Assessment of Genetic Polymorphisms in DNA from Formalin Fixed Neurological Tissues

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Abstract: The ability to analyze the genotype of deceased affected members of pedigrees segregating inherited neurological diseases considerably augments the informativeness of such pedigrees. This information has direct application in attempts to isolate disease genes by positional cloning strategies, and for genetic counselling. We show that the genotype at polymorphic simple sequence repeat loci can be determined from genomic DNA isolated from 10 micron thick paraffin embedded, formalin fixed neurological tissues. The critical constraint on this method is the size of the template target bearing the simple sequence repeat, which should ideally be less than 165 base pairs.

Résumé: Évaluation de polymorphismes génétiques d'ADN provenant de tissus nerveux fixés par la formaline. La capacité d'analyser le génotype de membres atteints décédés de familles où ségrèguent des maladies neurologiques héréditaires augmente considérablement l'informativité de tels arbres généalogiques. Cette information a une application directe à l'isolation de gènes anormaux par des stratégies de clonage positionnel et pour le conseil génétique. Nous démontrons que le génotype peut être déterminé au niveau de sites polymorphiques constitués de séquences répétitives simples à partir d'ADN génotique isolé d'échantillons de tissus nerveux de 10 microns d'épaisseur fixés à la formaline et inclus dans la paraffine. L'élément critique dans cette méthode est la taille de la séquence répétitive ciblée qui, idéalement, devrait être inférieure à 165 paires de bases.

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A growing number of human neurologic disorders with onset in adult life are now known to have either a purely genetic etiology, or to have a genetic component to their etiology.[1] Recent efforts to map the human genome, and recent advances in statistical analysis of pedigree data for both simple and complex traits suggest that it should theoretically be possible to map and eventually isolate the genetic defects responsible for susceptibility to many of these diseases.

A major limitation to this approach arises from the fact that many of the adult onset inherited neurologic diseases either considerably shorten the life expectancy of affected individuals or have the onset of symptoms very late in life. The resulting sub-optimal structure of disease pedigrees hampers attempts both to isolate the actual disease gene and to predict genetic risk amongst at-risk family members.[2] However, tissue specimens have frequently been collected at diagnostic biopsy or post-mortem studies from deceased affected members in preceding generations of many pedigrees with late onset inherited neurological disorders. We report that DNA extracted from formalin-fixed, paraffin-embedded neuropathology specimens can be analyzed for informative simple sequence repeat (SSR) polymorphisms (also known as microsatellite or simple tandem repeat polymorphisms).

Methods and Materials

Source of tissues

Formalin fixed, paraffin embedded tissue specimens were obtained from brain tissue of eight patients dying with Alzheimer Disease (AD). The brain tissue had been fixed in buffered neutral formalin for at least ten days, embedded in paraffin, and stored at room temperature for five to fifteen years. For one subject genomic DNA was extracted both from freshly frozen brain tissue and from formalin fixed brain tissues to test the reliability of formalin fixed tissues as a source of genetic information. To confirm that the alleles observed in DNA extracted from fixed tissues showed Mendelian inheritance, we also investigated the segregation of alleles in an extended family with Familial Alzheimer Disease (FAD3) from

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which pathologic samples have been collected from several deceased affected subjects whose spouses and/or offspring were also available.[3,4] The primary genetic defect in this pedigree has recently been mapped to chromosome 14 near the marker D14S43.[5]

Extraction of DNA from paraffin embedded tissue

Two or three 10 micron sections were removed from the surface of a single paraffin block and discarded. Using a clean microtome, several additional 10 micron thick slices were collected either singly or in clusters of three sections in order to determine whether the quantity of initial starting material influenced the subsequent efficiency of polymerase change reaction (PCR) amplification. Both single sections (low concentration treatment) and clusters of three sections (high concentration treatment) were extracted twice in 1.0 ml xylene at 23° C X 30 minutes each, and then re-extracted twice in 1.0 ml absolute alcohol. The tissue residua were recovered by centrifugation, dried in a vacuum desiccator, broken up with a sterile pipette tip, re-suspended in 200 µl TNE (10 mM Tris, 1 mM EDTA, 100 mM NaCl, pH 7.5), 0.5% Tween 20 (Sigma), 400 µg/ml Proteinase K (Boehringer Mannheim) at 55° C for 18 h, and finally extracted with phenol-chloroform (1:1) and chloroform. Because potential contaminants from the fixation and embedding processes might influence PCR amplification efficiency, we also investigated three different methods for recovering the genomic DNA from the digestion buffer for both the "low concentration" and the "high concentration" treatments above namely, ethanol precipitation; microfiltration on a Centricon 30 (MW cut-off 30 kD); or microfiltration on a Centricon 100 column (MW cut-off 100 kD). The microfiltration steps were accomplished by diluting the digestion buffer to 2 ml in sterile H_2O, concentration to 50 µl, followed by a second round of dilution and reconcentration to effectively accomplish the microdialysis. The DNA from each of the six "purification variants" from the same paraffin block was resuspended in 50 µl sterile distilled H_2O and stored at –80° C without quantifying the amount of DNA recovered (previous work suggests that A_{260} measurements on these specimens are frequently erroneous due to the presence of nucleic acid breakdown products and other contaminants[6]).

