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Application of FISH method for preimplantation genetic diagnostics of reciprocal and Robertsonian translocations

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Abstract

Introduction. Carriers of reciprocal (RCP) and Robertsonian (RT) translocations are known to be at risk for reproductive difficulties. Preimplantation genetic diagnosis (PGD) is one of the options these carriers have to try to fulfill their desire to have a child. The FISH technique is one of the best method to detect RCPs, and, together with the Next Generation Sequencing, to diagnose RTs. The aim of the present study was to assess the usefulness of the FISH method for rapid diagnosis of translocations in our center to improve the reproductive counseling.

Material and methods. From 2008 to 2012 one hundred and twenty seven fresh cycles of the *in vitro* fertilization (IVF; without freezing embryos) were performed in 42 couples with an RCP and 35 couples with an RT translocations. The patients were diagnosed before IVF as translocation carriers and therefore they opted for PGD. The classical FISH protocol has been applied with specific oligonucleotide probes.

Results. In total 521 blastomeres were tested in order to determine the presence or absence of genetic anomalies resulting from one of the parents being a translocation carrier. Despite the large number of abnormal embryos (407 embryos — 78.1% of all examined embryos), 19.4% of blastomeres appeared to come from a normal or balanced embryos that may have been transferred to the uterus. In 63 of the 127 cycles embryo transfer (ET) was feasible and 24 women had a successful singleton or twin pregnancy. Thus, a live delivery rate of 18.9% per started cycles and 38.1% per cycle with ET was obtained.

Conclusion. FISH should be regarded as an optimal preimplantation genetic diagnosis method for specific RCP and RT translocation carriers to increase the chance of successful IVF procedure. *(Folia Histochemica et Cytobiologica 2015, Vol. 53, No. 2, 162–168)*

Key words: preimplantation genetic diagnosis; Robertsonian translocations; reciprocal translocations; IVF; embryo transfer; FISH

Introduction

Difficulties associated with conception are an important and common problem of the modern world. It is estimated that it affects approximately 15% of couples in reproductive age. About 35% of the issues involved with infertility are due to the man, another 30% due to the woman, and 10% result from complications associated with both partners [1]. There are many causes of infertility, however, approximately 30% of cases are associated with genetic factors such as translocations [2].

Balanced chromosomal translocations involve breaks in two chromosomes and abnormal repair of the chromosomal fragments resulting in the trans-

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position of genetic material from one chromosome to another one without loss of any genetic material. Carriers of balanced translocations are phenotypically normal, unless one of the translocation breakpoints interrupts an important gene or position effects take place. It has been estimated that 1 in 625 individuals carries a balanced chromosomal translocation [3]. Even phenotypically normal carriers of balanced chromosomal translocations may experience reduced fertility, spontaneous abortions or birth defects [4]. In couples suffering from recurrent miscarriage, the incidence of the couple being a carrier of a structural chromosome abnormality is approximately 4–5%, mainly including reciprocal (RCP) and Robertsonian (RT) translocations [5]. Reduced fertility in translocation carriers may in part be the result of formation of a quadrivalent or trivalent structure (RCP and RT translocations, respectively) during meiosis what enables homologous chromosomes to pair. Theoretically, chance of producing normal or balanced gametes is 4:32 for RCP translocation, and 4:16 for RT translocations. However, the actual percentage depends on several factors, including chromosomes involved, the breakpoints, and carrier's gender [6, 7]. It should be noted that levels of unbalanced gametes will be significantly higher than the empiric risk of having a chromosomally unbalanced live birth. This is due to the fact that many, if not most, of the segregate products, will be spontaneously aborted early in development (depending on the chromosomes and size of the segment involved) [4, 8].

Preimplantation genetic diagnosis (PGD) is an option for couples carrying balanced translocations. Fluorescence *in situ* hybridization (FISH) allows for analysis of metaphase nucleus from biopsied embryos. However, the number of chromosomes studied by FISH is limited to the number of chromosome-specific probes available. The strategy of FISH analysis depends on chromosomes involved in the structural rearrangement and the size of the translocated segment, and usually involves a combination of the locus-specific, centromeric, and/or subtelomeric probes.

The aim of the present study was to use the FISH method for rapid diagnosis of translocations during *in vitro* fertilization (IVF) fresh cycle (without freezing embryos). We retrospectively analyzed the results of 5 years (2008–2012) of PGD for RCP and RT carriers to improve the reproductive counseling.

