Title page

Manuscript Title: The impact of mosaicism in preimplantation genetic diagnosis (PGD): Approaches to PGD for dominant disorders in couples without family history.

Running Head: PGD for dominant disorders without family history.

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What’s already known about this topic:

- Preimplantation genetic diagnosis (PGD) is a reproductive option available to patients that are carrier of an autosomal dominant disorder and seeking to have an “unaffected” child.
- The transmission risk where the patient is a mosaic with no family history for the disorder can deviate from the mendelian 50% which can lead to confusion at the level of diagnosis.

What’s does this study add:

- Careful pre-clinical analysis and follow up studies on embryos in some cases of autosomal dominant disorders has detected mosaicism for the causative mutation.
- Knowledge that the patient undergoing PGD for an autosomal dominant disorder with no family history is a potential mosaic for the causative mutation will render the diagnosis more robust, thereby, reducing the risk of misdiagnosis.

Ethics statement: PGD for the disorders presented in this study is licenced by the Human Fertilisation & Embryology Authority (HFEA). The IVF clinic (Centre for Reproductive and Genetic Health) providing the embryonic samples has an HFEA licence to conduct PGD. All patient consent forms where obtained for any post PGD/PGD follow-up studies on untransferred embryos covered by the HFEA licence. No additional ethical approval was required for this study and the authors declare that no ethics was breached.

Abstract

Objectives

Mosaicism in certain dominant disorders may result in a “non-Mendelian” transmission for the causative mutation. Preimplantation genetic diagnosis (PGD) is available for patients with inherited disorders to achieve an unaffected pregnancy. We present our experience for two female patients with different dominantly inherited autosomal disorders; neurofibromatosis type 1 (NF1) and tuberous sclerosis complex type 2 (TSC2).

Methods

PGD protocol development was carried out using single cells from the patients. PGD was carried out on polar bodies and different embryonic cells.

Results

Protocol development for NF1 using lymphocytes from the patient suggested mosaicism for the mutation. This was supported further by quantitative fluorescent-PCR performed on genomic DNA. During PGD, polar bodies and blastomeres lacked the mutation that probably was absent

or present at very low levels in the patient's germline. Single lymphocyte analysis during protocol development for TSC2 did not indicate mosaicism, however, analysis of single buccal cells and multiple embryo biopsies across two consecutive IVF/PGD cycles confirmed gonosomal mosaicism.

Conclusions

The trend in PGD is for blastocyst biopsy followed by whole genome amplification, eliminating single cell analysis. In the case of certain dominantly inherited disorders, pre-PGD single cell analysis is beneficial to identify potential mosaicism that ensures robust protocols.

Introduction

Mosaicism is a condition where an individual has two (or more) genetically distinct cell types. Mosaicism may be limited to somal tissues (somatic mosaicism), or the gonads (gonadal mosaicism) or present in both (gonosomal mosaicism). The precise timing of post-zygotic mutations that occur during development determines the distribution of mutant cells in the individual. Mutations that occur before the primordial germ cell (PGC) differentiation, before ~15 mitotic divisions, can be present in both somatic and germ tissues[1]. Mutations that occur after PGC differentiation will only be present in either the somatic or germline lineages. For autosomally inherited disorders an affected individual who has no previous family history has a *de novo* mutation that arose in the individual themselves as a post-zygotic event or in the germ cells of a parent of the individual. Depending upon when the *de novo* mutation occurred, the individual will have a transmission risk ranging from 0-50%; therefore careful counselling is necessary when considering reproductive options.

Neurofibromatosis type 1 (OMIM# 162200) is a neurocutaneous disorder with autosomal dominant inheritance, complete penetrance and variable expressivity[2]. It is caused by mutations in the *NF1* gene located on 17q11.2 and encodes the neurofibromin protein, a negative regulator of Ras GTPases[3].. The *NF1* gene has a very high mutation rate[4]; approximately 50% of all patients with NF1 have no family history of the disease[5]. A high rate of sporadic mutations resulting in mosaic occurrence may explain a milder clinical phenotype known as segmental NF1[6]. Germline mosaicism in NF1 has been reported in cases where affected children with a characterised mutation were born to healthy parents not showing the mutation in their lymphocytes[7].

