Preimplantation genetic diagnosis of chromosome translocations by analysis of polymorphic short tandem repeats

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INTRODUCTION We aimed to develop and implement a short tandem repeat (STR) polymerase chain reaction alternative to fluorescence *in situ* hybridisation (FISH) for the preimplantation genetic diagnosis (PGD) of chromosomal translocations.

METHODS Selected informative STRs located on translocated arms of relevant chromosomes were used to discriminate between normal and unbalanced chromosome states in each embryo.

RESULTS PGD cycles were performed on five couples where one spouse carried a balanced translocation. 27 embryos were analysed, of which 12 were normal/balanced, 12 were abnormal/unbalanced and three were indeterminate. Four PGD cycles proceeded to embryo transfer, of which two led to pregnancy. The first pregnancy showed a normal male karyotype, and a healthy baby was delivered at term. A second pregnancy unexpectedly miscarried in the second trimester from unknown causes.

CONCLUSION STR analysis is a simple and suitable alternative to FISH for detecting unbalanced chromosomal states in preimplantation embryos.

Keywords: capillary electrophoresis, chromosome translocation, polymerase chain reaction, preimplantation genetic diagnosis, short tandem repeat
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INTRODUCTION

In the general population, balanced structural chromosomal rearrangements are common and present in approximately one in 500–1,000 births.[1] Structural chromosomal rearrangements play an important role in the majority of chromosomal abnormalities, with a high recurrence risk. Although carriers of balanced translocations usually do not show any adverse phenotypic effects, they are known to be at risk for infertility, repeated miscarriages and/or having offspring with unbalanced karyotypes.[2] Risks associated with chromosome translocations are dependent on the gender of the translocation carrier, the chromosomes involved and the position of the chromosomal breakpoints.[1,3]

Chromosomal abnormalities, such as balanced translocations, are known to interfere with gametogenesis. Individuals with chromosomal abnormalities are known to have high rates of unbalanced gametes and severe meiotic disturbance, with spermatogenic arrest in males.[2,4,5] A reported 12% of azoospermic and severely oligozoospermic males carry a karyotype abnormality, mainly an XXY constitution or a Robertsonian or reciprocal translocation.[6] In addition, chromosomes involved in reciprocal or Robertsonian translocations form quadrivalents or trivalents at meiosis, respectively. Quadrivalents can segregate, with or without recombination, to yield 32 possible meiotic outcomes, only two of which are genetically balanced.[7] The remaining 30 genetically unbalanced meioses likely lead to recurrent spontaneous abortions or offspring with congenital anomalies. Likewise, trivalents can segregate to generate 17 possible meiotic outcomes, again with only two genetically balanced outcomes.

Preimplantation genetic diagnosis (PGD) offers the opportunity to preselect and transfer only chromosomally balanced embryos, thereby significantly increasing the chances of achieving a successful and healthy pregnancy. PGD of chromosomal translocations has traditionally been accomplished by fluorescence *in situ* hybridisation (FISH).[8-11] Most commonly, PGD of reciprocal translocations involves the use of commercially available distal/telomeric probes in combination with proximal/centromeric probes in a tricolour FISH assay,[10,12] or the use of specific breakpoint spanning probes that allow balanced and normal embryos to be differentiated. Unfortunately, the development of case-specific breakpoint spanning probes is laborious and expensive. For Robertsonian translocations, PGD usually involves the use of commercially available locus-specific enumerator probes.[11]

Preimplantation diagnosis or screening by FISH is associated with several inherent technical limitations, including cell loss, signal

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overlap, signal splitting and poor probe penetration/hybridisation, which could confuse signal interpretation and eventually lead to a failure to transfer normal/balanced embryos or an erroneous transfer of chromosomally unbalanced embryos. Although technical improvements have been achieved for FISH, the average error rate of FISH has been reported to be 6%.[10]

Since the essential requirement of translocation PGD is the discrimination between balanced and unbalanced chromosomal states, we explored the use of short tandem repeat (STR) markers as an alternative to FISH. STR markers have been used extensively in PGD for single gene disorders, either for direct mutation detection and indirect mutation detection by linkage/haplotype analysis (as a confirmation of the direct mutation analysis results), or as a means of detecting biparental genetic contribution and extraneous DNA contamination. Selection of appropriately located and informative STR markers would enable the identification of balanced translocation states based on inheritance of one allele from each parent at each marker locus. In contrast, unbalanced states would be identified by the absence of an allele or presence of both alleles from the carrier parent at one or more marker loci. Due to the single nucleotide resolution of capillary electrophoresis (CE), this platform has been used extensively in STR marker analysis, especially when multiple markers are being used in a single assay.

