

New Recommendations for Robertsonian Translocation Management After Preimplantation Genetic Testing of Structural Rearrangement (PGT-SR) Attempts: What Should Be Done With Mosaic Embryos?

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Abstract

Robertsonian translocation (RT) carriers are phenotypically normal, but they are known to be at increased risk of repeated miscarriages compared with the general population estimated at about 15% of pregnancies, and also resulting in the birth of a child with a mental retardation or congenital anomalies. Preimplantation Genetic Testing (PGT) is therefore a solution for RT carriers. An appropriate probe strategy allows to differentiate balanced embryos, unbalanced embryos, and mosaic embryos. We performed the first comparative analysis between two or three probes FISH strategies to analyze if a probe strategy choice for PGT-SR studies of Robertsonian translocations (RT) influences the fate of embryos? Our investigations present 13 years of experience of PGT for Robertsonian translocation carriers to improve the accuracy of abnormality detections. A deeper analysis of 283 PGT-SR attempts by comparing two strategies of probes highlighted the irrelevance of using a third probe for FISH diagnosis and above all a significant difference of mosaic embryo rates between probe strategies. These findings could be used as new recommendations of Robertsonian translocation management in many laboratories to improve their practices. It could be readily run, less expensive, reliable and accurate. Furthermore, the propounded strategy of mosaic embryo transfer should be considered after a detailed genetic counseling.

Introduction

During the last decade, the application of new technologies based on array comparative genome hybridization (aCGH) or next generation sequencing (NGS), to preimplantation genetic testing (PGT) analysis replaced fluorescence in situ hybridization (FISH) technique in many laboratories. This has meant a broader investigation focused on aneuploidy screening¹⁻⁶. However, the establishment of these approaches remains relatively expensive and sometimes unsuited for laboratories performing diagnosis of specific chromosomal abnormalities. Furthermore, preimplantation genetic testing of aneuploidy (PGT-A) is prohibited by law in many countries.

To date, FISH technique represents the method of choice to study chromosomal rearrangements in preimplantation genetic testing of cleavage stage embryos⁷. Despite technical difficulties associated with fixation technique, FISH analysis has been developed to provide an accurate diagnostic, by improving the nuclei fixation and FISH probe protocols⁸. Since it was introduced in clinical diagnosis, different strategies have been established to improve FISH analysis. Colls et al. (2007) focused on enhancing FISH probe applications⁹. This report suggested that centromeric probes were more prone to give inconclusive results than locus specific probes. Thereby, reanalysis with additional probes binding a subtelomeric or telomeric locus specific improved dubious results. Mir et al, (2010) indicated that an additional hybridization with a locus specific probe improved the accuracy of diagnosis and found that centromeric probes were more likely to have a low confirmation rate for monosomy¹⁰. Furthermore, this study concluded that FISH analysis performed on one or two biopsied cells showed similar confirmation

rates of diagnosis between Day 3 and Day 5. These studies converge towards specific probes rather than an addition of probes which might add interpretation ambiguities.

The aim of our investigations was to improve the accuracy of meiotic segregation pattern analysis of RB chromosomes in 1631 embryos provided from 148 Robertsonian translocation carriers. We questioned whether the use of two or three probes was more efficient. Exploration of embryo status after PGT-SR was performed to establish FISH recommendations for laboratories using this technique. In addition, our concern was with the establishment of a strategy transfer for mosaic embryos mainly inspired by recent studies offering mosaic embryo transfer after PGT-A¹¹⁻¹³.

Materials And Methods

Patients

96 male and 52 female patients referred for a RT were included in this retrospective study. These patients were directed to our center to undergo a PGT between 2005 and 2018 after several years of miscarriages, abortions, primary infertility or already had an affected child. Products of conception (POC) karyotype analyses were performed in external establishments.

