COMPREHENSIVE GENOMIC STUDY IN PATIENTS WITH IDIOPATHIC AZOOSPERMIA AND OLIGOASTHENOTERATOZOOSPERMIA

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ABSTRACT
Infertility affects 10% to 15% of all couples. In about 40% of the cases the male factor is reported to be the main reason for unsuccessful fertilization. Although in 70% of the male factor infertility cases the etiology is recognizable, in the remaining 30% the reason is unknown and the male infertility is named idiopathic. In the current study, we selected 10 patients with idiopathic infertility, affected by azoospermia and oligoasthenoteratozoospermia. All patients were subjected to DNA analysis for deletions of the Y chromosome and cytogenetic analysis for chromosomal aberrations. After these analyses, the patients without such kind of abnormalities were further analyzed by microarray-based comparative genomic hybridization (array CGH). The cytogenetic analysis revealed two patients with chromosomal mutations – inv(9)(p11;q13) and t(Y;9)(q12.3;q21.1). Two other patients were detected to have deletions of the AZFc region of the Y chromosome. Thus, we applied array CGH analysis for the six patients without chromosomal or Y-chromosome aberrations. Apart from the presence of known polymorphisms, we established copy number alteration in 17q12-17q21.2 – a region containing the gene for zona pellucida binding protein ZPBP2. This protein plays a crucial role for the proper spermatogenesis. Our results prompted for investigation of this gene and protein as a potential candidate for spermatogenic failure.


Keywords: azoospermia, oligoasthenoteratozoospermia, microarray-based comparative genomic hybridization (array CGH), zona pellucida binding protein ZPBP2

Introduction
Infertility affects 10% to 15% of all couples. In about 40% of the cases the male factor is reported to be the main reason for unsuccessful fertilization (WHO, 2007). Although in 70% of the male factor infertility cases the etiology is recognizable (16), in the remaining 30% the reason is unknown and the male infertility is named idiopathic (14).

Different genomic anomalies are quite frequent in some types of infertility, leading to various spermatogenic abnormalities, and this increases the risk for the offspring and the outcome of infertility treatment (11, 22). Diagnostics of the genomic and chromosome anomalies, especially microstructural aberrations, can diminish the bad consequences, as well as the overwhelming financial and emotional costs of repeatedly unsuccessful IVF procedures (31).

Genetic or hereditary diseases and specific alterations in the Y chromosome are the main factors in the genetic etiology of infertility, known so far (12). In about 10% to 20% of the men without mature sperms in the ejaculate, some deletion of the Y chromosome is discovered (8). The deleted regions involve the locus of the azoospermic factor (AZF) located in Yq11, which is separated in four non-overlapping sub-regions – AZFa, AZFb, AZFc and AZFd. Each of these regions could be connected to specific histology of the testes and some candidate-genes have been identified in the regions. It is reported that the Deleted in Azoospermia (DAZ) gene family is the most common deleted AZF candidate-gene and it is located in the AZFc region. Recently, a small new deletion of the Y chromosome (1.6°Mbp) was described in infertile men with different kinds of spermatogenic failure. The DAZ gene has an autosomal homologue, DAZl (DAZ like), on the short arm of chromosome 3 (3p24), and it is possible that an autosome defective locus DAZl could be responsible for the spermatogenic defect as well. The genetic complexity of the AZF locus on the long arm of the Y chromosome could be uncovered only by fine mapping of its sequence (24).

Comparative genomic hybridization (CGH) is a technique which improves the study of genomic imbalances, since it allows to investigate simultaneously all chromosomes at a higher resolution. The modification of the method with using DNA microarrays instead of metaphase chromosomes facilitates the investigation of thousands of sub-microscopic loci, thus increasing the chance to discover microstructural aberrations and to precisely determine the aneuploidy (6, 25, 27).

In the present study, we selected 10 patients with idiopathic infertility, affected by azoospermia and oligoasthenoteratozoospermia. They were diagnosed as
idiopathic after performing extended spermograms, hormonal, laboratory and clinical examinations, as well as after consulting an endocrinologist and andrologist. All patients were subjected to DNA analysis for deletions of the Y chromosome and cytogenetic analysis for chromosomal aberrations. After these analyses, the patients without such kinds of abnormalities were further analyzed by microarray-based CGH.

To our knowledge, this is the first report on the analysis of whole genome array CGH of men with idiopathic azoospermia and after oligoasthenoteratozoospermia cytogenetic and Y-microdeletions screening.

Materials and Methods

Patients
The study was approved by the local Ethics Committee of the Medical University of Sofia. All 10 participants were asked for and provided their informed consent. The patients included in the study were selected in ART-Center “Tednobios”, Sofia. All of them suffered from non-obstructive azoospermia or oligoasthenoteratozoospermia, diagnosed as idiopathic.