We document here the results of our first six STR PGD cycles, involving five couples where one spouse was a carrier of a balanced chromosome translocation. Encouragingly, in the case of a couple whose husband had severe male factor infertility and was a carrier of a reciprocal translocation, a healthy baby boy with a normal chromosome constitution has been born. However, in another couple whose husband was diagnosed with oligozoospermia and carried a Robertsonian translocation, an uneventful first trimester of pregnancy unexpectedly miscarried in the second trimester. The cause could not be ascertained, as karyotyping of the products of conception was declined.

METHODS

Between April 2008 and September 2010, four carriers of Robertsonian translocations and one carrier of a reciprocal translocation underwent translocation PGD testing via STR polymerase chain reaction (PCR) and CE. The PGD procedures were performed under the Health Services Development Programme of the Ministry of Health, Singapore, and written informed consent was obtained from all patients.

To detect chromosomal imbalances in embryos, a set of STR markers was tested to determine their informativity in each couple (Table I). For patients with Robertsonian translocation, informative STR markers in the q arm were chosen. In one patient who had a low level mosaicism for monosomy X (Turner syndrome), informative STR markers on the chromosome X as well as the amelogenin locus on chromosomes X and Y were included (Table I). For the sole patient with reciprocal translocation, informative STR markers in both arms of the sub-metacentric chromosome were used.

In vitro fertilisation (IVF) procedures were carried out at two centres in Singapore, namely the Clinic for Human Reproduction at the National University Hospital and the KKIVF at the KK Women's and Children's Hospital. Oocytes obtained from female partners after controlled ovarian hyperstimulation with recombinant follicle stimulating hormone were fertilised by intracytoplasmic sperm injections (ICSIs). On Day 3, embryos at the 5–10 cell stage were biopsied by zona drilling using a 1.44-µm diode laser (Research Instruments, Falmouth, UK) after a five-minute incubation in Ca++/Mg++-free biopsy medium (Irvine Scientific, Santa Ana, CA, USA).[13] One blastomere was removed from each five-cell embryo through the zona opening while two blastomeres were removed from the more developed embryos.

Each blastomere was transferred into a 0.2 mL reaction tube containing 5 µL of lysis buffer (0.2 M potassium hydroxide, pH 14.0). Reaction tubes were incubated at 65°C for ten minutes, after which 5 µL of neutralisation buffer (0.2 M Tricine, pH 5.7) was added. Each assay was designed as a single round multiplex PCR in a final reaction volume of 50 µL containing 2–7 pairs of fluorescently-labelled PCR primers at concentrations ranging from 0.1 µM to 0.5 µM, 0.2 mM of each deoxyribonucleotide triphosphate (Roche Diagnostics, Mannheim, Germany), 2.5–4 units of HotStarTaq DNA polymerase (Qiagen, Hilden, Germany) in 1x PCR buffer containing 1.5 mM magnesium chloride. Thermal cycling was performed in the GeneAmp® PCR System 9700 (Applied Biosystems, Foster City, CA, USA), with an initial 15-minute enzyme activation at 95°C, followed by 45 cycles of denaturation at 98°C for 30 seconds, annealing at 60°C ranging from 30 seconds to 1 minute, and extension at 72°C ranging from 30 seconds to 1 minute 30 seconds, that culminated in a final five-minute extension at 72°C. PCR products were resolved in an ABI PRISM® 3130XL Genetic Analyzer together with the GeneScan™-500 ROX-labelled size standard using the 36-cm capillary and POP-4™ polymer system. The electrophoretically separated amplicons were analysed using GeneMapper® software v4.0 (Applied Biosystems), and the fragment size for each allele of each STR marker was assigned manually by identifying the peak on the electropherogram.

