GENETICS

Primer System for Single Cell Detection of Double Mutation for Tay-Sachs Disease¹

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Purpose: Nearly 100% of infantile Tay-Sachs disease is produced by two mutations occurring in the alpha chain of the lysosomal enzyme beta-N-acetylhexosaminidase (HEXA) in the Ashkenazi Jewish population. Although others have described primer systems used to amplify both sites simultaneously, few discuss the allele dropout problems inherent in this test. Our goal was to construct a more robust test enabling stronger signal generation for single cell preimplantation genetic diagnosis and to investigate the occurrence of allele dropout.

Methods: New nested primers were designed to optimize detection of both major Tay-Sachs mutations. Four hundred fifty-seven single cells, including normal cells and those carrying mutations of either the 4bp insertion exon 11 or splice-site intron 12 defects, were used to screen a new primer system.

Results: Based on PCR amplified product analysis, total efficiency of amplification was 85.3%, (390/457). The allele dropout rate for the 4bp insertion mutation in exon 11 and splice-site mutation in intron 12 was 4.8% and 5.8%, respectively.

Conclusions: Multiple mutation detection and analysis within the Tay-Sachs disease gene (HEXA) is possible using single cells for clinical preimplantation genetic diagnosis. Alternative PCR primers and conditions offer various methods for developing systems compatible to specific program requirements.

KEY WORDS: preimplantation genetic diagnosis; single cell PCR; Tay-Sachs disease.

INTRODUCTION

Tay-Sachs disease is a lysosomal storage disease which, in its most severe form, leads inexorably to death during the age of 3–4 years. Tay-Sachs disease is most frequent in the Ashkenazi Jewish population and non-Jewish individuals of French-Canadian ancestry, including the Cajun population in Louisiana, where the carrier frequency is 1/30. In the general population, this frequency is 1/170 (1).

In the Ashkenazi population, nearly 100% of infantile Tay-Sachs disease is produced by two mutations. The first major mutation accounting for approximately 70% of the mutations, is a 4bp insertion (TATC) in exon 11 of the Tay-Sachs gene (HEXA) (2,3). The second mutation, accounting for nearly 30%, is a splice-site mutation at the 5' end of intron 12 where a G is converted to a C (4). Our purpose in this study was to construct a more robust test than has previously been described (6,7), generating stronger signals for single cell preimplantation genetic diagnosis. We report a new single reaction primer system used to amplify both sites simultaneously and discuss the problem of allele dropout.

MATERIALS AND METHODS

Cell Lines

Fibroblast cells, containing the Tay-Sachs disease gene 4bp insertion mutation in exon 11 (GM11853 homozygous and GM03052 heterozygous) and lymphoblast cells, with Tay-Sachs disease gene intron 12

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(G > C) splice-site mutation (GM03461 heterozygote), were obtained from the Human Genetic Mutant Cell Repository at the Coriell Institute for Medical Research (Camden, NJ). Compound heterozygote cell lines for both mutations were not available.

KOH Cell Treatment

A single cell was placed in 6 μ l of KOH solution (200 mM potassium hydroxide/50 mM dithiothreitol), centrifuged and heated at 65°C for 20 min. Six μ l of a neutralizing solution (900 mM Tris-HCL, pH 8.3, 300 mM potassium chloride, 200 mM hydrochloric acid) was then added and brought to 52 μ l with sterile ultra pure water (5).

Polymerase Chain Reaction (PCR)

Primer Design. A novel single reaction nested primer system utilizing 3 outer primers was designed using Primer Designer3 (Scientific & Educational Software) and sequences obtained from Genbank (S62047911 and S62047912). The extra primer (#22) was used to selectively enhance the product for and detection of the exon 11 mutation sequence (see Ref. 14).

The outer-primers were: #16–GCCAGACACAAT-CATACAGG; #18–TCCTGCTCTCAGGCCCAACC-CTC (6); and #22–CAACAACAGTCTGGTGATGG (7).

The nested primers for the 4 base pair insertion mutation in exon 11 were: #16 and #43–CTTTGG-TTAGCAAGGAGAG.

The primers for the intron 12 (G > C) splice-site mutation were: #20–CCCCTGAGCAGAAGGCTCT-GGTG and #18 (Figure 1).

