

Article

Proof of principle and first cases using preimplantation genetic haplotyping – a paradigm shift for embryo diagnosis



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Abstract

Preimplantation genetic haplotyping (PGH) proof-of-principle was demonstrated by multiple displacement amplification (MDA) of single buccal cells from a female donor and genotyping using 12 polymorphic markers within the dystrophin gene; the known paternal genotype enabled identification of the paternal haplotype in the MDA products despite 27% allele dropout. MDA amplified DNA from 49 single human blastomeres with 100% success. The MDA products were genotyped using a total of 57 polymorphic markers for chromosomes 1, 7, 13, 18, 21, X and Y; 72% of alleles amplified providing results at 90% of the loci tested. A PGH cycle was carried out for Duchenne muscular dystrophy. One embryo was biopsied: PGH showed a non-carrier female, which was transferred with no resulting pregnancy. A PGH cycle was carried out for cystic fibrosis. Seven embryos were biopsied and PGH allowed the exclusion of 2 affected embryos; a carrier and a non-carrier embryo were transferred resulting in an on-going twin pregnancy. PGH represents a paradigm shift in embryo diagnosis, as one panel of markers can be used for all carriers of the same monogenic disease, bypassing the need for development of mutation-specific tests, and widening the scope and availability of preimplantation genetic testing.

Keywords: *haplotype analysis, multiple displacement amplification, PGD, PGH, single gene disorders, whole genome amplification*

Introduction

Preimplantation genetic diagnosis (PGD) is a reproductive option for couples at substantial risk of conceiving a pregnancy affected with a known genetic disease who wish to avoid the emotional burden associated with an affected child, termination of pregnancy or recurrent miscarriages (Verlinsky and Kuliev, 1998; Braude *et al.*, 2002; Ogilvie *et al.*, 2005). PGD has been applied clinically for three groups of genetic disorders: (i) single gene diseases, such as cystic fibrosis (OMIM 219700) and spinal muscular atrophy (OMIM 253300), using polymerase chain reaction (PCR) (Verlinsky *et al.*, 1998; Wells and Sherlock, 1998; Sermon *et al.*, 2004) (ii) X-linked disorders, such as Duchenne muscular dystrophy

(OMIM 310200) and X-linked incontinentia pigmenti (OMIM 308300), which can be avoided by sex selection using fluorescence in-situ hybridization (FISH) (Pettigrew *et al.*, 2000); (iii) chromosome rearrangements such as reciprocal or Robertsonian translocations, where FISH can be used to avoid pregnancies with chromosome imbalance (Munné *et al.*, 2000; Scriven *et al.*, 2001; Pickering *et al.*, 2003; Scriven, 2003). In-vitro fertilization (IVF) techniques are used for generally fertile couples in order to generate embryos *in vitro* from which a single cell can be removed as a biopsy for genetic testing on day 3 of culture. Only those embryos diagnosed as free of the disease are transferred to the woman's uterus on day 4 or 5 with the aim of generating an unaffected pregnancy.

Current application of PGD for single gene defects is limited by the need to develop family-specific single-cell PCR mutation tests. These tests simultaneously amplify the familial mutation(s) and one or two closely linked polymorphic markers to control for contamination, or derive human leukocyte antigen (HLA) haplotypes for HLA matching (Rechitsky *et al.*, 2004). The very small amounts of DNA targeted and analysed by these procedures means not only that they are extremely vulnerable to contamination by extraneous DNA, but also that allele dropout (ADO; where one or both of the two alleles at any locus fails to amplify) compromises the accuracy of the diagnoses (Rechitsky *et al.*, 1998; Lewis *et al.*, 2001; Thornhill *et al.*, 2005). In order to offer a robust clinical service, it is necessary to spend lengthy periods designing and optimizing single cell PCR multiplex reactions to minimize ADO, and to carry out PCR testing in designated areas away from other DNA diagnostic work in order to reduce the risk of contamination. These limitations mean that only a few centres have developed the facilities and staffing to carry out these tests, and that the number of disease tests offered by any one centre is usually small, and generally restricted to diseases where there is a common mutation. Some large private centres have overcome these problems by employing sufficient staff to work up new tests efficiently, and to carry out the labour-intensive protocols they have developed for nested PCR analysis of linked markers with family-specific mutations (Verlinsky *et al.*, 1998). Elsewhere, such as in the UK, where PGD is offered by publicly funded services, economic restraints have limited development and application of similar protocols.

Multiple displacement amplification (MDA) utilizes bacteriophage ϕ 29 polymerase for efficient amplification of the whole genome (Dean *et al.*, 2002), and provides large quantities of DNA from small samples (Lasken and Egholm, 2003; Luthra and Medeiros, 2004). Routine use of MDA on single cells is still being evaluated; in initial studies, this application of MDA showed levels of ADO in subsequent PCR assays that varied from 5%–31% (Handyside *et al.*, 2004; Hellani *et al.*, 2004). Nevertheless, it has been used clinically in PGD of cystic fibrosis and β -thalassaemia; MDA products were used as a target for specific PCR assays to detect the familial mutations, alongside simultaneous testing of unlinked short tandem repeats (STR), allowing DNA fingerprinting to monitor contamination and identification of the embryo (Hellani *et al.*, 2005).