Cytogenetic analysis
G-banded chromosomes were prepared from whole blood samples using standard laboratory protocols.

DNA extraction and evaluation
DNA was extracted from blood of the patients by the phenol-chloroform procedure. The yield was estimated by Nanodrop, as the necessary concentration of 100 ng/µl was obtained for each sample and 260/280 ratios for protein/RNA free were in the range of 1.8–2.0. As an additional quality check, DNA was run on a 1 % agarose gel: DNA of high molecular weight (>50 kbp) indicated it suitable for use.

Polimerase chain reaction (PCR) for detection of Y-chromosome deletions
We used primers amplifying a region of the SRY gene, a segment from the pseudoautosomal region of Y-chromosome (ZFY), as well as three pairs of primers for loci AZFa (sY84, sY86), AZFb (sY127, sY134) and AZFc (sY254, sY255) – Table 1. The length of the amplification products was as follows: ZFY – 495 bp, SRY – 472 bp, sY254 – 400 bp, sY255 – 126 bp, sY84 – 320 bp, sY86 – 326 bp, sY134 – 301 bp, sY127 – 274 bp. The conditions for multiplex PCR are given in Table 2. Initial denaturation – 5 min at 95 °C, each next cycle – 30 s at 94 °C (Table 1 and Table 2). The PCR products were loaded on a 2 % agarose gel. The separation was done in 1x TBE buffer at 150 V. The fragments were visualized by UV at 315 nm. The results were documented by digital camera Kodak/EDAS 290.

Genomic arrays
We used genomic arrays CytoChip (BlueGnome, Cambridge, UK) covering the entire genome at a median 565 Kb, a resolution optimised to detect pathogenic imbalances while minimizing polymorphisms. In addition, it investigates sub-

telomeres at a median 250 Kb resolution, reliably detects mosaicism and examines 90 known genetic conditions at a median 100 Kb resolution. This resulted in an average density of 1 clone/0.5 Mb.

Array-CGH probe labeling, hybridization, image capture and data analysis
Test and reference male genomic DNA from a donor with successful reproduction (400 ng) was labeled by random priming using BlueGnome Fluorescent Labelling System. The labeled products were purified by AutoSeqTM G50 columns, and incorporation of dyes was evaluated by Nanodrop. Incorporation in the range of 6 pmol/µl to 15 pmol/µl and DNA yield in 180 ng/µl to 325 ng/µl were considered suitable for further analysis. A mix of Cy5 and Cy3 labeled probes and a mix of Cot-1 and Herring sperm DNA were ethanol precipitated at -80 °C for at least 30 min. Hybridization processing was done by dissolving precipitated probes in hybridization buffer. Arrays were washed in SSC solutions with decreasing concentrations and scanned by GenPix 4100A.

The images were analyzed by BlueFuse for Microarrays 3.5 software (BlueGnome, Cambridge, UK). In data processing log2 ratios of Cy3 and Cy5 intensities were generated for all hybridized clones. Normal copy numbers were considered in a log2 ratio between -0.3 and +0.3; values above +0.3 were evaluated as gain/amplification and those under -0.3, as losses (deletions). Genomic profiles were represented with log2 ratios on the Y-axis and along the 23 chromosomes on the X-axis. Individual chromosome profiles were represented with clone positions on the Y-axis and log2 ratios on the X-axis.

Results and Discussion
The cytogenetic analysis revealed two chromosomal rearrangements involving chromosome 9. Eight of the patients in our study were diagnosed to have normal karyotype. In two of them, the cytogenetic analysis detected the following karyotypes:

i) male karyotype with pericentric inversion of chromosome 9 – 46, XY, inv (9) (p11;q13) – Fig. 1A;

ii) male karyotype with balanced translocation between chromosome 9 and Y with breakpoints in 9q21 and Yq12 - 46,XYt(9;Y)( q21.1;q12.3) – Fig. 1B.

Deletion of the AZFc region of the Y-chromosome was detected in two patients of our group. From all Y-chromosome regions targetly investigated in our study by PCR, we detected AZFc deletion in two of the patients (Fig. 2).

The array CGH analysis revealed genetic gain of 17q12-q21. The remaining 6 patients were subjected to array CGH analysis. We considered as specific and significant only changes that affect more than two adjacent BAC clones and display log2 ratio outside the normal threshold after correction with the standard deviation. Thus, we revealed a significant aberration in one of the patients – gain of 17q12-17q21 (Fig. 3), encompassing the genes for zona pellucida binding protein ZPB2.

