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A novel Y chromosome microdeletion potentially associated with defective spermatogenesis identified by custom array comparative genome hybridization

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KEY MESSAGE

We designed a high-resolution aCGH assay to check for chromosome aberrations in men with defective spermatogenesis. The assay identified a novel Y chromosome microdeletion confined to patients that was not revealed by conventional methods. This finding may help provide further insights into the causes of male infertility.

A B S T R A C T

Male infertility is a major health problem worldwide. Oligospermia and azoospermia are the most common symptoms of this disorder. Despite recent advances, the aetiopathogenesis of defective spermatogenesis remains largely uncertain. The aim of this study is to discover unknown or novel chromosome aberrations associated with male reproductive failure. We developed a high-resolution custom array comparative genomic hybridization for initial screening of copy number variations in 10 patients with idiopathic oligozoospermia and azoospermia and eight normal fertile men. We found that deletions were mainly located in the deleted-in-azoospermia subregion and were confined to patients. More importantly, an interesting microdeletion of the Y chromosome designated as D01 was detected in four out of 10 patients with oligozoospermia and azoospermia. We validated this recurrent deletion in nine out of 100 additional infertile men using polymerase chain reaction assays, whereas, it was not present in 100 proven fertile controls

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(P = 0.002). Furthermore, a bioinformatics analysis demonstrated that the 5′ terminal of D01 is situated proximal to several conserved transcription factor binding sites within the Y chromosome. Our study indicated that this newly identified Y chromosome deletion might be potentially associated with impaired spermatogenesis and it is worthy of further investigations in larger cohorts.

Introduction

According to the World Health Organization, 15% of couples are infertile. Male factors constitute roughly one-half of cases, predominantly oligospermia or azoospermia (De Kretser, 1997; Forti and Krausz, 1998). Over 30% of male infertility cases resulting from spermatogenic problems are associated with genetic abnormalities, the remainder are caused by varicocele, genital tract inflammation, endocrine disorders and immune abnormalities (Bhasin et al., 1994; Chang et al., 1999). It has been shown that Y chromosome microdeletions are important genetic factors linked to male infertility (Krausz et al., 2011; Pryor et al., 1997). Moreover, the loci of azoospermia factors are in three recurrently deleted subregions of the Y chromosome: AZFa, AZFb, and AZFc. These three loci are believed to be responsible for human spermatogenesis (Hopps et al., 2003; Plaseski et al., 2008).

Conventional molecular methods for Y chromosome analysis include multiplex polymerase chain reaction (PCR) and karyotyping. Currently, six sequence tag sites (STS) are commonly used to detect deletions of AZF in most clinical laboratories (Simoni et al., 2004). Array comparative genome hybridization (aCGH) is a high throughput approach and can simultaneously detect a large number of copy number variations (CNV) in genetic material, permitting precise analysis of genotype–phenotype correlations. Several studies have explored the deletions and duplications in the human genome using aCGH, and some have linked Y chromosome alterations to male fertility. Most of them, however, have focused on known CNV in western populations (Karcanias et al., 2007; Osborne et al., 2007; Stouffs et al., 2012). More recently, Yuen et al. (2014) used Agilent's Human Genome 8 × 15K aCGH format as a template to construct a custom microarray for interrogating the genetics of male infertility involving the Y chromosome. As a proof of principle, the study presented detailed data of array performance and feasibility of CNV analysis.

In the present study, we describe a high-resolution custom aCGH that was developed to screen important CNV of the Y chromosome in 10 Chinese patients with oligozoospermia and azoospermia. In contrast to previous studies, our focus is on discovery, validation and clinical application of new candidate markers for male infertility. A microdeletion of the Y chromosome [D01] was detected in four out of 10 infertile participants. In the second part of this experiment, we conducted a validation study reaching a total of 100 patients and 100 fertile controls for the deletion D01, which seemed to be specific to patients based on the aCGH profiling. As expected, this recurrent deletion was further confirmed in nine out of 100 additional infertile men; it was not, however, present in the normal fertile group (n = 100) with PCR and gel electrophoresis assays. In the present study, we show that the novel Y chromosome deletion is potentially associated with defective spermatogenesis, and this observation needs further examination in larger cohorts.

