Efficient combined FISH and PRINS technique for detection of DAZ microdeletion in human sperm

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Abstract Intracytoplasmic sperm injection (ICSI) now offers an effective therapeutic option for men with male infertility and is believed to allow transmission of genetically determined infertility to the male offspring. Transmission of DAZ (Deleted in Azoospermia) microdeletion is one of the major concerns for oligo and severe oligozoospermia patients. Screening of the Y chromosome microdeletion in the diagnostic work-up of infertile men is mainly done using polymerase chain reaction (PCR) on blood leukocytes. However, there are evidences showing that presence of DAZ in somatic cells might not be indicative of its presence in germ cell lineage. In this report we are going to describe a combined Primed in situ labeling (PRINS) and fluorescence in situ hybridization (FISH) technique to show the localization of DAZ gene as well as Y chromosome centromere on sperm nuclei. PRINS is a combination of FISH and in situ polymerization provides another approach for in situ chromosomal detection. In the present study the PRINS primers specific for DAZ genes and traditional direct labeled centromere FISH probes for Y and X chromosomes were used in order to simultaneously detect DAZ genes and sex chromosome aneuploidy in sperm samples.

Keywords Human sperm · Y chromosome · DAZ · PRINS · FISH

Capsule Using combined techniques of PRINS and FISH, both DAZ localization and Y chromosome centromere on human sperm can be visualized easily on single cells.

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Introduction

Infertility affects 10–15 % of the human couples and spermatogenic failure is among the main reason in 50 % of these cases [1]. In more than 60 % of cases the origin of reduced testicular function is unknown [2, 3]. Evidence exists that microdeletions of the Y chromosome play a causal role in male infertility [4]. Molecular and cytogenetic studies from infertile men have shown that microdeletions within the azoospermia factor region (AZF) [5] are prevalent causes of male factor infertility. Analysis of these microdeletions resulted in the identification of three loci in Yq11 involved in the control of spermatogenesis, corresponding to three non-overlapping regions: AZFa, AZFb, AZFc [6–8]. About 10–15 % of azoospermia and severe-oligozoospermia patients have microdeletions in AZF region of Y chromosome [9]. A great part of microdeletions happen in AZFc region, which includes 80 % of them. About 15–20 % of azoospermia and 7–10 % of oligozoospermia patients have microdeletion in AZFc region [10]. There is a variety of genes in this region from which DAZ (Deleted in Azoospermia) gene is the most susceptible candidate for deletion in oligozoospermia and azoospermia males [11]. Since the development of assisted reproduction technology, intracytoplasmic sperm injection (ICSI) now offers an effective therapeutic option for these men and is believed to allow transmission of genetically determined infertility to the male offspring [12].

Therefore, analysis of microdeletions should be performed in all patients who are candidates for ICSI [13]. Detection technologies have emerged as important components of health care and are increasingly used in ART procedures. Microdeletion detection has been included in the recommended guidelines for male infertility [14–16]. Screening of the Y chromosome microdeletion in the diagnostic work-up of infertile men [17] is mainly done using

polymerase chain reaction (PCR) on blood leukocytes [18]. However, there are evidences showing that presence of DAZ in somatic cells might not be indicative of its presence in germ cell lineage. Sperm DNA damage is clearly associated with male infertility and abnormal spermatogenesis [19, 20]. Reactive oxygen species (ROS) and oxidative stress might play a critical role in induction of sperm DNA damage and the etiology of defective sperm function and male infertility [21]. It was shown previously that men exposed to high background natural radiation [22–24], 137-Cesium irradiated men [25, 26] and recently in vitro irradiation of leukocytes [27] led to genome instability in AZF region of Y chromosome especially in the DAZ genes. Therefore DAZ genes might be deleted during or after either spermatogenesis or spermiogenesis. These changes are certainly not detectable with somatic cell analysis. Moreover, some patients with severe oligozoospermia could be mosaic for the Y chromosome deletion in leukocytes but normal in the germ cell lineage [28]. If these subjects undergo ICSI, they will produce healthy male offspring with normal Y chromosome. On the contrary, higher frequencies of Yq microdeletions in sperm DNA was reported as compared to DNA isolated from blood [29, 30]. Therefore it would be reasonable for all couples opting for ART, to undergo sperm DNA Yq screening in order to provide the most adapted therapies and counseling. PCR based DAZ analysis does not show situation of DAZ gene in individual cells. There has been attempt to show DAZ gene by the use of FISH on sperms or metaphase lymphocytes [31–33] or PRINS [33–35] in lymphocytes either as interphase or metaphase cells. However, the PRINS method was not used on sperms for DAZ localization. Also, the availability of DAZ probes for FISH analysis is not universal. In this report we describe a combined PRINS and FISH technique to show the DAZ gene on sperm nuclei. PRINS, Primed in situ labeling, [33, 35, 36] is a method of target DNA sequence detection and localization which combines features of fluorescence in situ hybridization (FISH) and polymerase chain reaction (PCR). In this procedure, introduced by Koch et al. (1989) [37] the chromosomal identification is performed by in situ annealing of specific and unlabeled oligonucleotide primers to complementary sites on denatured chromosome spreads or nuclei. The annealed primers provide initiation sites for chain elongation catalyzed by a Taq DNA polymerase in the presence of free nucleotides, of which at least one is labeled. The in situ visualization of generated fragments results from the incorporation of the labeled nucleotide [38]. Here we have used PRINS technique with primers specific for DAZ genes, in combination with direct labeled centromere probes for Y and X chromosomes using traditional interphase FISH technique. This combined method allows simultaneous detection of DAZ genes and sex chromosome aneuploidy in sperm samples.