PCR reactions

Aliquots of DNA samples derived from each of the six purification variants from the same paraffin block were amplified using six published primer pairs which yield PCR amplimer products of 67-91 base pairs (bp) (D9S56/D9S12),[7] 88-106 bp (D19S47),[8] 106-122 bp (D19S49),[9] 123-143 bp (D9S110),[10] 165-197 bp (D9S55),[11] and 195-209 bp (D9S15).[12] PCR amplifications were carried out using annealing temperature and buffer conditions previously optimized for each set of primers using renewable sources of DNA. Typically, two microlitres of each template sample were amplified using 35 cycles in a final reaction volume of 20 µl (1.5 mM MgC_{l2}, 10 mM Tris pH 8.3, 50 mM KCl, 0.01% gelatin, 0.1% Triton X-100, 200 µM dNTPs, 10 pM each primer, 0.75 µCi ^{32}P-dATP or ^{32}P-dCTP, and 0.6 units of Taq DNA Polymerase). The products were diluted 2 - 3 fold with formamide loading dye, denatured at 98°C for 5 minutes, and resolved according to size by electrophoresis on a 6% denaturing polyacrylamide gel. The genotype at each locus was determined for each individual from the number and size of the resultant autoradiographic bands.

Results

Simple Sequence Repeat sequences were successfully amplified from 10 micron thick sections of paraffin embedded tissues; however the efficiency of this amplification was variable (Table 1). Previous studies have suggested that PCR amplification of DNA from fixed tissues was inhibited by contaminants co-purified with the template DNA and recommended low concentrations of initial starting material (usually a single 10 micron section). However, we observed that PCR targets of less than 150 bp (D9S56, D19S47, D19S49 and D9S110) showed consistently successful amplification of both alleles regardless of preparative technique or quantity of starting material. Conversely, inconsistent amplification was observed (3 out of 6 trials) for primer pairs with amplification products greater than 165 bp (D9S55 and D9S15). The efficiency of amplification for primers with larger amplimers (D9S55 and D9S15) was not related to the method of template preparation or amount of starting material (Table 1).

Amplification of six separately extracted sections from the same tissue block generated identical genotype information on all trials for D9S56, D19S47, D19S49 and D9S110, indicating that the amplification results are reproducible. Furthermore no differences were observed between genotypes assessed using DNA from frozen tissue sources and those determined using formalin fixed tissue specimens from the same subject. More importantly, we were able to show Mendelian inheritance of alleles for the D9S110 and D9S55 loci in an extended pedigree (Figures 1a and 1b respectively).

Discussion

Our results demonstrate the feasibility of using formalin fixed, paraffin embedded neurological tissue samples as a source of template DNA for genetic analyses using PCR based SSR polymorphisms. Although previous studies have suggested that contaminants in the extractate may inhibit PCR

Table 1. Factors Potentially Affecting PCR Amplification Efficiency.

		DNA Preparation Method					
LOCUS ID	SIZE (bp)	1	2	3	4	5	6
D9S56	67 - 97	+	+	+	+	+	+
D19S47	88 - 106	+	+	+	+	+	+
D19S49	106 - 112	+	+	+	+	+	+
D9S110	123 - 143	+	+	+	+	+	+
D9S55	165 - 197	+	–	+	–	–	+
D9S15	195 - 209	–	+	–	+	+	–

The effect of the method of DNA preparation (Column 1 = Ethanol precipitation, single section; Column 2 = Ethanol precipitation, three sections; Column 3 = 30 kD microfiltration, single section; Column 4 = 30 kD microfiltration, three sections; Column 5 = 100 kD microfiltration, single section; Column 6 = 100 kD microfiltration, three sections), and of PCR product size on reliability of PCR amplification of SSR sequences are examined. Results are depicted as (+) = specific amplification of both alleles; (-) = non-specific amplification or no amplification. Loci with high molecular weight PCR products (> 165 bp) were less reliably amplified than sequences with lower molecular weight products.

