Analysis of mtDNA variant segregation during early human embryonic development: a tool for successful NARP preimplantation diagnosis

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Background: Diseases arising from mitochondrial DNA (mtDNA) mutations are usually serious pleiotropic disorders with maternal inheritance. Owing to the high recurrence risk in the progeny of carrier females, “at-risk” couples often ask for prenatal diagnosis. However, reliability of such practices remains under debate. Preimplantation diagnosis (PGD), a theoretical alternative to conventional prenatal diagnosis, requires that the mutant load measured in a single cell from an eight cell embryo accurately reflects the overall heteroplasmy of the whole embryo, but this is not known to be the case.

Objective: To investigate the segregation of an mtDNA length polymorphism in blastomeres of 15 control embryos from four unrelated couples, the NARP mutation in blastomeres of three embryos from a carrier of this mutation.

Results: Variability of the mtDNA polymorphism heteroplasmy among blastomeres from each embryo was limited, ranging from zero to 19%, with a mean of 7%. PGD for the neurogenic ataxia retinitis pigmentosa (NARP) mtDNA mutation (8993T→G) was therefore carried out in the carrier mother of an affected child. One of three embryos was shown to carry 100% of mutant mtDNA species while the remaining two were mutation-free. These two embryos were transferred, resulting in a singleton pregnancy with delivery of a healthy child.

Conclusions: This PGD, the first reported for a mtDNA mutation, illustrates the skewed meiotic segregation of the NARP mtDNA mutation in early human development. However, discrepancies between the segregation patterns of the NARP mutation and the HV2 polymorphism indicate that a particular mtDNA nucleotide variant might differentially influenced the mtDNA segregation, precluding any assumption on feasibility of PGD for other mtDNA mutations.

Oxidative phosphorylation disorders are considered to be one among the most common inborn errors of metabolism, and around 15% of them are ascribed to maternally inherited mtDNA mutations.¹ Because of the high recurrence risk in the progeny of carrier females, at-risk couples often ask for prenatal diagnosis. However, the value of prenatal diagnosis of an mtDNA mutation for predicting the clinical phenotype in the postnatal period is questionable, partly owing to the absence of data on the rules that govern the segregation of wild type and mutant mtDNA species (heteroplasmy) among tissues in the developing embryo. Three factors are required to ensure the reliability of such practices: a close correlation between the mutant load and the disease severity, a uniform distribution of mutant load among tissues, and no major change in mutant load with

time, so that the determination of the mutant DNA ratio in a prenatal sample (for example, a chorionic villous sample or amniotic fluid) may be a good indicator of the mutant load in most tissues at birth.² These criteria are suggested to be fulfilled for the NARP 8993T→G mutation, as shown by two studies on mtDNA nucleotide 8993 segregation in several families,^{3,4} but any similar assumption cannot be done for other mtDNA disorders.^{5,6}

Preimplantation genetic diagnosis (PGD) is regarded, theoretically at least, as a suitable alternative to conventional prenatal diagnosis of mtDNA disorders for two reasons. First, it is feasible on single cells soon after fertilisation (eight cell stage). Second, a rapid genetic drift and markedly skewed segregation of mtDNA populations are thought to occur (bottleneck theory) during oogenesis, so that early embryos are expected to carry predominantly either mutant or wild-type mtDNA species. This might be true particularly for the NARP 8993T→G mtDNA mutation, as a study in a female NARP carrier showed that most oocytes inherited either a very low level or a very high proportion of mutant mtDNA.⁷ This feature of mtDNA segregation could be very attractive for PGD, as only embryos showing a very low mutant load should be regarded as suitable for implantation. However, three potential limitations of this approach have to be taken into account. First, the bottleneck size remains largely under debate, and it seems to depend on the type of mtDNA mutation.⁸ Second, PGD for women carrying pathogenic mitochondrial DNA (mtDNA) mutations requires that the proportion of mutant mtDNA species diagnosed in the biopsied cells of the embryo (blastomeres) is an accurate indication of the mutant load in the remaining embryo. That this criterion might be fulfilled could be anticipated from data on mtDNA polymorphic variants in mouse embryos,⁹ but hitherto such data have not been available in humans, irrespective of the type of mtDNA variant. Third, the comments on the predictive value of prenatal diagnosis are also valid for PGD. Probably because of these uncertainties, no PGD for an mtDNA mutation has yet been reported. The only available guidelines on the choice between prenatal diagnosis and PGD were provided during the 74th ENMC International Workshop dedicated to mitochondrial diseases,² as follows: for asymptomatic women with low levels of mutant mtDNA (<50% mutant mtDNA and hence a relatively low recurrence risk), chorionic villus sampling is appropriate, while for symptomatic women and those with levels above 50%, oocyte donation and PGD should be seriously considered.

