J Assist Reprod Genet (2011) 28:77–83
DOI 10.1007/s10815-010-9483-7

GENETICS

Preimplantation genetic haplotyping a new application for diagnosis of translocation carrier's embryos- preliminary observations of two robertsonian translocation carrier families

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Received: 8 July 2010 / Accepted: 13 September 2010 / Published online: 25 September 2010

Abstract
Purpose Preimplantation genetic diagnosis using fluorescence in-situ hybridization (PGD-FISH) is currently the most common reproductive solution for translocation carriers. However, this technique usually does not differentiate between embryos carrying the balanced form of the translocation and those carrying the homologous normal chromosomes. We developed a new application of preimplantation genetic haplotyping (PGH) that can identify and distinguish between all forms of the translocation status in cleavage stage embryos prior to implantation.
Methods Polymorphic markers were used to identify and differentiate between the alleles that carry the translocation and those that are the normal homologous chromosomes.
Results Embryos from two families of robertsonian translocation carriers were successfully analyzed using polymorphic markers haplotyping.
Conclusions Our preliminary results indicate that the PGH is capable of distinguishing between normal, balanced and unbalanced translocation carrier embryos. This method will improve PGD and will enable translocation carriers to avoid transmission of the translocation and the associated medical complications to offspring.

Keywords Chromosomal translocation · Fertility problems · Fluorescence in-situ hybridization · Polymorphic markers · Preimplantation genetic diagnosis

Capsule PGH differentiates between normal, balanced and unbalanced translocation carriers' embryos and enables the avoidance of translocation's transmission and the associated medical complications to offspring.

This work was performed in partial fulfillment of the requirements for a Ph.D degree of Jana Shamash, Sackler Faculty of medicine, Tel Aviv University, Ramat Aviv, Israel.

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Introduction

Robertsonian translocations are frequent chromosomal structural abnormalities, with an incidence of 1.23/1,000 newborns [1]. Robertsonian translocation involves two acrocentric chromosomes that fuse near the centromere region with loss of the short arms. Despite this loss, usually an adverse phenotype is not associated with this type of translocation, since the short arms contain mainly tandemly repeated satellite DNA sequences and the ribosomal RNA genes, which are present on all five pairs of acrocentric chromosomes [2]. Although translocation carriers are phenotypically normal, there is a high risk of producing

unbalanced gametes due to complex segregation modes during meiosis. A trivalent structure is formed at meiosis I through pairing of translocated chromosome and the two corresponding normal acrocentric chromosomes. This trivalent structure undergoes one of three modes of segregation: alternate, adjacent or a rare 3:0 segregation patterns. Only alternate segregation pattern results in normal or balanced gametes, the other two segregation patterns generate unbalanced types of gametes. This high proportion of unbalanced gametes is the reason for translocation carriers to experience difficulties in achieving and maintaining pregnancy, and as a result, they frequently suffer from spontaneous abortions and are at a significant risk to deliver a phenotypically abnormal offspring with major congenital anomalies [3].

Here we present two families with Robertsonian translocations. One parent in the first family carries the most common Robertsonian translocation involving chromosomes13 and 14 [4]. A potential live born with an unbalanced outcome of this translocation will have trisomy 13 -Patau syndrome the empirical risk of occurrence is 0.4% [5]. The second family, the parent carries a translocation that involves chromosomes 15 and 21. The potential live born with an unbalanced outcome from this translocation will have 15/21 is trisomy 21- Down's syndrome (OMIM190685) with 10% empirical risk of live born [3]. Furthermore, nonhomologous Robertsonian translocation carriers are at risk (0.6–0.8%) for having an offspring with uniparental disomy (UPD) following trisomy rescue [6–8]. UPD has been described for all the acrocentric chromosomes, but an abnormal phenotype has been associated only with UPD 14 and 15, probably since they harbor imprinted genes [9, 10]. The risk of an abnormal phenotype in Robertsonian translocations involving the rest of the acrocentic chromosomes, is limited to the manifestation of recessive disorders due to partial or complete isodisomy.

Preimplantation genetic diagnosis (PGD) is an alternative to the prenatal diagnosis and termination of affected pregnancies [11–14]. PGD by interphase fluorescence in-situ hybridization (FISH-PGD) analysis provides an opportunity to detect and select normal or balanced embryos for transfer. Despite the significant advantages provided by FISH- PGD, there are still technical limitations and one of them is that FISH technique for PGD does not differentiate between embryos carrying the balanced form of the translocation and the normal embryos carrying homologous normal chromosomes without the translocation. Therefore, using FISH-PGD does not omit the risk for the translocation carriers to have pregnancies with an unbalanced translocation and also can't secure them the prevention of transmitting reduced fertility to their offspring.

