



Identification of the origin of marker chromosomes by two-color fluorescence *in situ* hybridization and polymerase chain reaction in azoospermic patients

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ABSTRACT. Y chromosomal microdeletions at the azoospermia factor locus and chromosome abnormalities have been implicated as the major causes of idiopathic male infertility. A marker chromosome is a structurally abnormal chromosome in which no part can be identified by cytogenetics. In this study, to identify the origin of the marker chromosomes and to perform a genetic diagnosis of patients with azoospermia, two-color fluorescence *in situ* hybridization (FISH) and polymerase chain reaction (PCR) techniques were carried out. The marker chromosomes for the two patients with azoospermia originated

in the Y chromosome; it was ascertained that the karyotype of both patients was 46,X, ish del(Y)(q11)(DYZ3+, DXZ1-). The combination of two-color FISH and PCR techniques is an important method for the identification of the origin of marker chromosomes. Thus, genetic counseling and a clear genetic diagnosis of patients with azoospermia before intracytoplasmic sperm injection or other clinical managements are important.

Key words: Azoospermia factor; Azoospermia; Marker chromosome; Fluorescence *in situ* hybridization; Polymerase chain reaction

INTRODUCTION

Marker chromosomes are structurally abnormal chromosomes in which no part can be identified by cytogenetic techniques alone. They are morphologically distinctive and incompletely characterized abnormal chromosomes. Marker chromosomes account for 0.06-0.12% and 0.07-0.15% of the genetic material in newborn babies and fetuses, respectively, and their origins cannot be ascertained morphologically by G-banding (Sachs et al., 1987). The fluorescence *in situ* hybridization (FISH) technique is a very important tool for cancer cytogenetic studies, genetic gene mapping, and chromosome disease diagnosis at the DNA level (Fu et al., 1996; Yung, 1996). Since intracytoplasmic sperm injection (ICSI) was first reported in 1992 (Palermo et al., 1992), numerous cases of the highly successful use of ICSI have been reported (Balaban et al., 2001; Ludwig et al., 2001; Madureira et al., 2014). ICSI has become the treatment of choice for male factor infertility. However, it has been reported that patients with azoospermia and severe oligozoospermia may present chromosomal abnormalities or microdeletions in the Y chromosome (Reijo et al., 1996; Vogt et al., 1996; Kent-First et al., 1999; Foresta et al., 2001; Fu et al., 2002; Han et al., 2013; Mierla et al., 2014). Therefore, it is important to identify genetic defects in patients with azoospermia and severe oligozoospermia before ICSI treatment.

In this report, we successfully identified the origin of the marker chromosome for two patients with azoospermia by two-color FISH using X and Y chromosome centromere-specific repeat sequence probes and polymerase chain reaction (PCR) amplification using specific primers at different regions of the two arms of the Y chromosome.

MATERIAL AND METHODS

Case data

The patients with azoospermia were recruited from infertility and genetic clinics. Both had abnormally small testes, no history of adnexitis, cryptorchidism, or severe mumps, and neither had recently consumed cotton oil. Routine sperm analyses were performed and no sperm was found. Abnormal chromosomes smaller than the G-group cannot be used to ascertain the origin of the marker chromosomes by using G-banding analysis in these two patients. The karyotype was 46,X, +mar.

Fluorescent *in situ* hybridization

The X and Y chromosome centromere-specific α DNA repeat sequence probes were purchased from Cytocell Ltd. (Cambridge, UK); the probe positions were Xp11.1-q11.1 and Yp11.1-q11.1, respectively, and they were labeled by green and red fluorescence, respectively. The hybridization conditions were according to protocols specified by Cytocell Ltd., with slight modifications. After hybridization and washing, the slides were counterstained with 4',6-diamidino-2-phenylindole (DAPI; 0.125 μ g/mL). The results were then observed under a fluorescence microscope and photographically recorded (Fu et al., 1996).

DNA extraction

Approximately 2-5 mL peripheral blood specimens were collected for each patient or normal males. Five to ten volumes of 1X blood lysis buffer [150 mM NH_4Cl , 10 mM KHCO_3 , 0.1 mM ethylenediaminetetraacetic acid (EDTA)] were added to the specimens, which were put on ice for 30 min and centrifuged at 3000 rpm for 10 min at room temperature. The cell pellets were resuspended in 3 mL 1X nucleic lysis buffer (1 mM Tris, pH 8.2, 40 mM NaCl, 0.2 mM EDTA) and vortexed. Sodium dodecyl sulfate (300 μ L, 10%) and proteinase K (10 μ L, 20 mg/mL) were added, mixed, incubated at 55°C for 3-5 h or 37°C overnight, and finally the DNAs were routinely extracted by phenol/chloroform (Fu et al., 2002; Fu, 2012; Long et al., 2014; Khan et al., 2015; Yang et al., 2015).

