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Is the Bottleneck Cracked?

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Is the Bottleneck Cracked?

Since the development of molecular diagnosis of mtDNA disease, there has been increasing pressure on clinical geneticists for genetic counseling of this uniquely difficult group. However, these advances have revolutionized neither prognostication nor prenatal diagnosis of mitochondrial diseases. This inability to predict risk of affected offspring in mtDNA diseases is largely due to the uniparental inheritance of multiple copies of mtDNA. Recently, however, the prospects for prenatal diagnosis of mtDNA diseases have taken a turn for the better. Until now, the unique segregation of mtDNA mutations and the current state of knowledge have made prenatal diagnosis risky in these diseases: Is the mtDNA in a chorionic villus sampling (CVS) truly representative of the fetus? How closely would the level of mtDNA mutant in either CVS or fetus relate to prognosis? Recent data derived from a mouse model and direct evidence from human oocytes are helping to answer these questions.

The Problem

Despite major advances in our understanding of the mutations and pathogenesis of mtDNA disease, genetic counseling is at best imprecise, and prenatal diagnosis is risky. The late Anita Harding was a reluctant pioneer in this field (A. E. Harding, personal communication). A woman in the original NARP (neurigenic weakness, ataxia, and retinitis pigmentosa, associated with a point mutation at bp 8993) family requested CVS in two pregnancies (Harding et al. 1992). In both cases, analysis of mtDNA indicated that the dose of 8993 mutant mtDNA in the CVS was >95%. Since this suggested that the fetus would be severely affected, both the pregnancies were terminated. Similarly, prenatal diagnosis and termination of a fetus at high risk of NARP has also been performed once in the United States (Bartley et al. 1996). However, until now, many clinical geneticists would feel that the uncertainties are too great to offer prenatal diagnosis.

Every nucleated cell contains thousands of copies of mtDNA, and it is widely held that, in normal individuals, these mtDNAs are substantially identical (homoplasmic). mtDNA is maternally inherited. When a point mutation arises, there may be complete switching to the new mtDNA variant, within a single generation (Hauswirth and Laipis 1982, 1985). Because oocytes contain ~100,000 mtDNAs and yet the mutation presumably occurs once, it appears that only a small number of mtDNAs ultimately populate the organism. This suggests that there is first a restriction in the numbers of mtDNA to be transmitted, followed by amplification, or a so-called genetic bottleneck. Switching, when it occurs, is probably incomplete more often than it is complete, generating individuals who are heteroplasmic (i.e., who have more than one mtDNA type). Heteroplasmy is also a feature of mtDNA disease in which homoplasmy for severe pathogenic mutants may be lethal. Again, large differences in the proportions of variant mtDNAs between offspring suggest that a restriction/amplification event has occurred at some stage. Further accumulation of mtDNA mutants in postmitotic tissues may underlie the progressive nature of mtDNA disease. Hence, in addition to the mitochondrial bottleneck during oogenesis, segregation of mutants in developing tissues could contribute to variation in the level of mutant mtDNA between offspring. Figure 1 illustrates how variation in the level of mutant mtDNA might be generated in children by alternative combinations of bottleneck and postconception segregation of mutant mtDNAs. It demonstrates that the relative importance of bottleneck and segregation of mtDNA after fertilization are critical to the feasibility of prenatal diagnosis of mtDNA diseases. If the bottleneck is wide and has little effect, so that segregation of mutants is the major source of variation (fig. 1, *left panel*), then CVS remains problematic. If, however, a bottleneck during oogenesis causes most of the variation (fig. 1, *right panel*), then sampling either products of conception or oocytes will be representative of

