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ORIGINAL INVESTIGATION

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Quantitative DNA pooling to increase the efficiency of linkage analysis in autosomal dominant disease

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Abstract DNA pooling is an efficient method to rapidly perform genome-wide linkage scans in autosomal recessive diseases in inbred populations where affected individuals are likely to be homozygous for alleles near the disease gene locus. We wanted to examine whether this approach would detect linkage in autosomal dominant (AD) disorders where affected individuals may share one allele identical by descent at loci tightly linked to the disease. Two large outbred pedigrees in which the AD diseases familial venous malformation (FVM) and hereditary hemorrhagic telangiectasia (HHT1), linked to 9p and 9q, respectively, were investigated. Separate pools of DNA from affected (n = 21 for FVM and 17 for HHT1) and unaffected family members (n = 9 FVM and HHT1), and 25 unrelated population controls were established. Polymorphic markers spanning chromosome 9 at approximately 13.5-cM intervals were amplified using standard PCR. Allele quantitation was performed with a fluorimager. Visual inspection of allele intensities and frequency distributions suggested a shift in frequency of the most common allele in the affecteds lane when compared to control lanes for markers within 30 cM of the FVM and HHT1 loci. These subjective assessments were confirmed statistically by testing for the difference between two proportions (one-sided; $P \leq 0.05$). When using population controls, the true-positive rates for FVM and HHT1 were 5/5 and 2/5 markers, respectively. False-positive rates for FVM and HHT1 were 3/9 and 2/9, respectively. In both AD diseases investigated, quantitative DNA pooling detected shifts in allele frequency, thus identifying areas of known linkage in most cases. The utility of this technique depends on the size of the pedigree, frequency of the disease-associated allele in the population, and the choice of appropriate controls. Although the false-positive rate appears to be high, this approach still serves to reduce the amount of overall genotyping by about 60%. DNA pooling merits further investigation as a potential strategy in increasing the efficiency of genomic linkage scans.

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Introduction

DNA pooling (Smith 1953; Lander and Botstein 1987; Sheffield et al. 1995), has been shown to be a powerful and efficient method to rapidly perform genome-wide linkage scans in autosomal recessive diseases in inbred populations where affected individuals are likely to be homozygous for alleles at loci surrounding the disease gene (Ben Hamida et al. 1993; Pollak et al. 1993; Sheffield et al. 1994; Carmi et al. 1995). This strategy exploits the fact that affected individuals in inbred families share a chromosomal region inherited from a common ancestor surrounding the disease locus. Traditionally, genotype results are generated for individual DNA samples using a given marker or markers. In the DNA pooling strategy, DNA from multiple affected and unaffected individuals from each family as well as population controls are pooled in separate lanes and simultaneously amplified by PCR with a given marker. Markers linked to (LOD score > 3.0) and in association with a disease gene will show loss of heterozygosity and hence a different pattern of alleles from appropriate control groups. Unlinked markers should demonstrates an allele distribution that is similar to control lanes. This strategy considerably reduces the number of PCR reactions and gel lanes needed to screen a marker while conducting a genome-wide or candidate gene scan. We investigated whether this approach would detect linkage in autosomal dominant (AD) disorders, as suggested

by Kanis et al. (1995), where affected individuals within a family may share one allele identical by descent at loci tightly linked to the disease.

Patiens and methods

Hereditary hemorrhagic telangiectasia (HHT1) and familial venous malformation (FVM) were selected for study because we had access to large outbred AD pedigrees that are linked to opposite arms of chromosome 9 (McDonald et al. 1994; Gallione et al. 1995), thus permitting a single chromosome scan as a strategy for a limited simulated genome scan. For the pooling experiments, DNA was quantitated rapidly with a Molecular Dynamics fluorimage SI using a picogreen protocol supplied by the company. Separate pools of DNA from affected ($n = 21$ for FVM and 17 for HHT1) and unaffected ($n = 9$ for both FVM and HHT1) family members, and 25 unrelated population controls were established by combining equal amounts of DNA from each individual. Phenotypically unaffected relatives were selected in the line of descent to act as a control within the family. Individuals with uncertain phenotype were excluded from this group, assuming that some individuals might be non-penetrant gene carriers. The population control group consisted of ethnically and geographically matched individuals who were randomly selected healthy spouses from other pedigrees in our laboratory.