DNA primers for PCR

The primers were from sY84, the USP9Y gene exon/intron 7, sY143, and sY254, corresponding to regions AZFa, AZFa, AZFb, and AZFc, respectively, on the long arm of the Y chromosome (Sun et al., 1999; Fu et al., 2002). The sizes of the amplified fragments were 326, 249, 311, and 350 bp, respectively. The primers for USP9Y gene exon/intron 7 were as follows: 5'-CTTCACACAAATGCGTTTCA-3' (left primer) and 5'-TGCAATTATTTGAACAAACATGA-3' (right primer). The primers for sY84 were as follows: 5'-AGAAGGGTCTGAAAGCAGGT-3' (left primer) and 5'-GCCTACTACCTGGAGGCTTC-3' (right primer). The primers for sY143 were as follows: 5'-GCAGGATGAGAAGCAGGTAG-3' (left primer) and 5'-CCGTGTGCTGGAGACTAATG-3' (right primer). The primers for sY254 were as follows: 5'-GGGTGTTACCAGAAGGCAAA-3' (left primer) and 5'-GAACCGTATCTACCAAAGCAGC-3' (right primer). The marker of sY254 was located in the DAZ gene region. As a positive control, the amplified SRY (the sex determination region on the Y chromosome) gene (609 bp), located on the short arm of the Y chromosome, was used. The primers for SRY were as follows: XES7 5'-CCCGAATTCGACAATGCAATCATATGCTTCTGC-3' (left primer) and XES2 5'-CTGTAGCGGTCCTGTGCTGCGGTG-3' (right primer).

PCR amplification

PCR was performed in a 10- μ L reaction volume containing 50-80 ng genomic DNA, 50 mM KCl, 10 mM Tris, pH 8.3, 0.01% gelatin, 2 mM MgCl_2 , 250 μ M of each dNTP, 0.2 μ M of each primer (Sagon, Canada) and 0.6 U Taq polymerase (Sagon). The cycling reaction was performed in a programmable thermal cycler, PE9600 (Perkin-Elmer Applied Biosystems), at

94°C for 40 s, 59°-66°C for 25-40 s, and 72°C for 25-40 s for 35 cycles. The denaturing step in the first cycle was 90 s and the extension step in the last cycle was 5 min. The products were stored at 4°C until required for electrophoresis (Fu et al., 2002).

Agarose gel electrophoresis

The PCR products were added to 2 µL of 6X loading buffer, mixed, and run on a 2% (w/v) agarose gel in a 0.5X TBE (Tris/borate/EDTA) buffer. After electrophoresis at 100 V for 40 min, the gel was stained with ethidium bromide (0.5 µg/mL), and the images were documented using a ChemiDoc XR (Bio-Rad, USA) (Fu, 2012).

RESULTS

Hybridization using X and Y chromosome centromere-specific repeat sequence probes and DAPI staining showed a green fluorescence signal with a blue background on the X chromosome centromere in the metaphase chromosome, and a red signal with a blue background on the small marker chromosome (Figure 1A). Green and red hybridization signals were also visible in the nucleus (Figure 1B). This result indicates that the smallest marker chromosome originated in the Y chromosome.

To ascertain from which part of the Y chromosome the marker chromosome originated, PCR amplification was conducted using the primer pairs of the SRY gene located on the short arm of the Y chromosome and the primer pairs corresponding to AZFa, AZFb, and AZFc regions located on the long arm of the Y chromosome. The results showed that PCR amplification of the SRY gene was positive, whereas PCR amplification of the AZFa, AZFb, and AZFc regions was negative in the two patients (Figure 2). Therefore, the marker chromosome originated in the Y chromosome including the whole short arm, the centromere, and a few parts of the long arm, and was absent from Yq11 to the terminal of the long arm.

The marker chromosome, therefore, was as found to originate in the Y chromosome in two patients with azoospermia using the FISH and PCR technique. The karyotype was determined to be: 46,X, ish del(Y)(q11)(DYZ3+, DXZ1-) according to the results of molecular detection and the rules according to the International System for Human Cytogenetic Nomenclature (ISCN), 1995 (Mitelman, 1995).

