obtained at the WRGL laboratory being concordant with the results from the MRC-Holland testing laboratory.

Conclusion

In order to know if partial AZFc deletions have an effect on fertility, an easy detection method for such deletions is required. Our results show that the P360 MLPA probemix provides a quick and simple method of detecting these deletions which would otherwise have been missed by conventional multiplex PCR. As the MLPA probemix covers all three AZF regions, the detection of partial AZFc deletions is performed simultaneously with the detection of larger AZF region deletions, meaning that the entire range of Y-chromosome deletions can be identified in a single reaction. The use of such methodology will allow the collection of accurate, fully-characterised microdeletion data. Once this is obtained, detailed genotype/phenotype correlations may provide a definitive answer to the question of causality of AZFc partial microdeletions.

Conflict of Interest

David Bunyan and Jonathan Callaway have no conflict of interest to declare. Nadja Laddach is the designer of the P360 MLPA probemix at MRC-Holland. As she does not receive payments for any future sales of the P360 probemix we do not consider her employment by MRC-Holland to represent a conflict of interest.

References

1. Tiepolo L, Zuffardi O. Localization of factors controlling spermatogenesis in the nonfluorescent portion of the human Y chromosome long arm. Hum Genet. 1976;34(2):119-24.
2. Navarro-Costa P, Gonçalves J, Plancha CE. The AZFc region of the Y chromosome: at the crossroads between genetic diversity and male infertility. Hum Reprod Update. 2010;16(5):525-42.
3. Fernandes S, Huellen K, Goncalves J, Dukal H, Zeisler J, Rajpert De Meyts E, et al. High frequency of DAZ1/DAZ2 gene deletions in patients with severe oligozoospermia. Mol Hum Reprod. 2002;8 (3):286-98.
4. Hucklenbroich K, Gromoll J, Heinrich M, Hohoff C, Nieschlag E, Simoni M. Partial deletions in the AZFc region of the Y chromosome occur in men with impaired as well as normal spermatogenesis. Hum Reprod. 2005;20(1):191-7.
5. Zhang F, Li Z, Wen B, Jiang J, Shao M, Zhao Y, et al. A frequent partial AZFc deletion does not render an increased risk of spermatogenic impairment in East Asians. Ann Hum Genet. 2006;70(Pt 3):304-13.
6. Giachini C, Guarducci E, Longepied G, Degl'Innocenti S, Becherini L, Forti G, et al. The gr/gr deletion(s): a new genetic test in male infertility? J Med Genet. 2005;42(6):497-502.
7. Krausz C, Giachini C, Xue Y, O'Bryan MK, Gromoll J, Rajpert-de Meyts E, et al. Phenotypic variation within European carriers of the Y-chromo-

somal gr/gr deletion is independent of Y-chromosomal background. J Med Genet. 2009;46(1):21-31.

