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Non-disclosing preimplantation genetic diagnosis for Huntington disease

Harvey J. Stern[1,2]*, Gary L. Harton[1], Michael E. Sisson[1], Shirley L. Jones[1], Lee A. Fallon[1], Lilli P. Thorsell[1], Michael E. Getlinger[1], Susan H. Black[1,2,3] and Joseph D. Schulman[1,2,3]

[1]*Genetics & IVF Institute, Fairfax, Virginia, USA*
[2]*Department of Human Genetics, Medical College of Virginia, Virginia Commonwealth University, Richmond, Virginia, USA*
[3]*Department of Obstetrics and Gynecology, Medical College of Virginia, Virginia Commonwealth University, Richmond, Virginia, USA*

Objectives Individuals at risk for Huntington disease face difficult decisions regarding their reproductive options. Most do not wish to pass on the gene for Huntington disease to their children, but may not be prepared themselves to undergo presymptomatic testing and learn their genetic status. For these reasons, many at-risk individuals with a family history of HD would choose a method of genetic diagnosis that would assure them that they can have children unaffected with HD without revealing their own genetic status (non-disclosing). We have shown that, with a carefully designed and executed programme of non-disclosing preimplantation genetic testing, one can successfully assist at-risk couples to have their own biological children who are free from Huntington disease, without forcing parents to confront knowledge of their own genetic status.

Methods Couples where one partner was at 50% risk for Huntington disease underwent *in vitro fertilization* with preimplantation embryo biopsy and molecular analysis for Huntington disease where appropriate.

Results After extensive counselling and informed consent, 10 couples underwent 13 *in vitro fertilization* and two frozen embryo transfer cycles in a programme for non-disclosing preimplantation genetic diagnosis for Huntington disease. In 11 cycles, embryos determined to be free of Huntington disease were transferred, resulting in five clinical pregnancies. One set of twins and three singleton pregnancies have delivered. One pregnancy resulted in a first-trimester loss.

Conclusions The option of non-disclosing preimplantation genetic diagnosis should be reviewed, along with other relevant medical options, when counselling at-risk Huntington disease families. Copyright © 2002 John Wiley & Sons, Ltd.

KEY WORDS: Huntington disease; preimplantation genetic diagnosis; non-disclosing prenatal diagnosis

INTRODUCTION

Huntington disease (HD) is a chronic degenerative disorder of the central nervous system characterized clinically by motor, cognitive, behavioural and affective abnormalities (Craufurd, 1996). HD is inherited as an autosomal dominant genetic trait with complete penetrance. The mutation responsible for HD is an expansion of a $(CAG)_n$ trinucleotide repeat near the 5′ end of the *Huntington* gene located on chromosome 4p16.3. Most individuals have 11–31 copies of the repeat with affected individuals having 38 or greater repeats (Huntington's Disease Collaborative Research Group, 1993). The age of onset of symptoms varies but is typically between 30–50 years, although 4% of patients can have onset before age 20 (Walker *et al.*, 1981).

In many families, the diagnosis of HD is made in a parent when their children are already at reproductive age, and this places many at-risk individuals in a very difficult position regarding their own reproduction (Wexler, 1979). They must cope with the fact that their parent has a fatal disease that they also have a 50% risk of inheriting. In addition, until the diagnosis of HD is ruled out in the individual at-risk, they have a 25% risk of having children who will also eventually be affected with HD.

Most parents do not wish to pass on the HD gene mutation to their children, but may not themselves be prepared to undergo presymptomatic testing and learn about their own genetic status, and potentially the fact that they will develop an incurable disease. In addition to the medical implications of a diagnosis of HD, an affected individual and their family must also face very significant emotional, financial and social issues, including the potential for insurance and job discrimination. For these reasons, many at-risk individuals with a family history of HD would choose a method of genetic diagnosis that would assure them that they can have children unaffected with HD without revealing their own genetic status (non-disclosing).

In 1996, Schulman *et al.* (1996), described a strategy of preimplantation genetic testing for HD and potentially other serious, late-onset dominant genetic

*Correspondence to: H. J. Stern, Genetics & IVF Institute, 3020 Javier Road, Fairfax, VA 22031, USA. E-mail: hstern@givf.com

disorders. Couples where one partner is at 50% risk for HD would be offered the option of having *in vitro fertilization* (IVF) with preimplantation embryo biopsy and molecular testing for HD, without disclosure of the genetic status of the at-risk partner. Couples receive no information regarding the outcome of the embryo testing and are told that only HD-free embryos will be replaced in the uterus or cryopreserved. We report the successful implementation of such a non-disclosing preimplantation genetic diagnosis (PGD) programme at our Institute, and demonstrate that with meticulous attention to confidentiality, careful genetic counselling and fully informed consent, one can successfully assist at-risk couples to have their own biological children who are HD-free, without forcing parents to confront knowledge of their own genetic status.