Tuberous sclerosis complex type 2 (TSC2; OMIM# 191092) is also a neurocutaneous multisystem disorder with autosomal dominant inheritance and variable expressivity[8]. It is caused by mutations in *TSC2* located on the short arm of chromosome 16 (16p13.3) and encodes tuberin[9].. Molecular genetic studies have shown that approximately 65% of all patients with TSC2 have no family history of the disease[10]. Mosaicism leading to a variable clinical phenotype in TSC2 has been described[9] and germline mosaicism has been reported[11].

Preimplantation genetic diagnosis (PGD) is a reproductive option for couples which, unlike prenatal diagnosis, allow genetic analysis prior to establishment of a pregnancy[13]. The couple undergoes assisted reproduction so that the embryos are produced by *in vitro* fertilisation (IVF). Embryo biopsy is usually performed at the cleavage stage where 1-2 blastomeres are removed or at the blastocyst stage where clumps comprising of 3-5 cells are removed for genetic testing. Embryos without the causative mutation are transferred to the uterus so that if a pregnancy ensues the fetus is expected to be unaffected for the indication being tested.

Here we present two cases of PGD where mosaicism for the causative mutation was detected.

In the first case, mosaicism of an *NF1* mutation in a female patient was identified in lymphocytes during development of a single-cell PCR protocol prior to PGD. This was supported by analysis of polar bodies and blastomeres during PGD.

In the second case, mosaicism for a mutation in *TSC2* was suspected in a female patient during the first cycle of PGD on blastomeres. This was confirmed from further analysis of buccal cells from the patient, in addition to the analysis of blastomeres, trophectoderm cells and whole untransferred embryos of a second cycle of PGD.

Materials and methods

Patient description

NF1

The patient was a female aged 29 diagnosed with NF1. The referral clinical genetics report indicated a heterozygous seven base pair (7bp) duplication in the *NF1* gene which caused a frameshift in the subsequent coding sequence. No family history of NF1 was reported indicating a *de novo* mutation. Based on a 50% transmission risk of the causative mutation, the patient was referred to our centre for PGD.

TSC2

The patient was a female aged 31 diagnosed with TSC at the age of 21. The referral genetics report indicated that she was heterozygous for a substitution mutation C>T resulting in a stop codon and leading to a premature termination of the protein. No family history of TSC2 was reported indicating a *de novo* mutation.

Genomic DNA extraction, lymphocyte separation and buccal epithelial cell preparation

Genomic DNA was extracted from peripheral blood collected in tubes containing EDTA from the patient couples and female patients' relatives using the QIAamp DNA Blood Maxi Kit (QIAGEN, Manchester, UK).

Lymphocyte separation was performed on peripheral blood collected in tubes containing lithium heparin from the patient couples using Ficoll-Paque PLUS (GE Healthcare Life Sciences, Little Chalfont, UK).

Buccal epithelial cells were collected from the patients with a buccal swab, and resuspended in 1x PBS (Invitrogen, Paisley, UK).

Single cell isolation

Single cell isolation was performed on the separated lymphocytes and buccal epithelial cells under an inverted microscope using a 0.2-mm polycarbonate microcapillary (Biohit, Cheshire, UK). Single cells were washed three times in 1x PBS drops, which contained 0.1% polyvinyl alcohol (Sigma, Dorset, UK). The cells were then transferred to RNase-, DNase-free 0.2-ml PCR tubes containing alkaline lysis buffer (50mM dithiothreitol, 200mM NaOH) and stored at -20^{o}C until cell lysis at 65^{o}C for 10 min and further amplification.

Confirmation of mutational status

DNA from the affected female and her partner for both the NF1 and TSC2 cases were analysed by Sanger sequencing to confirm the presence of the reported mutation. DNA from the parents of the affected female with NF1 and from one parent and one sibling from the patient with TSC2 was available. These samples were used to establish the female haplotypes at linked STR markers for the *NF1* and *TSC2* genes respectively.