Testing family members for multiple polymorphic markers that lie within a disease gene and/or closely flanking it enables the deduction of the high-risk (i.e. mutation carrying) haplotypes(s) that have been inherited by the affected individual(s), and the low-risk haplotypes. Once the high- and low-risk haplotypes within a family have been established they can be used to determine the genetic status of embryos for that family. By testing sufficient markers this approach would overcome: (i) difficulties associated with particular markers being uninformative for a given family; (ii) problems of ADO, since sufficient results will be generated from other markers to compile the haplotypes; and (iii) potential problems that might result from undetected recombination between a mutation and a linked marker, since the large number of loci tested will increase the likelihood of identifying such recombinants and identify the location of recombinations with greater precision.

A proof-of-principle of preimplantation genetic haplotyping (PGH) is presented here. It is a novel approach to embryo diagnosis for single gene disorders that uses the products of MDA to generate extended haplotypes spanning the disease locus in order to determine the genetic status of single cells, and the application of this approach to clinical PGD cycles for Duchenne muscular dystrophy and Cystic Fibrosis.

Materials and methods

IVF protocols

For the PGH treatment cycles, down-regulation, ovarian stimulation, oocyte retrieval, fertilization by intracytoplasmic sperm injection (ICSI), embryo culture and single cell biopsy were as previously described (Pickering *et al.*, 2003).

For the cystic fibrosis carrier couple, the 37-year-old patient was stimulated in her second cycle of treatment on 150 IU recombinant FSH (r-FSH) daily for 12 days. She produced 47 eggs of which 41 were suitable for ICSI. Due to the anticipated risk of ovarian hyperstimulation syndrome (OHSS) transfer was deferred and the fertilized eggs frozen at the two-pronucleate (2PN) stage. The patient only developed mild hyperstimulation symptoms so 2 months later 13 2PN fertilized eggs were thawed, 10 survived and seven embryos were biopsied on day 3.

Preparation and lysis of single cells

Proof of principle

Forty-nine blastomeres were collected from eight cleavage stage embryos (including three sibling pairs) that were not suitable for treatment or freezing during IVF, and had been donated to research as part of an Human Fertilisation and Embryology Authority (HFEA) approved research project (HFEA research licence R0075). Parental samples were not available from the donated embryos so to demonstrate the feasibility of haplotyping on single cells, single buccal cells were collected from a female donor whose father was available to provide a DNA sample. Single cells were washed through polyvinylpyrrolidone/phosphate-buffered saline and collected in 2.5 μ l 200 mM NaOH + 50 mM dithiothreitol using a finely drawn out polished glass capillary. Samples were heated to 65°C for 10 min to lyse the cells then neutralized with 2.5 μ l 200 mM tricine. A series of eight blanks for the blastomeres and four blanks for the buccal cells were included as negative controls.

PGH treatment cycles

The single biopsied cells were lysed as described above and two blanks were included in each cycle as negative controls.

Multiple displacement amplification

Cell lysates were used directly for MDA (Repli-g kit; Qiagen, UK) by adding 45 μ l of 1 \times Reaction Master Mix provided in the kit. Samples were incubated for 16 h at 30°C, the enzyme inactivated at 65°C for 3 min and then held at 4°C. The MDA

PCR analysis

followed by 25 cycles of 94°C for 30 s, 57.5°C for 1.5 min, 71°C for 2 min; then 72°C for 20 min (Mann *et al.*, 2004); (ii) two multiplex PCR assays: to amplify 12 polymorphic di-nucleotide repeat markers across the *DMD* gene on the X-chromosome and four sex chromosome markers (5'DYSII, STRMP, STR2, STR4, STR25, STR44, STR45, STR49, STR62, DXS1214; STR79GT2, STR79GT3, AMEL, SRY, DYS448 and DYSA72); (iii) two multiplex PCR assays: to amplify 10 polymorphic di-nucleotide repeat markers and three tetra-nucleotide repeat markers across and flanking the cystic fibrosis transmembrane regulator (*CFTR*) gene, along with the p.Phe508del (commonly known as deltaF508) mutation on chromosome 7 (D7S523, D7S2554; D7S2502; D7S486, D7S2460; IVS1CA, IVS8CA, Phe508, CFSTR1, D7S2847; D7S480, D7S643, D7S650, D7S2490); (iv) two multiplex PCR assays: to amplify 11 polymorphic di-nucleotide repeat markers and one tetra-nucleotide repeat marker across and flanking the β 3 chain of laminin 5 (*LAMB3*)

embryo cell

Amel
SR Y
DYS448

5DYSII
STR MP
STR2
STR4
STR25
STR44
STR46
STR40
STR62
DXS1214
Str79at2
Str79at3

D1S2872
D1S2885
D1S471
D1S491
D1S246
Lamb3in14
Lamb3in5
D1S205
D1S425
D1S414
D1S2703
D1S419

D7S623
D7S2554
D7S2502
D7S486
D7S2480
MS1CA
MSRCA
Phy10R
CFHr1
h7S7847
D7S480
D7S643
D7S650
D7S2490

D13S742
D13S252
D13S634
D13S306
D13S628

D18S978
D18S391
D18S36
D18S300
D18S819
D18S635

D21S1435
D21S1409
D21S11
D21S1270
D21S1411

key: 3 alleles 2 alleles 1 allele 0 alleles

gene on chromosome 1 (D1S2872; D1S2685; D1S471, D1S491, D1S245, LAMB3in14, LAMB3in5, D1S205, D1S425, D1S414, D1S2703; D1S419).