Materials and methods

Samples

Participants were recruited with informed consent. This study was approved by the Human Research and Ethics Committees of Guangdong Women and Children Hospital on 28 June 2013 (reference number: 201301012). The screening samples, including five patients with oligospermia, five patients with azoospermia and eight normal fertile men (proven fathers) as references were enrolled between June 2013 and October 2013 in our hospital. Each patient underwent comprehensive andrological examinations, including medical history, scrotal sonography, hormone profiles and semen analysis. In addition, genetic testing for karyotype and Y chromosome deletions was conducted. The duration of infertility was at least 1 year, and infertile men with obstructive azoospermia, varicocele, cryptorchidism, hypogonadism, parotitis, iatrogenic infertility and any endocrine disorders were excluded. Semen analysis in accordance with the guidelines of the WHO Laboratory Manual (5th edition) (Cooper, 2010) revealed a motile sperm count less than 5×10^6/ml in men with oligospermia and no spermatozoa in azoospermia samples. Normal karyotypes and absence of known Y chromosome deletions were identified in all patients. Detailed data from these individuals are given in **Table 1**. Healthy controls from the same ethnic background (Han

Table 1 – Clinical features and experimental data from 10 infertile patients.

Patient	Age (years)	Motile sperm count ($\times 10^6$/ml)	Diagnosis	Karyotype	STS–PCR	Endocrine disorders
s527	24	0	Azoospermia	46, XY	Normal	Not found
s615	28	0	Azoospermia	46, XY	Normal	Not found
s528	37	0	Azoospermia	46, XY	Normal	Not found
s612	42	0	Azoospermia	46, XY	Normal	Not found
s480	28	0	Azoospermia	46, XY	Normal	Not found
s640	33	<5	Oligospermia	46, XY	Normal	Not found
s587	29	<5	Oligospermia	46, XY	Normal	Not found
s559	34	<5	Oligospermia	46, XY	Normal	Not found
s539	33	<5	Oligospermia	46, XY	Normal	Not found
s532	33	<5	Oligospermia	46, XY	Normal	Not found

STS–PCR = sequence tag sites and polymerase chain reaction.

Chinese) were recruited from the Reproductive Medicine Center, Guangdong Women and Children Hospital. According to Krausz and McElreavey (2001), a male and his female partner who have one or more children conceived naturally without the use of assisted reproductive technology are regarded as fertile. To ensure proof of fertility, paternity testing was carried out for all of the fertile men included in this study. They received the same check up as the patients, except semen analysis owing to the practical difficulties in obtaining semen specimens for reproductive studies from healthy volunteers. Given that the aim of this study was to define pathogenic CNV related to defective spermatogenesis, the control group only included men presenting with normal results from andrological examinations. An additional 121 men with azoospermia from the Reproductive Medicine Center, Guangdong Women and Children Hospital underwent work-up that included medical history, semen analysis and Y chromosome analysis using STS–PCR in our hospital. From these patients, a second panel of 100 patients without known Y chromosome deletions were enrolled. In addition 100 proven fathers (with paternity testing) were recruited from the the Reproductive Medicine Center. The additional participants were used for the validation of candidate CNV.

Karyotyping and multiplex polymerase chain reaction

The karyotyping assays in 10 patients were conducted with minor modifications according to the procedure described elsewhere (Schnedl, 1971; Seabright, 1972).

For Y chromosome analysis, STS–PCR primer sets include sY84 and sY86 for AZFa, sY127 and sY134 for AZFb, sY254 and sY255 (both in the *DAZ* genes) for AZFc, respectively. In brief, the 5′ end of either forward or reverse primer was labelled with 5′FAM, JOE or TAMRA fluorescent dye for each pair of primers. To establish multiplex PCR, ZFX/Y and SRY were used as control loci. The PCR products were separated and analysed by the ABI 3500XL genetic analyzer.

Polymerase chain reaction

Conventional PCR assays were used to validate the suspected Y chromosome deletions in additional samples. The mixture contained Promega PCR Master Mix buffer, forward primer, reverse primer and genomic DNA template in a volume of 25 µl. Hot start PCR was initiated by 10 min incubation at 95°C, ended after a 10 min extension at 72°C, 40 cycles of denaturation at 95°C for 15 s, annealing at 56°C for 45 s, and extension 72°C for 1 min. Amplification of D01, D02 and D03 fragments shared the same cycling procedure. The PCR products were separated by electrophoresis on a 1.5% agarose gel. The primer sequences, location of primers and the expected sizes of the amplicons are shown in **Supplementary Table S1**.

