A High Prevalence of HLA-H 845A Mutations in Hemochromatosis Patients and the Normal Population in Eastern England

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ABSTRACT: We have examined normal individuals and all the patients currently being treated for hemochromatosis at the Norfolk and Norwich hospital for mutations in the HLA-H gene. We found a gene frequency in 200 normal subjects for the 845A (C282Y) allele of 0.085, corresponding to a carrier frequency of 17% which is among the highest reported anywhere in the world. The frequency for the less penetrant 187G (H63D) allele was 0.16 among 58 of the normal subjects, which corresponds to a carrier frequency of 32%. All 18 hemochromatosis patients were homozygous for the 845A allele which is not significantly different from other reports in our subset of 12 unrelated patients. These findings present a snapshot of a relatively stable population containing a predicted 3,500 individuals homozygous for the 845A allele but not diagnosed with hemochromatosis. This population will be an excellent model for studies on the penetrance of the 845A homozygous genotype and population screening.

Keywords: hemochromatosis, haemochromatosis, iron, penetrance, Norfolk, genetics, screening

INTRODUCTION

The discovery of the HLA-H gene (1) may have provided molecular geneticists with an unparalleled opportunity to reduce mortality and morbidity by applying a simple DNA-based test. This supposition is based on the observation that hemochromatosis is one of the commonest genetic diseases in Caucasians (1,2,3) and the fact that a simple and effective treatment already exists (4,5). Detection of mutations in the HLA-H gene (1) provides an excellent and unchallenged test for chromosome 6-linked hemochromatosis, either because these are the causative changes in hemochromatosis or because they are in extreme proximity. We chose to examine the prevalence of HLA-H mutations (1) in a Caucasian population in eastern England where hemochromatosis was apparently under-diagnosed, suggesting the potential value of an additional test that could be applied both to patients referred with a suspicion of hemochromatosis and in screening the normal population. Two questions must be addressed before the cost and benefit of screening of a particular population can be determined. First the

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frequency of the mutant alleles must be determined and secondly the penetrance of the genotypes with respect to disease manifestations must be determined. We have determined the frequency of the mutant alleles in this population with sufficient accuracy to justify a pilot screening program. The question of penetrance is much harder to answer, but we suggest that this stable and relatively untreated population with a high prevalence of 845A homozygotes is ideal for studies of penetrance.

MATERIALS AND METHODS

Patients

DNA was extracted by standard methods from the blood of 200 normal individuals and the 18 patients being treated for hemochromatosis by phlebotomy at the Norfolk and Norwich Hospital, England which has a catchment area of about 500,000 people. For our normal group we chose patients who were referred to this hospital for reasons unrelated to known manifestations of hemochromatosis so that our sample would be representative of the hospital population. The normal group was screened anonymously. To identify bias that might be introduced by overrepresentation of particular genotypes among particular patients groups, we included several different patient groups including pregnant women and negative diagnoses in our sample and recorded patient age, sex and in most cases diagnosis for subsequent analysis. Diagnostic criteria for haemochromatosis patients were fasting transferrin saturation above 60% in 2 samples and hepatic iron index above 2 where appropriate.

DNA Amplification

DNA was amplified in 50 µl PCR reactions containing 25 µl of 2X PCRmaster (Boehringer Mannheim) reagents with up to 100 ng of DNA and 6 µg/ml of the relevant primers described by Feder et al. (1). Thermal cycling was carried out in a Progene (Techne) programmable heating block as follows: 94C for 4 minutes then 35 cycles of 94C for 40 seconds, 55C for 40 seconds and 70C for 40 seconds. This was followed by incubation at 70C for 10 minutes. The PCR amplification was tested by running 10 µl of the product on a TBE/3% MetaPhor agarose (FMC Bioproducts) gel containing 0.5% ethidium bromide. All PCR work was carried out in rooms dedicated to either preparation or product analysis.

Allele-Specific Oligonucleotide Hybridization for Nucleotide 845 Alleles

Five µl of each PCR reaction product was mixed with 500 µl of 0.4 M NaOH, 40 mM EDTA and applied to Hybond N+ (Amersham) membranes using a slot blot manifold. The membranes were rinsed in 50mM tris-HCl pH 7 and then incubated at 50C in Rapid-Hyb buffer (Amersham) for 15 minutes. A ^{32}P-end labeled oligonucleotide probe specific for either the wild-type 845G allele (GATATACGTGCCAGGT GGAG) or the 845A mutant allele (GATATACG TACCAGGTGGAG) was then added to 50 fmol/ml to the hybridization solutions of duplicate blots and allowed to hybridize for 30 minutes. Membranes were then washed four times for two minutes each in SSPE at room temperature. The membranes were then washed in SSPE for the apparatus temperature equilibration time plus 10 minutes at 58C (845G) or 53C (845A) and then twice as before at room temperature. Membranes were exposed to Biomax film (Kodak) with a Biomax MS intensifying screen (Kodak) at -70C for 2 hours.