8. Simoni M, Bakker E, Krausz C. EAA/EMQN best practice guidelines for molecular diagnosis of y-chromosomal microdeletions. State of the art 2004. Int J Androl. 2004;27(4):240-9.
9. Schouten JP, McElgunn CJ, Waaijer R, Zwijnenburg D, Diepvens F, Pals G. Relative quantification of 40 nucleic acid sequences by multiplex ligation-dependent probe amplification. Nucleic Acids Res. 2002;30(12):e57.
10. Noordam MJ, Westerveld GH, Hovingh SE, van Daalen SK, Korver CM, van der Veen F, et al. Gene copy number reduction in the azoospermia factor c (AZFc) region and its effect on total motile sperm count. Hum Mol Genet. 2011;20(12):2457-63.
11. Wu B, Lu NX, Xia YK, Gu AH, Lu CC, Wang W, et al. A frequent Y chromosome b2/b3 subdeletion shows strong association with male infertility in Han-Chinese population. Hum Reprod. 2007;22(4): 1107-13.
12. Yang Y, Ma M, Li L, Zhang W, Xiao C, Li S, et al. Evidence for the association of Y-chromosome haplogroups with susceptibility to spermatogenic failure in a Chinese Han population. J Med Genet. 2008;45(4):210-5.
13. Giachini C, Laface I, Guarducci E, Balercia G, Forti G, Krausz C. Partial AZFc deletions and duplications: clinical correlates in the Italian population. Hum Genet. 2008;124(4):399-410.
14. Repping S, Skaletsky H, Brown L, van Daalen SK, Korver CM, Pyntikova T, et al. Polymorphism for a 1.6-Mb deletion of the human Y chromosome persists through balance between recurrent mutation and haploid selection. Nat Genet. 2003;35(3): 247-51.
15. Repping S, van Daalen SK, Korver CM, Brown L G, Marszalek JD, Gianotten J, et al. A family of human Y chromosomes has dispersed throughout northern Eurasia despite a 1.8-Mb deletion in the azoospermia factor c region. Genomics. 2004;83 (6):1046-52.
16. Bunyan DJ, Eccles DM, Sillibourne J, Wilkins E, Thomas NS, Shea-Simonds J, et al. Dosage analysis of cancer predisposition genes by multiplex ligation-dependent probe amplification. Br J Cancer. 2004;91(6):1155-9.
17. Archer HL, Whatley SD, Evans JC, Ravine D, Huppke P, Kerr A, et al. Gross rearrangements of the MECP2 gene are found in both classical and atypical Rett syndrome patients. J Med Genet. 2006;43(5):451-6.
18. Benito-Sanz S, Gorbenko del Blanco D, Huber C, Thomas NS, Aza-Carmona M, Bunyan D, et al. Characterization of SHOX deletions in Leri-Weill dyschondrosteosis (LWD) reveals genetic heterogeneity and no recombination hotspots. Am J Hum Genet. 2006;79(2):409-14.
19. Girirajan S, Mendoza-Londono R, Vlangos CN, Dupuis L, Nowak NJ, Bunyan DJ, et al. Smith-Magenis syndrome and Moyamoya disease in a patient with del(17)(p11.2p13.1). Am J Med Genet A. 2007;143A(9):999-1008.
20. Bakrania P, Robinson DO, Bunyan DJ, Salt A, Martin A, Crolla JA, et al. SOX2 anophthalmia syndrome: 12 new cases demonstrating broader phenotype and high frequency of large gene deletions. Br J Ophthalmol. 2007;91(11):1471-6.
21. Blyth M, Foulds N, Turner C, Bunyan D. Severe Marfan syndrome due to FBN1 exon deletions. Am J Med Genet A. 2008;146A(10):1320-4.
22. Wyatt A, Bakrania P, Bunyan DJ, Osborne RJ, Crolla JA, Salt A, et al. Novel heterozygous OTX2 mutations and whole gene deletions in anophthalmia, microphthalmia and coloboma. Hum Mutat. 2008;29(11):E278-83.
23. Foley P, Bunyan D, Stratton J, Dillon M, Lynch S A. Further case of Rubinstein-Taybi syndrome due to a deletion in EP300. Am J Med Genet A. 2009; 149A(5):997-1000.
24. Thomas NS, Harvey JF, Bunyan DJ, Rankin J, Grigelioniene G, Bruno DL, et al. Clinical and molecular characterization of duplications encompassing the human SHOX gene reveal a variable effect on stature. Am J Med Genet A. 2009;149A (7):1407-14.
25. Bunyan DJ, Skinner AC, Ashton EJ, Sillibourne J, Brown T, Collins AL, et al. Simultaneous MLPA-based multiplex point mutation and deletion analysis of the dystrophin gene. Mol Biotechnol. 2007; 35(2):135-40.
26. Barber JC, Maloney VK, Huang S, Bunyan DJ, Cresswell L, Kinning E, et al. 8p23.1 duplication syndrome; a novel genomic condition with unexpected complexity revealed by array CGH. Eur J Hum Genet. 2008;16(1):18-27.
27. Glancy M, Barnicoat A, Vijeratnam R, de Souza S, Gilmore J, Huang S, et al. Transmitted duplication of 8p23.1-8p23.2 associated with speech delay, autism and learning difficulties. Eur J Hum Genet. 2009;17(1):37-43.
28. Barber JC, Bunyan D, Curtis M, Robinson D, Morlot S, Dermitzel A, et al. 8p23.1 duplication syndrome differentiated from copy number variation of the defensin cluster at prenatal diagnosis in four new families. Mol Cytogenet. 2010;3:3.