Probes designed for custom array comparative genome hybridization

A custom aCGH was developed to detect CNV on the human genome. First, genomic sequences (hg19) of the whole Y chromosome, *AR* gene on chromosome X, *CFTR* gene on chromosome 7, *PLOG* gene on chromosome 15, *INSL3* gene on chromosome 19, *RXFP1* gene on chromosome 4 and mitochondrion were downloaded from NCBI (http://www.ncbi.nlm.nih.gov/). Then, 60-mer probes with matched melting temperature were selected to remove those probes with secondary genomic alignments on the genome. After the most stringent filtering, 56,205 probes were generated. Specifically, there were 50,615 probes on chromosome Y, 1694 probes covering *AR* gene on chromosome X, 2124 probes covering *CFTR* gene on chromosome 7, 253 probes covering *POLG* gene on chromosome 15, 527 probes covering *INSL3* gene on chromosome 19, 1161 probes covering *RXFP1* gene on chromosome 4 and 1,331 probes covering mitochondrial DNA. All of the probes with average probe spacing of 500 bp were submitted to Agilent's E-array system (Agilent, Santa Clara, CA, USA) to produce the custom array.

Array comparative genome hybridization assay

Genomic DNA was isolated from peripheral blood with QuickGene SP kit DNA whole Blood (Fujifilm, Tokyo, Japan). The aCGH experiments were conducted according to the manufacturer's protocol (Agilent Technologies, Palo Alto, CA). Briefly, 0.5 µg genomic DNA from the test and the universal reference (pooled gDNA from normal fertile men) were digested with *Alu*I (5U) and *Rsa*I (5U) (Promega, R6281 and R6371) at 37°C for 2 h. Digestion fragments were verified by agarose gel electrophoresis. Patient samples were labelled with Cy5-dCTP whereas the pooled reference sample was labelled with Cy3-dCTP using Agilent DNA Labelling kit (Agilent Technologies, Palo Alto, CA, USA). Labelling efficiency was determined by NanoDrop ND-1000 UV-VIS spectrophotometer. Labelled DNA was denatured and pre-annealed with Cot-1 DNA and Agilent blocking reagent before hybridization at 65°C for 40 h in an Agilent hybridization oven. Arrays were then washed with Agilent Oligo CGH wash buffer 1 and 2 (5188–5226), acetonitrile (Sigma-Aldrich, 271004-1L), and Stabilization and Drying Solution (Agilent, 5185–5979), according to standard procedures. Finally, slides were scanned with an Agilent scanner, and image analysis was conducted using default aCGH settings of Feature Extraction Software 10.7.3.1 (Agilent Technologies, Palo Alto, CA, USA).

Statistical analysis

Data were segmented using the circular binary segmnetation (CBS) algorithm as implemented in the R-package 'DNAcopy' (Olshen et al., 2004). The algorithm starts with the whole chromosome and segments recursively by testing for change points; it stops when no changed points can be found in any of the segments. The test statistic was used to detect a narrow changed segment in the middle of a large segment. The CBS algorithm parameters were used as follows: the number of permutations was 5000, window size was 500 and overlap was 0.5. Next, thresholds for gain and loss were defined at ± 1 on a log2 scale. Potential novel CNV regions were filtered by database of genomic variants (Iafrate et al., 2004) using BEDTools (Quinlan and Hall, 2010). The chi-squared test was used to compare the suspected deletions between patient samples and normal controls. P = 5% or lower was considered to be statistically significant.

Results

Discovery of Y chromosome copy number varations in patients with spermatogenic failure

To evaluate performance of the custom aCGH, infertile men who had been previously genotyped were first tested using STS–PCR, karyotyping, or both, with known Y chromosome aberrations. As expected,