PCR protocols and fragment analysis

The assessment of informativity and haplotyping for linked microsatellite polymorphic markers was performed on genomic DNA from the couples and family members using PCR with fluorescently labelled primers (Eurogentec, Liège, Belgium). Fragment analysis was performed on ABI 3130xl (Life Technologies, Dorset, UK) and the data was analysed using the Genemapper analysis software v3.5 (PE Applied Biosystems, Warrington, UK).

NF1

Multiplex fluorescent PCR for simultaneous direct mutation detection and linkage analysis was carried out using the following: 1) Primers for the mutation locus encompassing the 7bp duplication and 2) Linked polymorphic STR markers ivs27AC28.4, D17S1166, NF1int29 intragenic and centromeric, and D17S1800 0.26Mb telomeric to the 7bp duplication in the *NF1* gene. The detection of a 250bp PCR product at the mutation locus indicated the presence of the of the 7bp duplication within the *NF1* gene whereas presence of a 243bp fragment identified the normal allele.

TSC2

Multiplex fluorescent PCR for simultaneous direct mutation detection and linkage analysis was carried out using the following 1) Primers for the mutation locus encompassing the C>T mutation, 2) Linked polymorphic STR markers D16S3024 0.44Mb telomeric, D16S664 0.26Mb and D16S663 0.36Mb centromeric to the C>T mutation. A second round PCR with the mutation locus primers and subsequent minisequencing was used to detect the mutant T allele. Minisequencing was performed using the SNaPshot® Multiplex Kit (Life Technologies).

Optimisation of single cell protocols

To optimise the protocols, single cells were tested under different PCR conditions to achieve an amplification efficiency of ≥ 95% and an allele-drop-out (ADO) rate of ≤ 5% at each locus.

Efficiency of the marker was defined as the proportion of cells tested that resulted in amplified product at the mutation locus. ADO was defined as the proportion of cells known to be heterozygous that appeared to be homozygous at a specific locus. This included changing the annealing temperature of the protocols and concentration of the primers for the mutation loci. In addition, two rounds of PCR were carried out such that the mutation locus was split into a singleplex PCR and the remaining STR markers into a quadruplex (in the case of NF1) and triplex (in the case of TSC2) for the second round of amplification. The optimised multiplex PCR protocol was performed on more than 30 lymphocytes (in the case of NF1) and 50 lymphocytes (in the case of TSC2) from the female affected partner. Both protocols used the QIAGEN Multiplex PCR Kit (QIAGEN) at an annealing temperature of 60^{o}C for 40 cycles.

Singleplex PCR for the different mutation markers were also performed using the QIAGEN Taq PCR Master Mix Kit (QIAGEN).

Quantitative fluorescent-PCR (QF-PCR)

Quantitative fluorescent PCR was performed on duplicate genomic DNA samples from the patient with NF1 and her partner with the optimised multiplex protocol for a reduced number of cycles; 20 and 25. QF-PCR was also performed using a singleplex protocol with the mutation primers for the same number of reduced cycles. The QF-PCR was repeated on genomic DNA extracted from the peripheral blood of the patient collected seven months after the first sample. Quantification was performed after measuring the peak areas of the normalized mutant and normal alleles for the mutation locus.

PGD/IVF treatment

The IVF treatment was conducted at the Centre for Reproductive and Genetic Health (CRGH). The CRGH has a license to conduct PGD from the Human Fertilisation & Embryology Authority

(HFEA). Both NF1 and TSC2 are disorders licensed by the HFEA. All consent forms were obtained from the patients regarding PGD and the use of results/findings in research.

Following controlled ovarian stimulation human chorionic gonadotropin was administered and oocytes were collected transvaginally 37 h later.

For the NF1 case, oocytes were denuded and the first polar body (PB1) was biopsied and then intra-cytoplasmic sperm injection (ICSI) was performed. The second polar body (PB2) was biopsied post ICSI. At cleavage stage two blastomeres were removed from each embryo that had more than six cells and one blastomere when the embryo had six cells or fewer.

For the TSC2 case, oocytes were denuded and subjected to ICSI for each of two PGD cycles. A cleavage stage biopsy was carried out as described above. A trophectoderm biopsy was also performed on all embryos that developed into blastocysts. All untransfered embryos from both cycles were collected for follow-up/confirmation analysis.

