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Short Communication

Genome-wide screening of severe male factor infertile patients using BAC-array comparative genomic hybridization (CGH)☆

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ABSTRACT

Male factor infertility is present in up to 50% of infertile couples, making it increasingly important in their treatment. Although most research into the genetics of male infertility has focused on the Y chromosome, male factor infertility may result from other genetic factors. We utilized the whole genome array comparative genomic hybridization (CGH) to identify novel genetic candidate associated with severely impaired spermatogenesis. We enrolled 37 patients with severe male factor infertility, defined as severe nonobstructive type oligozoospermia ($\leq 5\times10^6$/ml) or azoospermia, and 10 controls. Routine cytogenetic analyses, Yq microdeletion PCR test and whole genome bacterial artificial chromosome (BAC)-array CGH were performed. Array CGH results showed no specific gains or losses related to impaired spermatogenesis other than Yq microdeletions, and there were no novel candidate genetic abnormalities in the patients with severe male infertility. However, Yq microdeletions were detected in 10 patients. Three showed a deletion in the AZFb-c region and the other 7 had deletions in the AZFc region. Although we could not identify novel genetic regions specifically associated with male infertility, whole genome array CGH analysis with higher resolution including larger numbers of patients may be able to give an opportunity for identifying new genetic markers for male infertility.

1. Introduction

Male factor infertility is present in up to 50% of infertile couples, making it increasingly important in their treatment (Bhasin et al., 1994; Brugh et al., 2004). Many aspects of male infertility, however, remain poorly understood, and medical therapy for these patients is largely unsuccessful. Up to 60% of individuals with male factor infertility may have the condition as a result of genetic factors, including cytogenetic abnormalities and microdeletions of the Y chromosome (Lilford et al., 1994). Detailed genetic studies such as chromosomal abnormality, Y chromosome microdeletion have been conducted for less than two decade and many cases of male infertility are still diagnosed as idiopathic (Kleiman et al., 1999). Although most research into the genetics of male infertility has focused on the Y chromosome, recent studies indicate that the Y chromosome is not the only chromosome containing genes associated with spermatogenesis (Brooks, 2000; Silber and Repping, 2002; Wang et al., 2001). Moreover, microdeletions in the Y chromosome are observed in only 10-15% of men with non-obstructive azoospermia and severe oligozoospermia. Over the past 30 years, the treatment of infertility has seen the development of revolutionary new assisted reproductive technologies such in vitro fertilization (IVF) or intracytoplasmic sperm injection (ICSI). These methods of assisted reproduction are now used to treat most couples with severe male factor infertility. Despite the worldwide diffusion of ICSI procedures, however, there have been concerns about the risk of transmission genetic abnormality in these patients. Therefore, future research into genetic abnormalities on the human chromosome at whole genome level and their role in male infertility is mandatory and will possibly provide new insights into the genetic basis of the infertility phenotype. Recently, array-CGH has emerged as a powerful new molecular tool for high-resolution genome analysis. Although multiplex PCR is restricted to the analysis of a limited number of loci, array-CGH can potentially analyze thousands of different loci simultaneously. We therefore utilized whole genome array-CGH to identify novel genetic candidate in men with severe male factor infertility.

2. Material and methods

2.1. Patients

We prospectively evaluated 37 men diagnosed with severe male factor infertility at our andrology clinic between March 2008 and August

Abbreviations: CGH, comparative genomic hybridization; BAC, bacterial artificial chromosome; IVF, in vitro fertilization; ICSI, intracytoplasmic sperm injection; PHA, phytohemagglutinin; AZF, azoospermic factor; CNV, copy number variant; STS, sequence tagged site.

☆ Potential conflicts of interest: None.

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2009; for comparison, we evaluated 10 normal controls (Table 1). The study protocol was approved by our institutional review board.

Men were included if they had severe nonobstructive type oligozoospermia, defined as a sperm concentration $\leq 5\times10^6$/ml, or azoospermia. Patients who underwent any medical or surgical treatment during the follow-up period were excluded. Other exclusion criteria included a previous history of genital infection, varicocele, cryptorchidism, exposure to gonadotoxin, hypogonadism, or an insufficient period of sexual abstinence. Patients with any structural or numerical chromosome abnormalities were excluded, as were patients with obstructive type oligozoospermia, as defined by semen volume, testis size, serum FSH level and obstructive lesion on imaging such as transrectal ultrasonography.

Evaluation of men consisted of a thorough personal and family history, physical examination, semen analyses, and laboratory tests including hormonal profile and genetic tests. Statistical analyses were performed using a commercially available software program (SPSS 11.5, Chicago, Illinois). The Mann–Whitney U test was used to compare the patient group and control group. Statistical significance was defined as a p-value$<$0.05 and all statistical tests were 2-sided.

2.2. Semen analysis

Sperm concentration, motility and morphology were assessed according to the WHO manual (WHO, 1999). All semen samples were obtained by masturbation into a wide-mouthed plastic container in a separate room after sexual abstinence of $\geq$3 days; the samples were allowed to liquefy for at least 20 minutes at 37C before analysis. If sperm was not detected by conventional microscopic Makler chamber evaluation, the sample was centrifuged at 1500 *g* for 10 min to detect any viable sperm.