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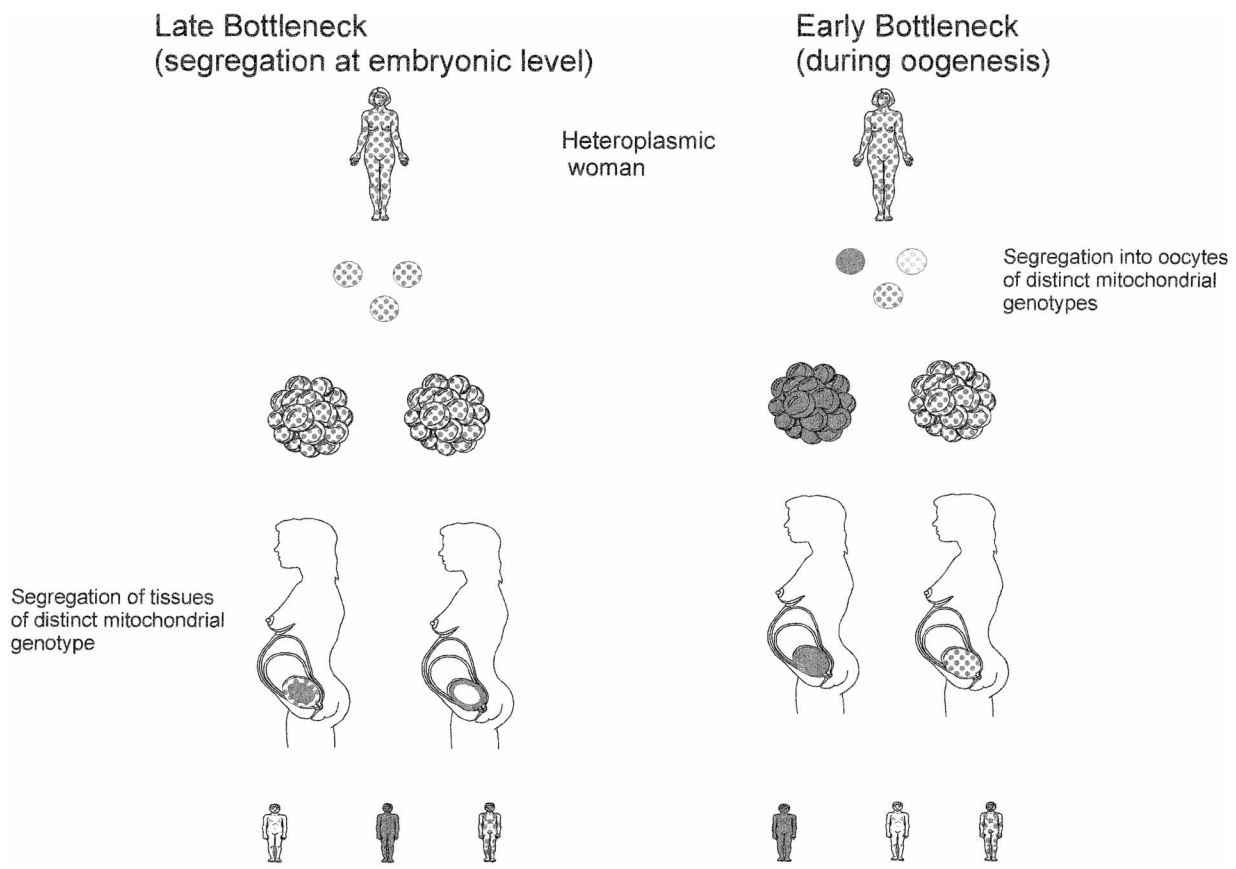

Figure 1 Timing and magnitude of the bottleneck, which are important for prenatal diagnosis of mtDNA diseases. This diagram illustrates two different scenarios in which variation between generations arises either mainly by segregation during development, with a wide bottleneck during oogenesis (*left panel*), or mainly by narrow bottleneck with minimal effect of segregation prenatally (*right panel*). If the bottleneck has little effect, and if segregation of mutants is the major source of the variation (*left panel*), then CVS remains problematic; if, however, a bottleneck during oogenesis causes most of the variation (*right panel*), then sampling either products of conception or oocytes will be representative of the dose of mutant mtDNA to be transmitted.

the dose of mutant mtDNA to be transmitted. Indeed, preimplantation diagnosis, as well as CVS, might be possible. Furthermore, sampling oocytes (e.g., after diagnostic superovulation) might be useful in any effort to advise individual women about the likelihood of bearing an unaffected fetus, which may have been low in the case of Harding's patient (Harding et al. 1992). The two alternatives illustrated in the figure are extremes. The true position for transmission of most mtDNA mutations may lie somewhere between. New data are clarifying the uncertainties about the size and timing of this bottleneck, which have been a major impediment to genetic counseling.