We report here our first attempt at PGD for the NARP disorder.

Abbreviations: MtDNA, mitochondrial DNA; NARP, neurogenic ataxia retinitis pigmentosa; PGD, preimplantation genetic diagnosis

Table 1 Levels of HV2 polymorphism heteroplasmy in blastomeres from 15 embryos collected from four women

Mother		Embryo		Per cent of 13C allele		Dispersion of heteroplasmy mean value
Identification No	Per cent of 13C allele (WBC)	Identification No	No of blastomeres tested	Mean*	Range†	
1	90	1	4	57	57 (0)	98%
		2	4	98	97 to 100 (3)	
		3	5	0	0 (0)	
2	92	4	2	92	83 to 100 (17)	14%
		5	3	86	77 to 96 (19)	
		6	4	98	94 to 100 (6)	
		7	5	91	83 to 99 (16)	
3	82	8	7	100	97 to 100 (3)	17%
		9	3	91	89 to 93 (4)	
		10	3	93	88 to 95 (7)	
		11	4	91	88 to 93 (5)	
4	91	12	6	74	73 to 78 (5)	15%
		13	3	87	84 to 90 (6)	
		14	6	87	83 to 91 (8)	
		15	9	72	68 to 75 (7)	

The HV2 tract consists of a run of 12 to 18 cytosines interrupted by a thymine at nucleotide 310. Heteroplasmy is common in individuals with more than eight cytosines before the thymine. The maternal HV2 genotype was 13C/14C (patients 1, 2, 4) or 12C/13C (patient 3).

*Average value of the heteroplasmy rates detected in all blastomeres from a given embryo.

†Lowest and highest levels of 13C allele percentage detected among blastomeres of a given embryo (dispersion of heteroplasmy in parentheses).

WBC, white blood cell.

METHODS

DNA was extracted from blood, muscle, amniotic fluid, and cord blood using the Nucleon Bacc3 kit (Amersham Biosciences, Amersham, UK).

Control embryos

Fifteen 4-day embryos were donated to research by four unrelated couples after informed consent. All embryos tested came from our PGD programme and were affected with various genetic diseases without any known relation to mtDNA. Two to nine blastomeres per embryo were available. After removing the zona pellucida, 68 individual blastomeres were collected in individual tubes to be analysed. Blood cell DNA samples from the four mothers were also collected.

Patients

PGD for NARP was requested by a couple who had a eight year old son affected by Leigh syndrome (mutation load = 100% in lymphocyte and muscle DNA samples) and wanted to avoid a pregnancy termination. The proband had an early onset progressive neurodegenerative disorder. Lactic acidosis suggested a mitochondrial disorder, and tomodesintometric investigation revealed symmetrical hypodensity of the basal ganglia and lenticular nuclei associated with cortical atrophy. The mother was an asymptomatic carrier of the NARP 8993T→G mutation (mutation load 18% in lymphocyte DNA) and showed a broad distribution of the NARP mutation load among 30 single lymphocytes isolated from her blood (from zero to 44%, mean 18%).¹⁰

A standard IVF protocol was used and four oocytes were collected and inseminated by intracytoplasmic sperm injection (ICSI), resulting in three embryos at day 3 (two 7-cell and one 8-cell).