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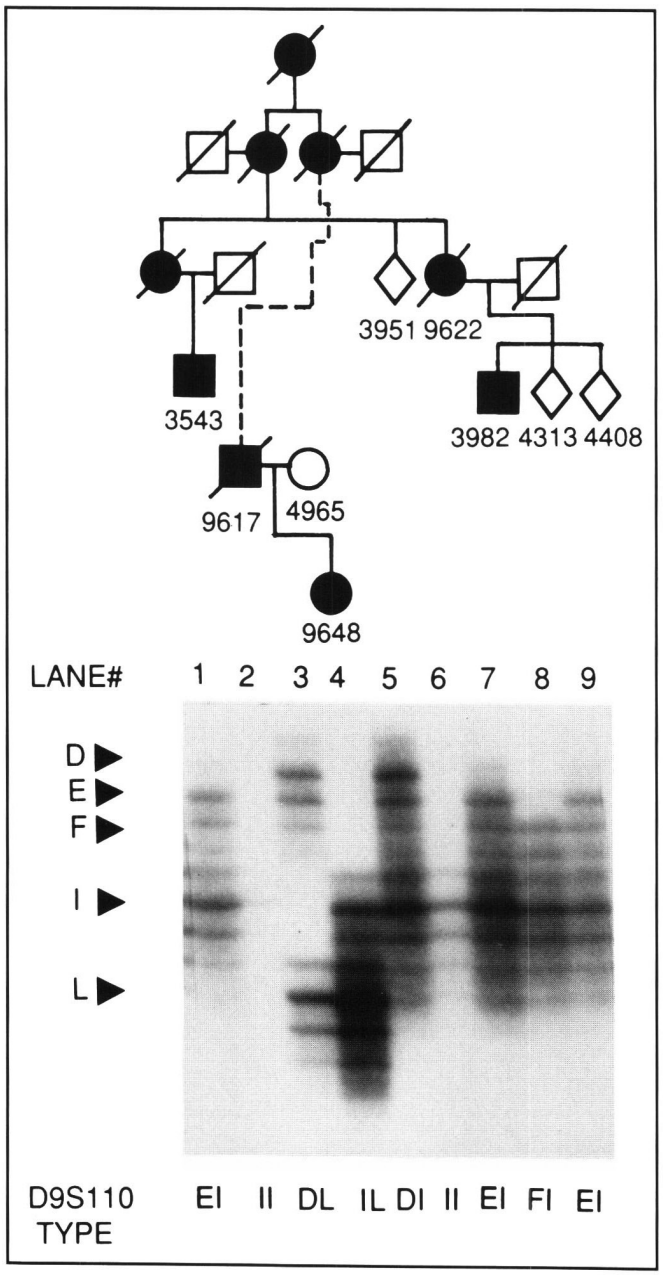

Figure 1a: *Oligonucleotide primer pairs for the D9S110 locus were used to amplify DNA from 10 micron paraffin sections from two deceased subjects (lane 2: 9617; lane 6: 9622) and DNA from buffy coat leukocytes from their other living affected and unaffected relatives (lane 1: 3543; lane 3: 4965; lane 4: 9648; lane 5: 3951; lane 7: 3982; lane 8: 4313; lane 9: 4408). The genotype of each individual is depicted below the autoradiogram. Mendelian patterns of inheritance of alleles are observed. Filled symbols in the pedigree diagram represent individuals affected with familial Alzheimer Disease. A complete diagram of this pedigree has been published elsewhere .*[3-5]