MATERIALS AND METHODS

Description of programme

Couples interested in non-disclosing PGD for HD undergo a medical intake and genetic counselling with a physician/medical geneticist and programme coordinator (nurse practitioner) prior to initiation of a cycle. The details of an IVF/PGD cycle are reviewed, and other reproductive options are discussed. It is explained that PGD offers an alternative to more traditional methods of prenatal genetic testing such as chorionic villus sampling or amniocentesis, and allows genetic analysis to be performed on early embryos prior to implantation and pregnancy. The information gained by PGD is used to select for replacement in the uterus only those embryos considered unlikely to have the $(CAG)_n$ repeat expansion associated with HD. The preimplantation genetic diagnosis programme at our Institute has been approved by the Genetics & IVF Institute and Inova Fairfax Hospital institutional review boards.

The key aspects of the non-disclosing nature of the cycle are also reviewed during the consultation. Couples are informed that in order not to reveal any information from which they can infer their genetic status, they will not be informed of certain details of their cycle including number of follicles, number of eggs retrieved, number of fertilized eggs, or the number of embryos biopsied, transferred or frozen. Every effort is made to anticipate the potential for any administrative, clinical or laboratory staff to unknowingly provide a means by which the couple could infer their genetic status. Among the clinical staff, only the programme's physician director and nurse-coordinators are aware of the genetic status of the couple. Since PGD patients are treated in the same clinical area as infertility patients, special precautions are used to prevent the inadvertent revealing of information regarding the cycle to the couple. The clinical charts have special markings on the cover and cycle monitoring sheets are colour coded to remind the sonography and nursing staff not to discuss cycle progress with the couple.

Included in the informed consent that couples sign prior to the initiation of a non-disclosing HD PGD cycle is a section which requires the couple to designate (1) the *maximum* number of unaffected embryos they wish to transfer (assuming that number is available), and (2) the disposition of embryos not transferred (cryopreservation, donation, disposal). With the signed informed consent, it is understood that the programme director will act as the couple's agent and make all necessary medical decisions during the cycle. Couples are informed that an IVF/PGD cycle may be cancelled for a number of medical and/or technical reasons relating to the woman's ovarian response, oocyte retrieval, fertilization, embryo culture and biopsy, or the molecular analysis, and that the specific reason for cancellation will not be shared with the couple. It is also discussed that to maintain the non-disclosing nature of the cycle, if no unaffected embryos are available, a transfer of no embryos will be performed. If, on preliminary testing by the PGD laboratory, it is determined that the couple is not at-risk for HD, actual embryo biopsy is not performed, but embryo transfer will occur on the same day as other HD programme transfers (day 4–7 post-oocyte retrieval).

Finally, couples are counselled that at no point in time will the results of their HD status be provided to them by an Institute staff member. The molecular results for the at-risk individual may, however, be provided to a counsellor associated with a comprehensive HD presymptomatic testing and treatment programme upon written, signed request of the couple.

Description of patients

Ten couples underwent 13 IVF and two frozen embryo transfer cycles as part of the non-disclosing HD PGD programme at our Institute. All couples contained a partner at 50% risk for inheriting HD, and in 8 of the 15 cycles one partner was demonstrated to have a $(CAG)_n$ expansion. These were equally distributed between the male and female partner, and all were asymptomatic. A summary of patient and cycle information is presented in Table 1. We have purposefully elected to present cycle information in aggregate rather than provide information regarding individual patients. It was felt that, with the relatively small number of patients, it would be possible for some individuals to gain information about their HD status. If desired, relevant medical individuals can obtain this information by contacting the corresponding author.

Table 1—Patient and IVF cycle details

Age of female partner	Mean 32.5 (range 27–41)
Number of eggs retrieved	Mean 11.5 (range 2–34)
Number of fertilized eggs	Mean 8.5 (range 1–22)
Number of embryos transferred	Mean 2.2 (range 0–6)
Size of expanded CAG allele	Mean 43.1 (range 41–46)

IVF and embryo biopsy

Luteal down-regulation with leuprolide acetate and ovarian stimulation with human menopausal gonadotrophin was performed as in conventional IVF. After administration of 10 000 IU of human chorionic gonadotrophin, oocytes were recovered by transvaginal ultrasound-guided follicle aspiration. All oocytes were inseminated by intracytoplasmic sperm injection (Sherins *et al.*, 1995) to reduce the risk of sperm contamination of extracted blastomeres. The resultant embryos were grown for 72 hours *in vitro* prior to biopsy.