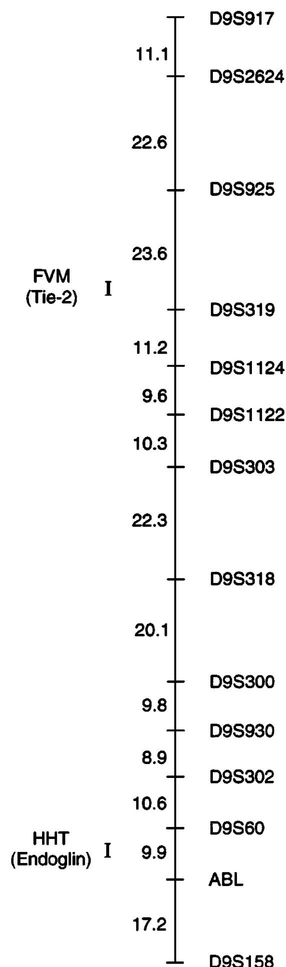


Fig. 1 Map of chromosome 9 demonstrating location of markers used in this study (Murray et al. 1994) relative to the location of the genes for familial vascular malformation (*FVM*; Vikkula et al. 1996) and hereditary hemorrhagic telangiectasia (*HHT1*; McAllister et al. 1994). Distances shown are in centimorgans

Polymorphic tetranucleotide markers were chosen to span chromosome 9 (Fig. 1). These markers were noted in preliminary studies to produce much less stutter artifacts than dinucleotide markers, thus making allele identification and quantitation easier. In order to fill a gap towards the telomeric end of chromosome 9q, where tetranucleotide markers are not represented, three dinucleotide markers were also selected as part of the chromosome scan. In total, 14 markers were chosen, spaced at an average interval of approximately 13.5 cM. Markers were amplified using standard PCR (Carmi et al. 1995) with 50 ng of total DNA from each pool.

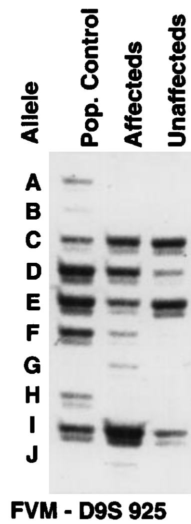
Allele quantitation was performed with a Molecular Dynamics fluorimager SI, which generated frequency distribution curves. Allele quantitation was performed by determining the area under each allele peak and dividing this area by the total area under all allele peaks. The frequency of the most common allele in the affecteds lane was then compared to the frequency of that same allele in unaffected and population control lanes by testing for the difference between two proportions (one-sided). A significance level of 0.05 was utilized. Since this approach is intended to be utilized as an initial screening procedure, no corrections for multiple comparisons are necessary.

Visual inspection was carried out in the following manner. The allele that was most intense in the affecteds lane was identified (since this is most likely to be the linked allele) and then compared to the relative intensity of the same allele in the adjacent unaffecteds and population control lanes (Fig. 2a). Next, the allele quantitation plot obtained via fluorimager analysis of the gel was examined (Fig. 2b). Once again, the affecteds lane was examined first, looking for the tallest peak. A comparison of the height for the same allele in adjacent control lanes allows one to determine if the difference looks significant.

In order to generate LOD scores, we utilized MLINK at θ values of 0, 5, 10, 15, 20, 30, and 40. The maximum LOD score was obtained by inspection of the profile of linkage and examining 1% intervals between the region where it appeared the maximum would be, i.e., if the profile looked as though the maximum was between 10 and 15, LOD scores were calculated at θ values of 11, 12, 13, and 14. If the maximum appeared to fall beyond 20 cM, the maximum LOD score at the nearest θ value of 20, 30 or 40 was reported.