Assays (ii)–(iv) were set up utilizing a universal tagged primer approach (Heath *et al.*, 2000; Pagan *et al.*, 2004). Each 10 µl reaction consisted of 5 µl 2× Qiagen multiplex PCR buffer, 2 µl of a 4/50 dilution of MDA product, 0.2, 0.4 or 0.8 pmol non-fluorescent tagged primers, 4 pmol of fluorescently labelled tag primers. PCR was carried out on a Dyad thermocycler using the following conditions: 95°C for 15 min; followed by 29 cycles of 94°C for 30 s, 56°C or 58°C for 1 min, 72°C for 1 min; then 72°C for 5 min, followed by 60°C for 30 min. PCR products were run on either an ABI 3100 or 3730 genetic analyser using POP-6 polymer with a Genescan-500 LIZ size standard and analysed using either Genotyper software (Applied Biosystems, Warrington, UK) or GeneMarker (Biogene, Cambridgeshire, UK).

Haplotype analysis

Proof of principle

Buccal cells from the female donor's father were genotyped using the *DMD* multiplex assays to identify the paternal alleles and thus assign the paternal *DMD* haplotype in the donor (data not shown). Samples for genotyping were not available from the couples who had donated the research embryos; haplotypes could not therefore be assigned in the blastomeres.

PGH treatment cycles

For the couple presenting with a family history of Duchenne muscular dystrophy, the *DMD* multiplexes were used to genotype both reproductive partners, and the carrier female's affected brother. The allele sizes obtained from the affected male family member identified the high-risk *DMD* haplotype; nine markers were fully informative (i.e. the two reproductive partners had different sized alleles) (see **Figure 1**).

For the couple presenting with a previous child affected with cystic fibrosis, the *CFTR* multiplexes were used to genotype both reproductive partners and their child. The allele sizes obtained from the affected child identified the high-risk *CFTR* haplotypes; the paternal low-risk haplotype could be identified by seven alleles and the maternal low-risk haplotype by 10 alleles (see **Figure 2**).

Results

Proof of principle

The average yield of DNA from the single buccal cells was 7 µg (range 1–21 µg). MDA blanks gave average readings of 3.7 µg but no alleles were detected in any subsequent PCR. Apparent presence of MDA products was therefore not an indicator as to whether MDA had been successful, and assessment of DNA concentration in further amplified products was not carried out.

The PCR results obtained using the MDA products from single buccal cells were compared with DNA extracted from the donor's blood, from which a 27% ADO was determined,

and compared with the father's genotype to obtain the paternal haplotype (see **Figure 3**). The donor was heterozygous at eight *DMD* markers. One buccal cell had a third allele at the *DMD* marker STR4, consistent with slippage during either MDA or PCR. MDA and haplotype analysis with the eight informative markers provided a mean of six alleles per haplotype, which allowed the *DMD* paternal haplotype to be identified, despite the high ADO at individual loci.

Each of 49 single human blastomeres was subjected to MDA followed by PCR using the multiplexes described; the results are shown in **Table 1**. All MDA blanks were negative in subsequent PCR assays. Ample MDA product remained for any further testing required. Parental samples were not available, but the alleles observed for blastomeres obtained from the same embryo were mostly consistent, and the alleles shared between sibling embryos were consistent with the family relationship. Out of a total of 3415 alleles, only seven (0.2%) alleles with inconsistent sizes were observed, which may have been due to mitotic replication error.

Embryos 1, 2 and 3 were found to be male, embryos 5, 6, 7 and 8 were female, and embryo 4 gave results consistent with XXY. In addition, the results obtained for embryo 4 suggested trisomy for chromosomes 18 and 21, and several instances of mosaic aneuploidy, including monosomy and nullisomy, were observed in the other embryos; this finding is not unexpected, as these blastomeres were from embryos that had arrested in development.

Extreme preferential amplification of some alleles was observed; however, the identification of alleles in the presence of preferential amplification was easier using tetra-nucleotide repeat markers as PCR stutter bands were greatly reduced. Preferential amplification and ADO at specific loci were consistent when a PCR assay was repeated using the same MDA products (**Figure 4**), suggesting that the preferential amplification of alleles occurred during MDA and was not a PCR artefact.

The ADO rate was defined as the number of alleles that failed to amplify from the total number of expected alleles at the heterozygous loci (zero alleles at a locus were counted as two ADO events). Results were compared between cells amplified from the same embryo and within marker panels in order to identify informative (heterozygous) loci to calculate ADO. The following data were excluded from calculating the ADO rate: (i) markers where the same single allele was seen for all cells from an embryo as this marker was likely to be homozygous; (ii) markers for a chromosome where aneuploidy was suspected, for example, only one allele at all markers on a specific chromosome suggested the blastomere may be monosomic for that chromosome; (iii) all data from embryo 4 as this appeared to be the result of partial trisomy rescue; (iv) markers where three alleles were seen as this may be due either to a microduplication event that can not be verified as parental samples were not available, or to slippage of the *Taq* polymerase during PCR.