Embryo biopsy was performed as previously described (Fallon *et al.*, 1999), with removal of two blastomeres wherever possible. IVF stimulation and oocyte retrieval for blastocyst biopsy was as for embryo biopsy, except that the resultant embryos were grown *in vitro* for between four to six days using G1 and G2 sequential medium (Zandaer IVF, Vero Beach, FL). Blastocyst biopsy was performed as described by Fields *et al.* (1992).

Molecular analysis

An attempt is made in all families to confirm at the molecular level the diagnosis of HD in the affected parent. If prior DNA testing has been performed elsewhere, the records are obtained and reviewed. If not, it is strongly recommended that the symptomatic parent be tested, since a negative result in an at-risk individual assumes that HD is the correct diagnosis. The informed consent emphasizes that if the diagnosis of HD cannot be confirmed at the molecular level, and another neurological disorder is actually responsible for the condition segregating in the family, then HD PGD will provide incorrect information regarding their genetic risks.

The strategy that is used is to first determine the $(CAG)_n$ repeat number in the at-risk individual and their partner. If no $(CAG)_n$ expansion is seen in either, the couple is considered to be not at-risk and the cycle will proceed with a day 4–7 transfer *without embryo biopsy*. When a $(CAG)_n$ expansion is discovered, the normal allele of the affected partner is compared with the two normal alleles of the other partner. If the repeats differ by two or more, the result is considered informative, and the strategy is used to determine the genetic status of the embryo by testing for presence or absence of the normal allele from the affected parent (Figure 1). Although, in many cases, the expanded $(CAG)_n$ repeat in single cell blastomeres from an affected embryo is amplified, allele dropout is frequently seen, particularly with larger expansions. Allele dropout occurs when different sized $(CAG)_n$ repeats are amplified in a single cell PCR reaction and there is preferential amplification of only one allele (Wells and Sherlock, 1998). This makes a diagnostic strategy based solely on detection of the expanded allele unacceptable for HD PGD. Allele dropout in our series was determined from examination of the blastomere results as well as internal controls using

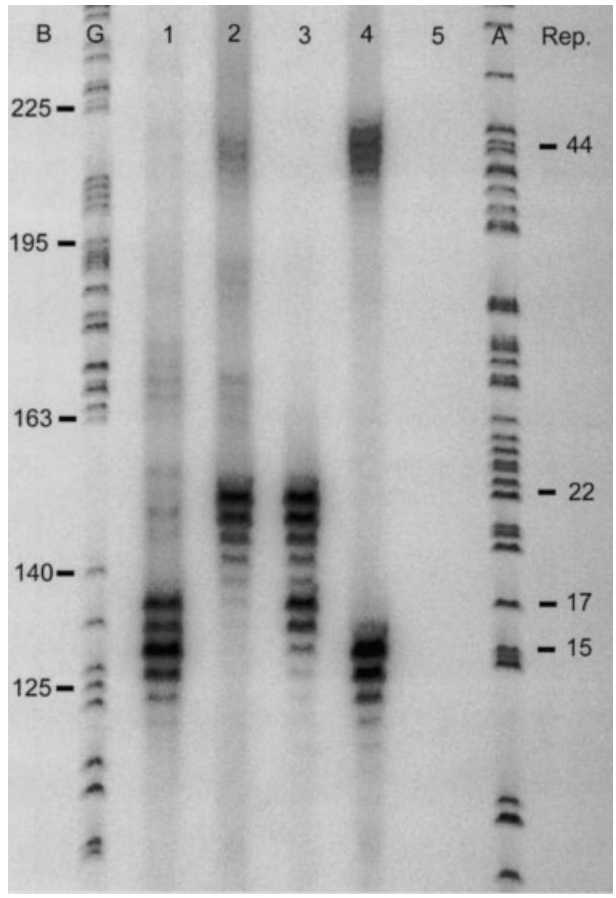

Figure 1—Autoradiograph of polymerase chain reaction (PCR) products from the Huntington gene on maternal and paternal DNA as well as single blastomeres from a preimplantation genetic diagnosis cycle for Huntington disease. B: number of bases; G: G-track from M13mp18 sequencing reaction; lane 1: unaffected partner DNA sample (15 repeats and 17 repeats); lane 2: affected partner DNA sample (22 repeats and 44 repeats); lane 3: single blastomere from an unaffected embryo (17 repeats and 22 repeats); lane 4: single blastomere from an affected embryo (15 repeats and 44 repeats); lane 5: cell-free fluid wash negative control from embryo biopsy; A: A-track from M13mp18 sequencing reaction; Rep: number of (CAG)n repeats

single cell fibroblasts with known $(CAG)_n$ repeats. Overall, the allele dropout rate was approximately 20% (including expansions greater than 35 repeats).