First Run PCR. The initial 90 μ l reaction volume containing DNA (single cell) and consisting of PCR buffer [(2.5 mM MgCl₂, 10 mM Tris-HCl, pH 8.3, 33 mM KCl, 0.01% wt/vol gelatin), 200 μ M dNTP (Perkin Elmer), 0.1 μ M outer primers, and 3 U “AmpliTaQ Gold” polymerase (Perkin Elmer)] was placed in a DNA thermal cycler (Perkin Elmer PCR system 9600). After an initial denaturation of 96°C for 12 min, two programs were used in succession for the first run PCR: (1) five cycles were carried out using 97°C denaturation for 30 s, 68°C annealing for 45 s, and 72°C extension for 45 s; (2) twenty cycles were carried out using 95°C denaturation for 20 s, 68°C annealing for 30 s, and 72°C extension for 45 s. Final product extension was carried out at 72°C for 12 min.

Second Run PCR. A PCR reaction mix was used for the second run PCR consisting of 1.5 mM MgCl₂, 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 0.001 wt/vol gelatin, 200 μ M dNTP (Perkin Elmer), 0.3 μ M inner primers, and 0.5 U “AmpliTaQ Gold” polymerase (Perkin Elmer). Two microliters of first run PCR product were added to 23 μ l of the second run reaction mix. After an initial denaturation of 96°C for 12 min, 35 cycles were performed as follows: 95°C of denaturation for 20 s, 52°C annealing for 30 s, and 72°C extension for 45 s, followed by 72°C extension for 12 min. For Intron 12 mutations the annealing temperature was 64°C.

In addition to the tubes containing DNA from samples being tested, reaction tubes containing only master mix reagents plus water (without added DNA) were run simultaneously as negative controls against possible contamination from extraneous DNA. Because these controls contained no added DNA, no amplification signal should be seen. Any amplification observed in these tubes would indicate a contaminating DNA source of unknown origin. Evidence of contamination will invalidate the experimental set.

Analysis of PCR Product

Sizes of the expected second run amplified products are 206bp (exon 11) and 135bp (intron 12).

Heteroduplex Formation

In order to specifically identify the amplification products in the presence of the 4bp insertion exon 11 mutation, reannealing of the PCR products was performed to produce an artifact-heteroduplex formation. PCR product from both a known normal DNA (6.5 μ l) and unknown DNA sample (6.5 μ l) was placed in a reaction tube. The mixture was denatured at 95°C for 5 min, and then allowed to reanneal at 65°C for 5 min. Electrophoresis of 10 μ l of the reaction was performed using 10% polyacrylamide gels which were then stained with SYBR Green I (8). The PCR product of the sample cell with an unknown DNA status was run on two lanes of the gel, alone and with the known homozygous normal DNA PCR product. The following results were observed (Fig. 2). If the two lanes both formed a single band, then the sample was diagnosed as normal. If the unknown sample (run by itself) forms a heteroduplex-banding pattern (extra two higher bands), then the unknown sample contains both a normal and abnormal allele and is therefore a heterozygote-carrier. If the untreated sample lane forms a single band and

ORIGIN exon 11

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#16-----
1 ccaggaatct ctcagcttt gtgtccttac tgccattga cctttataa cagattcaGC
61 CAGACACAAT CATAcAGGtg tggcgagagg atattccagt gaactatag aaggagctgg
121 aactgggtcac caaggccggc ttccgggcc ttctctctgc cccctggtac ctgaaccgta
181 tatic*ctatgg cctgactgg aaggatttct acgtagtga acccctggca ttgaagggtg
-----#43
241 aaagcagaga gCTCTCCTTG CTAACCAAAG gaggct //

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(unknown intervening sequences)

ORIGIN intron 12

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-----#22                                #20-----
1 agttacocca CCATCACCAG ACTGTTGTTG cttgtttcc ctcaggtacc CCTGAGCAGA
61 AGGCTCTGGT Gattggtgga gagccttgta tgtggggaga atatgtggac aacacaaacc
-----#18
121 tggccccag gctctg(G)taa gggttttcgg gggggagggtg GAGGGTTGGG CCTGAGAGCA
181 GGAggttctcc ctagagacc caa //

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Fig. 1. Primer map for the simultaneous amplification of two mutations for Tay-Sachs disease. First round PCR amplification uses primer sets #16, #22, and #18 to amplify sequences encoding exon 11 and intron 12 mutation sites. Second round amplification uses primer sets #16, #43, #20, and #18 to separately amplify exon 11 and intron 12 mutations respectively. Hatch marks (///) represent unknown intervening sequences. "Mutation *" marks the site of the two Tay-Sachs mutations. Arrows denote the 5' to 3' direction of synthesis. See Materials and Methods for the sequences of each primer numbered from beginning to end within the primer box. The entire sequence may be obtained from Genebank.