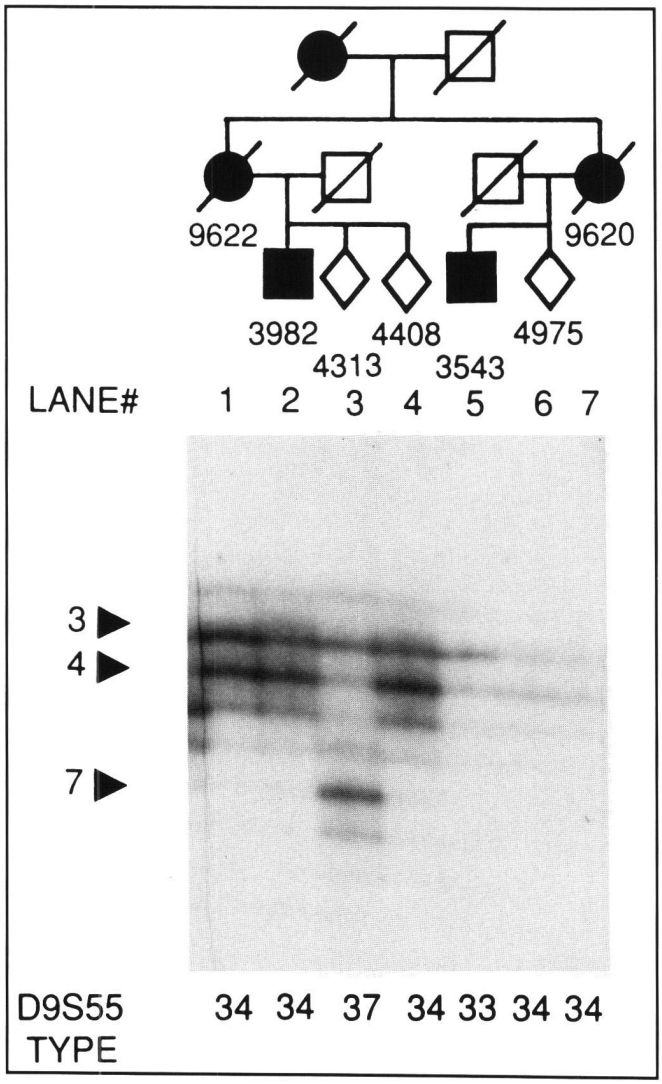

Figure 1b: *Published oligonucleotide primer pairs for the D9S55 locus were used to amplify DNA from 10 micron paraffin sections from two deceased subjects (lane 1: 9622; lane 7: 9620) and DNA from buffy coat leukocytes of their respective living affected and unaffected offspring (lane 2: 3982; lane 3: 4313; lane 4: 4408; lane 5: 3543; lane 6: 4975).*

amplification,[6] we observed that the major limiting factor for reliable amplification of SSR sequences from fixed neurologic tissues is the size of the PCR target to be amplified (targets greater than 165 bp in size were not amplified on all trials). The small size of the target sequences which could be reliably amplified from fixed neurologic tissues is in sharp contrast to other studies which have reported successful PCR amplification of sequences up to 1 kb in length from fixed tissues.[6,13] The smaller size of amplifiable targets reported here probably arises from two sources. First, previous studies have examined the amplification of non-repetitive sequences. Amplification of SSR sequences is often more difficult, being complicated by constraints on the choice of optimal PCR primers near the SSR sequence and by other technical problems such as "slippage" of the DNA polymerase during amplification of the SSR sequence, etc.[8] Second, previous studies (refs. 6 and 13) have used non-neurologic tissues which are typically fixed in formalin for 12 - 24 hours. In comparison, neurologic tissues are typically fixed for at least seven days. This difference in fixation procedures is likely to be important because prolonged fixation in formalin significantly reduces the size of amplifiable products from 989 bp after 72 hours of fixation to 268 bp after 30 days of fixation.[13] One other study examining the amplification of single copy, non-repetitive templates from formalin fixed neurologic

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tissues also reported difficulty in amplification of templates of more than 300 bp in size.[14] We do not think that the small size of the PCR templates which could be reliably amplified from formalin fixed neurologic tissues reflect an artifact peculiar to the markers we have used because the markers we chose are representative of most other microsatellite loci in terms of nucleotide composition and Tm of the oligonucleotide primers and GC content of the sequences surrounding the microsatellite array.

The size constraints imposed by the nature of the target sequence and by the source of tissues typically available for the analysis of inherited neurologic diseases will require careful selection of genetic markers to be used for the analysis of fixed tissues from deceased members of such pedigrees. Optimally, such markers should have PCR products close to or below 165 bp in size. Markers with PCR products above this size obviously may still be used because microsatellite markers do differ in the ease with which they can be amplified and scored. However, our results suggest that they may have a higher failure rate, which will result in wastage of a non-renewable resource. Furthermore, if markers with PCR products of a higher molecular weight are employed, it will be important to bear in mind the theoretical possibility that high molecular weight alleles may not be amplified as well as lower molecular weight alleles. Under these circumstances heterozygotes may appear to be homozygous for the low molecular weight allele. While we draw attention to this caveat, we do not preclude the use of markers with allele sizes greater than 165 bp, but we do suggest cautious interpretation of the results. Indeed, we have been able to generate useful data for the D9S55 locus with amplimers between 165 and 197 bp in one family (Figure 1b) even though this same locus could not be amplified from three of six other specimens.

In addition to these caveats, four other practical considerations must also be borne in mind. First, the accuracy of the archival source of pathological tissues needs to be assured. Second, pathologic tissue specimens are usually handled by multiple personnel, and great care must therefore be exercised to minimize the possibility of amplification of contaminant sequences. This objective can most easily be achieved by using a different, clean microtome to cut each tissue block, and by discarding the first few sections cut from the surface of a new block. Third, the putative clonal nature and genomic instability of some tumours,[15-17] argues that where possible sections should be used from tumour free zones. Finally, the PCR products generated from fixed tissue templates are rarely as pure as those generated from conventional sources, and as a result autoradiographs frequently contain minor spurious bands. This might be minimized by using two rounds of amplification with a second set of internal primers.

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