The amplified alleles ranged in size from approximately 130–490 base pairs and the ADO between markers was similar and therefore independent of allele size. Each single blastomere collected had a visible single nucleus. However, some of these cells may have

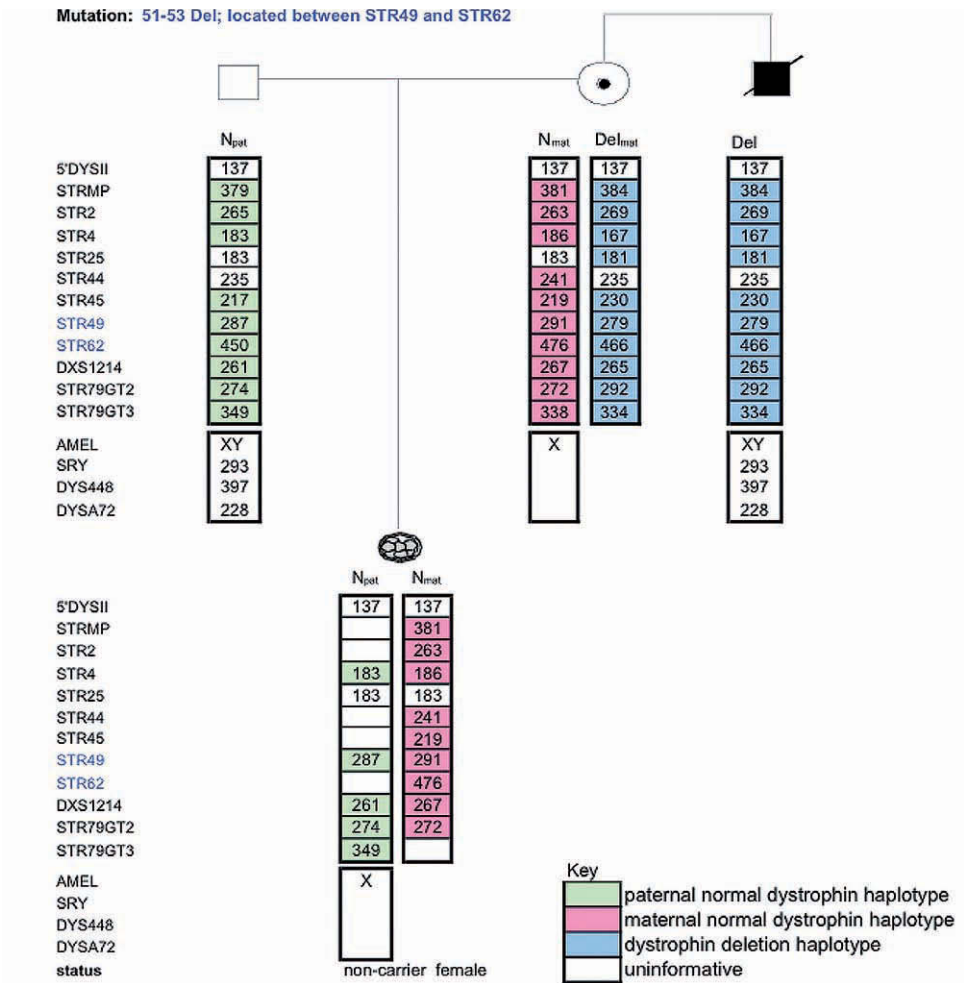


Figure 1. Preimplantation genetic haplotyping (PGH) case for Duchenne muscular dystrophy (DMD) in a woman known to be a carrier of a deletion of exons 51–53. The familial deletion is located between markers STR49 and STR62. The DMD multiplexes were run and an additional marker DYSA72 was included. The affected brother's genotype (solid square) is shown, from which the maternal haplotype associated with the deletion is deduced. Shared paternal and maternal alleles are uninformative for the construction of haplotypes. The single embryo (represented as diagrammatic embryo with internal cells) tested in the PGH case had the paternal and normal maternal haplotypes and was identified as a non-carrier female.