Compliance with Ethical Standards

The patients were informed about investigations and gave their consent before participation in the study, which was approved by the Internal Ethical Board of Montpellier University Hospital on March 08, 2005, under the reference number N °2006-45. In addition, patients were informed about the whole PGT procedure and an informed consent was obtained from all the patients involved in each medical care of the present study. PGT-SR management including all technical steps were conducted on the basis of ESHRE recommendations^{7,14}

Sperm Preparation and Controlled Ovarian Stimulation (COS)

Semen preparation and COS technique were performed as described in Zenagui and collaborators study¹⁵. After two to five days of sexual abstinence, sperm samples were collected in a sterile container. Sperm selection for ICSI treatment was performed by discontinuous PureSperm (Nidacon, Göteborg, Sweden) gradient. 1 ml of semen sample was layered upon a 40/60/80/90% PureSperm density gradient and centrifuged at 300 g for 10 min. This procedure was followed by resuspension in 1 mL of gametes handling medium (G-IVF PLUS, Vitrolife, Göteborg, Sweden) and a second centrifugation at 300 g for 10 min. Ovarian stimulation was carried out by one of the following protocols depending on estimated ovarian reserve using a long desensitizing protocol or a gonadotrophin-releasing hormone (GnRH) antagonist protocol. The oocytes were then fertilized using ICSI (intracytoplasmic sperm injection) procedure. Fertilization was evaluated 22 h following the sperm injection procedure. Embryo quality was assessed at 2 and 3 days post-microinjection. According to the percentage of anucleated fragments and to the number of blastomeres, embryos were biopsied and one (embryos ≤ 6 cells) or two cells (embryos

≥ 7 cells) were removed ¹⁶. After biopsy, embryo transfers were carried out if the embryos had continued their development. Embryo vitrification in case of a freeze-all policy was performed using Vit Kit-Freeze (Irvine Scientific, Santa Ana, CA, USA). On the day of biopsy, embryos were warmed using Vit Kit-Thaw (Irvine Scientific) in accordance with the manufacturer's instructions. Up to two embryos were transferred on Day 4 of in vitro culture and extra healthy embryos were cryopreserved, if available. Frozen embryo transfers (FET) with supernumerary embryos were achieved on a natural cycle, stimulated cycle or with hormonal replacement treatment.

FISH Procedures

Two and three colour FISH assays were used to detect all chromosome segments involved in RT and to identify transferred normal/balanced embryos. The FISH techniques on blastomeres were performed as recently described in our recent study ^{15,17}. The embryos included in this study were obtained over a period of 14 years and two different strategies have been used for FISH analysis. The first strategy involved two relevant probes, each probe is located within the terminal long arm. The second strategy required a third probe addition on one of the two translocated chromosomes. The additional probe is located in paracentromeric regions. The slides were observed using an epifluorescence Zeiss Imager Z2 microscope equipped with adequate filter sets (Zeiss, Le Pecq, France). Image acquisition was performed using a digital camera and the Isis FISH imaging system (Metasystems, Altussheim, Germany). FISH interpretation on one (embryos ≤ 6 cells) or two blastomere nuclei (embryos ≥ 7 cells)

Embryo Chromosome Pattern Classification

According to the scoring criteria previously published ⁷, FISH interpretation on blastomere nuclei was assigned as follows:

“Normal or balanced” if the two nuclei showed two distinct signals for each chromosome tested.

“Mosaic” if the first nucleus appeared balanced and the second unbalanced, “Unbalanced” if the two nuclei showed an abnormal fluorescence pattern. “Undetermined combination” when a diagnosis was not possible because of technical problems. FISH interpretation on one cell nucleus was assigned as above, except for mosaic embryos since it is necessary to have two nuclei to attribute this status.

Statistical Analysis

An independent X^2 test was used to compare PGT results of male and female carriers. The differences were considered statistically significant when $P < 0.05$.

