Technical report

Development of a multiplex ARMS test for mutations in the HFE gene associated with hereditary haemochromatosis

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Abstract

Genetic testing for hereditary haemochromatosis is likely to be a significant workload for diagnostic laboratories. The C282Y and H63D mutations in the HFE gene associated with hereditary haemochromatosis have previously been detected using a number of methods including alterations in the restriction digest pattern of polymerase chain reaction (PCR) amplified products. An amplification refractory mutation system (ARMS) has been developed that will simultaneously detect both hereditary haemochromatosis mutations. Comparison of the results obtained from the analysis of 46 samples referred for hereditary haemochromatosis testing showed no discrepancies between ARMS and restriction enzyme digestion. Furthermore, consistent results were obtained by ARMS from both blood and buccal mouthwash samples. The ARMS test is quicker and less expensive in terms of consumables and scientist time than restriction enzyme analysis, and is therefore suited to the routine diagnostic analysis of hereditary haemochromatosis.

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Keywords: hereditary haemochromatosis; C282Y mutation; H63D mutation; multiplex ARMS analysis

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Hereditary haemochromatosis is one of the most common single gene disorders in northern European populations, affecting approximately 1 in 200–300 people.[1] This autosomal recessive condition is characterised by the progressive accumulation of excess iron in a variety of organs, where it may eventually cause cirrhosis of the liver, diabetes, arthropathy, and cardiomyopathy.[2] However, the development of symptomatic iron overload is very variable and clinical manifestations typically do not appear until middle age following decades of iron deposition. Phlebotomy can be used to control amounts of stored iron, and is most effective if instituted early.[3]

Two mutations have recently been identified by Feder *et al* in HLA-H (now designated HFE),[4] a candidate gene localised to chromosome 6p22.1. A G to A transition at nucleotide 845 results in a cys to tyr substitution at codon 282 (C282Y), while a C to G change at position 187 causes a his to asp mutation at codon 63 (H63D). There is a clear association between C282Y and hereditary haemochromatosis, with over 90% of UK patients homozygous for this mutation.[5] In contrast, the significance of the H63D mutation remains unclear.[6 7]

While previous screening procedures for hereditary haemochromatosis have relied on the measurement of serum transferrin saturation and ferritin concentrations, with subsequent liver biopsy to determine hepatic iron deposition or quantitative phlebotomy to estimate iron overload, the identification of mutations in HFE now offers the possibility of a presymptomatic screen for genetic status. Both C282Y and H63D have previously been detected by allele specific oligonucleotide–ligation assay,[4] alterations in restriction enzyme digestion patterns of polymerase chain reaction (PCR) amplified products,[7 8] first nucleotide change,[8] and allele specific oligonucleotide hybridisation.[9] To develop a test that would require less sample manipulation than restriction digestion of PCR products, avoid the use of radioisotopes, and allow the simultaneous detection of both mutations, we used an amplification refractory mutation system (ARMS)[10] that has been successfully used to screen for mutations in the CFTR gene.[11 12] Having developed a multiplex ARMS test, we compared its performance with that of restriction enzyme digestion of PCR amplified products.

Materials and methods

SAMPLES

Forty six patient samples referred on the basis of either raised serum transferrin saturation or ferritin concentration, or a family history of hereditary haemochromatosis were analysed for the C282Y and H63D mutations by both restriction enzyme digestion and multiplex ARMS. DNA was extracted from blood by standard proteinase K digestion followed by ethanol precipitation. Buccal cells from mouthwash samples were pelleted by centrifugation and lysed with 50 mM NaOH at 100°C.

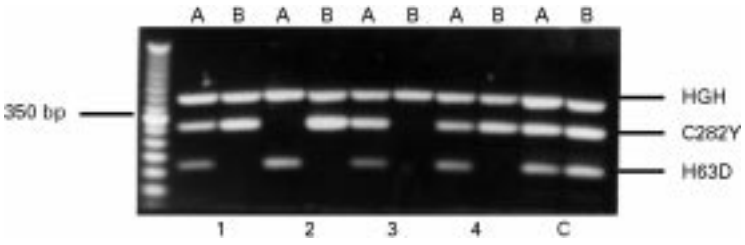

Figure 1 Multiplex ARMS detection of the C282Y and H63D mutations. Each ARMS test comprised two reaction tubes: tube A, mutant C282Y and normal H63D; tube B, normal C282Y and mutant H63D. First lane, 50 base pair DNA ladder; patient 1, C282Y carrier; patient 2, non-carrier; patient 3, C282Y homozygote; patient 4, C282Y carrier; C, C282Y/H63D control.

Following neutralisation with Tris-HCl pH 7.7, cell debris was pelleted and the supernatant removed for analysis. Either 50 ng of DNA or 2.5 µl of buccal cell supernatant was used as template in subsequent PCR amplifications.

RESTRICTION ENZYME DIGESTION

PCR amplification of the C282Y and H63D loci was performed using primers described by Feder *et al*.[4] The products were digested with Rsa1 or Bcl1 to detect the presence of the C282Y and H63D mutations, respectively. Digested fragments were separated on 1.5% agarose stained with ethidium bromide.