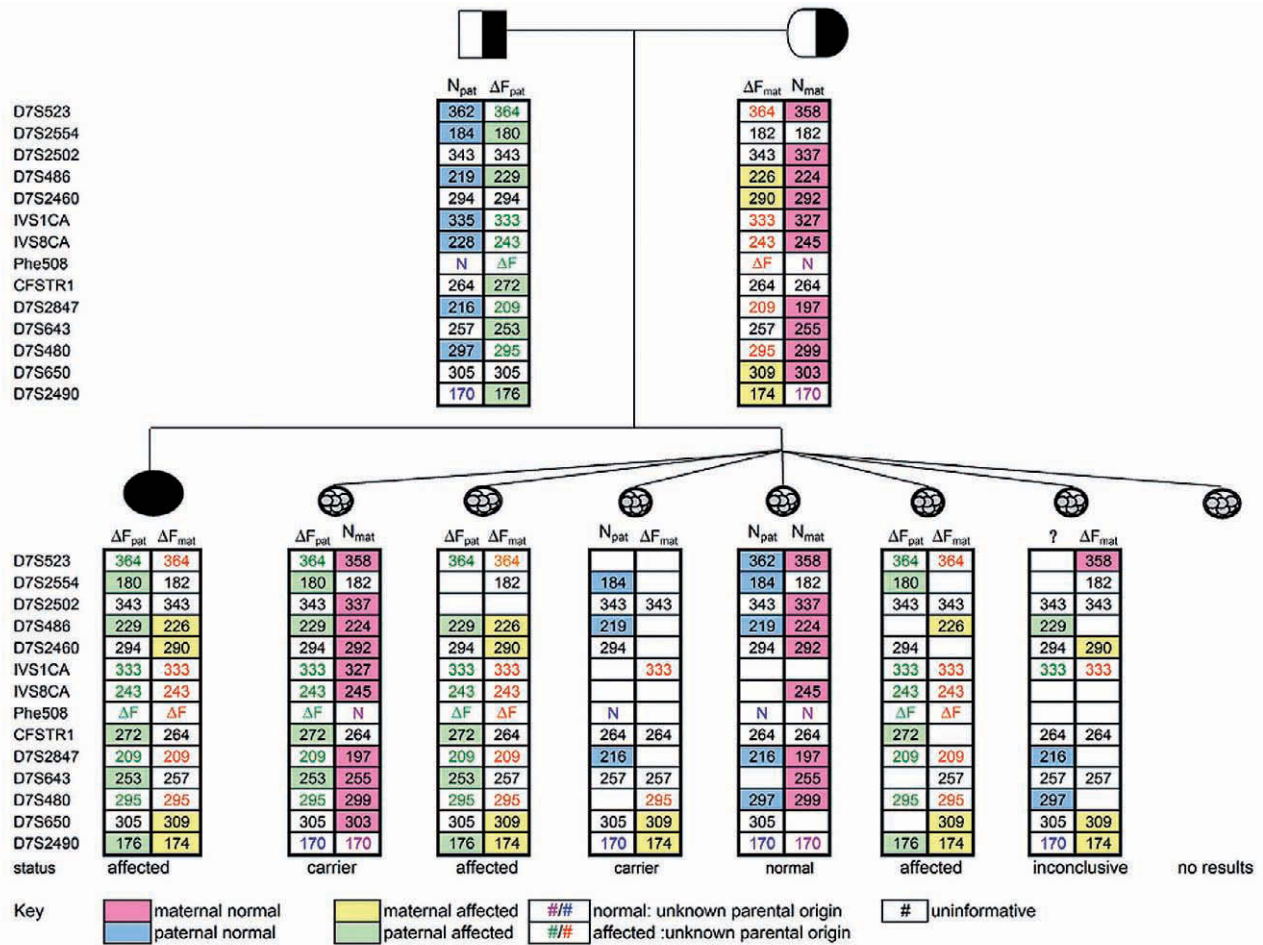


Figure 2. Preimplantation genetic haplotyping (PGH) case for cystic fibrosis in a couple known to be carriers of the p.Phe508del mutation who have a previous affected child, from whose genotype the high- and low-risk haplotypes are deduced. Fully informative alleles are shown in coloured boxes; partially informative alleles with known risk, but of unknown parental origin are shown as coloured numbers. Alleles that are shared between high- and low-risk haplotypes are uninformative for the construction of haplotypes and shown in white boxes. Seven embryos were tested in the PGH case and identified two carriers, one non-carrier, two affected, one inconclusive and one 'no results obtained'.

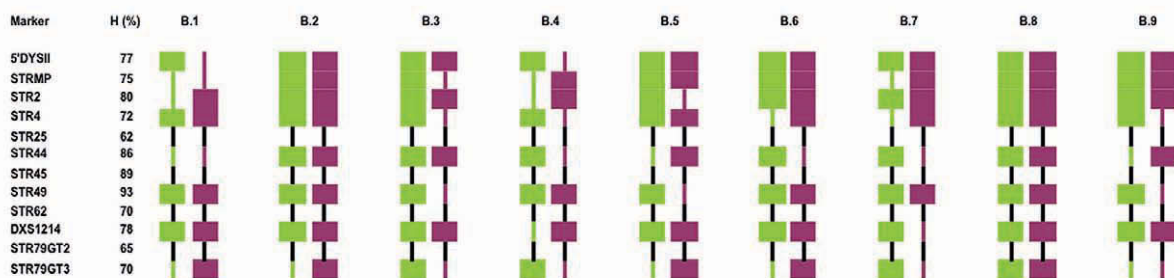


Figure 3. Construction of *DMD* haplotypes from marker analysis of multiple displacement amplification products obtained from nine buccal cells: the markers are shown on the left in their relative order across the *DMD* gene, along with their respective heterozygosity values (H). The paternal green and maternal pink haplotypes are shown for each buccal cell. The coloured boxes represent alleles that were successfully assigned to a haplotype and the coloured line represents allele dropout at an informative marker. The black line represents homozygous markers that are uninformative for the purposes of haplotyping.

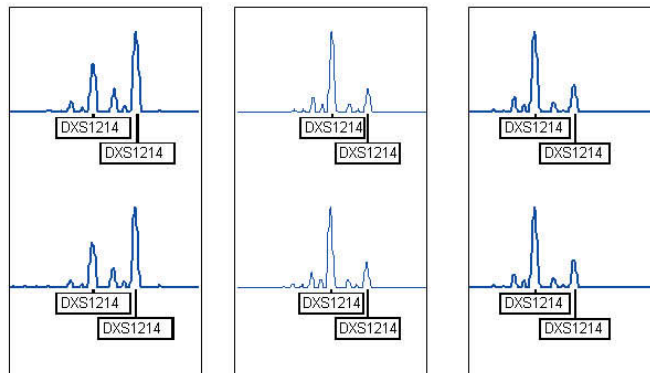


Figure 4. Example of preferential amplification at marker DXS1214. The polymerase chain reaction (PCR) assay was duplicated on multiple displacement amplification (MDA) products from three different buccal cells, shown in separate boxes. Preferential amplification at this locus was consistent between duplicated PCR assays, demonstrating that preferential amplification occurs during MDA.

been degenerate; such cells give significantly lower amplification efficiencies by PCR (Cui and Matthews, 1996). 72% of alleles amplified, giving results at 90% of the markers, amplification rate would be expected to vary, dependent on the quality of the DNA.