Results

Visual inspection of gels and allele frequency distributions suggested a shift in the frequency of the most common allele in the affecteds lane when compared to population control lanes for most of the markers within 30 cM of the FVM and HHT1 loci. Statistical significance ($P \leq 0.05$) was found for all markers within 30 cM of the FVM locus (D9S925, D9S319, D9S1124, D9S1122, D9S303) and for two markers within 30 cM of the HHT1 locus (D9S930, D9S158), yielding true-positive rates of 5/5 and 2/5, respectively. For example, marker D9S925 is tightly linked to FVM, and this is apparent by visual inspection of the gel (Fig. 2a) as well as the allele frequency distribution (Fig. 2b) when comparing allele I in the affecteds versus unaffected and population control lanes. These results were confirmed by statistical analysis comparing the frequency of this allele in the affecteds and control lanes (Table 1). One marker linked to the HHT1 locus (D9S60) did not demonstrate an association because of an ancestral recombination which led to two different alleles segregating in separate portions of the pedigree. The marker D9S302, which is within 30 cM of HHT1, did not give consistent PCR products despite multiple PCRs and hence allele frequencies may not be accurate.



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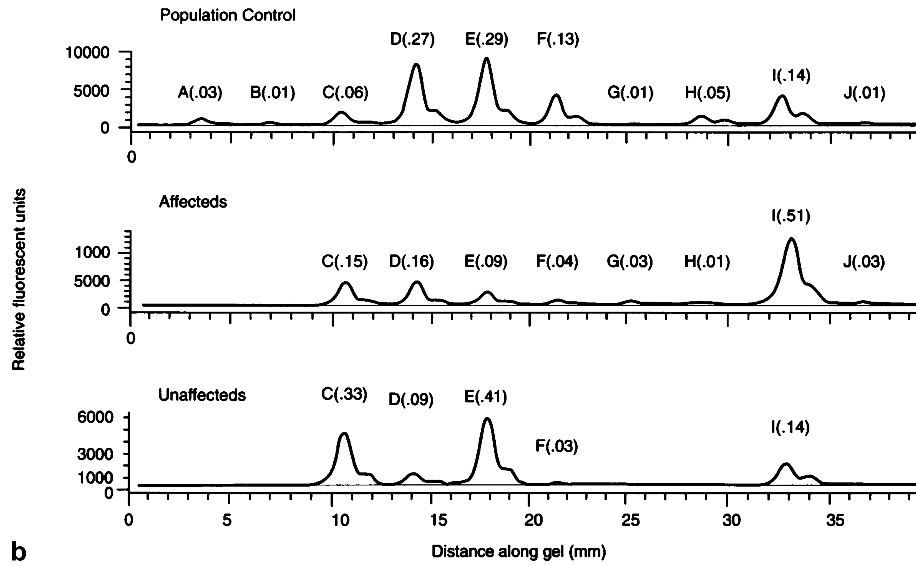

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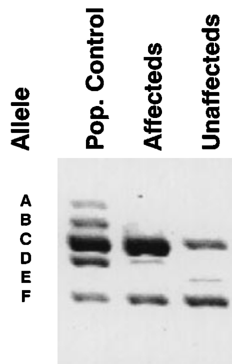