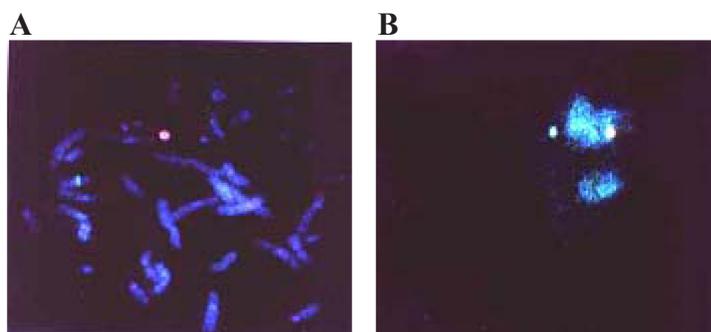


Figure 1. Two-color FISH detection for patient using X and Y chromosome centromere-specific repeat sequence probes. **A.** Metaphase chromosomes. **B.** Interphase nuclei. Green and red indicate X chromosome and Y centromere-specific fluorescent signals, respectively.

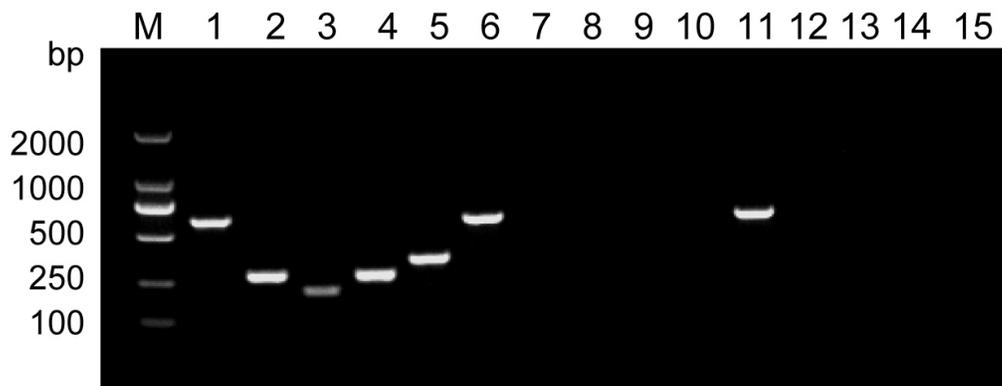


Figure 2. PCR detection for SRY, AZFa, AZFb, and AZFc. Lanes 1-5 indicate PCR detection of SRY, AZFa (sY84 and USP9Y), AZFb and AZFc for normal male. Lanes 6-10 indicate PCR detection of SRY, AZFa (sY84 and USP9Y), AZFb, and AZFc for one patient with a marker chromosome, whereas lanes 11-15 indicate PCR detection of SRY, AZFa (sY84 and USP9Y), AZFb, and AZFc for another patient with a marker chromosome. Lane "M" shows DL2000 DNA marker with indicated molecular weight size (bp).

DISCUSSION

The FISH technique is a very important tool for cancer cytogenetic studies, gene mapping, chromosome disease diagnosis, and as a supplemental tool for conventional chromosomal banded techniques. Four different types of FISH probe are available (Yung, 1996): centromeric probes, whole chromosome painting probes (WCPPs) or chromosome band-specific painting probes, chromosome-specific unique sequence probes (USPs), and telomeric probes. Centromeric probes contain repetitive DNA sequences found in the centromeric or pericentromeric regions of human chromosomes. Because of these repetitive sequences, these probes require a short hybridization time to generate strong signals, and can be utilized for both metaphase chromosome and interphase nucleus hybridization. This makes centromeric probes especially desirable for prenatal and neonatal cases that require rapid diagnostic results for aneuploidy (abnormality in the number of chromosomes in the nucleus, including sex chromosomes). The drawback is that a structurally imbalanced chromosome cannot be distinguished from a normal one.

Each WCPP is composed of a mixture of many probes, complementary in sequence to a specific site along the length of a single chromosome. When many such sequence-specific probes are used together in a single hybridization reaction, the whole chromosome of interest lights up and is therefore 'painted'. WCPPs are especially useful for distinguishing structurally imbalanced chromosomes from normal ones. A limitation of this class of probe is that they may not completely cover the whole chromosome, especially cryptic regions.