RESULTS

Customised assays for each couple were preclinically validated on 32–48 single lymphocytes from both husband and wife. Amplification efficiencies averaged 94% (range 91%–98%), while the allele dropout (ADO) rate ranged from 5.4% to 7.8%. Three cases involved female Robertsonian translocation carriers, one case had a male Robertsonian translocation carrier, and one case involved a male reciprocal translocation carrier (Table I). A total of 83 oocytes were retrieved from six IVF cycles (5–33 oocytes per IVF cycle), of which 53 were fertilised after ICSI (4–19 zygotes per cycle) and 40 cleavage-stage embryos were

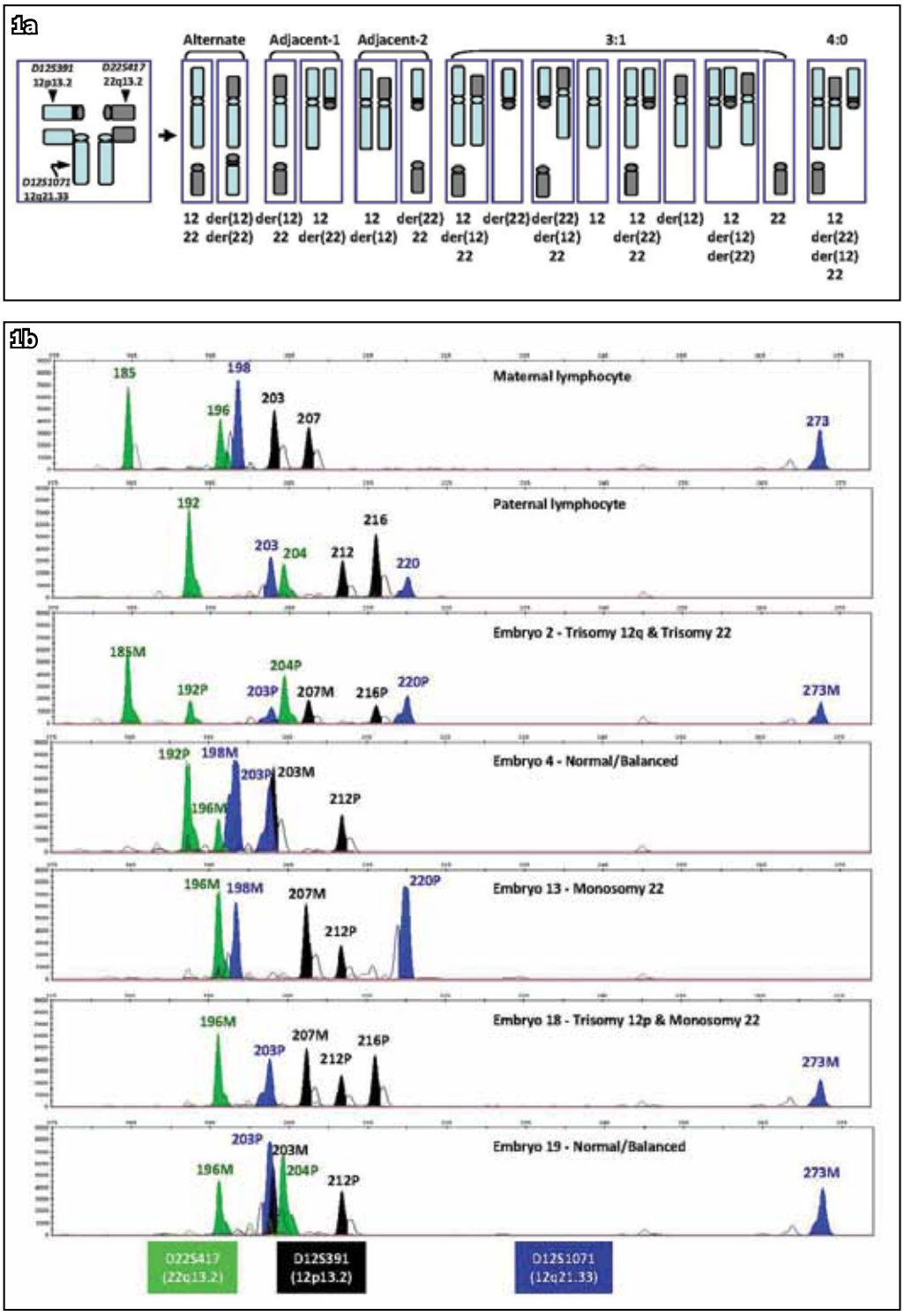

Fig. 1 Results of single-cell short tandem repeat (STR) polymerase chain reaction analysis in a couple where the husband carried a t(12;22) translocation showing (a) possible gamete chromosome complements produced by the reciprocal translocation carrier; and (b) electropherograms of STR amplicons from maternal lymphocyte, paternal lymphocyte and embryo blastomeres. Only results from embryos with definitive diagnoses are shown. Maternal and paternal alleles are indicated with an 'M' and 'P', respectively, after allele sizes (in base pairs).

obtained (0–15 embryos per cycle). 27 embryos were biopsied on Day 3, and a total of 49 blastomeres were isolated. All biopsied blastomeres produced amplification products, and a definitive diagnosis was obtained for 21 of the 27 embryos (78%). In four of the five cases, embryos with a normal/balanced chromosome constitution were identified and embryo transfers were initiated. From these transfers, successful pregnancies were achieved in two cases (Table I). The first successful case involved a couple where the husband had severe male factor infertility and carried a balanced t(12;22) reciprocal translocation. Definitive diagnoses were obtained for five of the eight embryos tested, of which two were diagnosed as chromosomally normal/balanced and three were chromosomally unbalanced due to paternal meiotic malsegregation of the 12/22 quadrivalent (Table I, Fig. 1). A