Restriction Digestion for Nucleotide 187 Alleles

Duplicate aliquots of 5 and 10 µl of PCR product were digested in 20 µl reactions containing 20 and 10 units respectively of Bcl 1 (New England Biolabs) at 50C for 3 hours. The products were run on a TBE/3% MetaPhor agarose (FMC Bioproducts) gel containing 0.5%

ethidium bromide. The bands in apparent heterozygotes were analysed using a GDS8000 image analysis system (Ultra Violet Products) to confirm equal ratios of intensity of the two products in the two digests.

RESULTS

Ten of the patients have no relatives diagnosed with hemochromatosis and the remaining 8 came from families with 2 and 6 affected members. All 18 hemochromatosis patients were homozygous for the 845A mutant allele. This frequency of 100% is the same as that observed in Australian hemochromatosis patients (6) and among the 12 unrelated patient groups is not significantly different ($p>0.05$) from the frequencies of 83% and 92% observed in large groups of American (1,2) and French (7) hemochromatosis patients. All the hemochromatosis patients were homozygous for the normal 187C nucleotide.

Among the normal group of 200 individuals, 32 were heterozygous and one homozygous for the 845A mutant allele. This allele frequency of 0.85% (95% confidence intervals 0.058-0.112) is higher than the frequencies of 6.0% observed in British (3) and 0. 64 (1) and 0.75 (2) in American Caucasians but this does not reach significance at the $p = 0.05$ level. Analysis of subsets of the normal group by age, sex or diagnosis showed no significant differences in the gene frequency.

Among a subset of 58 of the normal individuals, 17 were heterozygous and 1 homozygous for the 187G allele. This allele frequency of 0.16 is similar to previous reports in American Caucasians (1,2) and not significantly higher ($p>0.05$) than the frequency observed in Europeans (3).

The 845A and 187G alleles were in linkage disequilibrium on all 26 informative chromosomes from unrelated individuals.

DISCUSSION

Our finding in this small sample of hemochromatosis patients of complete association between hemochromatosis in this population and the 845A homozygous genotype confirms the 845A mutation as the likely causative change in most hereditary hemochromatosis.

Our study of normal individuals drawn from the catchment area of the Norfolk and Norwich Hospital represents the first published characterization of the frequency of HLA-H mutant alleles within a defined and relatively stable population. We are therefore able to predict the numbers of expected genotypes and compare this with clinical observation. The 0.085 frequency of the 845A allele is among the highest yet reported and would correspond to about 3,500 homozygotes within the hospital catchment area of about 500,000. This figure is in marked contrast to the 18 patients currently being treated. Taking a mean survival for symptomatic patients of 15 years (8) and a normal life expectancy of 77 years (9) we calculate that only about 3% of 845A homozygotes are diagnosed with hemochromatosis. This suggests that, in this region, hemochromatosis may be massively underdiagnosed and that the homozygous 845A genotype may not be fully penetrant with respect to disease manifestations. Incomplete penetrance is suggested by the observation that hemochromatosis is consistently more frequently diagnosed in men than women (4,5,10) in populations where most affected individuals are likely to be 845A homozygotes (1,2,7).

It is significant ($p<0.001$) that 7 of the diagnosed hemochromatosis patients are from 2 families. This may reflect either the greater likelihood of diagnosis of an affected individual in a family with an affected member or a tendency for hemochromatosis to be more penetrant in certain families or both these possibilities. Greater concordance of both biochemical markers (11,12) and manifestations of hemochromatosis (12) has been observed within families than between families in HLA-A-identical individuals.

We therefore present the hypothesis that the 845A homozygous genotype may vary in its degree of penetrance in different families and in different regions. Both these situations could be explained by shared cultural dietary habits or shared secondary genetic factors.

Within our population the predicted number of individuals homozygous for the 845A/187C genotype is 6800 but none of these individuals has been diagnosed with hemochromatosis. This confirms the finding that this genotype is dramatically less penetrant than the homozygous 845A genotype in American populations (1,2) and suggests that the disease risk to an individual with this genotype is similar to the population disease risk.

It is now important to address the question of penetrance by determining the numbers of individuals from predicted populations of 845A homozygotes and 845A/187C compound heterozygotes who develop hemochromatosis-associated manifestations such as hepatocelullar carcinoma and diabetes. This could be done retrospectively using histology tissue archives and DNA archives. This approach would be complemented by studies of the iron physiology of individuals with the 6 known different HLA-H genotypes.

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