The USP class of FISH probes contains locus-specific DNA segments and is suitable for hybridizing both metaphase and interphase nuclei. Since each USP is gene-specific and is composed mainly of unique sequences, it is perfect for detecting microduplication and microdeletion of a specific chromosome region that is undetectable by cytogenetic means. Although a marker chromosome can be identified by its chromosome of origin, the structural abnormality of a small marker chromosome cannot be ascertained by centromeric probes alone. It is possible to identify such marker chromosomes completely using PCR or USPs, which can further ascertain which

parts of the marker chromosome originated from where, and where the deletions (if any) are.

Approximately 10-15% of married couples of child-bearing age are infertile. One of the important reasons is impairment of spermatogenesis, including azoospermia and severe oligozoospermia, which account for 10% of male infertility (Fu et al., 2002). As early as 1976, a cytogenetic study by Tiepolo and Zuffardi found that azoospermia patients exhibited the long arm deletion of the Y chromosome, including the distal fluorescence and the adjacent non-fluorescence part. Therefore, it was postulated that genes or gene families were located in the distal Yq11, defined as the azoospermia factor (AZF). Recently, studies have indicated that a defect in the spermatogenesis genes or a microdeletion in that part of the Y chromosome may lead to azoospermia and severe oligozoospermia. These genes on the Y chromosome are the USP9Y gene (chromosome Y ubiquitin-specific protease 9) and the DBY gene (DEAD/H box 3 in the Y chromosome) at the AZFa region (Sun et al., 1999; Foresta et al., 2000), the RBM (RNA-binding motif) gene at the AZFb region (Ma et al., 1993), and the DAZ gene (deleted in azoospermia) at the AZFc region (Reijo et al., 1995). Therefore, we can detect microdeletions on the Y chromosome by PCR amplification of these genes at the AZF regions of the Y chromosome. However, the genes related to spermatogenesis also exist on autosomes (Yen et al., 1996; Seboun et al., 1997; Van Golde et al., 2001). Therefore, exact identification of the origin of marker chromosomes by the method described above may improve molecular pathology diagnosis for patients with azoospermia.

Hybridization using X and Y chromosome centromere-specific repeat sequence probes and DAPI staining resulted in a green fluorescence signal arising from the X chromosome centromere in the metaphase chromosome, and a red signal from the small marker chromosome. Green and a red hybridization signals were also observed in the nucleus. This indicates that the smallest marker chromosome originated in the Y chromosome (Figure 1). To ascertain which part of the marker chromosome originated from which part of the Y chromosome, PCR amplification was performed using the primer pair of the SRY gene located on the short arm of the Y chromosome and the primer pairs corresponding to AZFa, AZFb, and AZFc regions located on the long arm of the Y chromosome. The results showed that the PCR amplification of the SRY gene was positive, whereas the PCR amplifications of AZFa, AZFb, and AZFc regions were negative (Figure 2). Therefore, the marker chromosome originated on the Y chromosome including the whole short arm, the centromere, and a few parts of the long arm, and was absent from Yq11 to the terminal of the long arm; the karyotype was determined to be: 46,X, ish del(Y)(q11)(DYZ3+, DXZ1-).

ICSI provides the only sufficient treatment to overcome severe forms of male infertility using the primary spermatocyte, round spermatid, elongating spermatid, elongated spermatid, or spermatozoa stages (Hourvitz et al., 1998; Kahraman et al., 1999; Balaban et al., 2001; Madureira et al., 2014). However, male infertility is linked to several genetic problems such as an increased number of chromosomal aberrations, cystic fibrosis transmembrane conductance regulator gene mutations, Y chromosome microdeletions, and androgen receptor mutations. Therefore, couples that are affected by these issues are at a higher risk of transmitting genetic problems to their offspring compared with the general population. There is growing evidence that the short-term health of offspring resulting from ICSI is relatively unremarkable, but our growing understanding of the genetic basis of much of male subfertility and of the impaired genomic integrity that characterizes the oligozoospermic phenotype suggest a cautious approach to the long-term health of ICSI offspring would be wise. Patients with azoospermia who have chromosomal abnormalities may also father children with the help of ICSI treatment

using the primary spermatocyte, round spermatid, elongating spermatid, elongated spermatid, or spermatozoa stages. Genetic counseling and accurate genetic diagnosis are important before ICSI treatment.

The combination of FISH with PCR, therefore, is another very important method for the identification of a marker chromosome for patients with chromosomal abnormalities, especially those resulting in azoospermia and severe oligozoospermia.

Conflicts of interest

The authors declare no conflict of interest.

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