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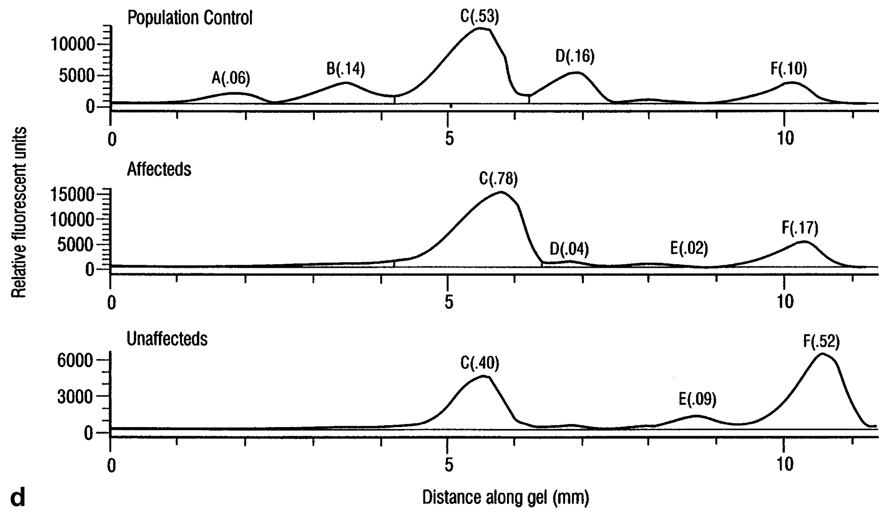

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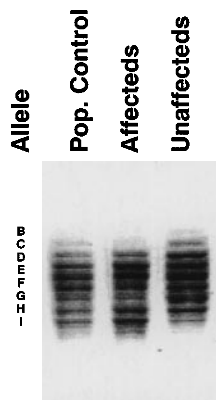

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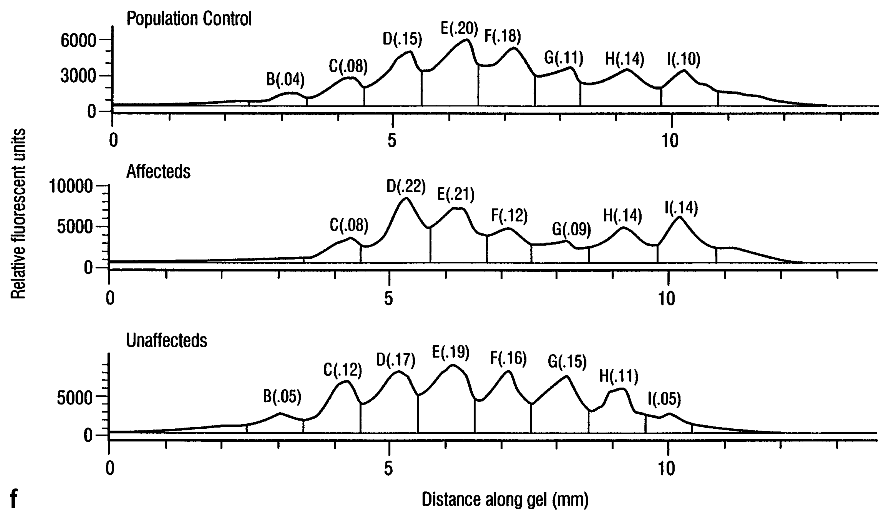

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◀ **Fig. 2** **a** Gel image from a fluorimager demonstrating allele intensities for FVM unaffected, affected, and population control pools with tetranucleotide marker D9S925. A clear difference is seen with the linked allele (I) which is much more intense in the affecteds lane when compared to control lanes. **b** Allele frequency distributions of the same marker shown in **a**. Area under each peak was quantitated by the fluoroimager to arrive at an estimate of the frequency of each allele. A striking difference can be seen in the appearance of the linked allele (I) in affecteds when compared to unaffected and population control plots. **c** Gel image for the unlinked marker D9S318 in FVM pooled samples. Allele C is the most intense in the affecteds and relatively more intense than in the population and unaffected controls. This is an example of a false-positive marker (i.e., appears to be linked by visual inspection and quantitation but the LOD score is < +3.0). **d** Allele frequency distribution for the marker D9S318 shown in **c.** **e** Gel image for the linked marker D9S60 in HHT1 pooled samples. This is an example of a false-negative result. An ancestral crossover is present and multiple, intense bands are visible in the affecteds lane, making it difficult to decide which is the most intense allele. Stutter bands from each allele also complicate visual inspection. **f** Allele frequency distribution for marker D9S60 shown in **e**. Allele quantitation is extremely difficult due to stutter bands overlapping with alleles

Some unlinked markers also demonstrated false positives (defined as P value ≤ 0.05 outside 30 cM of the disease locus). Specifically, using population controls, D9S2624, D9S318, and D9S930 yielded significant P values for a false-positive rate of 3/9 in FVM. In HHT1, the false-positive rate was 2/9 (D9S319 and D9S1124). Utilizing the unaffected family members as controls, FVM generated a lower true-positive rate of 3/5 and a false-positive rate of 2/9. Analysis of the HHT1 data utilizing unaffected family members as controls yielded a true-positive rate of 0/5 and false-positive rate of 1/9.