All biopsied samples and collected embryos were placed in separate tubes containing alkaline lysis buffer. The tubes were stored at -80^{o}C before the cell lysis step and subsequent analysis was performed at the UCL Centre for PGD.

Results

NF1

Single-cell multiplex protocol

To determine the efficiency of diagnosis prior to clinical application, > 30 single lymphocytes from the affected female were tested using the optimised multiplex PCR protocol, with the acceptance criteria of ≥ 95% for amplification efficiency and ≤ 5% for ADO at each locus. Even after many modifications to the multiplex PCR protocol, we observed an unusually high apparent

ADO (29%) for the mutation locus only (Table 1). This high apparent drop-out was solely for the mutant allele and not the normal one (Figure 1 A, B, C).

The high apparent ADO was observed for both lymphocyte (Table 1) and buccal epithelial cells (data not shown) of the female patient. The ADO rates of all other markers were well within the acceptance criteria. In addition, singleplex PCR for the mutation locus on either cell type also showed high apparent ADO of the mutant allele for the affected female patient (data not shown).

Quantitative fluorescent-PCR

To determine if mosaicism was the cause of the high apparent drop-out observed for the mutant allele, QF-PCR was performed. Results obtained from duplicate female genomic DNA samples showed that the mean mutant peak area was considerably lower compared to that of the normal at two different PCR cycles (mean ratio = 0.46 at 20 cycles and 0.655 at 25 cycles). In addition, the allele peak areas of the other heterozygous microsatellite markers present in the multiplex were of comparable size (Figure 1 D). The QF-PCR was repeated on genomic DNA extracted from the peripheral blood of the patient at another time point with similar results (data not shown).

PGD strategy

Polar body 1, PB2 and two blastomeres were biopsied sequentially from each embryo This approach of multiple biopsies was undertaken to ensure a robust diagnosis in spite of the suspected mosaicism for the *NF1* mutation in the female patient.

Embryo diagnosis

Nineteen embryos (designated 1 to 19) were biopsied and a total of 74 cells were collected. All but nine cells (12%) gave results. Seven embryos (3, 4, 7, 8, 9 11 and 17) gave complete results from PB1, PB2 and two blastomeres. These embryos were used to determine the parental

haplotype and deduce the chromosomal segregation at meiosis (Figure 2). Embryo 19 (not included in Figure 2) showed external contamination therefore no diagnosis could be reached. The mutation was not detected in any of the 63 cells that had successfully amplified and that inherited either homologue of maternal chromosome 17.

The female had an embryo transfer that ensued in a pregnancy and a live birth of an unaffected baby. Also, ten embryos that had reached the blastocyst stage were cryopreserved.

TSC2

Single-cell multiplex protocol

With the same acceptance criteria described before for efficiency and ADO, > 50 single lymphocytes from the affected female patient were tested using the optimised multiplex PCR protocol. All tested markers were within the acceptable ranges except for that of the mutation locus which was slightly out of range for ADO (5.9% > 5%) despite many modifications to the PCR multiplex protocol and subsequent minisequencing reaction. However, this slightly high apparent ADO rate did not alert any suspicion of mosaicism at this pre-clinical single cell analysis stage.

Embryo diagnoses at PGD and follow-up

Cycle one of PGD

Six embryos (designated 1 to 6) were biopsied at cleavage stage with a total of 12 blastomeres taken and analysed. Figure 3 shows the expected transmission of the parental haplotypes to the embryos with the exception of embryo 2 that showed only one paternal chromosome. Minisequencing did not detect the mutation in any of the cells from embryos that inherited either homologue of maternal chromosome 16. In order to rule out errors due to technical problems, a rebiopsy was performed at day 4 post-fertilisation for embryos 1, 3 and 6. Embryo 4 had

arrested at this stage and the whole embryo was tubed. Analysis was performed on these samples and minisequencing confirmed the results obtained before. Embryo 5 was a hatching blastocyst on day 6 that was rebiopsied and cryopreserved. The remaining whole embryos (1, 3 and 6) which had arrested and the trophectoderm biopsy from Embryo 5 were analysed by both minisequencing and Sanger sequencing. The results reconfirmed the diagnosis obtained from previous analyses. Figure 3 shows the sequential mutation detection results obtained for embryo 4 as compared to the patient couple.