Results

Pregnancies and PGT Outcomes

In all, 1,631 embryos reached at least a six-cell stage and were of sufficient quality to undergo a biopsy were analyzed. 283 cycles were performed in 148 couples with RT, including 96 males and 52 female

carriers. A total of 258 embryos continued to develop and were successfully transferred at Day 4 post fertilization. 17 pregnancies were biochemical and 12 spontaneously terminated and weren't considered in clinical pregnancies. A clinical pregnancy was achieved in 106 patients (35.8% pregnancy rate per embryo transfer). 109 healthy babies were delivered (38.4% birth rate per embryo transfer). Despite our recommendations about POC analyses, only 4/89 couples underwent karyotype analysis to avoid any risk of imbalance. PGT and POC data analyses were summarized in Table 1.

Since the probe strategies might influence PGT analysis, results were observed for two and three probes separately and regardless of carrier sexes. The accuracy of probes was ~ 97%. The risk of error after probes testing was acceptable as described in PGT-SR study ¹⁸. Global analysis of balanced and unbalanced embryo rates found no difference observed according to the FISH probe strategies (40.49% for two probes and 40.19% for three probes, P=1) (Table 2). PGT attempts were carried out on two biopsied cells if the embryo had more than 6 cells, on one biopsied cell if the embryo had fewer than 6 cells or when a diagnosis was not possible because of technical problems. The analysis of balanced embryos involving one or two blastomere cells showed no difference observed in balanced embryo rates (p=0.1) according to the FISH probe strategies (Table 3). Interestingly, mosaic embryo rates observed with two probes were significantly higher to the one observed with three probes (34.4 % versus 19.7 %, p=0.0001). Inversely, abnormal embryo rates observed with two probes had a lower percentage than observed with three probes (Table 4).

Discussion

RT carriers are phenotypically normal, but they are known to be at increased risk of repeated miscarriages compared with the general population estimated at about 15% of pregnancies, and also resulting in the birth of a child with congenital anomalies or a mental retardation ¹⁹. Preimplantation Genetic Testing (PGT) is therefore a solution for RT carriers. Although the advent of new molecular technologies provided an accurate PGT-A analysis, FISH technique is widely used in PGT-SR and is considered as the technique of choice for structural abnormality detections in embryonic samples ^{10,14,20-24}. The objective of our study was to minimize the risk of transferring a viable unbalanced embryo by adopting a reliable FISH strategy.

Our approach was based on a comparison of the efficiency of two FISH probe strategies. During PGT-SR attempts, similar rates of balanced embryos were observed with two versus three FISH probes used (40.4% and 40.5% respectively). As mentioned, embryos were classified as normal/balanced if the two nuclei showed two distinct signals for each probe tested. We showed clearly that an addition of a third paracentromeric probe didn't improve the rate of balanced embryos and consequently embryo transfer rates. Several reports had suggested that centromeric probes were tending to give inconclusive results and were more prone to be involved in No Result (NR) than locus specific probes ⁹. These observations were confirmed by Mir et al. (2010) ¹⁰. Moreover, when one or two blastomere cells were analyzed, embryos showed similar confirmation rates of balanced embryos either with two or three probes. The

biopsy of two cells did not increase the accuracy of FISH technique. Our results confirm these reported data ¹⁰. The authors revealed similar rates of diagnosis between Day 3 and Day 5 when one or two blastomeres had been analyzed on Day 3 and thus, they concluded, that biopsy of two blastomeres did not increase the accuracy of FISH technique.

On the basis of ESHRE recommendations ^{7,14}, balanced embryos are consistently and reliably detected when probes set contain sufficient probes to detect all expected unbalanced forms of rearranged chromosomes. In this study, we showed that two-FISH probes allowed the detection of all imbalances. The addition of the third probe was not essential for PGT-SR analysis and detection of imbalances (Table 2), but mainly to strengthen physician interpretations. Given the results achieved through this study, we might conclude that an accurate diagnosis may be performed on RT carriers with a set of two subtelomeric FISH probes as well as a diagnosis performed with a set of three FISH probes. A single cell biopsy is adequate and will not compromise the 'confidence,' of the result given by the 2 probes strategy.