Our goal was to develop a new application of preimplantation genetic haplotyping (PGH) that can identify chromosomally normal embryos and distinguish them from embryos carrying balanced or unbalanced translocation prior to implantation. For this purpose, haplotypes of the chromosomes involved in the translocation and the normal homologous chromosomes are established using informative polymorphic markers, in the parents and in another related individual with a known karyotype result. Once the alleles that correlate with the translocated chromosomes are characterized, PGH of the embryos is possible and only embryos carrying the low risk haplotypes are selected for transfer.

Materials and methods

Patients

Two couples at high risk of having embryos with an unbalanced translocation underwent FISH-PGD. Both couples were counseled about the PGD procedure and the necessity of confirming the diagnosis with prenatal diagnosis. They consented to donate the embryos that were found to be unbalanced by FISH-PGD. The study was approved by the Ethics committee for genetic testing in Israel.

Couple 1

Couple 1 was referred to PGD due to a maternal balanced Robertsonian translocation 45, XX rob(15;21)(p10;p10) detected after three spontaneous abortions and two elective terminations of pregnancy, due to fetuses affected by trisomy 21. Maternal age was 25. Following the translocation detection she underwent one IVF treatment followed by PGD using FISH analysis with two direct labeled probes (Abbott Molecular, AbbotPark, IL) for chromosomes 15 and 21 and a pregnancy was achieved. Amniocentesis revealed that the embryo carried the balanced maternal translocation (later the DNA extracted from the amniotic fluid was used for setting the segregation mode of the translocation alleles). Uni-Parental Disomy (UPD) was excluded and a healthy child was born.

Two years later the couple returned and following standard IVF treatment, 8 embryos were analyzed by FISH: 3 embryos were found to be normal/balanced, pregnancy was achieved and a healthy child was born. Two of the unbalanced blastocysts were collected and a DNA extraction and amplification were performed.

Couple 2

Couple 2 was referred to PGD after a paternal balanced Robertsonian translocation 45,XY,rob(13:14)(p10;p10) was diagnosed because of severe azospermia. Following stan-

dard PGD by FISH a pregnancy was achieved, and a healthy girl was born. Amniocentesis revealed a normal karyotype free of the paternal translocation (later we used the DNA extracted from the amniotic fluid for setting the segregation mode of the translocation alleles). The couple returned 2 years later, and following standard IVF treatment 13 embryos were analyzed by FISH, three of whom were found normal/balanced. Unfortunately a pregnancy was not achieved. Two unbalanced blastocysts were collected and a DNA extraction and amplification were performed.

Blastomeres biopsy and fixation

An ovarian stimulation protocol was followed by oocyte retrieval and insemination by ICSI or IVF. Day-3 embryos underwent blastomere biopsy using a micromanipulation system (Narashige, Japan) fitted on an inverted microscope (Diaphot 300, Nikon, Japan). A laser system (ZILOS-tk, Hamilton Thorne) was used for dissection of the zona pellucida prior to biopsy. A single blastomere was removed from each embryo. After the manipulated embryos were returned to the culture media, the biopsied blastomeres were fixed on a glass slide using 3:1 Acetic acid-Methanol solution (Merck KGaA, Darmstadt, Germany). The fixed nuclei were prepared for Fluorescent in-situ hybridization by dehydration in fresh 70%, 80%, and 100% ethanol (BioLab ltd., Jerusalem, Israel) for 2 min each at −20°C.

Embryos that were found to be abnormal by FISH-PGD according to signal scoring criteria (see FISH section) were donated for research and a second blastomere was extracted. The blastomere was aspirated into a polymerase chain reaction (PCR) tube with 3.5 μL PBSx1 (Repli-g midi kit, QIAGEN GmbH, Hilden, Germany) for genomic DNA amplification and haplotyping.

