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RUNNING TITLE: Meiotic studies using M-FISH

IDENTIFICATION OF MEIOTIC ANOMALIES USING MULTIPLEX FISH: PRELIMINARY RESULTS

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CAPSULE: A sequential protocol combining the analysis of classical preparations followed by Multiplex-FISH (M-FISH) in the same meiotic figures allowed us to identify all bivalents in Metaphase I and all chromosomes in Metaphase II, and to characterize chromosome abnormalities.

ABSTRACT

Objective: To characterize meiotic anomalies in infertile males by Multiplex-FISH (M-FISH) and to determine whether synaptic problems affect specific bivalents or whether anomalies are random.

Design: Analysis of meiotic preparations using standard techniques and Multiplex FISH.

Setting: Assisted Reproduction Centers and Universitat Autònoma de Barcelona.

Patients: Three fertile males undergoing vasectomy, four sterile patients with oligoasthenoteratozoospermia (OAT) and one patient with a Robertsonian translocation t(13;14).

Interventions: Unilateral testicular biopsy in controls and patients with OAT, and collection of a semen sample from the translocation carrier.

Main Outcome Measure: To identify all bivalents in Metaphase I (MI) and all chromosomes in Metaphase II (MII), and to characterize chromosome abnormalities.

Results: All bivalents in MI and all chromosomes in MII could be identified. In controls, and in one patient with OAT, meiosis was normal. Other patients with OAT showed different types of anomaly: desynapsis, breaks, precocious XY separation or cryptic reorganizations. The Rob t (13;14) was easily identified.

Conclusions: Results confirm the high incidence of synaptic errors in OAT patients. Bivalents in MI and chromosomes in MII were individually identifiable. Non-disjunctional errors or small reorganizations overlooked in classic meiotic preparations were identified. Synaptic anomalies seem to affect meiotic bivalents at random.

Key words: meiosis; synaptic anomalies; chromosome reorganizations; Multiplex FISH.

INTRODUCTION

Meiotic studies are seldom included in the protocol of exploration of sterile or infertile males, because testicular biopsies require minor surgery, and also because most laboratories lack the expertise to analyze meiotic preparations. However, meiotic studies (1,2) provide useful information on synaptic errors and on their influence in the production of univalents or of bivalents with a reduced chiasma count (1,3-6), on the meiotic behavior of balanced chromosomal reorganizations (7-9), and on the presence of “de novo” meiotic structural anomalies (10), all of which can produce genetically unbalanced spermatozoa (11).

Data obtained from meiotic studies are helpful not only to establish a prognosis regarding the reproductive possibilities of the couple, but also to decide on the most convenient assisted reproductive technique (ART) to use, and to provide the couple with more accurate genetic counseling which may include prenatal diagnosis, preimplantational genetic diagnosis (PGD) or even sperm donation.

The most important limitations of the technique using standard protocols (12) are: 1) since the bivalents affected by synaptic anomalies in MI and the bivalents possibly affected by an interchromosomal effect in carriers of structural reorganizations cannot be identified, a sperm FISH study of aneuploidies is impossible because there is no indication as to the probes to use; 2) the products of the segregation of structural reorganizations can only be inferred from the configurations observed, although these conclusions can later be confirmed by sperm FISH, and, 3) taking into account that MII figures are usually scarce and difficult to analyze (13), usually it is not possible to identify

segregation errors during MI, or to predict segregation errors in MII (even if they are not frequent).

For these reasons, it was decided to carry out a study of meiosis using a sequential protocol, combining the analysis of classical preparations followed by Multiplex fluorescent in-situ hybridization (M-FISH) (14) in the same meiotic figures.

MATERIALS AND METHODS

Testicular biopsies were obtained under local anesthesia from three fertile controls undergoing a vasectomy and four sterile patients with a severe oligoasthenoteratozoospermia (OAT). A semen sample from a carrier of a balanced Rob t(13;14) was obtained by masturbation after three days of abstinence of all sexual activity. Testicular biopsies were processed according to the method of Evans et al (12), and the semen sample following the technique described by Vidal et al (15). The study was approved by our institutional Ethics Committee and all individuals signed an informed consent form.