Material and methods

Patients. The study protocol was approved by the Institutional Review Board and written informed consent was given by each couple participating in PGD. During medical and genetic counseling, the procedure, the genetic risk of chromosome translocation, an advantages and limitations of PGD were explained to the couples. The risk of misdiagnosis attributable to embryonic mosaicism and the 1–2% technical error rate of the FISH procedure used in PGD were also detailed.

The retrospective analysis included 42 couples who were RCP and 35 couples who were RT carriers. Participants underwent 127 PGD cycles between September 2008 and May 2012. Parental age, type of translocation, family and reproductive history were recorded.

Oocyte retrieval, assessment of fertilization and embryo development. All PGD couples underwent IVF procedure after stimulation using long-term protocol of pituitary desensitization with the GnRH agonist (Diphereline 0.1 mg/d; Pharmacia Upjohn, Kalamazoo, MI), starting on day 14 of the oral contraception cycle as described previously [9]. The oocyte retrieval procedure was performed 35 hours after administration of human chorionic gonadotropin (hCG; Ovitrelle, Merck Serono S.p.A., Modugno, Italy), under the control of transvaginal ultrasound. Retrieved oocytes were fertilized by intracytoplasmic sperm injection (ICSI) and checked daily for the presence of pronuclei and polar bodies. Fertilized embryos were cultured in G1 medium (Vitrolife, Västra Frölunda, Sweden) at 37°C under the atmosphere of 6% CO_2 and 5% O_2 in air for 3 days. Embryo development was assessed daily and embryos that reached the 6–8-cell stage, were equal in size and had fewer than 30% of fragmentation, were biopsied on day 3.

Embryo biopsy and blastomere fixation for FISH. For biopsy, embryos were incubated in G1 medium. Perforation in the zona pellucida membrane has been done by chemical method using Tyrode's solution. A single blastomere was taken from each embryo. After biopsy, the embryo was washed, transferred to G2 medium (Vitrolife, Västra Frölunda, Sweden) and cultured for the next two days. Aspirated blastomere samples were prepared as described by Coonen et al. [10]. Briefly, samples were lysed in hypotonic buffer for 1–2 minutes. The cell nuclei were separated and fixed on glass slides using 0.01N HCl/0.1% Tween20. The slides were air-dried, washed with PBS for 5 min and dehydrated in a series of 70%, 80%, 100% ethanol solutions before FISH.

Fluorescent *in situ* hybridization. The FISH probes used included locus-specific (LSI; Interphase FISH LSI®-Fusion Probe; Abbott Molecular, Des Plaines, IL, USA) and subtelomeric regions probes (TelVysion probes; Abbott Molecular; Kreatech™ FISH probes, Kreatech Diagnostics, Amsterdam, The Netherlands), specific for investigated chromosomes and labeled with different colors. The specificity and sensitivity of the probes had been previously tested using patients' lymphocytes' cultures. All probes had specificities of 100% and efficiencies of 84–95% [11].

Mixtures of the oligonucleotide probes were prepared according to the manufacturer's protocol. The nuclei and probes were combined on the glass slides and denaturized by heating to 73°C. The slides were incubated in the humidified chamber at 37°C and the results were assessed on the next day. In urgent situations, our modified short protocol with 4 hour hybridization at 73°C was used which allowed the material to be analyzed in only one day. The slides were analyzed using a fluorescence microscope (Olympus BX61, Olympus, Tokyo, Japan) and images were captured using camera (F view, Olympus). Blastomeres presenting two signals for each probe were classified as normal or balanced, while any other combination was classified as unbalanced. FISH signals were independently scored and interpreted by two observers.

Embryo transfer and pregnancy evaluation. Embryos with normal and balanced FISH signals were transferred into the uterine cavity on the 5th day after oocyte retrieval. Serum β-hCG concentration was measured 3, 6 and 10 days after transfer. Clinical pregnancy was defined as the presence of a fetal heartbeat on vaginal ultrasonography at 4–5 weeks after embryo transfer. The fertilization rate was defined as the proportion of fertilized oocytes compared to the number of all cells undergoing fertilization. The implantation rate was defined as the percentage of embryos which successfully undergo implantation compared to the number of embryos transferred in a given time period. Cancellation rate was defined as the number of cancelled cycles compered to all cycles. Miscarriage rate was defined as the percentage of spontaneous abortions which occur up to 12 weeks of pregnancy and the number of pregnancies. Live birth rate was defined as the percentage of all cycles that lead to live birth.

Statistical analysis. Data comparisons were made using chi-square test. All statistical analyses were performed using STATISTICA data analysis software system, version 10 (StatSoft Inc., Tulsa, OK, USA). Statistical significance was defined as $p < 0.05$.