3530
cytogenetic screening is highly recommended in male infertility, especially in cases without recognizable reason where genetic etiology is suspected (15, 30). Whereas only about 1 % of the general male population is affected by cytogenetic abnormalities, this proportion highly rises to 15 % or 20 % in azoospermic men (7, 31, 32, 33). Apart from the Klinefelter syndrome, which is the most common aberration among azoospermic men, different balanced chromosomal translocations are also described. it is well known that balanced translocations could produce unbalanced gametes thus leading to reproductive failure or delivery of an affected child with severe malformations (19). Another hypothesis suggests that potential autosomal genes involved in male gametogenesis might be deregulated by chromosome breakpoints (2). in our study reciprocal translocation [t (9;Y)] was seen in one azoospermic man. A compilation study in 464 infertile males with a balanced rearrangement has identified the chromosomal bands with potential significance for male infertility. The commonest one was 1q21 and breakpoints at chromosome 9 were reported in 5 out of 132 (3.8 %) azoospermic cases (2). The involvement of the Y chromosome in translocation with autosome is reported to have a frequency of 1:2000 (10, 20, 25). Usually they are between Y and another acrocentric chromosome; Y/non-acrocentric autosome translocation is quite rare (1). in another patient from our study we revealed pericentric inversion of chromosome 9. this is considered to be one of the most frequent polymorphisms of human chromosomes, occurring in 0.9 % to 4 % of the general population (18). A study in infertile men has reported 3.4 % incidence of this chromosome alteration among them, i.e. frequency close to that of the normal population (29). The authors, however, found a correlation between this aberration and the response of plasma gonadotropin to the administration of LH-RH. This response was abnormal in all patients carrying pericentric inversion of chromosome 9, suggesting that it may affect the reproductive process.

### TABLE 1

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequence</th>
<th>Reaction mix containing the primers</th>
</tr>
</thead>
<tbody>
<tr>
<td>ZFX – F</td>
<td>5’ – ACC R*CT GTA CTA CTG ACT GTG ATT ACA C – 3’</td>
<td>A and B</td>
</tr>
<tr>
<td>ZFX – R</td>
<td>5’ – GCA CY<em>T CTT TGG TAT CY</em>G AGA AAG T – 3’</td>
<td>A and B</td>
</tr>
<tr>
<td>SRY – F</td>
<td>5’ – GAA TAT TCC CGC TCT CCG GA – 3’</td>
<td>A and B</td>
</tr>
<tr>
<td>SRY – R</td>
<td>5’ – GCT GGT GCT TCC TTC AG – 3’</td>
<td>A and B</td>
</tr>
<tr>
<td>sY86 – F</td>
<td>5’ – GTG ACA CAC AGA CTA TGC TTC – 3’</td>
<td>A</td>
</tr>
<tr>
<td>sY86 – R</td>
<td>5’ – ACA CAC AGA GGG ACA ACC CT – 3’</td>
<td>A</td>
</tr>
<tr>
<td>sY127 – F</td>
<td>5’ – GGC TCA CAA ACG AAA AGA AA – 3’</td>
<td>A</td>
</tr>
<tr>
<td>sY127 – R</td>
<td>5’ – CTG CAG GCA GTA ATA AGG GA – 3’</td>
<td>A</td>
</tr>
<tr>
<td>sY254 – F</td>
<td>5’ – GGG TGT TAC CAG AAG GCA AA – 3’</td>
<td>A</td>
</tr>
<tr>
<td>sY254 – R</td>
<td>5’ – GAA CCG TAT CTA CCA AAG CAG C – 3’</td>
<td>A</td>
</tr>
<tr>
<td>sY84 – F</td>
<td>5’ – AGA AGG GTC TGA AAG CAG GT – 3’</td>
<td>B</td>
</tr>
<tr>
<td>sY84 – R</td>
<td>5’ – GCC TAC TAC CTG GAG GCT TC – 3</td>
<td>B</td>
</tr>
<tr>
<td>sY134 – F</td>
<td>5’ – GTG TGC CTC ACC ATA AAA CG – 3’</td>
<td>B</td>
</tr>
<tr>
<td>sY134 – R</td>
<td>5’ – ACC ACT GCC AAA ACT TTC AA – 3’</td>
<td>B</td>
</tr>
<tr>
<td>sY255 – F</td>
<td>5’ – GTT ACA GGA TTC GGC GTG AT – 3’</td>
<td>B</td>
</tr>
<tr>
<td>sY255 – R</td>
<td>5’ – CTC GTC ATG TGC AGC CAC – 3’</td>
<td>B</td>
</tr>
</tbody>
</table>