Prior to the application of M-FISH, all samples were stained with Leishman (20%) for 8-10 minutes. Evaluation of the preparations was carried out using an Olympus BX60 equipped with a capture-and-image-analysis system (CytoVision 2.7; Applied Imaging Corporation.California, USA). Pachytene, Metaphase I and Metaphase II figures were captured, and coordinates were noted to facilitate the location and analysis after the M-FISH protocol.

Before application of the M-FISH protocol, preparations were de-stained in an ethanol series (70%, 80%, 90%) one minute each, incubated for 30 minutes at 37 ºC in an RNase A solution (0.01% RNase A, Dnase-free in 2XSSC) and for 5 minutes in a Pepsin solution (0.005% pepsin in 0.1N HCl) at 37 ºC. Slides were fixed with formaldehyde (5% buffered formalin with 1XPBS; 0.05M $MgCl_2 \cdot 6H_2O$) during 2 minutes at room temperature. Afterwards, the manufacturer's M-FISH protocol was applied (Spectra Vysion Assay, Vysis Inc; Downers Grove, IL, USA).

Analyses were performed using an Olympus BX60 epifluorescence microscope equipped with specific filter sets for Aqua (Spectrum Aqua; excitation peak: 433 nm, emission peak: 480 nm), Cy 5 (Spectrum FRed; excitation peak: 655 nm, emission peak: 675 nm), FITC (Spectrum Green; excitation peak: 497 nm, emission peak: 524 nm), Gold (Spectrum Gold; excitation peak: 530 nm, emission peak: 555 nm), TexasRed (Spectrum Red; excitation peak: 592 nm, emission peak: 612 nm), and DAPI (excitation peak: 367 nm, emission peak: 452 nm). Capture and image analyses were carried out using a CytoVision system (CytoVision 2.7; Applied Imaging Corporation.California, USA).

RESULTS

The results obtained in this preliminary study show that M-FISH allows for the identification of individual bivalents in Metaphase I (Fig. 1) and in Pachytene (Fig. 2.A) and of the chromosomes in Metaphase II (Fig. 2.B).

The three controls and one of the patients with a severe OAT had normal MI and MII figures.

One of the sterile patients showed a precocious separation of the XY bivalent in 33.3% of the MI figures analyzed.

The other two patients with an OAT had multiple anomalies. One of them showed a precocious separation of the XY bivalent, a partial desynapsis of bivalents number 15 and 18, and a univalency of bivalent number 19. These anomalies, as is usual, were not present in all MI figures; instead, several combinations of them were found, e.g., XY separation plus bivalent 19 univalency, XY separation plus desynapsis of bivalent 18 (Fig. 2.C), etc.

In the other patient with OAT, the anomalies observed included the precocious separation of the XY bivalent, breaks in bivalent 9 (a frequent observation in classical studies but not identified as a break until M-FISH was used), desynapsis of bivalents 5,13,16,17 and 18, a reorganization involving the sex chromosomes in one MI figure and an XY disomy in a MII. Some of these anomalies are shown in Figs. 3.A and 3.B.

In meiotic preparations from the semen sample of the carrier of the balanced Rob t(13;14), the trivalent was easily identified (Fig. 3.C). Unfortunately, no MII figures were found in this sample.

DISCUSSION

M-FISH is a technique that can be sequentially used after the analysis of meiotic preparations using the classical protocol of Evans et al (12). The use of M-FISH allows for the identification of each individual bivalent in Pachytene and Metaphase I, and of each individual chromosome in Metaphase II.

Pachytenes are easily found in meiotic preparations. Synaptic anomalies can be detected if present. Thus, this stage can provide useful information on the bivalents affected by pairing problems.

The number of cells in Metaphase I that can be analyzed in meiotic preparations, specially in infertile males, is always low (13). For this reason, even if at present meiotic (synaptic) anomalies seem to affect different bivalents at random (with the exception of the XY bivalent), more studies will be needed to determine if the anomalies are indeed random (in which case the M-FISH analyses would not be helpful to choose the most adequate probes for a sperm FISH study) or if all or some of them are more frequent than others (in which case at least some probes should always be used).