Discussion

DNA pooling offers an efficient strategy to simplify genome-wide screens when looking for linkage in large inbred autosomal recessive pedigrees. This strategy has also been applied successfully to a rare recessive disease in the face of heterogeneity (Gschwend et al. 1996). The authors caution, however, that a sufficiently dense genetic map is necessary for this approach to be successful when heterogeneity is present, and any false positives must be followed up by typing further markers in the region. DNA pooling has also been suggested as a strategy applicable to mapping genes in AD disorders segregating in large kindreds, and in polygenic disorders (Kanis et al. 1995). Recently, a susceptibility gene, inherited as AD with incomplete penetrance, was identified for atrioventricular canal defects using a combination of DNA pooling and shared segment analysis (Sheffield et al. 1997).

We investigated the DNA pooling approach in AD disorders where large outbred pedigrees are available. This strategy assumes a relatively low frequency of the linked allele in the population in order that a difference can be detected in affecteds versus control lanes. An allele with a frequency approaching 50% in the population may closely approximate a linked allele (i.e., appear as the most intense band) within a family. Two large pedigrees (McDonald et al. 1994, Gallione et al. 1995), one with FVM and the other with HHT1 linked to 9p and 9q, respectively, were studied using DNA pooling and polymorphic markers spanning chromosome 9 in order to simulate a partial genome scan. We were able to detect, by visual inspection, a shift in al-

Table 1 Frequency of the most common allele in familial venous malformation *(FVM)* and hereditary hemorrhagic telangiectasia *(HHT1)* affecteds lane in comparison to the frequency of the same allele in unaffected and population control lanes. (*NO* Allele not observed, *NC* not calculated since affecteds allele frequency was less than the frequency in the comparison group)

Locus	FVM frequency of allele			Controls	P	HHT1 frequency of allele			Controls	P	FVM $z(\hat{\theta})$, $(\hat{\theta})$	HHT1 $z(\hat{\theta})$, $(\hat{\theta})$
	Affecteds	Unaffecteds	P			Affecteds	Unaffecteds	P				
D9S917	0.81	0.85	NC	0.82	NC	0.58	0.50	0.398	0.82	NC	0.46 (0.20)	0.00 (0.40)
D9S2624	0.48	0.18	0.029	0.04	< 0.001	0.52	0.20	0.026	0.46	0.375	4.59 (0.04)	2.34 (0.10)
D9S925[a]	0.51	0.14	0.008	0.14	< 0.001	0.40	0.30	0.341	0.29	0.203	8.66 (0.00)	0.83 (0.15)
D9S319[a]	0.84	0.64	0.085	0.36	< 0.001	0.60	0.48	0.296	0.36	0.026	4.38 (0.00)	0.83 (0.20)
D9S1124[a]	0.56	0.27	0.037	0.22	0.001	0.43	0.43	0.384	0.20	0.021	3.49 (0.07)	1.11 (0.13)
D9S1122[a]	0.52	0.26	0.057	0.11	< 0.001	0.51	0.28	0.097	0.38	0.169	2.18 (0.10)	–0.02 (0.40)
D9S303[a]	0.36	NO	< 0.001	NO	< 0.001	0.29	0.24	0.477	0.42	NC	0.87 (0.20)	0.12 (0.30)
D9S318	0.78	0.40	0.005	0.53	< 0.001	0.41	0.76	NC	0.53	NC	1.30 (0.11)	0.96 (0.20)
D9S300	0.42	0.30	0.279	0.33	0.09	0.38	0.39	NC	0.33	0.405	1.11 (0.20)	0.61 (0.20)
D9S930[b]	0.48	0.24	0.074	0.25	0.019	0.53	0.50	0.466	0.25	0.008	0.34 (0.20)	0.77 (0.15)
D9S302[b]	0.34	0.39	NC	0.19	0.081	0.23	0.16	0.407	0.24	NC	0.46 (0.30)	1.29 (0.14)
D9S60[b]	0.18	0.16	0.427	0.20	NC	0.22	0.17	0.474	0.15	0.297	–	5.08 (0.03)
ABL[b]	0.29	0.27	0.438	0.33	NC	0.43	0.23	0.130	0.33	0.241	–	5.52 (0.00)
D9S158[b]	0.32	0.29	0.470	0.23	0.232	0.28	0.08	0.092	0.33	0.016	–0.04 (0.40)	2.46 (0.11)
Number of chromosomes	42	18		50		34	18	50				