Evidence for a Bottleneck in Oogenesis

The limited data gathered so far suggest that a significant bottleneck has occurred by the time that oocytes are mature and that there is little segregation between fertilization and birth. These data appeared in important studies directly investigating mtDNA in gametes in mice and humans.

Three groups have recently constructed heteroplasmic mouse models of mtDNA segregation, by introducing donor cytoplasm into a fertilized recipient egg, obtaining mice with 5%–80% (Laipis 1996), 0%–30% (Jenuth et al. 1996), and 16%–100% (Meirelles and Smith 1997) donor mtDNA in the resulting mouse lines. Two groups investigated the variance of the proportions of donor mtDNA at different developmental stages. Jenuth et al. (1996) found that most of the difference, in the level of mutant mtDNA, between mother and offspring was generated while primordial germ cells progressed to primary oocytes, in their mouse model. These data suggest that a major component of the mtDNA bottleneck has occurred by the time when oocytes are mature. We showed that individual human oocytes can be heteroplasmic for length variants in a homopolymeric C tract in the major

noncoding region of mtDNA. Tissues from normal individuals possessed one major length variant (>95%), but there was no difference, in the pattern of the length variants, among somatic tissues in any control individual, when bulk samples were taken. In two normal controls, the major length variants differed among oocytes from the same donor, suggesting that segregation of founder mtDNA molecules had occurred by the time when oocytes were mature (Marchington et al. 1997). Blok et al. (1997) identified almost complete segregation of mutant and wild-type mtDNA during oogenesis in a family carrying the pathogenic NARP mutation at position 8993. They found that 7/8 oocytes from a patient with the 8993 mutation were almost homoplasmic for mutant mtDNA and that 1/8 oocytes was homoplasmic for wild-type mtDNA. These studies suggest that a major bottleneck occurs during oogenesis.

Although it is not possible to investigate developing human fetuses longitudinally, the mouse models suggested that mtDNA variants remain relatively evenly distributed throughout developing fetal tissues (Jenuth et al. 1996, 1997). Furthermore, the limited data from studies on human fetuses with pathogenic mtDNA mutations also suggest that mutant mtDNAs do not segregate much during embryogenesis (Harding et al. 1992; Suomalainen et al. 1993; Matthews et al. 1994)—if so, then the process shown in the right-hand panel of figure 1 is the nearest to reality. However, this model would be an oversimplification if there were significant differences either between the behaviors of different pathogenic mutations or between pathogenic mutants and neutral variants in the developing embryo. There is good evidence for nonrandom segregation of mtDNA postnatally, both in mouse models (Jenuth et al. 1997) and in studies of pathogenic human mtDNA mutants in patients (Larsson et al. 1990; Poulton and Morten 1993; Poulton et al. 1995; Weber et al. 1997). Furthermore, several studies of pathogenic mtDNA mutants in cell culture suggest that segregation of mutant and wild-type mtDNA may occur (Hayashi et al. 1991; Yoneda et al. 1992; Dunbar et al. 1995; Holt et al. 1997), perhaps resulting from factors such as impaired mitochondrial function, replicative advantage to mutant mtDNA (Yoneda et al. 1992; Holt et al. 1997), and differences in nuclear background (Dunbar et al. 1995; Holt et al. 1997).

Models Describing the Mitochondrial Bottleneck

Can we use this information to better predict transmission from the mutant load in the mother and in her previous offspring? The equations that have generally been used to estimate mitochondrial bottleneck size are essentially the standard Wright equations for population drift (Howell et al. 1992). These require an accurate estimate of the variance V_n in the offspring, which is rarely available in the British families that we counsel. The difficulty of calculating V_n makes its use impractical in clinical genetics. In addition, most authors have set the number of generations, g, at 15, in an attempt to take the cell divisions of oogenesis into account ("repeated selection").