From classical data, as well as from our M-FISH results, it is possible to establish that bivalent 9 is often affected, specially by breaks, producing an asymmetrical image like the one shown in Fig. 3.A. In fact, it is well known that chromosome 9 is specially prone to breaks and pericentric inversions (16). On the other hand, a reduction in the number of chiasmata results in symmetrical images like the one seen for bivalent 5 in the same figure (Fig. 3.A). Bivalent number 18 was affected in both patients. And univalency, as seen for bivalent 19 in one of our patients, is also frequently observed in sterile patients. Thus, perhaps a combination adding a few more probes to the 13, 18, 21, X and Y set

now used for aneuploidy screening could be enough to cover most cases with synaptic problems.

Finally, the use of M-FISH allows to identify the segregation errors produced in MI when analyzing Metaphase II figures (see below).
When meiotic configurations resulting from structural reorganizations are analyzed, the classical method does not always allow to individually identify the chromosomes involved in the multivalents (trivalents, quadrivalents) observed. Identification of the position and probable orientation of each chromosome will contribute to predict its segregation and the anomalies that can be expected from each particular configuration.

Taking into account that analysis of the preparations with Leishman staining allows to identify the location of chiasmata, it will be possible, for instance, to determine if an interstitial chiasma is present in pair A or in pair B of, say, a quadrivalent from a reciprocal translocation. The duplications/deficiencies resulting if the chiasma is in A may have more (or less) severe consequences than if the chiasma is in B. To give another example, in a translocation producing a trivalent plus one univalent, the consequences may be quite different depending on whether the univalent is chromosome A or B, or on whether the univalent is a translocated chromosome or its non-translocated homologue.

The presence of “de novo" meiotic translocations is not frequent, but has been described (10). Usually, they are detected by the presence of a meiotic configuration corresponding to a reorganization. However, cryptic reorganizations such as the one shown in Fig 3.A would go unnoticed in classical preparations.

Finally, Metaphase II figures in classical preparations are usually quite difficult to analyze because the chromatids are wide open and often separated and mixed. As a result, even counting chromosomes may become impossible (13), as seen in the Metaphase II spreads of Fig 2.B or Fig 3.B. However, the use of M-FISH allows for the identification of each individual chromosome or chromatid and also the characterization of anomalies such as the XY disomy shown in Fig 3.B. This characterization would have been impossible without the use of M-FISH, specially because the chromatids of the Y chromosome are so wide apart.

The limitations of the technique, with respect to the classical meiotic procedure, are mainly related to the fact that the bivalents affected in cases with synaptic anomalies may be random. If this were the case, then M-FISH would not be of help to choose the most appropriate probes for sperm-FISH studies. Another problem is that it cannot predict the segregation of the chromosomes in MII and, in this sense, it does not have any advantage over the classical technique. Finally, the technique is expensive and time-consuming, but both cost and time will probably decrease continually in the future.