Table I. Summary of translocation preimplantation genetic diagnosis (PGD) cycles performed using short tandem repeat (STR) markers.

Translocation	STR markers tested			Oocytes		Embryos	
	Uninformative	Informative (maternal; paternal alleles [bp])	Total	Total	Fertilised	Total	Biopsied
45,XX,rob(15;21)(q10;q10)	NA	Chr. 15: *PentaE* (M: 128/138; P: 102/150) Chr. 21: *PentaD* (M: 155/176; P: 171/181)	2	7	7	7	7
45,XX,rob(13;21)(q10;q10)	Chr. 13: *D13S250, D13S742, D13S256, D13S1810, D13S634, D13S631, D13S628* Chr. 21: *D21S1414, D21S270, D21S1411, PentaD*	Chr. 13: *D13S258* (M: 245/285; P: 236/273) Chr. 21: *D21S1412* (M: 399/403; P: 379/395)	13	5 6	4 5	0 1	0 1
46,XY,t(12;22)(p11.2;q11.2)	Chr. 12p: *D12S1303, D12S1581, D12S1608* Chr. 12q: *D12S1061, D12S1824, D12S2076* Chr. 22: *D22S529, D22S535, D22S693*	Chr. 12p: *D12S391* (M: 203/207; P: 212/216) Chr. 12q: *D12S1071* (M: 198/273; P: 203/220) Chr. 22: *D22S417* (M: 185/196; P: 192/204)	12	20	13	13	8
45,XX,rob(13;15)(q10;q10)[48]/ 44,X,rob(13;15)(q10;q10)[2]	Chr. 13: *D13S631, D13S258, D13S628* Chr. 15: *D15S818, D15S660* Chr. X: *DXS1108, DXS8087, DXS6789, DXS7132, DXS7127, DXS1073, F8IVS13, F8IVS22*	Chr. 13: *D13S1810* (M: 159/188; P: 166/172), *D13S634* (M: 362/364; P: 358/366) Chr. 15: *D15S533* (M: 352/356; P: 374/378), *PentaE* (M: 113/150; P: 76/108) Chr. X: *DXS6797* (M: 270/278; P: 262), *DXS6810* (M: 214/219; P: 223) Chr. X&Y: *AMELX/Y* (M: 106; P: 106/112)	19	12	5	4	4
45,XY,rob(13;14)(q10;q10)	Chr. 13: *D13S1810, D13S258, D13S628* Chr. 14: *D14S302, D14S605, D14S1434, D14S305, D14S614, D14S124, D14S126, D14S140, D14S612, D14S618, D14S1424* Chr. 21: *D21S1411, D21S1414, D21S1412, PentaD*	Chr. 13: *D13S631* (M: 201/209; P: 193/205), *D13S634* (M: 367/371; P: 358/365) Chr. 14: *D14S543* (M: 254/257; P: 240/261), *D14S128* (M: 352/374; P: 359/367) Chr. 21: *D21S1270* (M: 294/301; P: 290/317)	23	33	19	15	7