The optimised PGD protocol was then performed on buccal epithelial cells isolated from the female patient. A high apparent ADO rate (12.5%) was observed for the mutant allele hinting that the female patient might be mosaic for the mutation in her buccal epithelial cell lineage.

Cycle two of PGD

The patient was counselled and opted for a second cycle of PGD. Five embryos (designated 1 to 5) were biopsied at cleavage stage with a total of nine blastomeres taken and analysed. Figure 3 shows the transmission of the parental haplotypes to the embryos with the exception of embryo 5 that showed only one paternal chromosome. Minisequencing detected the presence of the mutant allele T in Embryo 3, but not in Embryo 4, which had the same haplotype at the linked markers indicating that the female patient was mosaic in her germline for the *TSC2* mutation. This finding of germline/gonadal mosaicism was supported by the resultsobtained from the first cycle of PGD where the mutation was not detected in any of the embryonic samples having inherited either maternal haplotype. Having detected both somatic (buccal cells) and germline mosaicism we suggest that the female patient had gonosomal mosaicism for the mutation. Embryo 2 from the second cycle was transferred because it did not have the mutation and also had the other maternal homologue of chromosome 16 compared to embryo 3. The remaining untransferred

embryos were reanalysed by both minisequencing and Sanger sequencing confirming the results obtained in PGD (data not shown).

Discussion

For the NF1 case, the single cell analysis of lymphocytes performed prior to PGD indicated that the female patient was mosaic for the mutation at least in her lymphoblastic lineage. This was supported by QF-PCR performed on her genomic DNA where there was a decrease in the peak area for the mutant allele compared to the normal one. Results from all PBs and embryos did not detect the mutation identified in lymphocytes from the patient indicating that her germline is either mosaic or lacking the mutation.

For the TSC2 case, the single cell analysis performed prior to PGD using lymphocytes did not show a clear indication of mosaicism for the mutation. PGD in the first cycle showed absence of the mutation in spite of each maternal homologue of chromosome 16 being separately represented in the embryos. Testing of buccal epithelial single cells showed a high apparent ADO rate (12.5%) for the mutation and the analyses of untransfered embryos obtained from cycle one and two altogether indicated gonosomal mosacism in this individual.

Both NF1 and TSC are frequent autosomal dominant disorders. Up to 50% of patients with NF1[5-6] and 65 % of patients with TSC[10] have *de novo* mutations with no family history for the disease. This can result in germline mosaicism resulting in < 50% transmission risk, which is a major consideration that should be taken into account in diagnostic settings such as PGD. Clinically, it can be difficult to identify mosaicism for a mutation especially in the case of genetic diseases with variable expressivity.

Here we report mosaicism for a mutation in two disorders that are commonly considered for PGD. Somatic mosaicism was identified in one situation (NF1) through routine single cell

analysis conducted prior to PGD. This led to analysis of PBs and blastomeres in the clinical cycle to ensure that the protocol was sufficiently robust to give a conclusive diagnosis when there was evidence of mosaicism. In another situation (TSC2), both somatic and germline mosaicism for the causative mutation was identified through extensive embryo analysis and follow-up studies, which enabled appropriate counselling of the patient prior to embryo transfer.

PGD for dominantly inherited disorders where there is no family history relies on detection of the mutation in embryos. Once the phase of the mutation is established with the haplotype at linked markers the diagnosis is not only dependent on the mutation locus alone. When mosaicism is identified in somatic tissues, testing of many single sperm will identify germline mosaicism in males. In females however it is not possible to predict whether there is mosaicism or if the mutation is present in the germline.