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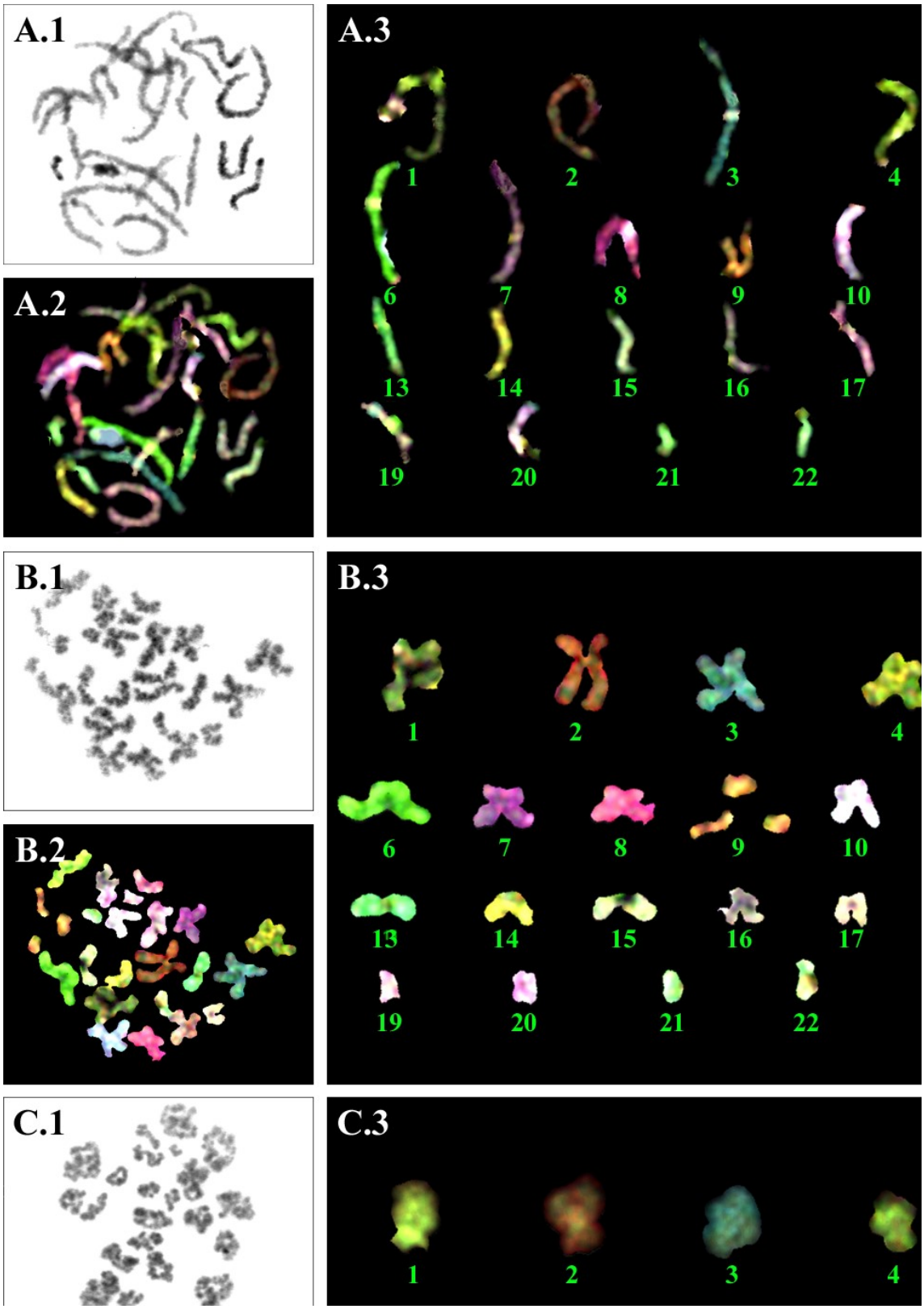
Figure 1. Metaphase I from a testicular biopsy. **A.** Leishman staining. **B.** Multiplex FISH image. **C.** Spectrum GOLD image. **D.** Spectrum AQUA image. **E.** Spectrum FRED image. **F.** Spectrum RED image. **G.** Spectrum GREEN image. **H.** DAPI image. **I.** Multiplex-FISH karyotype plus uniformly stained combined image allowing for bivalent identification together with an accurate chiasmata count.

Figure 2. A. Pachytene spread. **B.** Normal, X-bearing Metaphase II. **C.** Metaphase I showing precocious separation of the XY bivalent (C.2: arrows, lower left and upper right) and desynapsis of bivalent 18 which would be expected to show two chiasmata instead of a single one as seen in C.1 (C.2: arrow, upper left). (**A.1/B.1/C.1** Leishman staining. **A.2/B.2/C.2** M-FISH images. **A.3/B.3/C.3** Multiplex-FISH karyotypes.)

Figure 3. A. Metaphase I showing a reorganization involving the sex chromosomes; the Y chromosome includes material from the X (A.2: arrows, lower left and right / A.3: arrow), desynapsis of bivalent 5 which should show at least two chiasmata instead of a single one (A.2: arrow, upper right) and a break in bivalent 9 (A.2: arrow, upper left). **B.** Metaphase II showing an XY disomy (B.2: arrows) with a precocious separation of the chromatids of the Y chromosome (B.2: arrows, upper left and lower right). **C.** Metaphase I from a semen sample from the Robertsonian translocation t(13;14) carrier showing the translocation trivalent (C.2: arrow, lower right) and the precocious separation of the X (C.2: arrow, upper left) and Y (C.2: arrow, upper right) chromosomes.