The PCR results obtained using the MDA products from single buccal cells were compared with DNA extracted from the donor's blood, from which a 27% ADO was determined, and compared with the father's genotype to obtain the paternal haplotype (see **Figure 3**). The donor was heterozygous at eight *DMD* markers. One buccal cell had a third allele at the *DMD* marker STR4, consistent with slippage during either MDA or PCR. MDA and haplotype analysis with the eight informative markers provided a mean of six alleles per haplotype, which allowed the *DMD* paternal haplotype to be identified, despite the high ADO at individual loci.

PGH treatment cycles

For the Duchenne muscular dystrophy carrier, five eggs were collected, of which three fertilized successfully. Only one embryo was suitable for biopsy on day 3; successful MDA and *DMD* haplotyping of the biopsied cell showed that the embryo carried the low-risk maternal haplotype, as well as the paternal haplotype, and was therefore classified as a non-carrier female (**Figure 1**). The embryo was transferred on day 4 but did not result in a pregnancy.

For the cystic fibrosis carrier couple, MDA and *CFTR* haplotyping was successful for six of the biopsied cells and showed two embryos to be carriers, one non-carrier embryo, two affected embryos and one embryo gave an inconclusive result (**Figure 2**). In this last embryo the maternal high-risk haplotype was present with an extragenic recombination

between D7S2460 and D7S523, and the paternal haplotype also showed a recombination between D7S2847 and D7S486, but it was not possible to determine whether this was high or low risk since the Phe508 locus did not yield a result. A clear nucleus was not observed in the biopsied cell that gave no results. On day 5 the normal embryo with the low-risk maternal and paternal haplotypes was replaced along with the carrier embryo with the low-risk maternal haplotype, resulting in an on-going twin pregnancy. The remaining carrier embryo with the low-risk paternal haplotype was frozen. The embryos not selected for transfer or freezing were amplified by MDA and haplotyped; the results confirmed those obtained on the biopsied cells. The embryo that gave an inconclusive result was shown to have the normal Phe508 allele, implying the embryo had a recombinant low-risk paternal haplotype and was in fact a carrier. A low-risk maternal haplotype was identified in the embryo that gave no result on the biopsied cell.

Discussion

Haplotype analysis at the single cell level, either in conjunction with direct mutation analysis for PGD, or for HLA haplotyping, has previously been described (Verlinsky *et al.*, 1998) (Rechitsky *et al.*, 2004), and is in routine use in a small number of successful large PGD centres. These published protocols utilize 'nested PCR' – a method that results in the application and analysis of multiple PCR assays for each biopsied blastomere and wash drop. This is a labour-intensive and time-consuming process, which is generally only feasible in private centres that are able to charge appropriate fees to provide the staffing required. Most centres in state-funded health services are constrained by the cost of such an approach.

Haplotype analysis identifies family-specific high-risk and low-risk disease regions of the genome in individuals from families with inherited single gene disorders; a series of linked markers within, or in close proximity to, the gene of interest is tested. PGH can be applied to assign haplotypes in any mapped disorder. A minimum of two alleles is required for confident assignment of a haplotype and ideally, to reduce errors caused by recombination, informative markers should flank the disease gene or specific mutation. In the PGH proof-of-principle reported here, we have applied polymorphic markers from within the *DMD* gene, which are in routine use for carrier and prenatal testing for Duchenne and Becker muscular dystrophies. Genotyping of the donor buccal cells provided at least four informative alleles per *DMD* haplotype (see **Figure 3**), which is more information than actually needed for diagnosis and allowed *DMD* haplotypes to be identified despite the high ADO at individual loci. In the PGH cycle for Duchenne muscular dystrophy there was 30% ADO; however, the maternal low-risk haplotype was identified in the embryo by the presence of nine alleles and the paternal haplotype was identified with five alleles (see **Figure 1**). In the PGH cycle for cystic fibrosis there was a mean ADO of 28% (range 0–50%), resulting in a mean of 6.5 alleles (range 3–10) per low-risk haplotype (see **Figure 2**).

The number of markers required for any PGH multiplex to be universally applicable would depend on the heterozygosity of the markers chosen for inclusion. Our studies indicate that for loci with a mean heterozygosity of 85%, five markers would be sufficient. However, an additional factor influencing the number of markers to include is the likelihood of recombination within the region to be tested; 12 markers were incorporated into our *DMD* multiplexes because of the high intragenic recombination rate (10%) seen in the *DMD* gene (Abbs *et al.*, 1990).

The development of a generic multiplex for any given disease will be facilitated by existing routine diagnostic tests and commercially available primers. In addition, recent developments in the Human Genome Project and the availability of database resources means that the identification of polymorphic markers with high heterozygosity within or flanking the disease locus is a relatively straightforward and rapid process, as is designing the primers to amplify these sequences.