Materials and methods

Sample preparation

Semen samples were obtained randomly from 10 normal men (mean age 33.3 ± 5.6) without any male fertility problems referred to Fertility and Infertility Center of Shariati Hospital (Tehran, Iran) because of fertility problems of their spouse. In all cases, after 3 days of sexual abstinence, semen samples were collected by masturbation into sterile containers and were delivered to the laboratory immediately after ejaculation. The semen was allowed to stand at 37°C for 30 min to complete liquefaction, and then sperm count was done according to the World Health Organization criteria [39]. The mean sperm count of the samples was 68.6 ± 20.4 million/ml. The study was approved by the Ethical Committee of the School of Medical Sciences of the Tarbiat Modares University (Tehran, Iran). Patients gave their informed written consent. Samples were processed by swim-up techniques from pellet as described by Aitken and Clarkson (1988) [40]. The sperm sample was washed twice in 1X PBS by centrifugation (5 min at 2000 rpm) and fixed for 1 h in fresh fixative (3:1 methanol : glacial acetic acid) at 4°C. The sperm suspension was then dropped onto clean microscope slides and air-dried. Slides were aged for 1 day at room temperature.

Methodology used for PRINS and FISH on sperms

Before doing the PRINS procedure, the slides were immersed in a 0.5 M NaOH solution at room temperature for 5 min, washed in PBS for 2 min, passed through an ethanol series (70 %, 90 % and 100 %) for 2 min each and air dried. The use of NaOH solution allowed the simultaneous decondensation and denaturation of sperm nuclei [41]. Slides were then put in a 70 % formamide in 2x SSC at 73°C for 3 min for further denaturation of sperm DNA. Then slides were passed through ice cold ethanol series (70 %, 90 % and 100 %) for 2 min each and air dried. For each slide, the reaction mixture was prepared in a final volume of 50 µL containing: 10x PCR buffer containing 50 mM KCl, 10 mM Tris-HCl, pH 8.3, 1.5 mM MgCl₂, 0.2 mM dATP, dCTP, and dGTP, 0.02 mM dTTP, 0.02 mM fluorescent dUTP, 0.01 % BSA, 2.5U of Taq DNA polymerase (All reagents from Roche Diagnostics, USA) and 200 pmol of each oligonucleotide primer for DAZ. The four primers specific for DAZ were as follows: (5'-CTCTGCCTCTGGCTTTACCA-3', 5'-GAGGAGGCATCTGGAAATCATT-3', 5'-GGAAGCTGCTTTGGTAGATAC-3', 5'-TAGGTTTCAGTGTTCGATTCCG-3') [33, 34]. A blast search of the primer sequences showed that they were specific for their intended targets. Before transferring of PRINS reaction mixture, the prepared slide was placed on a PCR thermal

block (Eppendorf) and heated for 3 min at 76°C, then hold at 60°C. PRINS reaction mixture was transferred on the slide covered with a cover slip and sealed with rubber glue. The slide was left at 60°C for at least 1 min to allow the temperature of reaction mixture reach to annealing temperature. The PRINS was then performed by incubating the slide at 55°C for 20 min to allow primers to anneal, and then at 72°C for 40 min for primer extension.