Recently the blastocyst has become the preferred stage of biopsy although fewer embryos reach this stage of development[14]. ADO is low in trophectoderm samples derived from a blastocyst biopsy and single cell analysis prior to PGD is minimal with no protocol optimisation. In spite of the lower ADO rate in trophectoderm samples a conclusive diagnosis may be difficult in *de novo* cases when there are few embryos and it is uncertain if germline mosaicism for the causative mutation is present. For this reason and for cases of autosomal dominant disorders that show mosaicism, single cell analysis prior to PGD should include the cells of different lineages to identify mosaicism in order to minimise inconclusive results at PGD. Making a diagnosis using independent tests such as polar body analysis together with blastomere or trophectoderm analysis reduces the risk of misdiagnosis in these cases.

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Captions to table and figures

Table 1. Results of the optimised pre-PGD single cell PCR tests of the female patient with NF1 showing an unusually high level of apparent ADO (29%) for the NF1 mutation locus. The apparent drop-out at the mutation locus was only for the mutant allele and not the normal one. The patient was not heterozygous at the D17S1166 locus and thus % ADO was not applicable (N/A).

Figure 1. Electropherograms of the optimised multiplex PCR protocol performed on single lymphocytes (A, B, C) and QF-PCR at 20 cycles performed on genomic DNA (D) from the patient couple. (A) Lymphocyte from the affected female showing both mutant and normal alleles. (B) Lymphocyte from the affected female showing an apparent drop-out of the mutant allele. (C) Lymphocyte from the male partner showing only the normal allele. (D) A decrease in the mutant allele peak area relative to the normal one for the NF1 mutation locus is observed; for comparison, the peak areas of both alleles for STR marker D17S1800 are shown to be of similar size.

Figure 2. The transmission of the parental haplotypes on chromosome 17 to the embryos based on results obtained for the linked STR polymorphic markers IVS27AC28.4, D17S1166, NF1int29 centromeric and D17S1800 telomeric to the mutation locus. The mutant *NF1* allele

(250bp) was not detected in any of the biopsied cells. Embryos were colour-coded and the proposed segregation results are shown. Embryos 1, 2 and 15 showed homologous recombination in meiosis 1. There was a non-disjunction event at meiosis II for Embryo 5, whereas, for Embryo 14 premature segregation of sister chromatids in meiosis I may have occurred. Errors in meiosis I or II appear to have occurred in embryos 10 and 16. resulting in a possible trisomy of chromosome 17 in embryo 16. Each of the two blastomeres from embryo 12 showed a different and only one parental haplotype suggestive of 'embryonic mosaicism' for chromosome 17. E: embryo, PB1: polar body 1, PB2: polar body 2, B: blastomere, NR: no result, ADO: allele-drop-out.

Figure 3. The transmission of the parental haplotypes on chromosome 16 to the embryos for PGD cycles one and two of the patient couple with TSC2. This was based on results obtained for the linked STR polymorphic markers D16S3024 telomeric and D16S664, D16S663 centromeric to the *TSC2* C>T mutation. Compared to the affected female and normal male partners, the mutant T allele was not detected in any of the biopsied cells in cycle one of PGD when analysed by Sanger sequencing and minisequencing (left and right electropherograms respectively), shown here only for embryo 4. Minisequencing in cycle two of PGD detected the mutant T allele in embryo 3, which was used to establish the phase with the linked STR markers. SS: Sanger sequencing, MS: minisequencing, E: embryo, B: blastomere.

Table 1.

	IVS27AC28.4	D17S1166	NF1int29	Mutation locus	D17S1800
Efficiency (%)	32/32 (100)	32/32 (100)	32/32 (100)	31/32 (97)	32/32 (100)
Apparent ADO (%)	1/32 (3)	N/A	0/32 (0)	9/31 (29)	0/32 (0)