Results

The characteristics and clinical outcomes of the PGD cycles conducted in RCP and RT carriers are summarized in Table 1. During the 5-year time period, 127 cycles of PGD were performed in 42 couples with RCP, including 25 female (the mean age 32.3 ± 3.72; range 25–41 years) and 17 male carriers (the

Table 1. Patients' characteristics and PGD outcomes of investigated couples

	Reciprocal translocation	Robertsonian translocation	Total
Average age of women (mean ± SEM)	32.3 ± 3.72 y	33.9 ± 3.64 y	
Average age of men (mean ± SEM)	35.2 ± 5.03 y	35.5 ± 3.89 y	
Number of couples	42	35	77
Number of male/female carriers	17/25	21/14	38/39
Number of cycles	64	63	127
Number of retrieved oocytes	585	494	1079
Number of 2PN[a] embryos	414	330	744
Fertilization rate (%)	70.8	66.8	68.9
Number of biopsied embryos	328	220	548
Number of analyzed blastomeres	310	211	521
Number of normal or balanced embryos	48	53	101
Proportion of normal or balanced embryos to all embryos (%)	15.5	25.1	19.4
Number of embryo transfers (ET[b])	31	32	63
Implantation rate (%)	34.5	41.2	38.7
Cancellation rate (%)	64.1	61.9	63.1
Number of clinical pregnancies	10	14	24
Clinical pregnancy rate per ET[b] (%)	32.3	43.7	38.1
Miscarriage rate (%)	10	7.1	8.3
Live birth rate per couple (%)	21.4	37.1	28.6

[a]PN — pronuclei; [b]ET — embryo transfer
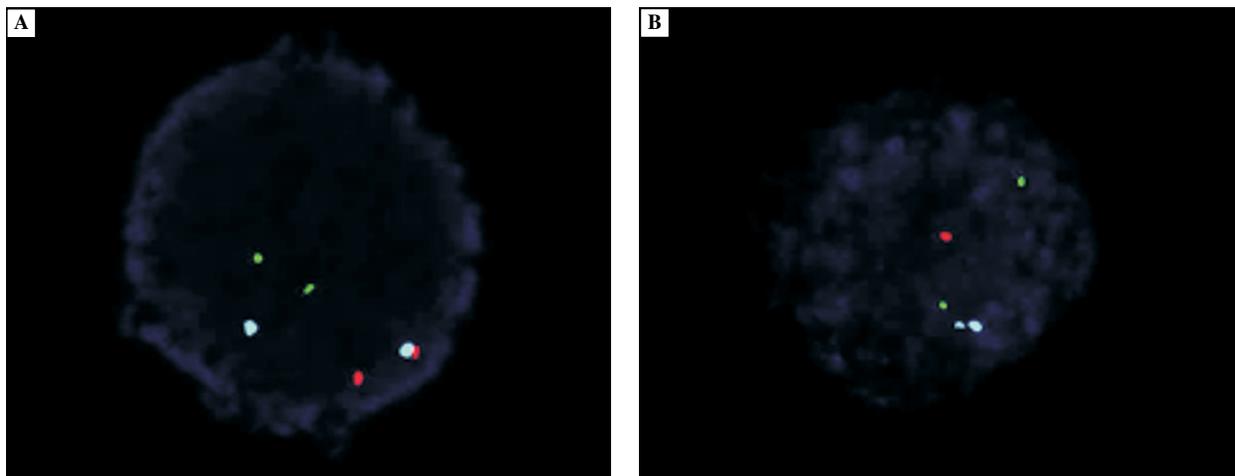
Figure 1. Blastomeres from two embryos of one couple analyzed by FISH probes specific for chromosomes 4 and 7. CEP7 (Spectrum Aqua) for centromere, 4p (Spectrum Green) and 7p (Spectrum Red) for appropriate telomeric regions. **A.** Genetically balanced (or normal) blastomere: nuc ish (CEP 7p11.1-q11.1x2) (4p02x2) (ST7pterx2); embryo was used for embryonic transfer; **B.** Genetically incorrect blastomere: nuc ish (CEP 7p11.1-q11.1x1) (4p02x2) (ST7pterx1); embryo was excluded from the embryonic transfer
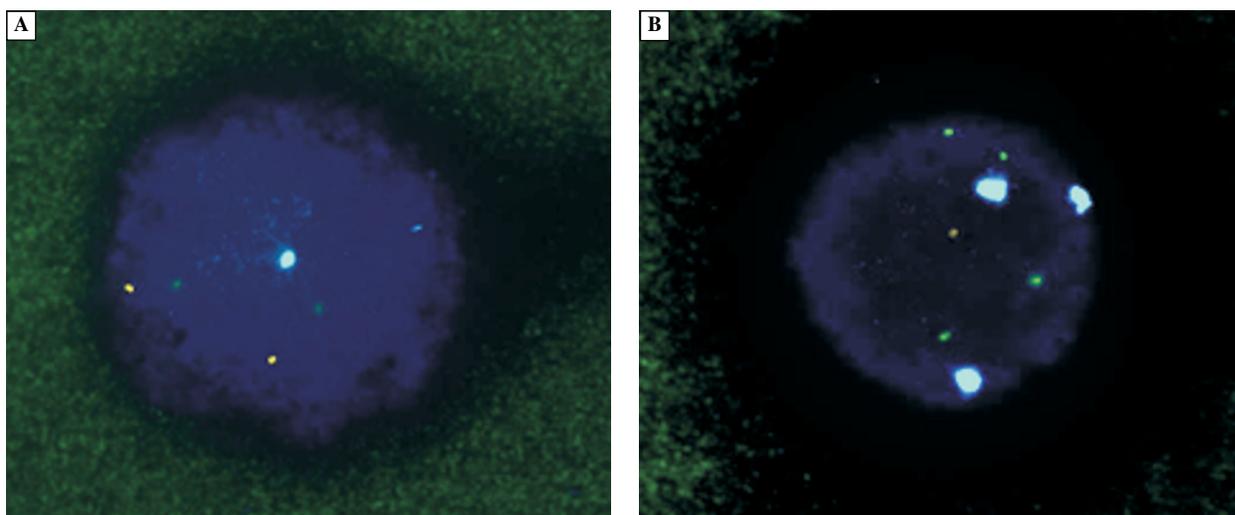
Figure 2. Blastomeres from two embryos of one couple analyzed by FISH probes specific for chromosomes 4 and 10. CEP 10 (Spectrum Aqua) for centromere, 10p (Spectrum Green) and 4q (Spectrum Orange) for appropriate telomeric regions. **A.** Genetically balanced (or normal) blastomere: nuc ish (CEP 10p11.1-q11.1x2) (4q D4S2930x2) (10p TEL006x2); embryo was used for embryonic transfer; **B.** Genetically incorrect blastomere: nuc ish (CEP 10p11.1-q11.1x3) (4q D4S2930x1) (10p TEL006x4); embryo was excluded from for embryonic transfer