Fluorescent in-situ hybridization (FISH)

Prior to FISH-PGD procedure, karyotypes of the patients were obtained by standard G-banding method, in order to map relevant probes for the translocation and to test probe combinations. A common strategy for translocation detection was employed using specific FISH probes that flank the breakpoint in order to detect all possible segregation patterns and to ultimately distinguish between chromosomally normal/balanced and unbalanced embryos [15]. FISH analysis was performed following the manufacturer's instructions. For translocation 15/21 three fluorescent probes were employed: CEP15 (D15Z1) specific for 15p11.2, LSI21 (D21S342,D21S341, D21S529) specific for 21q22.13-q22.2 and TelVision15q (D15S396) specific for15q26.3 (Abbott Molecular, AbbotPark, IL). For translocation 13/14 two fluorescent probes were used: TelVysion Probe13q (D13S327) for 13q34 and TelVysion Probe 14q (D14S1420) for 14q32.33 (Abbott Molecular, AbbotPark, IL)

Signal scoring was performed according to stringent criteria: blastomeres were scored as a 'normal/balanced' status if FISH clearly indicated two separate signals for each probe, while unbalanced blastomere showed deviation from the 'normal/balanced' signal pattern [16].

DNA samples

DNA was extracted from 9 ml peripheral blood from each parent and also from 15 ml of amniotic fluid from each couple's previous pregnancy, according to standard protocols.

Blastomere's whole genomic DNA amplification by multiple displacement amplification (MDA)

Isothermal DNA amplification method with 29 DNA polymerase was used (Repli-g midi kit, QIAGEN GmbH, Hilden, Germany) as described in the manufacturers' manual. The isothermal amplification was performed at 30°C for 16 h and the reaction was stopped upon incubation at 65°C for 3 min.

Polymorphic markers analysis

At least four fully informative markers, located on each translocated chromosomes, were required in order to identify the translocation related haplotype. A polymorphic marker is considered to be fully informative if each partner of the couple is heterozygote for that marker, and segregation of each allele can be determined. Markers close to the translocation breakpoint were preferable for confident assignment of a haplotype and ideal, to reduce misdiagnosis caused by chromosomal recombination.

Eight markers (Table 1) were used to haplotype both partners of couple 1 and the amniotic fluid with the balanced translocation karyotype. The allele sizes obtained from the amniotic fluid helped to identify the translocation chromosome's haplotypes.

Eleven markers were used to haplotype couple 2 (Robertsonian translocation 13/14), and the DNA from the amniotic fluid that presented normal karyotype free of the paternal translocation (Table 2).

Fluorescent primers for two polymorphic markers from clones AP001069, AL133493 (chromosome 21) were designed with the Primer3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi/). Primers for the remaining 17 markers were chosen from a commercial kit ABI PRISM® Linkage Mapping Set (version 2.5)(Applied Biosystems, Foster City, CA) (Tables 1 and 2).

PCR was performed in a 25 μL reaction containing 50 ng of DNA, 13.4 ng of each primer, and 1.5 mM dNTPs

Table 1 PCR results for eight markers on fetus, parents and two embryos from couple 1

Marker/clone name	Chromosome band (Mbp)	PCR product size				
		Mother	Previous pregnancy fetus	Father	Embryo No.1	Embryo No.2
D15S128	q11.2 (25.1)	***202***/204	***202***/204	204/200	***202***/200	***202***/200
D15S994	q15.1 (40.5)	***303***/280	***303***/307	307/305	***303***/305	***303***/305
D15S978	q21.1 (49.2)	***205***/201	***205***/203	203/189	***205***/203	***205***/189
D15S120	q26.3 (99.5)	***174***/166	***174***/176	176/176	166/176	***174***/176
D21S1922	q21.1 (22.2)	***252***/248	***252***/248	248/248	248/***252***/248	***252***/248
AP001069	q22.11 (31.9)	***260***/241	***260***/262	262/260	241/***260***/262	***260***/[a]
D21S1255	q22.2 (39.7)	***315***/313	***315***/319	319/315	***313***[a]	***315***/319
AL133493	q22.3 (46.9)	***313***/308	***313***/315	315/311		[a]/315
Karyotype		45, XX t(15;21)(p10;p10)	45, XX t(15;21)(p10;p10)	46,XY		
FISH results (ISCN format)		45, XX ish(D15Z1 ×3), (D15S396 ×2), (D21S342,D21S341, D21S529)×2[b]			nuc ish(D15Z1 ×2), (D15S396 ×2), (D21S342, D21S34, D21S529)×3[1]	nuc ish(D15Z1 ×3), (D15S396 ×2), (D21S342, D21S341, D21S529)×2[1][b]

Translocation related alleles are shown in bold and italic

[a] allele drop out defined as a restricted amplification of an allele to such an extent that it cannot be detected by ABI

[b] further FISH examination of the maternal lymphocytes methaphase revealed that chromosome 14 harbored a region homologues to centromere 15

Table 2 PCR results for 11 markers on fetus, parents and two embryos from couple 2