In contrast, we have used a "single-selection" model (Bendall et al. 1996) to infer the size of the intergenerational bottleneck n, assuming that it represents a one-time sampling of a small number of mtDNAs from a large pool. In this approach, Bayes's theorem is used to evaluate the posterior probability of n, given the observed proportions of the heteroplasmic variants in ovary/blood and oocytes, as well as the estimated experimental error in these proportions. This method is able to estimate a bottleneck size on the basis of the proportion of mutant mtDNAs in a single mother-child pair and is able to make good estimates when these differ, but it is not able to do so when they are similar (since the confidence intervals then become very wide).

Which is most applicable for predicting transmission, the single- or the repeated-selection model? Although, superficially at least, the repeated-selection model appears to represent the physiology more closely, the single-selection model appears to be more appropriate to the data available from small families. The repeated-selection model assumes that the variance in genotypic ratios of the progeny or developing oocytes is caused by an identical sampling event that occurs during each of the 15 or so cell divisions during oogenesis. This assumption may not be justified. Most of the 50-fold expansion of mtDNA that is seen as the primordial germ cells develop into mature oocytes (Chen et al. 1995) occurs after the final cell division of oogenesis. This expansion might be explained by four of five rounds of replication occurring in all mtDNAs. However, data from *Xenopus* oogenesis (Tourte et al. 1984) and from studies in cultured human somatic cells (Davis and Clayton 1996) suggest that a small subpopulation of mtDNAs may undergo numerous rounds of replication, to effect this expansion. If this applies to human oogenesis as well, the single-selection model would appear to be more suitable than the repeated-selection model. In the absence of a more accurate model, what is the size of the bottleneck in the published data cited above, and which of the alternative models generates the best fit to the distribution of heteroplasmic offspring?

Jenuth et al. (1996) used their heteroplasmic mouse model of mtDNA segregation in the female germ line (see above) to demonstrate that a major component of the mtDNA bottleneck has occurred by the time when oocytes are mature. Using a repeated-selection model, they estimated that the number of segregating units was ~200, but this falls to 6–60 (median 13) when a single

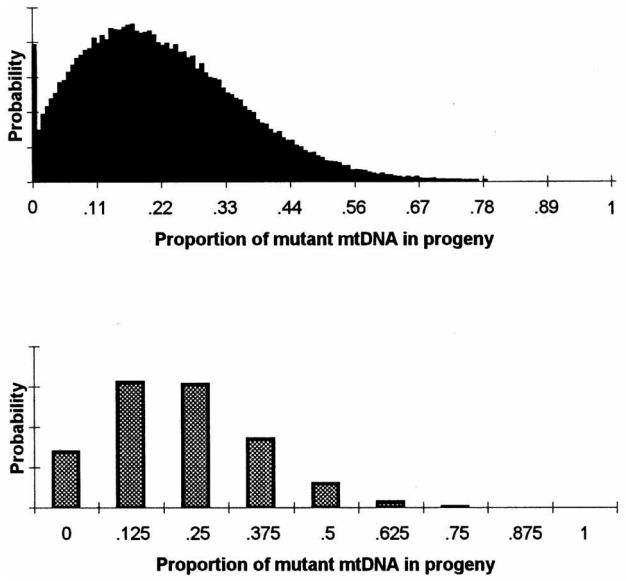

Figure 2 Fitting the repeated- and single-selection models to the data on mtDNA rearrangements: idealized plots for patient 1, for predicted percentage of mutant mtDNA in offspring, when 21% mutant mtDNA is detected in ovary, for repeated sampling ($g = 15$; $n = 135$; *upper panel*) and single selection ($g=1$; $n = 8$; *lower panel*). Both models fit the observed distribution reasonably well (D. R. Marchington and J. Poulton, unpublished data).

selection, rather than 15 selections, is used in the same equation.