During this study, we also observed that rates of mosaicism were ranged from 19.7% to 34.4%. Reassuringly, mosaicism rates performed at a cleavage stage were similar to the intervals cited in many previous studies (24% to 53%) in embryos at risk of Robertsonian translocation ^{20,25}. We thoroughly compared the incidence of mosaic chromosomal abnormalities according to FISH probe strategies. Interestingly, unlike the category of balanced embryos, mosaic embryo results showed different rates ($p < 0.001$) according to FISH probe strategies. Mosaic embryo rates were raised when FISH were performed with 2 probes compared to results obtained with a third paracentromeric probe. Inversely, the rate of unbalanced embryos decreased when diagnosis was performed with two than three FISH probes. We supposed that the addition of a third probe could disturb signal FISH interpretation. Mosaic embryos were more and more considered mainly for couples without euploid embryos ^{3,12,13,26}. Concern about transferring mosaic embryos arises from the apprehension that it could lead to abnormal pregnancies, although the following study showed that transfers of mosaic embryos resulted into healthy live births ²⁷, negative or biochemical pregnancies. Other recent studies by means of NGS based PGT-A performed an analysis of mosaic blastocyst transfers resulting in ongoing pregnancies and lead to babies that are healthy by routine examination ^{3,12,13,26}. However, pregnancy rates were directly related to the degree (low and high) and/or type (single or multiple segmental mosaics, whole chromosome mosaics, complex mosaics) of mosaicism. This has undoubtedly added a complexity to the PGT interpretation. Victor et al. (2019) Published that embryos with a single segmental mosaic fared better than the other types affected by one or two whole chromosomes, multiple segmental gains/losses, or complex mosaics ¹³. Even though authors described that cells harboring monosomies and trisomies result in similar clinical outcomes, we note that future larger studies are needed to refute or corroborate these conclusions. Through the present findings, we sought to propose an approach for mosaic embryo transfers resulting from Robertsonian translocations inspired by Grati et al. (2018) conclusions ²⁸. Our strategy of mosaic embryos transfer after a biopsy on Day 3 is based on risk evaluation to give a birth of an abnormal child. To this end, we established a classification according to chromosomal monosomies and trisomies embryo status. Mosaic euploid/monosomy embryos involving acrocentric chromosomes (13, 14, 15, 21,

22) could be considered for transfer with a lower risk of viability followed by a mosaic euploid/trisomy embryos involving chromosomes 14, 15, 22. Mosaic embryo euploid/trisomies (chromosomes: 13, 21) are the lowest priority for the transfer because it may lead to a viable affected birth (figure 1). Contrary to Grati's recommendations, we consider that the risk related to uniparental disomies (UPD) of chromosomes 14, 15 should be excluded. A recent study performed in 1747 UPD, demonstrated that the risk of UPD in a fetus carrying an inherited Robertsonian translocation was estimated ~0.06%, which was significantly lower than previous estimations²⁹.

To conclude, many diagnostic laboratories are still performing PGT-SR analysis with FISH probe strategies. This choice is explained by local regulations of certain countries. Further, the controversial contribution of the new technologies (NGS) in terms of costs and gains discouraged laboratories. Through the present study undertaken on the largest cohort of male and female RT carriers, a reliable and accurate FISH probe strategy could be proposed to PGT-SR laboratories to manage RT carrier attempts. This analysis allowed to demonstrate that an increase of FISH probes number from two to three didn't improve balanced embryo rates carried out on one or two biopsied cells but reveals a significant mosaic embryo number (34.4 % versus 19.7%).