mean age 35.2 ± 5.03; range 27–48 years), and in 35 couples with RT, including 14 female (the mean age 33.9 ± 3.64; range 22–42 years) and 21 male carriers (the mean age 35.5 ± 3.89; range 28–45 years).

The mean number of oocytes aspirated from the 77 couples, including couples with either a male or female RCP and RT carriers, was 11.7 per cycle. The percentage of fertilized embryos reached a developmental stage that permitted successful biopsy was 79.2% and 66.7% for RCP and RT translocation carriers, respectively. Out of the 521 blastomeres tested, only 101 (19.4%) were normal or balanced (Fig. 1A; 2A), 407 (78.1%) were abnormal (Fig. 1B; 2B) and 13 (2.5%) yielded inconclusive results. The fertilization rate was 70.8% for RCP and 66.8% for RT translocation carriers but proportion of normal or balanced embryos differed significantly and was higher in the RCP group ($p < 0.05$).

The number of clinical pregnancies determined by serum hCG levels and embryo's heart beats in

the 5–6th week was 26 (41.3%), out of 63 cycles of embryo transfer; however, two pregnancies were spontaneously terminated at 9 and 11 weeks of gestation.

No differences were evident during pregnancy and after delivery what was ascertained by the medical records.

Discussion

We present here for the first time the Polish experience on embryo-PGD use for rapid detection of RCP and RT translocations during IVF fresh cycle (without freezing embryos). Retrospective analysis of results obtained during 5 years revealed satisfying fertilization rate (68.9%) after exchange of genetic material derived from RCP and RT translocations carriers. With the use of FISH technique, we also identified and excluded 78.1% of abnormal embryos which can occur in couples who are carriers of chromosomal aberrations. This result is in agreement with the worldwide data which presents high rates of unbalanced embryos detected during PGD of RCP [12–16] and RT translocations [12–17].