Marker/clone name	Chromosome band (Mbp)	PCR product size				
		Mother	Previous pregnancy fetus	Father	Embryo no.3	Embryo no.4
D13S175	q12.11 (20.8)	106/106	106/114	***103***/114	114/***103***/106	106/114
D13S1236	q12.11 (22.6)	122/128	122/126	***120***/126	126/***120***/122	122/126
D13S1243	q12.12 (24.8)	249/253	249/249	***251***/249	249/***251***/249	249/249
D13S217	q12.3 (29.3)	250/252	250/252	***250***/252	252/***250***	250/252
D13S289	q12.3 (31.2)	168/162	168/156	***156***/156	***156***/156/168	162/156
D13S1296	q21.33 (70.6)	102/93	102/100	***106***/100	***106***/10010/2	102/100
D14S283	q11.2 (22.6)	135/148	148/146	***128***/146	***128***/148	146/148
D14S275	q12 (26.6)	147/147	147/154	***154***/154	***154***/147	154/147
D14S276	q22.3 (55.6)	243/243	243/239	***247***/239	***247***/243	247/243
D14S63	q23.2 (64.6)	187/183	183/193	***189***/193	***189***/183	189/183
D14S258	q24.2 (70.5)	200/200	200/198	***196***/198	***196***/200	196/200
Karyotype		46, XX	46, XX	45,XY,rob(13:14)(p10;p10)		
FISH results (ISCN format)					nuc ish(D14S1420×2),(D13S327×3)[1]	nuc ish(D14S1420×3),(D13S327×3)[1]

Translocation related alleles are indicated in bold and italic

in 1.5 mM $MgCl_2$ PCR buffer with 1.2U *Taq* polymerase (Bio-Line, London, UK). After an initial denaturation of 2 min at 95°C, 30 cycles were performed (94°C for 1 min, 59°C for 1 min, and 72°C for 1 min), followed by a 10-min final extension at 72°C.

PCR products were resolved in a 4% denaturing polyacrylamide optimized polymer (POP-4). Amplification products were sized by ABI PRISM® 3100 (Applied Biosystems, Foster City, CA) using internal control (GeneScanTM-500 LIZ®) (Applied Biosystems, Foster City, CA) and analyzed by Genescan and Genotyper software via the local southern algorithm.

Results

PGH results of couple 1

Haplotypes were assigned for mother, father, and fetus, which carried the balanced maternal translocation (detected by amniotic cell karyotype). In order to identify the alleles linked to the chromosomes involved in the translocation, 4 polymorphic markers from chromosome 15 and additional 4 markers from chromosome 21 were used (see Table 1). These markers were used to determine the translocation status of cleavage stage embryos in the PGD cycles. Four markers were fully informative, two were partially informative and two weren't informative. This combination was satisfying, especially with the information from the normal karyotype and the exclusion of UPD.

For embryo 1, all 4 polymorphic markers representing chromosome 21, showed three-allelic patterns consist of two maternal alleles and one paternal allele. In addition, results obtained using polymorphic markers representing chromosome 15, showed di-allelic pattern (one paternal and the other was maternal derivative chromosome 21/15) (Table 1) indicating unbalanced translocation with chromosome 21 trisomy compatible with adjacent segregation. This was in agreement with FISH results (Table 1).

Polymorphic markers analysis of embryo 2 (in couple 1), exhibited di-allelic pattern of chromosome 15 (one paternal and the other maternal derivative chromosome 21/15) and di–allelic pattern of chromosome 21 (one paternal and the other maternal derivative chromosome 21/15) (Table 1). These results indicated that embryo 2 was a carrier of balanced translocation. However, FISH analysis showed two signals of chromosome 21 (21q22.13-q22.2), three signals of centromere 15 while only two signals of subtelomere 15q (15q26.3) (Table 1). According to these results the embryo wasn't suitable for transfer due to abnormal FISH results. Further FISH examination of the maternal metaphases using chromosome 15 centromeric probe (D15Z1), revealed that one of her chromosome 14

harbored a region homologues to centromere 15. One in six individuals in general population will show one or more additional centromeric D15Z1 signal [17]. Hence two of the three centromere 15 signals revealed in embryo 2, were located on chromosomes 15 and the additional one on chromosome 14.

UPD was excluded in both embryos.

PGH results of couple 2

Haplotypes were assigned for mother, father and a fetus that did not carry the maternal translocation according to amniotic cell karyotype. Six polymorphic markers in chromosome 13 and five markers in chromosome 14 were applied. The same markers were used to determine the translocation status of cleavage stage embryos in the future PGD cycles. Four markers for both chromosomes involved in the translocation were fully informative and were used to identify the alleles linked to the chromosomes involved in the translocation (Table 2).