ARMS

Sense primers for both C282Y and H63D[4] were used in combination with the following specific antisense ARMS primers: C282Y normal/mutant (5'–GCTGATCCAGGCCT GGGTGCTCCACCTGCC/T–3') and H63D normal/mutant (5'–AGTTCGGGGCTCCA CACGGCGACTCTCAAG/C–3'). Primers for the human growth hormone gene were included as internal amplification controls.[13] Final primer concentrations for each set of amplimers were as follows: C282Y and HGH primers at 0.25 µM, H63D primers at 0.5 µM. Amplification was initiated by hot start after four minutes at 94°C, followed by 30 cycles of one minute at 94°C, 59°C, and 72°C. Products were separated on 1.5% agarose stained with ethidium bromide.

Results

Screening of the 46 samples revealed 16 C282Y homozygotes, 9 C282Y heterozygotes, 3 H63D heterozygotes, 8 compound heterozygotes, and 10 with neither mutation. Both screening methods were equally successful in analysing purified DNA and buccal cell extracts. Furthermore, there was complete agreement between the results for restriction enzyme digestion and the ARMS analysis.

All three sets of primers in the multiplex reaction gave relatively equal band intensities with no spurious or non-specific bands. Primer specificity was ensured by introducing a mismatch in each of the ARMS primers at the –2 position (penultimate 3' nucleotide: underlined in primer sequences) according to determined rules.[14] Figure 1 shows an ARMS result from four patients referred on the basis of raised serum ferritin concentration and a control sample from a healthy compound heterozygote. Each test consisted of two lanes, representing reaction tubes A and B.

Discussion

This multiplex ARMS system offers a reliable and robust method for the simultaneous detection of the C282Y and H63D mutations in the HFE gene. Comparison of the results obtained from the 46 samples tested with both ARMS and restriction enzyme digestion confirmed the accuracy of the ARMS system.

General population screening for hereditary haemochromatosis mutations will remain controversial until the penetrance (risk of tissue damaging iron overload) in C282Y homozygotes is established, and the role of the H63D mutation is clarified. Furthermore, a number of ethical considerations need to be addressed before such a screening programme can be considered.

However, the savings both in terms of consumables and operator time compared to restriction analysis of PCR products, make this ARMS test suited for the routine analysis of samples from individuals with symptomatic iron overload including those with sporadic porphyria cutanea tarda,[15] as well as the presymptomatic screening of at risk members of hereditary haemochromatosis families.

1 Merryweather-Clarke AT, Pointon JJ, Shearman JD, *et al*. Global prevalence of putative haemochromatosis mutations. *J Med Genet* 1997;**34**:275–8.
2 Powell LW. Primary iron overload. In: Brock JH, Halliday JW, Pippard MJ, Powell LW, eds. *Iron metabolism in health and disease*. Saunders: London, 1994:227–70.
3 Niederau C, Fischer R, Purschel A, *et al*. Long-term survival in patients with hereditary haemochromatosis. *Gastroenterology* 1996;**110**:1107–19.
4 Feder JN, Gnirke A, Thomas W, *et al*. A novel MHC class I-like gene is mutated in patients with hereditary haemochromatosis. *Nat Genet* 1996;**13**:399–409.
5 Worwood M, Shearman JD, Wallace DF, *et al*. A simple genetic test identifies 90% of UK patients with haemochromatosis. *Gut*. [In press.]
6 Beutler E, Gelbart T, West C, *et al*. Mutation analysis in hereditary haemochromatosis. *Blood Cells Mol Dis* 1996;**22**: 187–94.
7 Jouanolle AM, Gandon G, Jezequel P, *et al*. Haemochromatosis and HLA-H [letter]. *Nat Genet* 1996;**14**:251–2.
8 Jazwinska EC, Cullen LM, Busfield F, *et al*. Haemochromotosis and HLA-H [letter]. *Nat Genet* 1996;**14**:249–51.
9 Borot N, Roth M, Malfroy L, *et al*. Mutations in the MHC class I-like candidate gene for haemochromatosis in French patients. *Immunogenetics* 1997;**45**:320–4.
10 Newton CR, Graham A, Heptinstall LE, *et al*. Analysis of any point mutation in DNA: the amplification refractory mutation system (ARMS). *Nucleic Acid Res* 1989;**17**:2503–16.
11 Ferrie RM, Schwarz MJ, Robertson NH, *et al*. Development, multiplexing, and application of ARMS tests for common mutations in the CFTR gene. *Am J Hum Genet* 1992;**51**:251–62.
12 Schwarz MJ, Malone GM, Haworth A, *et al*. Cystic fibrosis mutation analysis: report from 22 UK regional genetics laboratories. *Hum Mut* 1995;**6**:326–33.
13 Kirschbaum NE, Foster PA. The polymerase chain reaction with sequence specific primers for the detection of the factor V mutation associated with activated protein C resistance. *Thromb Haemost* 1995;**74**:874–8.
14 Newton CR. In: McPherson MJ, Hames BD, Taylor GR, eds. *PCR 2: A practical approach*. Oxford: IRL Press, 1995: 219–53.
15 Roberts AG, Whatley SD, Morgan RR, *et al*. Increased frequency of the haemochromatosis Cys282Tyr mutation in sporadic porphyria cutanea tarda. *Lancet* 1997;**349**:321–3.