Fig. 2. Heteroduplex formation of PCR products from Exon 11 amplification. Lane M: 100bp DNA marker. Lane 1: normal cell PCR product control. Lanes 2, 4, 6, 8: product from amplified cell. Lanes 3, 5, 7, 9: equal volume mixture of normal cell PCR product and products shown in Lanes 2, 4, 6, 8, respectively. "A" indicates affected gel profile; "N" indicates a normal gel profile; "C" indicates carrier status. Assay was performed using 10% polyacrylamide gel electrophoresis.

the mixed lane forms the heteroduplex banding pattern, then the diagnosis of the unknown sample is homozygous affected.

Enzymatic Digestion

In order to determine the status of the exon 12 mutation, 5 μ l of PCR product was placed in 20 μ l 1X React₃ buffer containing 5 unit DdeI (BRL). Digestion occurred at 37°C for one hr. The digest was then submitted to electrophoresis on a 10% polyacrylamide gel that was then stained by SYBR Green as above. For a normal allele, the 135bp PCR product is cut into three fragments of 120, 11, and 4bp. In contrast, the mutated allele yields four fragments of 85, 35, 11, and 4bp. Heterozygotes give fragments of 120, 85, 35, 11, and 4bp (Fig. 3) (4). Fragments 11bp and 4bp are not observed under the conditions employed for electrophoresis. Control samples containing plasmid pBR-322 DNA (25ng) were run to confirm enzyme digestion activity.

RESULTS

Making use of the described primer sets, 108 fibroblasts (comprising 50 normal cells and 58 affected homozygous cells) were tested as single cells for the

Tay-Sachs 4bp insertion mutation in exon 11. In addition, 117 known heterozygous fibroblasts were tested for the same mutation. In other experiments, 232 lymphoblasts (heterozygotes) were tested for the intron 12 (G > C) splice-site mutation. The total efficiency of amplification for the combined tests was 85.3% (390/457). The allele dropout rate for the exon 11 carrier and the intron 12 splice-site heterozygote, was 4.8% and 5.8%, respectively. Sequencing data (not shown) of second run products verified the correct sequence amplification in the primer-guided reactions.

DISCUSSION

Two mutations are commonly linked to Tay-Sachs disease in the Jewish population. A 4bp insertion in exon 11 and a splice-site mutation at 5' end of intron 12 (G > C) account for almost all Tay-Sachs disease in Ashkenazi Jews. We have developed a new single reaction primer system that can be used to amplify these two Tay-Sachs mutations simultaneously. This system constitutes a more robust test for these mutations delivering stronger signal generation and lower ADO rates than those previously described for single cell preimplantation genetic diagnosis.

Although others have described primer systems used to amplify both mutation sites (6,7), those primers led

Fig. 3. Gel profile of Intron 12 mutation product treated with DdeI endonuclease. Lane M: 100bp DNA marker. Lane 1: normal cell PCR product control (135bp). Lanes 2: uncut pBR322 DNA. Lane 3: pBR322 DNA cut by DdeI (180bp band barely visible). Lane 4, 5: normal cell PCR product with pBR322 cut by DdeI. Lanes 6-9: single lymphoblast of affected Tay-Sachs (intron 12 G > C) splice-site mutation in one allele) PCR product with pBR322 DNA cut by DdeI. Assay products observed following electrophoresis on 10% polyacrylamide gel.

either to unconfirmed products or weak signals in our laboratory. We have employed a novel outer primer technique, utilizing three primers, to initiate amplification of initial products. This system helped generate specific first round amplification products while newly arranged inner primers enhanced signal strength. The third first round primer was used to selectively enhance the production of exon 11 sequences (14).