If the normal alleles cannot be distinguished (uninformative), blastocyst culture and biopsy is performed to directly test for the expanded $(CAG)_n$ repeat. Biopsy at the blastocyst stage provides multiple cells that can be used in a single PCR reaction. The risk of allele dropout for the expanded HD $(CAG)_n$ repeat decreases dramatically when five or more nuclei are included in the PCR reaction (G. Harton, unpublished observations).

Nested polymerase chain reaction amplification

Reagents

Denaturation mix was as follows: 50 mM NaOH, 0.06 mM EDTA, 10 mM DTT, with first-round PCR

primers IT1 and IT2 (Leeflang *et al.*, 1995) (0.75 μM each), AMXY1 and AMXY2 (Harton *et al.*, 1996) (0.18 μM each).

First-round PCR mix was as follows: 10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 100 μM each dATP, dCTP, dTTP, 100 μM deaza dGTP (Roche Molecular Products, Indianapolis, IN, USA), 10% (v/v) glycerol, 3% (v/v) dimethyl sulphoxide (DMSO), 0.025 IU/μl of freshly added rTth polymerase (Perkin Elmer Cetus, Foster City, CA, USA) and 0.025 IU/μl freshly added Taq DNA polymerase (Stratagene Inc., La Jolla, CA, USA).

Second-round PCR mix for Huntington $(CAG)_n$ repeat was the same as the first-round mix except using primers IT3 (Leeflang *et al.*, 1995) and end-labelled Hu3 (Reiss *et al.*, 1993) (^{32}P ATP, 6000 Ci/mmol) each at 0.25 μM final concentration, and 0.05 IU/ul of freshly added Taq DNA polymerase.

Blastomere preparation

Individual blastomeres were isolated from the embryo and transferred to a wash dish with phosphate buffered saline supplemented with 5% fetal bovine serum (5% FBS/PBS). The blastomere was washed through three areas of the dish prior to being transferred in 1–2 μl of 5% FBS/PBS to a silanized, 0.5 ml PCR tube containing 5 μl of denaturation mix. The sample was then overlaid with two drops of light mineral oil and incubated at 95°C for 10 minutes in a hot block. 1 μl of neutralization mixture (0.27 M ammonium acetate pH 5.4) was then added to the aqueous phase. The sample was then placed on ice until all samples had been collected. 8 μl of first-round PCR mix were then added to the aqueous phase of each sample. The samples were thermocycled as follows: initial denaturation, 96°C, 5 minutes, followed by 8 cycles of denaturation at 96°C, 1 minute 15 seconds, annealing at 64°C, 1 minute 30 seconds, and extension at 72°C, 2 minutes, followed by 18 cycles with denaturation temperature lowered to 94°C, followed by a final extension at 72°C, 5 minutes.

Blastocyst slice preparation

The trophectoderm slice was isolated from the rest of the blastocyst and washed as for a single blastomere. The slice was then transferred to a silanized, 0.5 ml PCR tube containing 10 μl of denaturation mix. For each sample, only the odd-numbered tube was used, the even numbered tube was saved for later. This sample was then treated to the same hot-block incubation as for the single blastomere, but 2 μl of neutralization mixture was used to neutralize the sample. The samples were then kept on ice until all samples had been treated. Prior to adding the first-round PCR mix to the tubes, each odd-numbered tube was mixed by pipetting the aqueous phase up and down three times and then removing approximately one half of the volume to the even-numbered tube that followed. The sample in the even numbered tube was then overlaid with two drops of mineral oil and placed back on ice. Once all samples had been split, the first-round PCR mix (8 μl) was added to the aqueous phase and the samples were thermocycled as for the single blastomere samples.

Second-round PCR for HD $(CAG)_n$ repeat was performed by adding 1 μl of first round PCR product to 14 μl of second round PCR mix and covering with two drops of mineral oil. Samples were then thermocycled as follows: initial denaturation, 94°C, 5 minutes, followed by 5 cycles of denaturation at 94°C, 30 seconds, annealing at 68°C, 1 minute 30 seconds, and extension at 72°C, 1 minute, followed by 30 cycles with denaturation temperature increased to 96°C, followed by a final extension at 72°C, for 5 minutes. In all PCR reactions, primers specific for amelogenin X and Y were included as internal controls as previously described (Levinson *et al.*, 1992). In addition, several external control DNA samples containing known HD $(CAG)_n$ expansions and normal alleles were co-amplified to assess the performance of the assay.