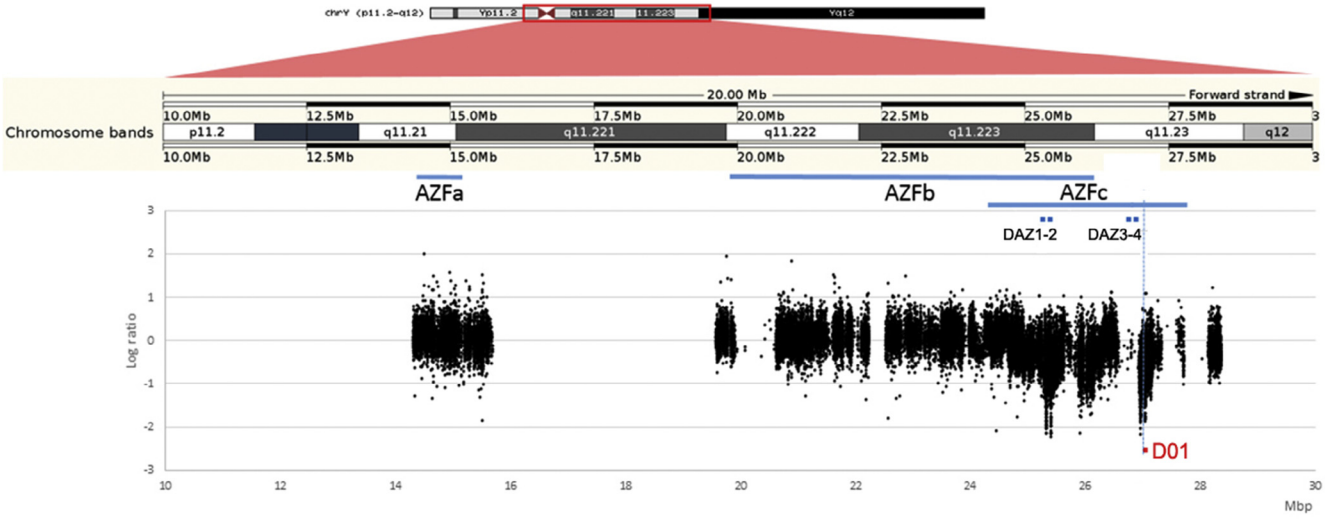

Figure 1 – Y chromosome abnormalities. The representative profile of the Y chromosome in a patient (s615) was established and multiple deletions determined using array comparative genome hybridization DNAcopy package analysis. The locations of AZFa/b/c, *DZF* gene cluster (DAZ1-4) and the deletion D01 on chromosome Y were labelled.

our aCGH successfully detected known AZFc and AZFb + c deletions (**Supplementary Figure S1**). Then, the high-resolution aCGH was applied to examine 10 patients with oligospermia and azoospermia without chromosome abnormalities identified by karyotyping and multiplex PCR (**Table 1**). Each case was determined to have a loss (deletion) or gain (duplication) corresponding to Y chromosomal coordinates. As shown in **Figure 1**, several deletions of the Y chromosome were found in patient s615. Interestingly, a number of deletions or duplications were found only on chromosome Y within patient samples. Here, the focus was on Y chromosome deletions because of their established association with defective spermatogenesis in men and all of these deletions in 10 patients are shown in **Figure 2**. Deletions were mainly located in the DAZ subregion, and one-half of them involved exons of the *DAZ* gene family, which represents the candidate responsible for the AZFc phenotype (Ferlin et al., 2004; Ferras et al., 2004). Moreover, three deleted fragments, designated as D01, D02

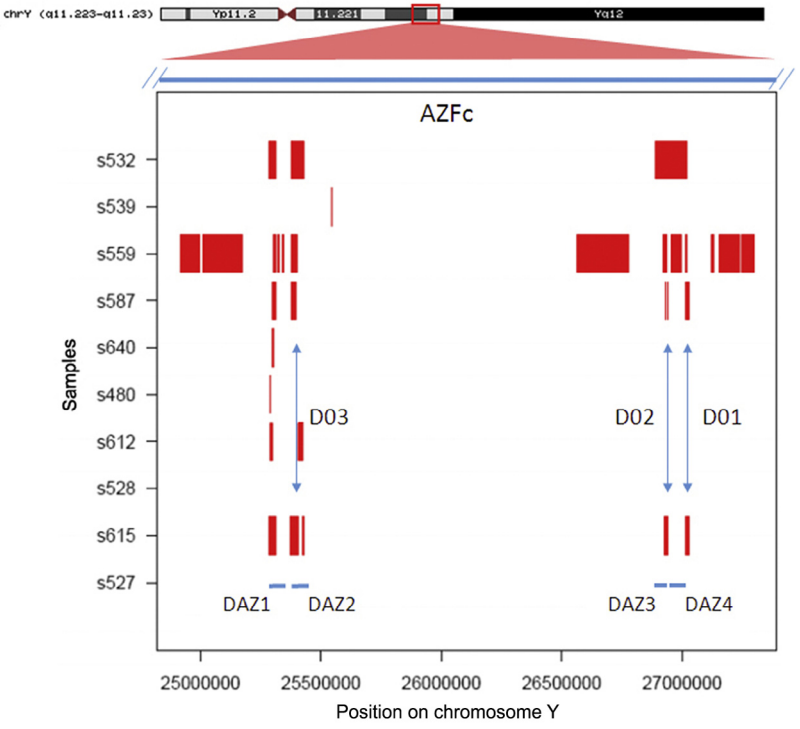

Figure 2 – Overview of observed Y chromosome deletions confined to 10 patients. Custom Array comparative genome hybridization revealed multiple deleted fragments (red blocks), including three suspected loci (blue arrows) of the Y chromosome in patients with oligospermia and azoospermia.