Along with generating specific tests for these mutations, it was important to determine the allele dropout rate. Other publications have also discussed the importance of allele dropouts in heterozygote and compound heterozygote situations and how they may affect the procedure clinically (9,10,11,12,13).

We screened 108 fibroblasts (including 50 normal and 58 homozygous cells) and 117 heterozygous fibroblasts for a 4bp insertion mutation in exon 11. We also tested 232 lymphoblast cells (heterozygotes) for intron 12 splice-site mutations. The combined efficiency of amplification was 85.3% in our setting and compares to 89.5% for those amplifying both Tay-Sachs mutations simultaneously (6). We also determined the allele dropout rates as 4.8% and 5.8%, respectively. Single cell amplification efficiencies and dropout rates can vary greatly depending on cell type, numbers reported, as well as, solubilizing conditions, and various PCR parameters.

There is virtually no danger of transferring an affected embryo due to allelic dropout when a cell is homozygous (including either affected or normal cells), since the remaining allele will correctly identify the cell (barring contamination of the sample by extraneous DNA). In the case of heterozygote or compound heterozygote cells however, there is a 4.8% ~ 5.8% chance that the embryo will actually be a heterozygotic

carrier, when the cell is diagnosed as normal, due to allelic dropout. Transferring a carrier embryo instead of a normal embryo in approximately 5% of embryos is a clinically acceptable situation since the offspring will not be affected by the disease. However, if the embryo is a compound heterozygote, and diagnosed as normal, there will be an additional 0.25% probability of the embryo actually being affected with the disease due to a double dropout occurrence. This can occur due to the separate amplification of each mutation during the second round of PCR amplification. Likewise, if the embryo is diagnosed as a heterozygote, there will be a 5% chance that it will actually be affected with the disease. Thus, it would never be clinically acceptable to transfer a carrier in the case of compound heterozygotes (Table I).

In order to reduce the risk of transferring a compound heterozygote embryo, which may be affected by Tay-Sachs after birth, two embryonic cells should be tested for each embryo. Only when both cells concur a normal status can there be assurance (99.875%) that the disease will not be transferred. If cells are diagnosed as carriers in this situation (two cells), then there is only a 0.25% chance that the embryo will be affected. However, since one half of those embryos will not concur as to the allele that dropped out and therefore not be transferred, this leaves only half the number of carriers, or 0.125% chance, that a diagnosed carrier will actually be returned affected with the disease (Table I).

It is important to determine allelic dropout rates with each new primer system in order to accurately assess the probabilities of transmitting disease with the embryos. Allelic dropout rates are also the basis of informing the patient of the chances of having an

Table I. Hypothetical Probability of Returning Designated Embryos Following Results Obtained from One or Two Cell Assays

Test type	Diagnosis	Probability ^a
One cell test	Normal cell (homozygote)	95% Normal 5% Carrier 0.25% Affected (compound) ^b 100% Carrier
	Carrier cell (heterozygote)	5% Affected (compound) ^c 99.75% Normal 0.25% Carrier 0.0025% Affected (compound) ^b 100% Carrier 0.125% Affected (compound) ^b
Two cell test	Normal cell/normal cell (homozygote)	
	Carrier cell/carrier cell (heterozygote)	

^a Percentages based on an allelic dropout rate of 5%.

^b Rate is not considered clinically significant and therefore embryo may be transferred.

^c Rate is unacceptable and embryo should not be transferred. Discrepant results from the testing of 2 cells from the same embryo would not permit the transfer of that embryo.

affected fetus even though the embryo was diagnosed as either being free of the disease or only being a carrier. This information helps the patient realize the importance of conducting prenatal testing during a PGD pregnancy. Further refinement of PCR primer sets may help to eliminate the incidence of unrecognized allelic dropouts in cases of compound-heterozygote cells in the future.

In conclusion, multiple mutation detection and analysis, within the Tay-Sachs disease gene (HEXA), is possible using single cells during preimplantation genetic diagnosis. The primer system described here permits reliable and robust signal generation for two prominent mutations in the Ashkenazi Jewish population. The allele dropout rate for these reactions have been determined and specific recommendations made to insure that every precaution is taken to avert the transfer of affected embryos. Patients must also realize the limitations of the procedure through patient consent.

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