Embryo 3 showed an unbalanced chromosome result, by FISH and by polymorphic markers methods. It showed unbalanced translocation resulting in trisomy 13, compatible with adjacent segregation at gametogenesis (Table 2).

Embryo 4 showed a bi-allelic pattern for both chromosomes 13 and 14 by polymorphic markers analysis correlating with normal alternate chromosomal segregation. This was incompatible with FISH results showing three signals of chromosome 13 and three signals of chromosome 14 (Table 2). The discrepancy is, probably due to our strict policy of interpretation of FISH signals, meaning that whenever there are unclear cut signal pattern, the embryo is considered as abnormal.

UPD was excluded in both embryos.

Discussion

FISH-PGD analysis is currently, the gold standard procedure for identifying unbalanced translocations in embryos. Although relatively successful, FISH is technically demanding and harbors some technical limitations that disrupt the diagnostic value of this method. One of the most significant limitations of the method is its incapability to differentiate between balanced and normal embryos. Transferring embryos with a balanced translocation perpetuates infertility and other abnormalities in the next generations. Previous attempts to overcome these limitations included a proposal to visualize chromosomes by fusing single blastomeres with enucleated or intact mouse zygotes, resulting in heterokaryons at the metaphase of the first cleavage division [18]. FISH with whole chromosome painting probes and FISH with specific probes spanning the chromosome breakpoints were offered for differentiating between normal, balanced and unbalanced embryos [19, 20]. Lately this method was modified with a chemical conversion procedure [21]. Although demonstrating relative success these techniques remain extremely complicated and are not suitable for routine clinical diagnosis.

PGH for single gene disorders and HLA typing was previously described and it is routinely used in many centers using multiplex nested PCR analysis [22]. In this study we demonstrated the capability of PGH to identify and differentiate between normal, balanced and unbalanced translocation in embryos. The limiting step in single cell embryo diagnosis is the small amount of DNA. MDA amplification using 29 DNA polymerase overcomes this obstacle by amplifying pikograms of genomic DNA into several micrograms [23]. Amplification of DNA increases the number of markers that can be tested and helps to overcome problems such as allele drop out, since sufficient results are generated from other markers to compile a haplotype. In addition, the large number of loci tested increases the probability of identifying correctly possible recombinations [24]. Furthermore, contamination is obviated as PGH provides an effective fingerprint of the MDA products and thereby identifies them as originating from the embryo and not from contaminating DNA.

Before performing PGH analysis for translocation carriers, the couple and another family member, have to be analyzed, both cytogenetically and by the polymorphic markers, in order to identify those markers that are linked to the translocated chromosomes. Nevertheless if the third individual is not available, embryos that weren't transferred in previous PGD cycles can be used.

The new application of PGH, presents several advantages over FISH analysis: First, it distinguishes between normal, balanced translocation, and unbalanced translocation carrier embryos. Second, substituting the technically demanding cell fixation step of the FISH method, with the simple transfer of cells into PCR tubes, can decrease the rate of analysis failures. Moreover, it might overcome some other FISH technical difficulties such as fluorescent signal overlaps or subjective interpretation of the signals. For example, the discrepancy between the FISH and the polymorphic markers results for embryo 4, is due to severe scrutiny in the interpretation of the FISH signals. Third, the use of several polymorphic markers simultaneously provides independent diagnosis and internal control, therefore increasing accuracy and efficiency. Fourth, PGH method allows the necessary UPD assessment of the embryo, to be obtained in preimplantation stage. And last, once implemented, PGH can be offered to couples carrying a gene or a regulatory element disrupting translocations.

A recent study describes a molecular strategy for routine preimplantation genetic diagnosis in translocation carriers

[25]. In this study polymorphic markers are used in a quantitative assay that allows distinction between di-allelic, mono-allelic and tri allelic status of the chromosomes involved in the translocation for each embryo. Their approach is more efficient then FISH but in contrast to our new approach it doesn't differentiate between balanced translocation carriers and normal chromosomes carriers embryos.

Conclusions

Our preliminary results in two Robertsonian translocations carries indicate that the polymorphic markers method is an advanced, relatively simple and efficient method for preimplantation genetic diagnosis. It is capable of distinguishing between normal, balanced translocation and unbalanced translocation carrier embryos. A larger cohort of translocation carriers including reciprocal translocations should be investigated in order to improve the crucial steps of this diagnostic tool and establish a reliable protocol.

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