Huntington $(CAG)_n$ repeat PCR products were visualized as previously described (Harton *et al.*, 1996). To aid in sizing of the PCR products, a G lane and an A lane from an M13mp18 sequencing reaction were run on all gels, along with PCR products from maternal and paternal DNA samples. After drying, gels were autoradiographed with XAR-5 film using an intensifying screen at −80°C for 1 to 2 hours.

RESULTS

Ten couples underwent 13 IVF and two frozen embryo transfer cycles for non-disclosing PGD for HD. In 11 cycles, embryos determined to be unaffected with HD were transferred, resulting in five clinical pregnancies, with one early loss, delivery of three singletons and one set of twins. Both HD mutation carrier and non-carrier couples successfully achieved pregnancy.

DISCUSSION

The localization of the HD gene to chromosome 4p in 1983 allowed predictive testing to be performed on at-risk individuals by linkage analysis (Gusella *et al.*, 1983). When the nature of the triplet repeat expansion in HD was identified, prenatal testing became even more accurate and available for all individuals without the need for family studies. The uptake of testing, however, was low due at least in part to the reluctance of at-risk individuals to suffer the burden of presymptomatic testing.

Two other approaches have been used for non-disclosing HD prenatal diagnosis. In one, the at-risk parent is not tested but analysis of the $(CAG)_n$ repeat is performed directly on the fetus after chorionic villus sampling at 10–12 weeks' gestation. The hope is that a normal $(CAG)_n$ repeat will be found in both alleles of the fetus. However, in 25% of cases, the fetus will demonstrate an increased $(CAG)_n$ repeat which will not only confirm the diagnosis of HD in the fetus, but will disclose the fact that the at-risk parent is affected

with HD. Another more widely used method is prenatal exclusion testing (Hayden *et al*., 1987) where linked DNA markers are used to determine whether the HD allele passed to the fetus from the at-risk parent orginated from the affected or unaffected grandparent. If the linkage analysis indicates inheritance from the grandparent with HD, the fetus shares the same 50% risk of being affected with HD as the intervening parent. Couples are then presented with the choice to either continue or terminate a pregnancy where the fetus has a 50% chance of either being affected or unaffected with HD. In this circumstance, experience indicates that many couples feel forced to proceed to direct fetal $(CAG)_n$ repeat analysis in which case, if abnormal, the HD status of parent and fetus will be revealed. In summary, both direct fetal $(CAG)_n$ analysis and exclusion testing in actual clinical practice provide non-disclosing prenatal diagnosis *only if the fetus is unaffected with HD.*

In contrast, the programme of non-disclosing PGD described here consistently assists couples at high risk for offspring with HD to have healthy children without revealing the parents' genetic status. Couples undergoing non-disclosing PGD are aware of the possibility that they will undergo IVF and may not be at-risk for HD, but accept this as necessary to maintain their choice of non-disclosure of their HD risk. Many couples are extremely grateful that the option now exists for them to have children who will not develop HD regardless of their own genetic risk. The clinical and laboratory staff have received information and education regarding the incidence, prognosis and burden of HD prior to the initiation of the non-disclosing HD PGD programme. Pre- and post-cycle conferences with staff have elicited strong support and respect for the at-risk HD couple's desire not to know information routinely shared with couples having IVF as a result of infertility. Administrative, clinical and laboratory staff do not perceive the necessary steps to prevent inadvertent disclosure of information to the at-risk couple as cumbersome or difficult. No breeches of the non-disclosure protocol have occurred.

Couples whose IVF cycle is unsuccessful, or who return for additional children, have an initial medical review with the programme director where the best approach for future cycles (either a new IVF cycle or frozen embryo cycle) is recommended to the couple without revealing the number of cryopreserved embryos available (if any). It is reviewed that since couples are unaware of the number of embryos produced in previous cycles, the ability to do a frozen transfer cycle does not indicate that they are not at-risk for HD.

CONCLUSION

As pointed out by Schulman *et al*. (1996), since nearly all cases of HD arise in pre-existing HD families rather than by new mutation, the use of PGD can eliminate the transmission of HD in a family and, on a broader scale, potentially decrease the population incidence of HD. Other significant late-onset dominant genetic disorders such as Charcot–Marie–Tooth disease and familial cancer syndromes may also prove suitable for prevention with similar strategy and tactics. We believe non-disclosing PGD offers an important new reproductive alternative for individuals faced with the prospect of a fatal neurodegenerative disorder and a desire to have healthy children, and that this approach should be reviewed, along with other relevant medical options when counselling at-risk HD families.

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