PGH specifically targets the familial disease by identification of the high-risk haplotype, and is not designed or intended to detect aneuploidy. However, due to the use of a panel of short tandem repeat markers, PGH may serendipitously also detect aneuploidy for the chromosome tested, but cannot be used to confirm a normal chromosome complement because of the possibility of ADO. ADO may cause only two alleles to be seen at individual markers but the results from all the markers together may allow the identification of three haplotypes, implying trisomy; the presence of one haplotype only would imply monosomy or uniparental disomy. Only embryos showing bi-parental inheritance will be considered for replacement.

For families with X-linked disease who are currently offered PGD by FISH sexing of embryos, PGH will allow the identification of normal males, thereby increasing the number of embryos available for selection in a PGD case, and bypassing the current practice of discarding all male embryos following sexing by FISH.

With the protocols described here, PGH can be applied immediately for carriers of Duchenne and Becker muscular dystrophies and for carriers of any cystic fibrosis mutation, not just p.Phe508del, to identify high- and low-risk genes in embryos. In the PGH cycle for Duchenne muscular dystrophy, the woman responded poorly to ovarian stimulation, and only five eggs were collected; after 3 days of embryo culture, only one embryo was suitable for biopsy. Had this embryo been male, it would have been discarded had FISH sexing been used for the diagnosis, while PGH may have identified a normal male, demonstrating that PGH is even more important in cases where there are a limited number of embryos for consideration. In this case, the single female embryo would have been diagnosed as suitable for transfer whichever approach had been used, but PGH provided the additional information that the embryo was not a carrier of the high-risk haplotype. In the PGH cycle for cystic fibrosis, the couple were both carriers of the p.Phe508del mutation and could have been offered conventional PGD by direct testing for the mutation, however the couple preferred to use PGH as it provides a genetic fingerprint confirming the identity of the embryos.

One drawback of PGH is the need for a pedigree analysis, with at least one affected family member, or sufficient unaffected members to enable categorical identification of high- and low-risk haplotypes. However, for most couples considering this option, DNA from appropriate family members is available at the laboratory where the diagnostic test was originally carried out, and can therefore be accessed for appropriate pedigree analysis and identification of the high-risk haplotype(s). In families where this material is not available, PGD by direct mutation test would be the only option.

The major advantage of PGH over direct mutation analysis is that the same test can be used for all families even when there is heterogeneity in the pathogenic mutations. Testing is no longer limited to common mutations only; for example, couples with any cystic fibrosis mutation can be offered the same linkage analysis test. PGH can also be applied even when the causative mutation has not been identified, such as is the case for some *DMD*/*BMD* families, or when the disease-causing change is not amenable to PCR amplification as in facio-scapulo-humeral muscular dystrophy (OMIM 158900), which can only be detected by Southern blot analysis. This will allow any family with a mapped single gene disorder access to preimplantation testing.

PGH represents a paradigm shift in embryo diagnosis, providing a generic approach that opens the way to the rapid, straightforward development and application of tests for any mapped single gene disorder. The approximately 10^6 -fold amplification of the DNA from a single cell using MDA removes most of the well-documented difficulties associated with single cell PCR. The microgram quantities of DNA obtained from the single buccal cells and blastomeres in this study allowed 57 loci to be analysed simultaneously, and *DMD* haplotypes to be confidently assigned, and removed the need to carry out PCR assays in laboratory areas dedicated to single cell analysis (Pickering *et al.*, 2003). In addition, the testing of a washdrop blank for each biopsied blastomere, an accepted part of PGD protocols to identify contamination (Thornhill *et al.*, 2005), is obviated, as PGH provides an effective 'fingerprint' of the MDA products, in contrast to the one or two linkage markers included in direct mutation tests, and thereby identifies them as originating from the embryo and not from contaminating DNA. Despite the acknowledged high ADO rate

following MDA, the number of linked markers tested meant that 93% (13/14) of haplotypes could be successfully constructed in the two PGH cases presented here. In line with current PGD protocols, following biopsy of day 3 embryos, PGH of blastomeres can be completed before embryo transfer on the following day.

The development of PGH represents a major step forward in widening the scope and availability of PGD for serious genetic disease. Application of PGH will mean that preimplantation testing could be offered by any assisted conception unit with appropriately trained biopsy practitioners, and access to and collaboration with a suitably accredited molecular genetic diagnostic laboratory. Waiting times for establishing new tests for specific disorders will be reduced substantially, and many more families with mapped single gene disorders will be able to benefit from this important alternative to prenatal diagnosis and termination of pregnancy.

Contributors

This study was initiated by Caroline Mackie Ogilvie and Jane Trussler; Stephen Abbs contributed the concept of using MDA products for PGD by haplotype analysis; this concept was extended by Pamela Renwick; laboratory protocols were developed and tested by Pamela Renwick, Jane Trussler, Elham Ostad-Saffari, Hiva Fassihi, Cheryl Black; Peter Braude provided the research embryos; Pamela Renwick, Caroline Mackie Ogilvie and Stephen Abbs managed the project and co-wrote the manuscript with contributions from Peter Braude.

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Conflicts of interest

Peter Braude is Chairman of the Scientific Advisory Committee for the Royal College of Obstetricians and Gynaecologists, and Advisor to the Scientific and Clinical Advances Group of the Human Fertilisation and Embryology Authority.

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Appendix. Primer sequences for markers used in the multiplexes for Duchene muscular dystrophy gene (*DMD*), cystic fibrosis transmembrane regulator gene (*CFTR*) and laminin 5 gene (*LAMB3*).