After the completion of PRINS reaction, the program of the thermo cycler was hold at 72°C, the cover slip was then removed, and slide was placed in 0.4X SSC, at 72°C for 2 min to remove unbound primers and unincorporated dNTPs, left in dark to air dry. The slide was put on the thermal block holding at 72°C, mixture of direct labeled centromeric probes for X and Y chromosomes (5 µl) (Cytocell, UK) placed on the slide, then covered with a cover slip and sealed with a rubber glue. The program was followed set the temperature at 73°C for codenaturation of the sample DNA and probes for 5 min, then 37°C for 5 min. After the completion of the program, the slide was placed in a humidified chamber at 37°C overnight. The following day, the cover slip was removed, and slide was placed in a jar containing 0.4X SSC at 72°C for 2 min followed by a further wash in 0.4X SSC, 0.05 % Tween 20 (AMRESCO Inc., USA) shaken for 30 s to remove unbound probes. Slides were mounted in Vectashield mounting media (Vector Laboratories, Inc, Burlingame, Calif) containing DAPI counterstain (0.3 µl/ml), covered with a 22 mm x 22 mm cover slip, and stored at 4°C in a light-free slide box until scoring. The slides were examined under the epifluorescence microscope (E800, Nikon, Tokyo, Japan), preferentially using first the triple band-pass filter, and confirming the coloration of the fluorescent spot with single band-pass (FITC or Rhodamin) filters.

Results

The sperm samples from 10 healthy male volunteers were collected. Using a modified PRINS method followed by FISH, detection of DAZ sequences and centromere of Y and X chromosomes were possible. In all sperm nuclei subjected to PRINS for DAZ, a green fluorescent signal at position Yq11.2 was observed (Fig. 1). Furthermore, the signals seen for Y and X chromosomes exhibited red and green fluorescent respectively (Fig. 2). The intensity of PRINS signals was the same as those obtained by FISH. Because of the chromatin status of the Y chromosome, the centromeric region shown by FISH and also DAZ region exhibited by PRINS were presented respectively red and green signals that were well separated (Fig. 3a) but in some instances due to the proximity of the studied regions, the red and green signals overlapped and appeared as yellow signal

(Fig. 3b) however with the aid of single band pass filter, the presence of each red or green signal was evident. For sperm nuclei bearing X chromosome, only one green signal was detected (Figs. 2 and 3a2).

Discussion

PRINS was used as a chromosome labeling technique to detect aneuploidy in various cell types including sperm [41, 42]. The traditional PRINS protocol utilized primers specific for repetitive alpha satellite sequences thus because of the large number of copies available for hybridization, interference with hybridization by chromatin or other proteins would still allow a strong enough signal to be visualized. PRINS was also used to localize single copy genes including DAZ and SRY on metaphase chromosome [33–35]. However, utilizing PRINS technique for the detection of single-copy genes, modifications must be made to the protocol to allow for efficient and accurate labeling [33, 35, 43]. Similar sets of primers were used to detect DAZ gene on Y chromosome at metaphase stage. However, in this study the DAZ signal detected on sperm nuclei was much larger and sharper than the signal observed on metaphase Y chromosome with the aid of commercially available kits for enhancing the fluorescent signals [33]. To be sure of the PRINS is showing DAZ gene on Y chromosome, alpha satellite probe for centromere of Y chromosome was used to show the presence of Y chromosome. Using a mixture of X and Y centromeric probes for this type of study might be useful to show probe hybridization efficacy and looking for chromosomal aneuploidy of sperm. However, using only Y centromeric probe suffice to show the status of DAZ gene in sperm nuclei. By using post hybridization washes in 0.4X SSC

Fig. 1 PRINS-labeled DAZ gene result. Green signal position is Yq11.2. Those sperms showing no signal are X chromosome bearing sperms

Fig. 2 Dual color FISH showing centromeric region of X (green signal) and Y chromosome (red signal) on sperm nuclei

alone and 0.4X SSC 0.05 % Tween 20, a minimal background was obtained for easy signal detection. All signals were visualized directly and recorded with video capture