We have recently applied the single-selection model to our study of length variants in mature oocytes from normal controls (Marchington et al. 1997) and have estimated that the bottleneck size is one to five segregating units (which could represent one to five mitochondria or mtDNAs). We have also examined oocytes from a patient with Kearn-Sayre syndrome caused by mtDNA rearrangements (D. R. Marchington and J. Poulton, unpublished data). Significant levels of rearranged mtDNA were detectable in the majority of the patient's oocytes, by means of multiplex PCR, with wide variation, in the levels of mutant and wild-type molecules, between individual oocytes. Length-variation polymorphisms in the D310 tract allowed us to identify founder subpopulations of mtDNAs in this patient's oocytes. Using a single-selection model, these respective methods generated most likely bottleneck sizes (n) of 1–31 (mode 8; median 9) and 6–11 in mutant and wild-type molecules. Using single selection in the Wright equation, for the data on the mtDNA rearrangements, we obtained a bottleneck size of 9; using a repeated-selection model we obtained a bottleneck size of 135 (D. R. Marchington and J. Poulton, unpublished data). This range overlaps with but is slightly lower than the data from mouse, suggesting that the apparent disagreement between the conclusions of these studies results more from the mathematical model used than from the different species studied.

Blok et al. (1997) found that 7/8 oocytes from a patient with the 8993 mutation were almost homoplasmic for mutant mtDNA and that 1/8 was homoplasmic for wild-type mtDNA. Using a repeated-selection model, they estimated a bottleneck size >20. However, under both a single-selection model and the assumption that their oocytes are virtually homoplasmic, the most likely bottleneck size falls to 1. This estimate is smaller than the one derived from our data on both controls and patients with mtDNA rearrangements. Previous authors who studied pedigrees with this mutation have noted that they were distinctive in rapid segregation to high levels of mutant mtDNA. It is possible that this is a result of nonrandom segregation of mtDNA, as discussed above. If so, the 8993 mutation may be unusual, because the nonrandom segregation appears to become significant in utero—that is, at a developmental stage earlier than that characterizing many of the other point mutations.

Predicting the Risk of Transmission

Can these data be applied to individual families? We suggest that these recent advances can be applied to the clinical dilemmas in two ways: in assessment of clinical risk and in a limited development of prenatal diagnosis.

Clearly, sufficient data are rarely, if ever, available for calculating the bottleneck size by means of the repeated-selection model. Similarly, it will usually be necessary to estimate rather than measure the level of mutant in ovary or oocytes. With these limitations in mind, we have tried fitting these predictions to observed transmissions in the following two examples. First, figure 2 illustrates the probability distributions predicted by the repeated-selection and the single-selection models for our patient with rearrangements discussed above (when bottleneck sizes of 135 and 9, respectively, are used) (Marchington et al. 1997; D. R. Marchington, V. A. Macaulay, G. M. Hartshorne, D. Barlow, and J. Poulton, unpublished data). The shapes of these two curves are similar, and both fit the observed distribution reasonably well. We conclude that, for practical purposes, either could have been used to advise the patient about the expected level of mutant mtDNA in her offspring at birth. Our ignorance both of how this could segregate postnatally and of the relationship between the dose of mutant mtDNA and the phenotype would, of course, limit its usefulness. For our second example (fig. 3), we have used the family reported by Larsson et al. (1992), because the level of the 8344 mutant (associated with myoclonic epilepsy and ragged red fiber [MERRF] syndrome; see Chomyn 1998 [in this issue]) generally exhibits less variation between tissues than is seen among some of the other, more