Marker	Forward	Reverse
<i>DMD</i> multiplexes		
5'DYSII	TAGCTAAAATGTATGAGTACTTGC	TCTGAAATAGTGTTCCTAAGGG
STRMP	ACTCACAAGGTTCCAGATATCTAC	CTATCTTTTTCATTCCAGTTGAG
STR2	CAGCCAGAATATTGACATTACTAC	TCTAGATCCAACTGAAGAGCCTAC
STR4	GCTTTGTCAGAACTTTGTCACCTG	AACTAAGAGTTACATTCCTGTAAAG
STR25	ATATCTCACCATACTTGAGCAGCT	GGACTAATGGAATTCAGCCAAGAC
STR44	TCCAACAATGGAAATCACATTTCAA	TCATCACAAATAGATGTTTCACAG
STR45	GAGGCTATAATTCTTTAACTTTGGC	CTCTTTCCCTCTTTTATTCATGTTAC
STR49	CGTTTACCAGCTCAAAATCTCAAC	CATATGATACGATTTCGTGTTTTGC
STR62	CACCTATGTTTCGCCATCTAGGACA	CAACCATAGTGTTATAAGGCAGAG
DXS1214	TCTGTAGAACCCAAATGACAACCA	AAGATAGCAGGCAACAATAAGA
STR79GT2	CCATAGCTTTAGATGTTGTCTGTG	GTTTGAGCAGCCTAGCAGATGTCC
STR79GT3	TAGATCATGGAACTTCTCAGCTTC	CAAAGAGCCCCAATATTCTCCAGAG
DYSA72	AGGCAGAGGATAGATGATATGGAT	TTCAGGTAAATCTGTCCAGTAGTGA
AMEL	CCCTGGGCTCTGTAAAGAATAGTG	ATCAGAGCTTAAACTGGGAAGCTG
SRY	AGTAAAGGCAACGTCCAGGAT	TTCCGACGAGGTCGATACTTA
DYS448	CAAGGATCCAAATAAAGAACAGAGA	GGTTATTTCTTGATTCCCTGTG
<i>CFTR</i> multiplexes		
D7S523	ATGCTTCATAAGCTCTCTATGGC	CATTTCCATTACCACTGCTATTATC
D7S2554	ATGGCTTTTTGCATACTAAATGC	CAGCATGCTACACCATGTATTG
D7S2502	CTGGAATTGTCTGAGCAGCTAG	CATGTATGCTCATGGTTGGA
D7S486	AGGAGGAAAGGCCAATGGTATATCCC	TTGCAATGAGCCGAGATCC
D7S2460	CAGGCTTAGGATCTCCCTGG	CTGGACTTTACGCTTCTAATATGCAT
IVS1CA	GCTACTCTCGTCAGTACAATGAGT	CTTCAGACTCAAACCTGGAACATTG
IVS8CA	ATCTATCTCATGTTAATGTGTAAGA	ACTAAGATATTTGCCATTATCAAGT
Phe508	TTCTCTGGATTATGCCTGGCACC	CTATATTTCATCATAGGAACACC
CFSTR1	TCTTTTTCTGTCTGTGCTGCATTC	GAACAATTGCATGCCAGCCT
D7S2847	GGTCACCTTCAGAAAGTATTGCC	TAAACTCAGAAGGAAACCATTCG
D7S480	TTCAGGTAGACAAGTTCCTGTC	CTACTCATTTCAACTTTGAGTCTCA
D7S643	GAAATCAAAGCTAATATTGCTCCC	GGAGATGGGTGTAGAGTGAATCTG
D7S650	TAGGCTGCTTAGCCCATAATCT	AAGACATGTGGAAGCGAACTC
D7S490	CTTGCGCAATAAGGTAAGACA	TGCAAGCAATTATGTGCTTGT
<i>LAMB3</i> multiplexes		
D1S2872	GACATTAGGTACATGGGGGATCG	CTACACTTCGGGACATCAGTGAG
D1S2685	CATCCACATCAGCACCTTCATCTAG	GCCATCTCCAAGGGCCTTTTATTGTG
D1S471	CCAGTAACACAGTGCCCAT	CCATTAAAATTGATGATATCGATT
D1S491	GAAAGTTCAAATCTCCCTGGCAATG	CAGACATGGGTTTGAGCTGTGAG
D1S245	GTTTCAGAACTTGCCAATTTAGC	CTCTTTACAATTCACTGTGATTGG
LAMB3in14	CGTGACTCACGATGTGCTAGA	GCCTGTACAATGCTTGTTCATC
LAMB3in5	TGCAGACTGATTGAAGCTCTCACAG	CTCCTACACTTCGCACAGTTGACC
D1S205	GAGAGGAATCCTGAGCACAGCAG	CGAGGCTAAGGGGTGAAGTGAG
D1S425	GCTCTAAAACAAAACACTCAGAACC	GTTTCTTTGTGCAACTGAAGGAC
D1S414	CAGTAAGAGCACAGTTCAACATC	CTCTGTCAATTTAGGTCTATTTCTG
D1S2703	AGCTCTCCAAGTCGGGATAGATG	CATCCATTTACCCAGTGTTGCAG
D1S419	TCCAGGACTGTACATTGGGTACC	CATGGCTTCGCATGTAAGTTGGGG