* R = A+G,  Y = c+t

### TABLE 2

<table>
<thead>
<tr>
<th>Reaction mix</th>
<th>Cycles</th>
<th>Hybridization</th>
<th>Synthesis</th>
</tr>
</thead>
<tbody>
<tr>
<td>A and B</td>
<td>30 cycles</td>
<td></td>
<td>3 min and 30 sec</td>
</tr>
<tr>
<td></td>
<td>1-29 cycle</td>
<td>1 min</td>
<td>55 °C</td>
</tr>
<tr>
<td></td>
<td>30 cycles</td>
<td>1 min</td>
<td>55 °C</td>
</tr>
</tbody>
</table>

Cytogenetic screening is highly recommended in male infertility, especially in cases without recognizable reason where genetic etiology is suspected (15, 30). Whereas only about 1 % of the general male population is affected by cytogenetic abnormalities, this proportion highly rises to 15 % or 20 % in azoospermic men (7, 31, 32, 33). Apart from the Klinefelter syndrome, which is the most common aberration among azoospermic men, different balanced chromosomal translocations are also described. It is well known that balanced translocations could produce unbalanced gametes thus leading to reproductive failure or delivery of an affected child with severe malformations (19). Another hypothesis suggests that potential autosomal genes involved in male gametogenesis might be deregulated by chromosome breakpoints (2). In our study reciprocal translocation [t (9;Y)] was seen in one azoospermic man. A compilation study in 464 infertile males with a balanced rearrangement has identified the chromosomal bands with potential significance for male infertility. The commonest one was 1q21 and breakpoints at chromosome 9 were reported in 5 out of 132 (3.8 %) azoospermic cases (2). The involvement of the Y chromosome in translocation with autosome is reported to have a frequency of 1:2000 (10, 20, 25). Usually they are between Y and another acrocentric chromosome; Y/non-acrocentric autosome translocation is quite rare (1). In another patient from our study we revealed pericentric inversion of chromosome 9. This is considered to be one of the most frequent polymorphisms of human chromosomes, occurring in 0.9 % to 4 % of the general population (18). A study in infertile men has reported 3.4 % incidence of this chromosome alteration among them, i.e. frequency close to that of the normal population (29). The authors, however, found a correlation between this aberration and the response of plasma gonadotropin to the administration of LH-RH. This response was abnormal in all patients carrying pericentric inversion of chromosome 9, suggesting that it may affect the reproductive process.
Azoospermia and oligozoospermia are evidently associated with deletions in loci of the AZF region of the Y chromosome (AZFa, AZFb, AZFc), which are introduced by multiplex PCR in infertile men (4, 21, 26, 28). The frequency of deletions in loci of the AZF region of the Y chromosome (AZFa, AZFb, AZFc) varies considerably between 3% and 55% in different studies (3). The deletions cause spermatogenic arrest, so their diagnostics is very important for successful treatment of infertility by ICSI and for preventing the transmission of the aberration to the next generation. The deletions cause spermatogenic arrest, so their diagnostics is very important for successful treatment of infertility by ICSI and for preventing the transmission of the aberration to the next generation. We detected, in two out of 10 patients (20%), deletion of the AZFc locus. The AZFc region is the location of the DAZ gene family and is known to be the most frequently deleted AZF candidate gene in infertile men (5, 9, 13, 15). Our results support this observation.

One interesting and new finding from our study is the genetic gain of 17q12-q21, detected by array CGH analysis. This region includes the gene for Zona pellucida binding protein 1 (ZPBP1), a spermatid and spermatozoon protein that localizes to the acrosome and is responsible for its binding to the oocyte zona pellucida (17). Abnormal proteins result in decreased fecundity and sperm head deformities. It disturbs the sperm maturation as well. Our results prompted for investigation of this gene and protein as a potential candidate for spermatogenic failure.

Including Y-chromosome microdeletions, chromosomal abnormalities and array CGH micro-structural defects, the total genetic abnormality rate in our study was 50% (5 out of 10 patients). It strongly recommends genetic testing and counseling before considering assisted reproduction in order to improve the successful outcome, eventually by elaborating preimplantation genetic diagnosis.

Conclusions

The genetic study of male infertility is a field with big potential and impact in reproductive medicine. In 50% of our patients we detected genetic aberrations – two cytogenetic mutations, two Y-microdeletions and 1 new and specific microaerration detected by array-CGH. The last contains the gene for zona pellucida binding protein ZPBP2. This protein plays a crucial role for the proper spermatogenesis. Our results prompted for...
investigation of this gene and protein as a potential candidate for spermatogenic failure.

REFERENCES