2.3. Cytogenetic analysis

Routine cytogenetic analyses were performed on lymphocytes from phytohemagglutinin (PHA)-stimulated peripheral blood cultures. Metaphase slides were prepared using standard protocols, and 20 GTG-banded metaphases per patient were examined. Metaphases were captured and karyotyped using the CytoVision System version 3.6 (Applied Imaging, UK).

2.4. DNA extraction and polymerase chain reaction for Yq microdeletion

Genomic DNAs were extracted from peripheral lymphocytes of patients using the QIAamp® DNA Blood Midi Kit (Qiagen, Germany), with DNA concentrations measured using the Nanodrop® spectrophotometer, ND-1000 (NanoDrop Technologies, Wilmington, DE).

Polymerase chain reaction (PCR) analyses were used to assess microdeletion in the long arm of the Y chromosome (Yq). DAZ genes and other sequences in the azoospermic factor (AZF) regions (AZFb, AZFc), including sY117, sY127, sY143, sY134, sY138, sY152, sY153, sY147, sY149, sY157, and sY158 were analyzed.

Table 1
Patient characteristics.

	Group1 (Patient)	Group 2 (Control)	p-Value
No. of patients	37	10	
Mean Age (range)	33 (22–44)	32 (25–38)	0.07
Sperm concentration ($\times10^6$/ml)	0.3 ± 1.0	99.2 ± 41.0	<0.05
Testis volume (ml)	11.1 ± 3.9	15.8 ± 2.4	<0.05
Serum FSH (mIU/ml)	19.9 ± 11.7	5.5 ± 3.5	<0.05

2.5. Whole genome BAC-array CGH

Test and reference DNA (Promega, Madison, WI) were labeled with Cy5- and Cy3-dCTP, respectively, and prepared for hybridization with the MacArray™ Karyo C-Chip (Macrogen, Korea), a whole genome-wide BAC array chip containing 4362 BAC clones with 1 Mb resolution. DNA labeling and hybridization were described, with some modifications (Pinkel et al., 1998). After hybridization for 48 hours at 37 °C in the slide chamber and post- hybridization washing, the arrays were scanned using a GenePix 4200 two-color fluorescent scanner, (MDS, Canada), and the scanned images were analyzed using MacViewer™ M software (Macrogen, Korea). The log2-transformed fluorescence ratios were calculated from background-subtracted median intensity values. For each spot, we used 0.25 and −0.25 as thresholds of gain and loss, respectively.

3. Results

Routine cytogenetic analyses showed that none of the 37 patients with severe male factor infertility had any structural or numerical chromosomal abnormalities. However, 10 patients had Yq microdeletions, with 3 having deletions in the AZFb-c region and the other 7 having deletions in the AZFc region.

Array CGH results showed no specific gains or losses related to spermatogenetic failure other than Yq microdeletions. All 10 Yq microdeletions identified by PCR were also detected by array CGH (Fig. 1). Several gains or losses were identified in all three groups, consisting of patients with Yq microdeletions (n = 10), patients without Yq microdeletions (n = 27) and controls (n = 10) (Fig. 2). The characteristics of these regions are summarized in Table 2. Several gains and losses were detected only in the patient groups (Table 3, Fig. 3). Gains of Xq23 and 5q11.1 and loss of 15q11.2 has not been listed as copy number variations in the databases. However, no known genes associated with impaired spermatogenesis were assigned in that region. The other gains and losses have been identified as previously known copy number variants (CNVs) (http://projects.tcag.ca/variation/),

4. Discussion

This study is the first to utilize whole genome array-CGH to identify new genetic markers of for severe male infertility. However, the sample size of this study is not large enough partly due to high cost of the whole genome-wide BAC array chip. The BAC-array used in this study was designed for genome-wide scanning of genomic imbalances with 1 Mb resolution. These arrays have been widely used in recent clinical and basic research to identify microdeletion syndromes and to characterize chromosomal abnormalities, especially marker chromosomes associated with prenatal diagnoses or cancer. Other than Yq microdeletions, we identified no specific gains or losses related to impaired spermatogenesis. Although we analyzed a relatively small number of patients, our results suggest that submicroscopic genome imbalances, except for Yq microdeletions, may not be a major cause of male infertility in patients with normal karyotype.

Since Yq deletions are the most significant pathogenetic cause of infertility in males, most major andrology and infertility centers now include Yq deletion PCR screening tests as part of the routine diagnostic investigation of severe male factor infertility. Since the first cytogenetic report of Y-deleted segments in 1976, mapping of the Y chromosome has been greatly facilitated by PCR amplification of sequence tagged sites (STSs) that span the deleted regions (Cram et al., 2000; Grimaldi et al., 1998; Oliva et al., 1998; van Golde et al., 2001). These PCR methods are based on the amplification of STSs specific to each AZF region. Array CGH systems can provide highly reliable and accurate diagnoses by the direct use of a gDNA target that can be easily prepared from the peripheral blood of infertile men. A recent study demonstrated that array-CGH may be an alternative to multiplex PCR