[a] Markers within 30 cM of the FVM locus
[b] Markers within 30 cM of the HHT1 locus

lele frequencies in the affecteds lane when compared to allele frequencies in the unaffected and population control lanes for all markers linked to FVM and for some markers linked to HHT1. We confirmed these differences statistically and were able to show that 5/5 and 2/5 markers within 30 cM of the FVM and HHT1 loci, respectively, demonstrated a statistically significant difference when population controls were utilized. One marker (D9S60) did not demonstrate an association because of an ancestral recombination which was previously demonstrated by haplotype analysis (McDonald et al. 1994). It is also important to note that 4/4 of the markers linked to FVM (LOD score > 3.0) were detected by the pooling method as being significant, while none (0/2) of the markers linked to HHT1 were detected by this method (D9S60 and ABL, both dinucleotide repeat markers). Unfortunately, at the time of the study, no tetranucleotide markers were available in the HHT1 region and we therefore utilized dinucleotide markers which made both the visual inspection and allele quantitation far more complicated due to the presence of superimposed stutter bands. We also compared allele frequencies in affecteds with genotypically unaffected individuals within the same family and obtained a true-positive rate of 3/5 and 0/5 for FVM and HHT1, respectively. Although in theory the comparison of allele frequencies in affected individuals to frequencies in unaffected family members is valid when the penetrance is high, in our pedigrees the power to detect significant differences was limited by the available sample size; furthermore, when the frequency of the disease-associated allele is common in the population, the probability that a mating is uninformative due to parental homozygosity is increased. Similarly, an identical difficulty will arise if the disease-associated allele is common in the pedigree by chance.

The choice of controls, as with any association study, is an important one. Unrelated controls should be matched at least geographically and ethnically, as in this study, since allele frequencies are known to differ in various populations. In general we prefer to use multiple types of controls, but place most weight on the "population controls" rather than "unaffected" family members. This is because in many cases the penetrance of the disease may not be high and hence disease status of older unaffected individuals may be uncertain. This problem, however, is not unique to the pooling strategy, and also plagues classical linkage analysis. In linkage, however, one can create penetrance classes which allow weighting of the contribution of the unaffecteds according to their age and likelihood of being correctly diagnosed. With pooling, this weighting cannot be taken into account, and therefore pooling is worse than linkage for analysis of diseases with low or age-dependent penetrance. Population controls also have the advantage of pooling more individuals and giving a more accurate estimate of the true allele frequencies but have the drawback of misrepresenting the allele distribution in the families. Where available, spousal controls may be helpful as in most cases spouses belong to the same population as the family under study. In most situations, however, spouses are limited in number (only one spouse per sibship) and may not be available for sampling.