ADO: allele dropout; N: no; NA: not available; Y: yes
* Where two blastomeres were analysed, results were concordant, unless indicated otherwise.

definitive diagnosis was not possible for the remaining three embryos due to conflicting/discordant results between the two blastomeres of the same embryo and the presence of exogenous DNA contamination or ADO of maternal alleles. The two normal/balanced embryos (#4 and #19) were transferred, which produced a singleton pregnancy. Amniocentesis revealed a 46,XY normal karyotype in the foetus, and a healthy baby boy was delivered at term.

The second pregnancy involved a couple where the husband was diagnosed with oligozoospermia and was a carrier of a rob(13;14) Robertsonian translocation. Definitive diagnoses were obtained for all seven embryos tested, of which five were diagnosed as chromosomally normal/balanced, one was chromosomally unbalanced due to paternal meiotic malsegregation of the 13/14 trivalent, and one was chromosomally abnormal due to other paternal and maternal meiotic non-dysjunctions (Table I, Fig. 2). Two normal/balanced embryos (#6 and #16) were transferred and a pregnancy ensued. The pregnancy was uneventful in the first trimester. However, a miscarriage unexpectedly occured in the second trimester. Karyotype analysis of the products of conception was declined. Therefore, the cause of the miscarriage could not be investigated.

Embryo ID	Blastomeres analysed	Result and remarks*	Embryo transfer	Pregnancy
1	2	Trisomy 21 arising from an adjacent segregation	N	N
2	2	Trisomy 21 arising from an adjacent segregation	N	
3	2	Normal/balanced embryo	Y	
4	2	Monosomy 21 arising from an adjacent segregation	N	
5	2	Normal/balanced embryo	Y	
6	2	Normal/balanced embryo	Y	
7	1	Normal/balanced embryo	N	
NA	NA	All embryos fragmented; PGD cycle aborted	NA	N
3	2	Normal/balanced embryo	Y	
2	1	Trisomies 12q and 22 arising from a 3:1 segregation	N	Y
3	2	Indeterminate embryo constitution, due to ADO of maternal *D12S391* and *D12S1071* alleles	N	
4	2	Normal/balanced embryo	Y	
6	2	Indeterminate embryo constitution, due to exogenous DNA contamination in both blastomeres, apparent monosomy 12 and monosomy 22 in one blastomere, and apparent normal/balanced constitution in the other blastomere	N	
13	1	Monosomy 22 arising from a 3:1 segregation	N	
16	2	Indeterminate embryo constitution, due to monosomy 12q and monosomy 22 in one blastomere consistent with a 3:1 segregation, and ADO of maternal *D12S391*, *D12S1071* and *D22S417* alleles in the other blastomere	N	
18	2	Trisomy 12p and monosomy 22 arising from an adjacent-1 segregation	N	
19	2	Normal/balanced embryo	Y	
1	2	Abnormal embryo constitution, due to presence in both blastomeres of only the *AMELY* and paternal *PentaE* alleles, absence of all other marker alleles in one blastomere, and absence of maternal *D13S1810*, *D13S634*, *D15S533*, and *PentaE* alleles in the other blastomere	N	NA
3	2	Abnormal embryo constitution, due to complete absence of *AMELX/Y* alleles in both blastomeres, maternal disomy of *D15S533* and *PentaE* alleles and absence of paternal genetic contribution in one blastomere, and absence of maternal *D13S1810*, *D13S634*, *D15S533* and *PentaE* alleles in the other blastomere	N	
4	2	Trisomy 15 arising from an adjacent segregation	N	
5	2	Trisomy 13 arising from an adjacent segregation	N	
2	1	Monosomy 13 arising from an adjacent segregation	N	Y
5	1	Normal/balanced embryo	N	
6	2	Normal/balanced embryo	Y	
7	2	Normal/balanced embryo	N	
8	2	Abnormal embryo constitution, due to concordant absence in both blastomeres of a paternal *D21S1270* allele and maternal *D14S543* and *D14S128* alleles	N	
16	2	Normal/balanced embryo	Y	
29	2	Normal/balanced embryo	N	