Procedures

The hypervariable region 2 (HV2) of mtDNA, consisting of a homopolymeric tract of cytosines (C) located between nucleotide 303 and 315 and showing length polymorphism,¹¹ was amplified by polymerase chain reaction (PCR) in conditions allowing amplification on single cells. Briefly, blastomeres were lysed by heating (65°C for 10 minutes), and mtDNA amplification was carried out using L155 and H389 primers¹² (0.5 µM) in a 30 µl reaction volume, containing

1.5 U of Expand Taq DNA polymerase, 10× PCR buffer 2 (3 µl, Roche Diagnostics, Mannheim, Germany), and 2 mM of dNTP mix (Roche Diagnostics). Thermal cycling consisted of an initial denaturation at 95°C for five minutes, followed by 22 cycles of 20 seconds at 97°C, 30 seconds at 56°C, and one minute 15 seconds at 68°C.

For NARP mutation analysis, two blastomeres were removed from each embryo, pipetted in a separate lysis buffer tube, and lysed by heating to 65°C for 10 minutes. For each embryo biopsied, a blank control was prepared by adding a small amount of the wash drops. PCR restriction analysis was undertaken as previously described.¹⁰ This test has a sensitivity of 2%, with a standard deviation shown to be less than 1.25%.¹⁰

RESULTS

Previous DNA tests on circulating leucocytes detected HV2 heteroplasmy in four control females (table 1). Striking variations in levels of HV2 heteroplasmy were found among patient 1 embryos, while dispersion of heteroplasmy mean values was low, both among all embryos from a given individual and among embryo–mother duplexes. In one case, a complete switch of HV2 mtDNA populations occurred in a single generation (from patient 1 to embryo 3). Dispersion of heteroplasmy was limited in blastomeres derived from a single embryo, ranging from zero to 19%, with a mean value of 7% (table 1). Again, these variations were individual, as patient 2 embryos showed the three largest differences compared with embryos from other individuals. Intercellular variations among blastomeres from a given embryo did not correlate with the stage of development (embryo 5 showed the greatest variation (19%) and was composed of three cells only, whereas embryo 15, composed of nine cells, showed a variation level of 8%).

Considering the variable degrees of heteroplasmy of this mtDNA polymorphism in human embryos, we investigated the degree of heteroplasmy for the 8993T→G NARP mutation in one immature oocyte and three in vitro fertilised (IVF) embryos at day 3 stage from a single NARP carrier (fig 1). The oocyte mutant load was 4% (data not shown). The 8-cell embryo was found to carry 100% of mutated species in the two blastomeres tested, and was therefore not transferred. Compaction of this affected embryo at day 4 prevented

Figure 1 Preimplantation and prenatal diagnosis of the NARP disease. Wild type and mutant 8993T→G mtDNA molecules were amplified and polymerase chain reaction (PCR) products (169 bp) were digested by *Ava*I(10U) for one hour at 37°C.¹⁰ The digestion generated three fragments (93, 70, and 6 bp) and two fragments (163 and 6 bp) for mutant and wild type species, respectively. Only the 93 and 163 bp fragments were fluorescent labelled. Digested products were separated by capillary electrophoresis using an ABI 3100 genetic analyser (Applied Biosystems). Results were analysed using Genscan and Genotyper softwares (Applied Biosystems). Lines 1–6: single blastomere analysis (1–2 and 5–6 from each of the 7-cell embryos, 3–4 from the 8-cell embryo; see “Patients” section above); lines 7 and 8: ensuing prenatal testing (7, amniocyte DNA analysis; 8, cord blood DNA analysis); line 9: undigested PCR product.

isolation and separate study of each of the remaining individual blastomeres, but quantification of mutant mtDNA species in the whole affected embryo consistently showed a 94% mutation load. No detectable amount of mutant mtDNA was found in the two remaining embryos, who were then transferred on day 4, resulting in a singleton pregnancy. Accordingly, no mutant mtDNA species was found in amniotic fluid, sampled as a control of the PGD result (fig 1). The male child was born at 38 weeks’ gestation (birth weight 4 kg). Cord blood DNA analysis, carried out on the parents’ request, detected no mutant mtDNA species (fig 1). This child is healthy at seven months of age.