Although DNA pooling is capable of detecting linked regions in our AD families, a number of other caveats, similar to those in any association study, need to be mentioned. First, our families represent large, multigenerational pedigrees, representing an ideal situation for pooling. The utility of pooling in multiple small families has not been established. In the latter situation, locus heterogeneity and/or lack of linkage disequilibrium may dilute the strength of the pooling approach. Secondly, we obtained a false-positive rate of 22–33% in our scan of chromosome 9. Relatively few markers were used in comparison to a genome-wide scan, and hence the false-positive rate we have obtained may not be entirely accurate. Recently, Sheffield et al. (1997) used 360 markers in a genome-wide scan to study a large family with a complex congenital heart defect that appeared to demonstrate an AD inheritance pattern. Twenty markers (6% false-positive rate) demonstrated a predominance of a shared allele in the affected pool by visual inspection when compared to control pools of unaffected individuals. The number of "interesting" markers was further reduced to five regions by limiting interesting regions to clusters of two or more positive markers. One of these regions subsequently demonstrated linkage. Hence, a combination of strategies, such as DNA pooling and shared segment analysis, may prove fruitful in future studies involving AD and/or complex disorders. Further studies involving screening a large portion of or the entire genome with markers in various AD disorders will be needed before it is clear what threshold one should set to determine what constitutes a positive marker, and before a clearer idea of the false-positive rate with the DNA pooling approach can be obtained.

As always, the acceptable type 1 error rates and power to detect a true association are intimately intertwined. In general, requiring a more stringent level of threshold for significance will tend to reduce the type I error rate but may lead to the failure to identify the true region of interest. In our families, reducing the threshold for significance from 0.05 to 0.02 did not alter the frequency of the true positives in FVM and HHT1 when population controls are used; however, the two false positives for HHT1 are excluded. Our preference is to suggest a less stringent significance level, since a false-negative result could undermine the entire linkage effort.

Several technical lessons have emerged from our study that are pertinent to future DNA pooling experiments. Accurate DNA quantitation is important so that pools with equal amounts of DNA from each individual can be established in order to permit the DNA sample from each patient equal opportunity for amplification. Tetranucleotide markers are superb for quantitation since they produce very little in the way of stutter artefact. Dinucleotides are much more difficult to quantitate, although one could incorporate a stutter correction algorithm (LeDuc et al. 1995; Perlin et al. 1995). We believe that quantitation of allele frequencies is important in the context of pooling for dominant disorders. Visual inspection is helpful but can be difficult due to the complex pattern of alleles in each lane (in contrast to recessive disorders where one is looking for loss of heterozygosity, i.e., a collapse of the

allele pattern to one intense allele band). For dominant disorders, one has first to pick out the most intense allele in the affecteds lane (difficult if two alleles are almost as intense) and then compare this relative intensity to the relative intensity of the same allele in control lances (see Fig. 2 a). Differences are much more easily appreciated with quantitation rather than visual inspection. For example, in Fig. 2 c, allele C for marker D9S318 is clearly the most intense in the affecteds lane, yet by visual inspection appears to be only slightly more intense than the same allele in the population control lane. With quantitation, however, it is easily apparent that the frequency of the alleles in affecteds (78%) is quite different from that in population and family control lanes (53% and 40%, respectively). A fluorescent system is ideal for quantitation since it offers a linear scale over many log units of fluorescence. We have tried densitometry with conventional autoradiographs and found it to be satisfactory, but only if the film threshold has not been exceeded (i.e., overexposure has not occurred). In addition, studies have shown that the allele frequencies obtained through pooling and fluorescent analysis are very close to frequencies obtained if individuals have been genotyped separately (Pacek et al. 1993; Darvasi et al. 1994; LeDuc et al. 1995). Differential allele amplification, a potentially important theoretical concern, does not appear to present a problem in practice (Kanis et al. 1995).

To summarize, in both AD diseases investigated, quantitative DNA pooling detected shifts in allele frequency of most linked markers, thus identifying the areas of known linkage. The utility of this technique depends on the size of the pedigree, frequency of the disease-associated allele in the population, and choice of appropriate controls. Although the false-positive rate appears to be high, this approach may serve to reduce the amount of overall genotyping by about 60%. DNA pooling merits further investigation as a potential strategy in increasing the efficiency of genomic linkage scans.

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