Table 2 – Description of three suspected deletions in infertile patients.

Deletion	Patients	Location	Size (bp)
D01	s615; s587; s559; s532	chrY: 27054209–27119777	65569
D02	s615; s587; s532	chrY: 26946158–27004161	58004
D03	s615, s559, s532	chrY: 25437106–25481363	44258

and D03, were found in at least three out of 10 patients, and these alterations were considered as suspected CNV related to impaired spermatogenesis. Detailed information of these three deletions are is presented in **Table 2**.

Validation of recurrent deletions in 100 independent samples

The recurrent deletions (D01, D02 and D03) were subjected to further examination with conventional PCR. Primer sets were designed to amplify specific products with the size of 899 bp, 219 bp and 534 bp, respectively (**Supplementary Table S1**). Additional samples were included for confirmation from 100 infertile patients and 100 normal fertile men. A total of nine cases with negative amplification of the expected fragment (899 bp) were found in 100 infertile samples, indicating the existence of the D01 deletion (**Figure 3**). Specific product, however, was present in all of the controls. These results suggested a significant difference in the frequency of D01 deletion between the infertile men and fertile controls ($P = 0.002$). As for D02 and D03 deletions, we did not find differences between the two groups, and therefore did not conduct any further investigation.

Bioinformatics analysis of the deletion D01

According to aCGH analysis, the D01 fragment locates in Yq11.23 with deletion range up to 65569 bp (27054209–27119778). Then, it was determined whether any functional genomic elements, such as protein coding genes, microRNAs and transcription binding sites were involved in this region. A bioinformatics analysis showed that the D01 region does not include or overlap any genes, consensus CDS, spliced expressed sequence tag, miRNA and small non-coding RNA. The 5′ terminal of it, however, is situated proximal to several conserved transcription factor (SRY, CEBP and NKX2-5) binding sites on chromosome Y.

Discussion

Because of its haploidy, the Y chromosome is prone to rearrangement, especially in the AZFc region (Skaletsky et al., 2003). Male infertility is a major reproductive health problem worldwide, and Y chromosome microdeletions largely account for the genetic causes of this condition. Compared with conventional methods used in male infertility analysis, aCGH possesses many advantages, especially for large-scale, high throughput and high resolution studies. The aCGH technique represents an effective tool for identification of CNV in the clinical setting.

Here, the focus was on the possible pathogenic CNV of the Y chromosome related to male infertility. Some genetic alterations have been identified by the aCGH, which was not identified by STS–PCR and karyotyping in 10 patients. In contrast, our custom array was able to accurately identify and refine known deletions. The novel deletions were mainly located in the *DAZ* gene cluster. The *DAZ* gene family constitutes a major candidate for the AZFc phenotype, but its specific roles and detailed mechanisms in human spermatogenesis require more research (Moro et al., 2000).

Because the deletion D01 was not only detected in four out of 10 screening samples (s615, s587, s559, s532) but also present in nine out of 100 additional infertile men, these data strongly indicated that this specific deletion (65569 bp) may be potentially associated with defective spermatogenesis. Although no genes or other functional elements are involved in this region, the 5′ terminal of it is situated proximal to some conserved binding sites for transcription factors (SRY, CEBP and NKX2-5). We are aware that structural abnormalities of the Y chromosome usually are not life-threatening, but affect primarily gamete production or sexual development. Recent studies have illustrated that regulatory elements in the human genome or non-coding RNAs could potentially affect spermatogenesis (He et al., 2009). Therefore, we speculate that the deletion D01 may influence transcription factors binding to functionally important sites on chromosome Y, and result in male reproductive failure by increased genomic instability or other unknown mechanisms (Jorgez et al., 2011; Krausz et al., 2009; Maduro et al., 2003). Our data suggest that this intergenic region might be a pathogenic CNV or represent a risk factor for defective spermatogenesis, but this new area needs further study.