To improve the possibility of pregnancy in absence of balanced embryos obtained, we propounded a strategy of mosaic embryo transfer after a Day 3 FISH analysis. This possibility should be considered after detailed counseling to ensure that the patient is aware of risks and potential benefits is of utmost importance to ensure informed decision-making by patients.

In addition to the considerations of embryo selection, it is essential that genetic counseling include a discussion about prenatal testing benefits and limitations. A prospective study on mosaic embryo transfer is currently discussed as part of a metacentric research project. Outcomes of pregnancies achieved following a mosaic embryo transfer will be decisive to confirm our proposition.

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Tables

Table 1: PGT and prenatal diagnosis outcomes of investigated couples

Clinical Parameters	PGD Outcomes	
	(Two probes)	(Three probes)
Number of couples	148	
Number of male/female carrier	96/52	
Number of Cycles	89	194
Number of analysed embryos	494	1137
Number of normal/balanced embryos	200	457
Number of Fresh embryo transfers (ET)	78	180
Pregnancy rates per ET	(27/78) 34.6%	(64/180) 35.5%
Number of frozen embryo transfers (FET)	11	22
Clinical pregnancy rate per FET transfer	(4/11) 36.4%	(11/22) 50%
Ongoing pregnancies	0	4
Pregnancies	31	75
Life-born children	109	
Prenatal diagnosis (PND): Yes	4: XX/XY and 3: balanced	
Prenatal diagnosis (PND): No	89	
Non-invasive prenatal diagnosis	5 normal	

Table 2: PGT analysis according to FISH probe strategies

PGT results	Two FISH probes	Three FISH probes
Normal/Balanced embryos	200 (40.49%)	457 (40.19%)
Abnormal embryos (unbalanced and mosaic embryos)	294 (59.51%)	680 (59.81%)

Table 3: Analysis of balanced embryo rates according to one or two biopsied cells and FISH probe strategies

Type of diagnosis	Two FISH probes	Three FISH probes
2 biopsied cells	171 (85.5%)	365 (79.9%)
1 biopsied cell	29 (14.5%)	92 (20.1%)

Table 4: Analysis of mosaic embryos according to FISH probe strategies

PGT results	Two FISH probes	Three FISH probes
Mosaic embryos	101 (34.4%)*	134 (19.7%)
Unbalanced embryos^a	193 (65.6%)	546 (80.3%)*

*The differences were considered statistically significant when $p < 0.05$; ^(a): balanced embryos were excluded from the analysis.