In our study, the number of retrieved oocytes and the fertilization rate did not differ between RCP and RT couples. However, proportion of normal or balanced embryos to all embryos analyzed differed significantly between analyzed groups. American Preimplantation Genetic Diagnosis Special Interest Group have published similar results regarding rates of unbalanced embryos distinguished between RCP and RT translocations carriers, where they observed more pregnancies in favor of patients with RT translocations [13, 15, 16, 18–21]. However, it has to be noted that other authors did not observed such discrepancies [14, 22]. These differences may result from application of divergent sets of FISH probes [14] or distinct timing of blastomere biopsy [14, 20] as the number of cells in the embryo is growing and the abnormality rate may change depending on the time of the day [20].

In both types of translocations analyzed in this study, we found high number of embryos which were suitable for blastomere biopsy, which is in agreement with other studies [11, 22]. In view of poorer assisted reproductive technology outcome in RCP and RT patients and slower growth profile of the embryos obtained from the translocation carriers [20], the number of biopsy acceptable embryos seems to be satisfying. However, we observed low percentage of transferable embryos (19.4%), which confirms observations of other authors [7, 14, 16, 22]. Moreover, the European Society for Human Reproduction and Embryology (ESHRE) PGD Consortium released

data collection X (2010) where they detailed the PGD results of 57 participating centers [23]. Considering oocytes that were successfully fertilized, biopsied and gave a successful diagnostic results (n = 53,652), the average of transferable embryos (normal/balanced) was only 26% (938/3,652) [23]. These data sets clearly provide compelling evidence that carriers of structural chromosomal rearrangements produce chromosomally unbalanced gametes, capable of fertilization. Indeed, the risk of having unbalanced sperm is 7–16% [12, 24–26] and the risk of having unbalanced oocytes 32–36% [13, 27] in transformation carriers as demonstrated by FISH method, reflecting higher maternal impact on the embryo [16, 22]. Combined with factors such as maternal age [28], abnormal embryo morphology [20, 29], lack of cell cycle check-points in the early embryonic mitotic divisions [30], all this issues cumulate and result in very high proportion of aneuploid embryos.

Clinical pregnancy rate in our study, in a group of patients who are < 35, is at the similar level (38.1%) as the clinical pregnancy rate reported by other authors (35.9% by Lim et al. [14]; 39% by Fischer et al. [15]). We also observed spontaneous abortion incidents. In contrast to other investigators [14], we did not perform the cytogenetic analysis of aborted tissue. It is common to observed mosaic embryos in translocation carriers [30–33]. Tetraploid FISH signal can be also visualized in unbalanced embryo [31, 32, 34]. Patients who are translocations carriers also suffer from antiphospholipid syndrome, luteal phase deficiency, factor V Leiden heterozygosity and Crohn's disease [33]. Thus, in the case of miscarriage which happened despite PGD, occurrence of unbalanced form of chromosomal aberration or parental effect cannot be ruled out.

IVF, when combined with PGD for structural rearrangements, is characterized by a very high probability of achieving a viable embryo and unaffected pregnancy. Close to a sevenfold reduction in pregnancy loss rate was obtained in translocation carriers couples who afforded PGD [15]. On the other hand, Franssen et al. [36] performed a systematic review of the published data and found that after natural conception, live birth rate per couple varied between 33% and 60% (median 55%). After PGD, live birth rate per couple varied between 0 and 100% (median 31%). Thus, PGD improves the live birth rate in couples with recurrent miscarriage [4, 7, 18, 22] but for those carrying a structural chromosome abnormality, its efficiency still seems to be insufficient [36]. However, for couples who are carriers of translocations with increased risk of chromosomally unbalanced offspring and increased risk of recurrent miscarriages, the PGD is still a chance

to exclude misdiagnosis and to choose the embryo that could be transferred preferentially [12].

FISH technique is a relatively good method of pre-implantation diagnosis of RCP and RT translocations. However, there are other possible approaches to RT translocation such as a multiplex fluorescent PCR using polymorphic microsatellite markers for the detection of numerical changes in chromosomes using their allelic fingerprints and Next Generation Sequencing (NGS). In the case of diagnostics of RCP translocations, progress was also reported in the development of single-cell comparative genome hybridization (CGH) technique and microarrays to enable PCR-based testing for translocations. However, the most important limitation of the present CGH protocol is still the three-day duration of the procedure, which is incompatible with the current laboratory framework for PGD. Therefore, for the moment, the best currently used method for the diagnosis of RCP translocation during IVF cycle is FISH.

In line with previously published PGD series describing application of FISH method for rapid diagnosis of translocations, we showed that this technique has a great potential to be a useful tool for embryos selection, especially in couples being a carriers of RCP and RT translocations or in improving reproductive counseling in couples with recurrent miscarriages.

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