Fig. 3 **a** Sperms showing two signals one for DAZ region (green) produced by PRINS and one for Y centromere (red) produced by FISH. Those cells carrying only one green signal are X-bearing sperms. **a1**: Y bearing sperm with normal DAZ; **a2**: X bearing sperm; **a3**: Y bearing sperm with deleted DAZ; **a4**: Y chromosome disomy, one with normal and one with deleted DAZ; **a5**: YY disomy, Y chromosomes with normal DAZ. **b** Sperm showing large yellow signals on sperms. Green signals showing DAZ localization is overlapped with red signal showing centromere position on sperm nuclei

without using any software to enhance the signal intensity or to reduce background. The overnight hybridization time needed for probe hybridization could be reduced to 4 h according to probe manufacturer's instruction. Therefore the combination of these two methods could easily lead to the detection of the presence or absence of DAZ gene in sperm nuclei bearing Y chromosomes (Fig. 3a1 and a3 respectively). As seen in Fig. 3, more than 95 % of sperms carry either green or green and red signals. Study of samples from all 10 individuals used in this study also showed similar pattern of signals, conferring the sensitivity and reproducibility of the method.

Techniques which have been used to assess DAZ microdeletion are PCR, real time PCR both used on blood and sperm DNA; and FISH and PRINS which were used only on metaphase preparations of lymphocytes. PCR and real time PCR are sensitive techniques, however, they are also affected by dilutional artifacts and the analysis is time consuming and labor intensive. They also do not show situation of DAZ on individual cells. PRINS and FISH were used to localize DAZ and SRY genes on metaphase Y chromosome previously [33, 34] but not on sperms with condensed nuclei. Also, the availability of DAZ probes for FISH analysis is not universal. Multi color PRINS for localization of DAZ and SRY genes need the arrest of first PRINS reaction by immersing the slide in stop solution and treatment of slide with dideoxynucleotides mix and klenow enzyme in order to block the free 3' end of the elongation fragments generated by the first PRINS reaction. Also if inappropriate washing procedure is adopted, the background signals might prevent efficient detection of labels.

However, the PRINS reaction used in this study to localize DAZ gene is a fast and low cost approach based on the use of short, unlabeled specific primers. The lengths of the PRINS primers range from 18 to 30 nucleotides. Compared to the size of DNA probes (250–600 bp), this small size greatly facilitates their in situ accessibility to the genomic target sequences. This is particularly significant in cells with highly condensed nuclei like sperm. The complementation process between the primer and its target will be so specific that a simple mismatch between the 3'-end of the primer and the genomic sequence will prevent initiation of the in situ elongation by the Taq DNA polymerase [42]. Based on the use of such primers, the PRINS reaction combines the high sensitivity of polymerase chain reaction (PCR) with the cytological localization of DNA sequences [37]. The FISH method for detection of X and Y chromosomes on sperms was based on the commercially available alpha satellite centromere probes. Combination of these two techniques not only allows detection of DAZ status in single cells but also makes it possible to assess sex chromosome aneuploidy in sperms (Fig. 3a4, a5).

Knowledge of the presence or absence of DAZ gene in sperm nuclei is very important concern for those individuals subject to ICSI. Screening for deletions of the Y chromosome involving the DAZ gene in severe oligozoospermia is suggested [18]. The estimated frequency of deletions involving DAZ locus is reported to be 3 % in azoospermia-severe oligozoospermia men consulting an infertility clinic when blood cells is screened for microdeletion using PCR technique [18]. However, screening for DAZ gene in blood leukocytes might not be representative of the status of DAZ gene in sperm nuclei which is used for sperm micro-injection into oocytes. It was shown that there is variation in DAZ copy number in infertile men when using real time PCR [44] and that copy number variation affect total motile sperm count [45]. On the other hand there are reports showing DAZ gene is vulnerable to damage by environmental genotoxic agents such as ionizing radiation [23–27], and spermatozoa are particularly susceptible to ROS-induced damage due to presence of large quantities of polyunsaturated fatty acids and low concentrations of scavenging enzymes in their plasma membranes [46]. Also there are reports showing absence of DAZ genes in spermatozoa of infertile men with somatic DAZ deletions [32]. Studies have shown higher frequency of Yq microdeletions in sperm DNA as compared to DNA isolated from blood [29, 30]. Therefore studying DAZ status using PCR in blood leukocytes might not be as accurate as showing DAZ gene on individual sperm nuclei. The combined methods of FISH and PRINS described in the present paper might be of help and value in this regard.

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Conflict of Interest statement The authors declare that there are no conflicts of interest.

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