Figures

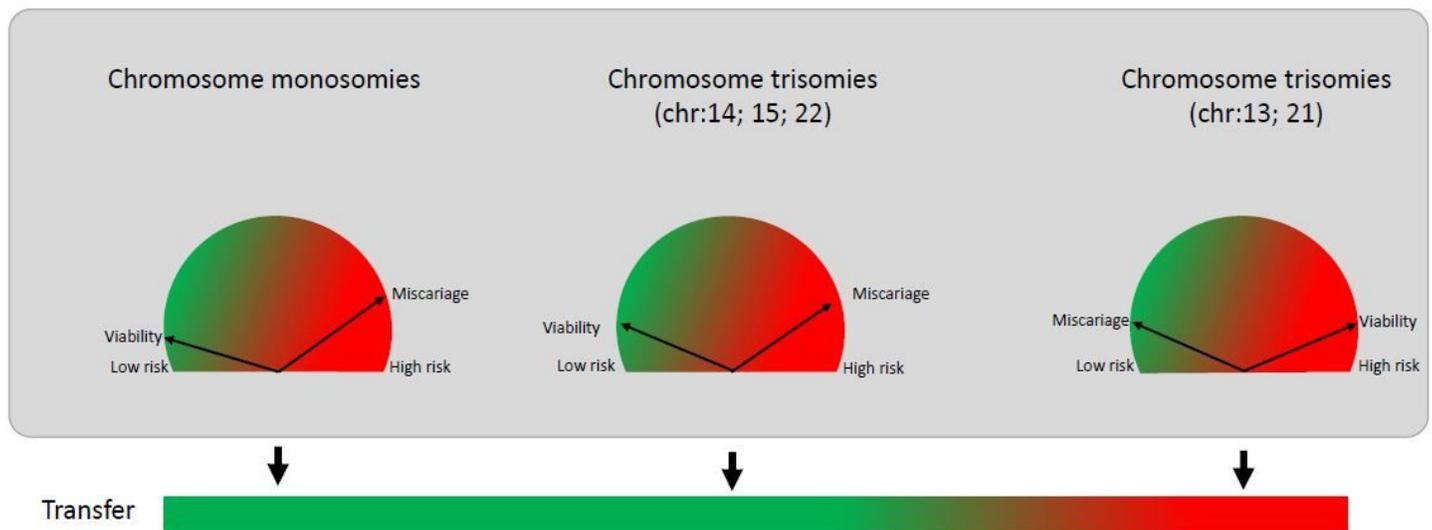


Figure 1

The prioritization of mosaic embryo transfer, based on the involved chromosomes. Euploid/monosomies are preferred over euploid/trisomies (chromosomes: 14, 15, 22) that are low priority. Mosaic embryo euploid/trisomies (chromosomes: 13, 21) are of the lowest priority for transfers. The balance between miscarriages and viability risks are based on an available data derived from pregnancy outcomes in which these chromosomes are involved.

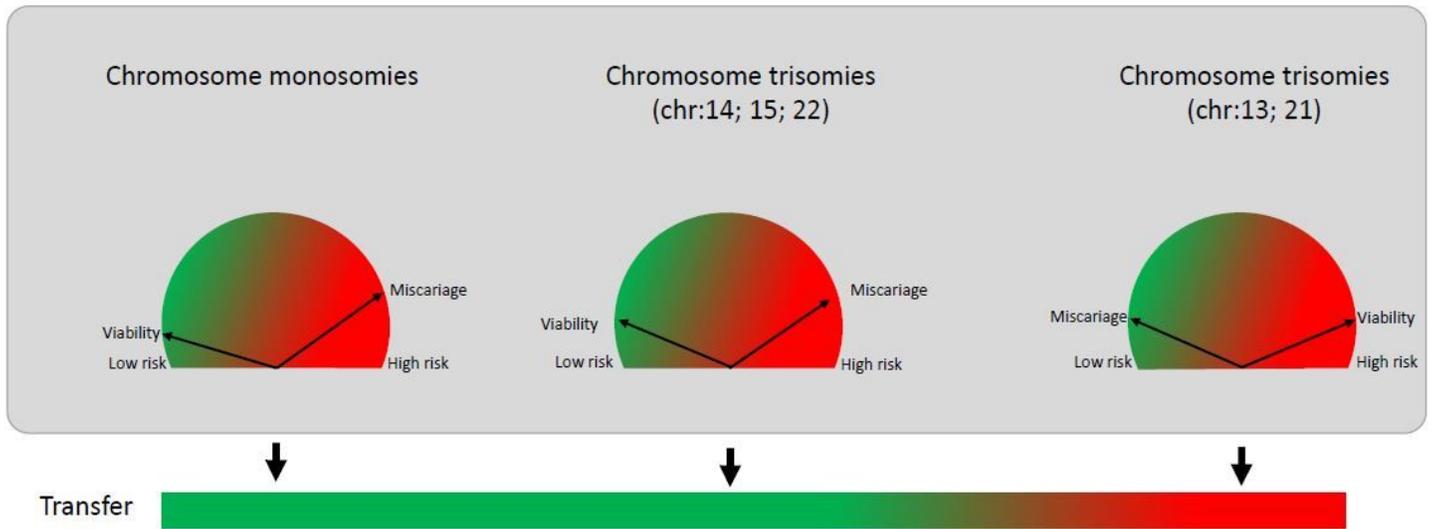


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