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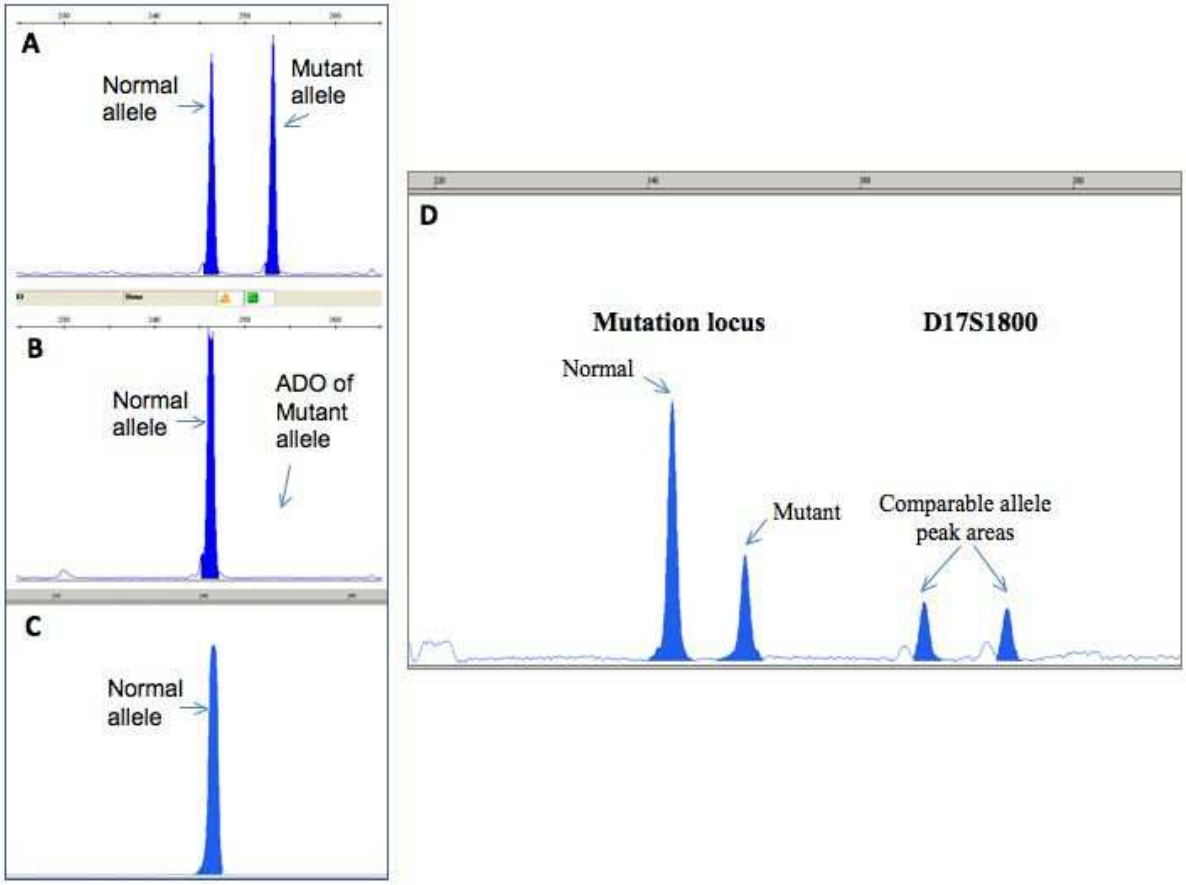
442 Figure 1.



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450 Figure 2.