It has been established that deletions in the AZF regions can cause spermatogenic impairment (Ferlin et al., 2005); however, Y chromo-

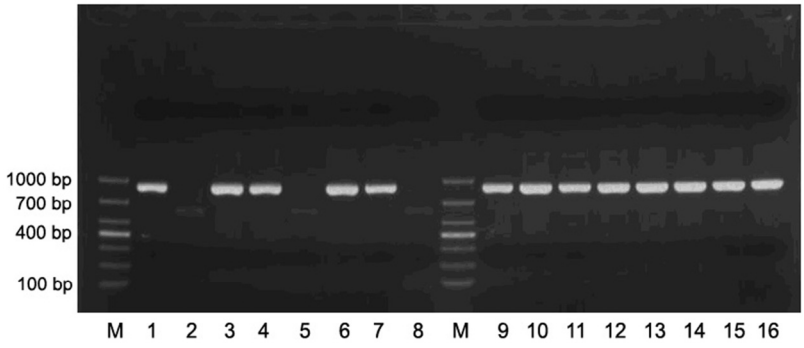

Figure 3 – Validation of a recurrent microdeletion D01 of the Y chromosome in additional samples. Specific primers were designed to amplify a smaller fragment (899 bp) within the deleted region (65569 bp) in 100 patients compared with 100 controls. Representative results were shown with agarose gel electrophoresis of the polymerase chain reaction products. Lanes 1–8: patients with azoospermia; lanes 9–16: normal fertile men. M = 1 kb base pair ladder.

some duplications seem to be common polymorphisms with selectively neutral or subtle phenotypes not usually associated with defective spermatogenesis and male infertility (Bailey et al., 2002; Blanco et al., 2000; Jobling, 2008). In a recent study, partial AZFc duplications were found to be related to spermatogenic failure in a Han-Chinese population. These segmental gains span 1.6–1.8 Mb and involve nine to 12 genes. Some of the AZFc genes are likely to be dosage sensitive, and their elevated expression may affect spermatogenesis (Lin et al., 2007). Also, another similar investigation reinforced the association between partial AZFc duplications and spermatogenic impairment, the authors demonstrating a significantly higher duplication load in infertile patients than in normozoospermic males. The DAZ1/2 cluster, constituting the main duplicated copies, rendered a risk factor in the non-deletion patients with male infertility (Lu et al., 2011). These findings, however, have not been validated in white people, indicating such a relationship might be population-specific (Giachini et al., 2008). In our study, only segmental gains (without deletions) of the Y chromosome were detected in two patients (s528 and s527). A total of seven duplications in AZFb and AZFc regions were identified and three of them contain genes, including CSPG4P1Y, FAM224A, FAM224B and TTTY13 (**Supplementary Table S2**). The functional significance of these duplications, however, should be interpreted with caution. More extensive studies are required to obtain a better understanding of Y chromosome gains.

In addition, thousands of probes were designed on the array for targeting candidate genes and regions, including *RXFP1* on chromosome 4, *CFTR* on chromosome 7, *POLG* on chromosome 15, *INSL3* on chromosome 19, *AR* on chromosome X and mitochondrion DNA according to previous reports (Cuppens and Cassiman, 2004; Ferlin et al., 2006, 2007; Rovio et al., 2001). We did not, however, find any CNV in these regions confined to patients. It is possible that mutations and polymorphisms of the above mentioned genes or loci contribute to male reproduction and spermatogenesis.

This study has several limitations. First, the 100 additional patients were simply used for validation of new CNV by a simple PCR. Since the power of aCGH is in its ability to find Y chromosome aberrations in a non-biased way, more findings would be obtained if we carried out this assay in the 100 infertility patients and 100 controls. Second, testing of the 100 additional men with impaired spermatogenesis confirmed the increased prevalence of the deletion D01. Although we could not rule out the presence of alternative CNV in these patients, we believe that the deletion D01 is potentially associated with male infertility.

In conclusion, we initially screened deletions or duplications of the Y chromosome confined to patients with oligospermia or azoospermia with high-resolution custom aCGH. Furthermore, we validated a recurrent microdeletion D01 being specific to men with impaired spermatogenesis in 100 additional samples in our second step analysis. A bioinformatics analysis demonstrated that the 5′ terminal of it is situated proximal to several conserved transcription factor binding sites on chromosome Y. Our observations indicated that the new deletion D01 of the Y chromosome relates to functional spermatogenesis and could possibly be useful in clinical practice.

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Appendix: Supplementary material

Supplementary data to this article can be found online at doi:10.1016/j.rbmo.2016.09.010.

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