DISCUSSION

Few data exist on mtDNA segregation during early human embryonic development. Because of technical improvements, analysis of mtDNA distribution has become possible at the single cell level.¹⁰ While various limitations such as allele drop out—a phenomenon whereby only one of the two alleles present is successfully amplified—may hamper the interpretation of PGD for nuclear DNA mutations, this issue should not arise during amplification of blastomere mtDNA, owing to the high copy number of mtDNA molecules in these cells (about 10^3 to 10^5 per blastomere).¹³

Using polymorphism analysis, we found that intercellular variation of mitochondrial heteroplasmy was fairly small among most preimplantation embryos ($\leq 8\%$ in 12 of the 15 embryos tested, table 1). These data are in agreement with results from studies in mouse embryos, showing that the

distribution of mtDNA substitution polymorphisms was virtually identical within blastomeres from a given embryo.⁹ It is noteworthy that in our study, the three remaining embryos with larger intercellular variation (16–19%) all originated from the same individual (patient 2). Though this small amount of data precludes any definitive conclusion on this point, this does suggest that genetic factors might affect mitochondrial segregation during early embryogenesis. Variation in the distribution of the HV2 polymorphism among embryos from a given mother was fairly small (14–17%) in three of the four women tested, while it was very large in patient 1. This variation cannot be ascribed to the level of maternal heteroplasmy, which was grossly identical (at least in white blood cells) in all four women.

Whether a particular feature of mtDNA polymorphism segregation could be generalised to disease causing mutations remains questionable. Thus investigation of mtDNA mutation load in oocyte and human preimplantation embryos is of major importance with respect to PGD for mtDNA disorders.

We investigated the level of heteroplasmy for the NARP mutation in three preimplantation embryos from a female carrier. The mutation load widely varied among embryos (from zero to 100%, fig 1) in agreement with data on oocytes from another group showing a very skewed segregation of the NARP mutation in gametes of a NARP carrier.⁷ The distribution of the mutant and wild type species was virtually identical between two blastomeres from each of the three embryos. To the best of our knowledge, such an analysis of mutant mtDNA segregation in early embryos has never been reported so far in humans or animals. Furthermore, we found a total absence of the 8993T→G mutation in amniocytes and cord blood in the ensuing pregnancy, in agreement with our data on blastomeres.

These results should be examined in the light of available data on NARP prenatal diagnosis. There are at least four reports on prenatal diagnosis for mutations at bp 8993 using DNA from chorionic villi (8 to 12 weeks’ gestation), and occasionally amniocytes (18 to 20 weeks’ gestation) or cord blood (at birth). Extreme mutant loads were observed in each case (undetectable mutation in three cases^{14–15} and 96%, 100%, and 100% mutant load in three affected fetuses).^{16–17} Thus, though additional data are needed before drawing definitive conclusions, PGD may be a valuable alternative to conventional prenatal diagnosis for this mutation. The finding of total skewing in segregation of mutations at bp 8993 during the preimplantation/prenatal period is difficult to reconcile with the intermediate values of NARP mutant load commonly observed in patients after birth. The hypothesis of a varied distribution of mutant load across different tissues is unlikely, as our study corroborates previous data showing apparent stability of the NARP heteroplasmy level throughout the entire human embryo.^{14–17} Should skewed segregation of the NARP mutation be the rule during preimplantation/prenatal periods, it can be hypothesised that intermediate values of heteroplasmy observed after birth result from a selection event occurring during the late stage of intrauterine life or in the postnatal period.

The results we achieved for the 8993T→G mutation cannot be extrapolated to other mtDNA disease causing mutations. As an example, assessment of the mtDNA mutant load in oocytes from an individual carrying the 3243A→G mtDNA mutation showed a random segregation, suggesting that distinct pathogenic mutations might behave in a different manner.⁸ In the current study, discrepancies between the segregation patterns of the NARP mutation and the HV2 polymorphism corroborate these data, indicating that the presence of a particular mtDNA nucleotide variant might differentially influenced the mtDNA segregation, thus

precluding any assumption on feasibility of PGD for other mtDNA mutations.

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