(**A.1/B.1/C.1** Leishman staining. **A.2/B.2/C.2** M-FISH images. **A.3/B.3/C.3** Multiplex-FISH karyotypes.)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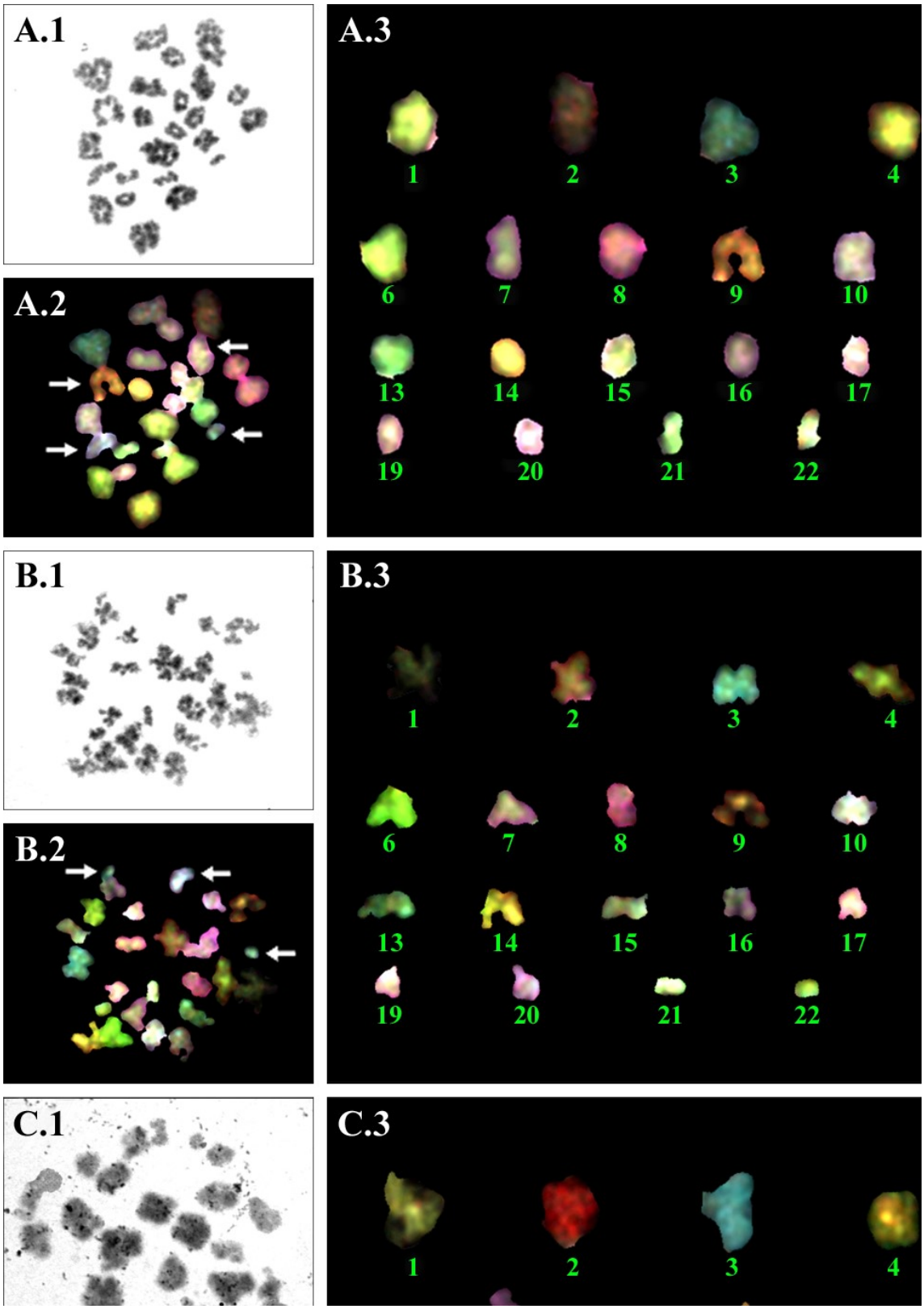
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