DISCUSSION

This report documents our successful application of an STR PCR-based strategy for PGD of chromosomal translocation carriers, which has traditionally been performed using FISH. Our initial pregnancy and live birth rate per embryo transfer of 25%, with an average maternal age of 35 years, is similar to the rate of 25.3% reported by the European Society of Human Reproduction and Embryology PGD Consortium using FISH-based assays, where data was generated from 3,524 cycles, with an average maternal age of 33.2 years.[14] We note that two other groups have also reported their independent development of PCR-based PGD assays for chromosome translocations.[15,16] This approach of STR PCR and allele sizing by CE, using informative STR markers residing on the translocated chromosome arms, enables all potential outcomes of meiotic segregations to be determined on the basis of the marker alleles present in the embryo. The STR-based approach is rapid (~4–5 hours) as compared to FISH (~6–16 hours) and allows a potentially greater number of embryos to be analysed. In addition, the inherent technical limitations of FISH, which could lead to interpretation errors, are avoided in the STR approach. Informative markers also allow determination of the parental origin of the tracked chromosomes in the embryo, enabling

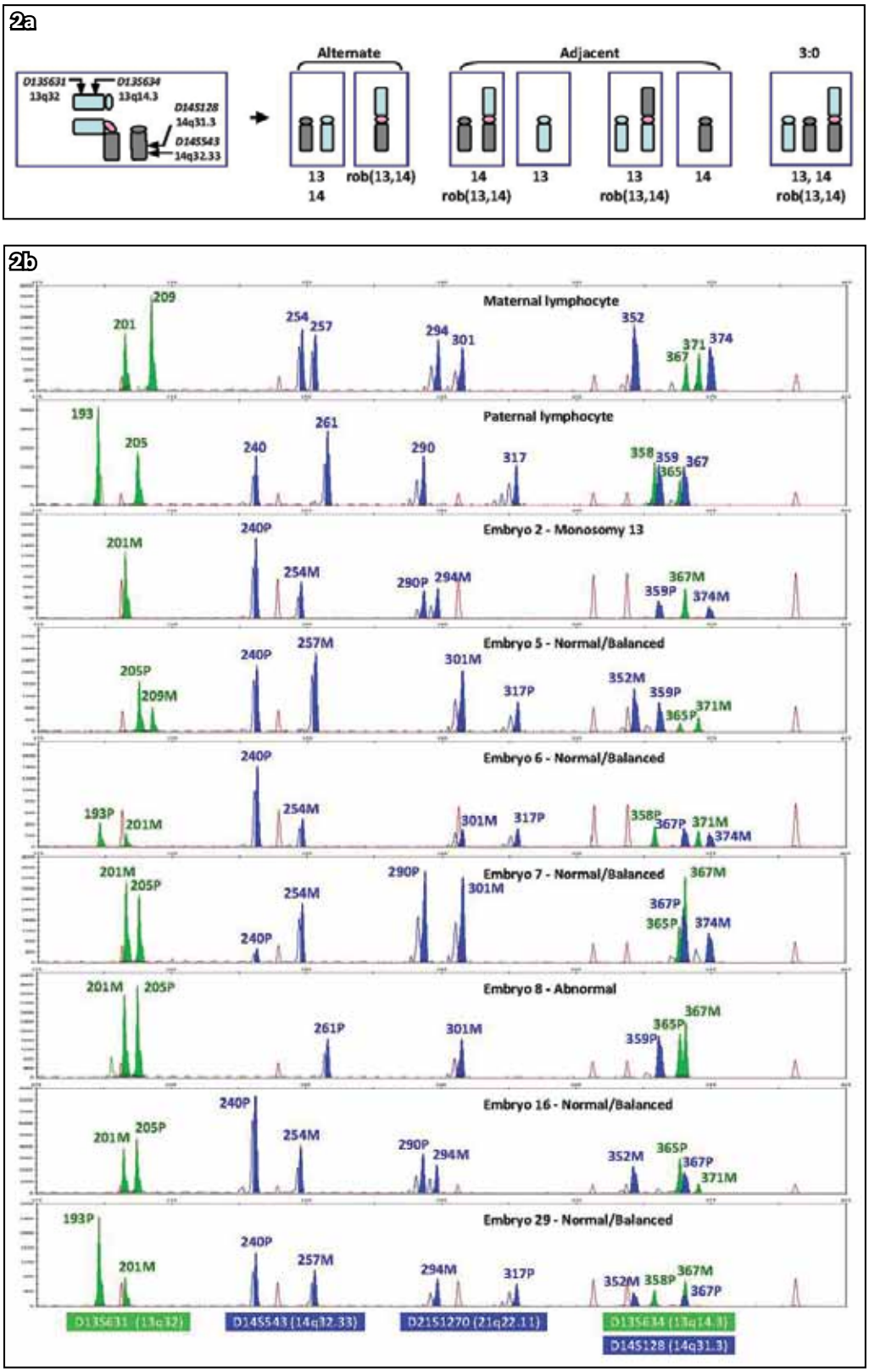

Fig. 2 Results of single-cell short tandem repeat (STR) polymerase chain reaction analysis in a couple where the husband carried a rob(13;14) translocation showing (a) possible gamete chromosome complements produced by the Robertsonian translocation carrier; and (b) electopherograms of STR amplicons from maternal lymphocyte, paternal lymphocyte and embryo blastomeres.

detection of rare abnormal events, such as uniparental disomy, and are also useful in detecting exogenous DNA contamination.

The total cost of an STR PCR assay mainly depends on the number of STR markers screened in order to identify informative markers for any couple. Among the five cases reported here, the most number of STRs screened for one case was 23. Each STR PCR primer pair (one untagged and one fluorescently tagged) costs ~ SGD 200. Therefore, the total primer cost for that particular case was ~ SGD 4,600. If tricolour FISH had been used for this case, each FISH probe would have cost ~ SGD 1,000–2,000 or

a total of ~ SGD 3,000–6,000. Then again, it should be noted that synthesised PCR primers are usually sufficient for ~ 2,000 reactions, whereas commercially available FISH probes come in aliquots that are sufficient for only 20 reactions. Thus, PCR primers can be used for more than one PGD cycle or used for other PGD cases involving the same translocations, while FISH probes are sufficient for only one PGD cycle.