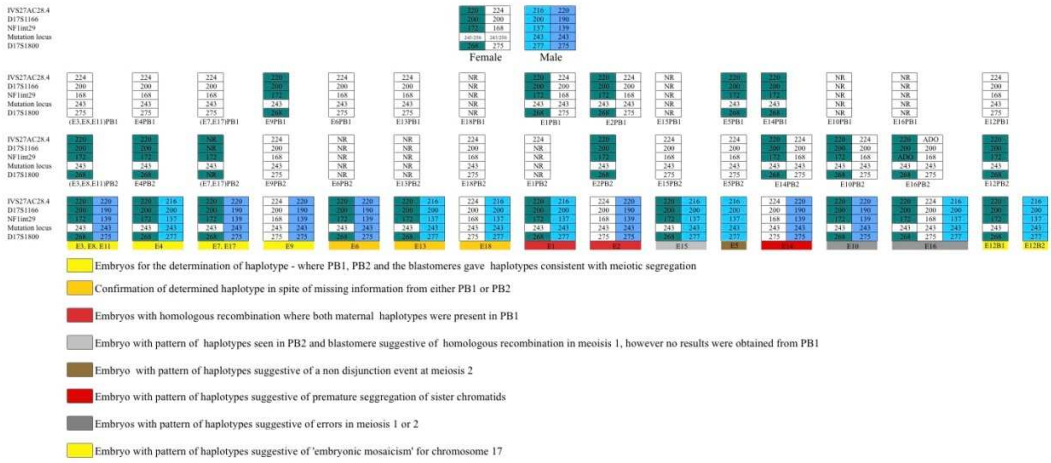
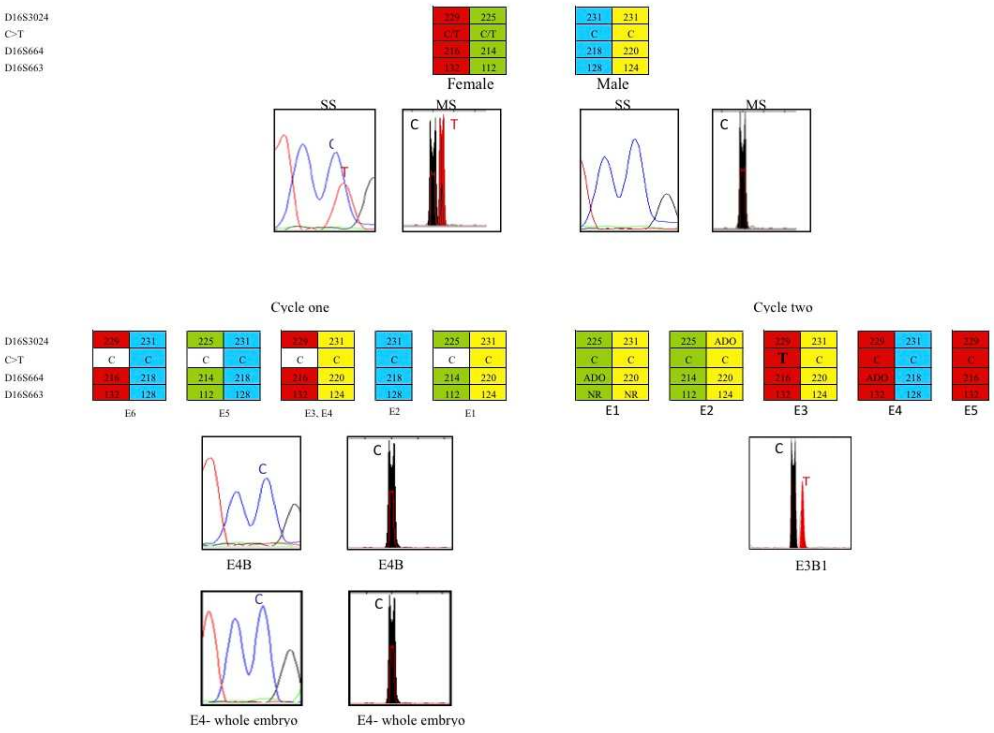

Female
Male
Embryos for the determination of haplotype - where PB1, PB2 and the blastomeres gave haplotypes consistent with meiotic segregation
Confirmation of determined haplotype in spite of missing information from either PB1 or PB2
Embryos with homologous recombination where both maternal haplotypes were present in PB1
Embryo with pattern of haplotypes seen in PB2 and blastomere suggestive of homologous recombination in meiosis 1, however no results were obtained from PB1
Embryo with pattern of haplotypes suggestive of a non disjunction event at meiosis 2
Embryo with pattern of haplotypes suggestive of premature seggregation of sister chromatids
Embryos with pattern of haplotypes suggestive of errors in meiosis 1 or 2
Embryo with pattern of haplotypes suggestive of 'embryonic mosaicism' for chromosome 17

Figure 3.


D16S3024
C>T
D16S664
D16S663
Female
Male
SS
MS
SS
MS
C
T
C
T
C
Cycle one
Cycle two
D16S3024
C>T
D16S664
D16S663
E6
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E3, E4
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E1
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E4
E5
C
C
C
T
E4B
E4B
E3B1
C
C
E4- whole embryo
E4- whole embryo