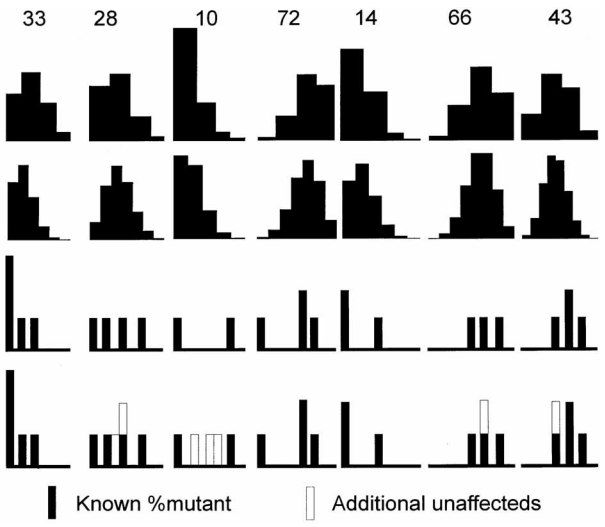

Figure 3 Fitting a single-selection model to data on an mtDNA point mutation. Predictions from data (Larsson et al. 1992) on a family with the MERRF mutation. *Top row,* Percent of mutant mtDNA in blood in the mother. *Second row,* Predicted probability of percent of mutant mtDNA in offspring, with a single-selection model and $n = 3$. *Third row,* Predicted probablity of percent of mutant mtDNA, with a single-selection model and $n = 8$. *Fourth row,* Observed percent of mutant mtDNA in offspring, *Bottom row,* Percent of mutant mtDNA in offspring, including unaffected offspring (*unblackened bars*), whose percentages of mutant mtDNA were estimated on the basis of values in the literature (Hammans et al. 1993). This estimate was necessary because empirical values were not available for all offspring. We suggest that the predictions fit the observations sufficiently well for clinical use in selected families.

common mtDNA mutations; hence, the level of mutant mtDNA in blood may reflect that in ovary. We have used a single-selection model and bottleneck sizes of 3 (a calculation that could readily be done in the clinic) and 8. In figure 3, the proportions of mutant mtDNA in the observed offspring are represented by blackened bars. Since the level of mutant mtDNA was not measured in all of the unaffected offspring, an estimate has been made for these missing values, on the basis of other data (fig. 3, *unblackened bars*). In 5/6 cases, the probability predictions for both $n = 3$ and $n = 8$ fit the observations reasonably well. Since there is a reasonable correlation between level of mutant mtDNA and severity of symptoms in this particular syndrome, we suggest that, in families with the MERRF mutation, this information could be usefully conveyed to informed patients.

We suggest that, once more data have been collected, such estimations will become usable in the medium term; reasonable fits may be more useful to patients than is the quality of information currently issued. Two pieces of information would be important for these calculations: the mean level of mutant mtDNA in oocytes and the bottleneck size. Although it may be reasonable to use blood levels of mutant mtDNA to estimate the level of mutant mtDNA in ovary in patients with the MERRF mutation, variable tissue distribution of mutant mtDNAs excludes this means of estimation in patients with either rearrangements or the 3243 mutation. A better estimate might be reached, in selected cases, by collecting the oocytes, which could be obtained after superovulation of affected women and estimating the level of mutant mtDNA in the individual oocytes (Blok et al. 1997). Collecting sufficient oocytes would enable assessment of the risk of transmission, without the need to estimate the bottleneck size. However, estimates of bottleneck size that are obtained from such studies and from large pedigrees will be critically important in establishing whether the size of n is specific to the characteristics of individual pathogenic mutations or to individual mothers. Second, these new data have implications for prenatal diagnosis. A major component of the variance between individuals seems to arise by the time when oocytes are mature, in all the cases studied so far. If so, prenatal diagnosis by sampling the embryo prior to implantation would seem a logical strategy, although it would not be one that is readily available in many places. The success of CVS in identifying the fetuses that are probably at high risk for developing NARP is encouraging, but it may be a consequence of the distinctive qualities of the 8993 mutation and, therefore, be less applicable to other mutations. Although most clinicians will feel that CVS is not yet widely applicable to mtDNA disease, there is clearly an urgent need to collect the human data needed to complete the picture. This includes quantitation of the mutant-mtDNA level in any products of conception that become available and documentation of the predictions made and of the outcome for families counseled.

In conclusion, new data are clarifying the uncertainties about the size and timing of this bottleneck, which have been a major impediment to genetic counseling. We anticipate that the mechanisms and dynamics of the bottleneck will be cracked during the next few years, provided that there is a concerted international effort toward collecting the information required. The use of donor cytoplasm (Cohen et al. 1997), which is now technically feasible and could theoretically be used to circumvent these difficulties, is unlikely to gain ethical approval in many countries.

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