Given the PCR-based strategy's dependence on the availability of informative markers flanking translocation breakpoints, problems may arise in cases where a translocation breakpoint is extremely telomeric, such that accurate breakpoint determination or identification of at least one informative distal marker is not possible. Similar to FISH, this PCR-based method also does not differentiate between balanced and normal chromosome constitutions.[17] While there has been some concern over whether mosaicism in cleavage-stage embryos affects diagnostic accuracy, a recent study has concluded that embryonic mosaicism does not significantly affect result calling, as most mosaic embryos contain 100% abnormal cells.[18]

Another potential pitfall of PGD translocation analysis by STR PCR is that ADO can lead to false positive (unbalanced result in a normal/balanced embryo) or false negative (normal/balanced result in an unbalanced embryo) conclusions. Analysis of two blastomeres (biological replicates) of the same embryo significantly minimises such risks of false negative-false positive diagnoses, as the probability of ADO of the same allele in both blastomeres is equal to the square of the individual blastomere's probability of ADO (that is, if the ADO rate is 10%, the probability of occurrence of ADO in both blastomeres is 0.1%). Additionally, the use of two (or more) markers (technical replicates) per chromosome arm or segment can also increase diagnostic confidence, as the probability of ADO in both (or all) markers is the product of individual STR ADO rates.

We started this programme using two blastomeres (for embryos with six or more cells) and one STR marker per chromosome arm for the first three cases. The assay's error rate was contributed by the ADO of STR markers (range 5.4%–7.8%). When we used only one STR marker per chromosome arm in our first three cases, the STR PCR assay showed no superiority compared to the 6% error rate of FISH. By switching to using two STR markers per arm in our next two cases, while retaining the analysis of two blastomeres per embryo, we were able to significantly lower the error rate compared to that of FISH, being the product of each marker's ADO rate. This would result in identifying the most suitable embryos for a successful normal pregnancy. Given the modest number of cycles performed thus far, more cycles will be needed to validate the benefits of using this approach.

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