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Polymorphism in intron 4 of *HFE* does not compromise haemochromatosis mutation results

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Two recent reports have described a polymorphism in *HFE* (refs 1,2) in the binding region of a PCR primer[3] widely used in diagnosis of hereditary haemochromatosis (HHC). This polymorphism, IVS4+48G/A (originally described by Totaro *et al.*[4]), is associated with the wild-type allele at the site of the common haemochromatosis mutation C282Y (845G→A), and may prevent amplification of this allele[1]. In population screening of 5,211 voluntary blood donors, Jeffrey *et al.*[1] found that this polymorphism caused 15 C282Y/IVS4+48G/A compound heterozygotes to be incorrectly assigned as C282Y homozygotes. In a regional referral laboratory, Somerville *et al.*[2] reported that 8 of 202 referrals were incorrectly assigned as C282Y homozygotes.

We have retyped 944 samples for the C282Y mutation by a new primer[1] external to IVS4+48G/A or by sequencing. We confirmed 575 previously diagnosed C282Y homozygotes using the new primer, as well as 192 C282Y wild-type homozygotes, including 10 IVS4+48G/A homozygotes, and 177 heterozygotes. Of the heterozygotes, 28 were C282Y/ IVS4+48G/A compound heterozygotes which had been correctly reported using the original Feder reverse primer. Three of these, however, had a mutant:wild-type band ratio of approximately 5:1. Re-extraction of DNA from these samples resulted in bands of expected intensity for heterozygotes. This indicates that DNA purity may have an effect on accuracy of diagnosis. We did not observe non-amplification of the polymorphic allele[1,2] under our various standard PCR conditions (http://genetics.nature.com/supplementary_info/).

IVS4+48G/A is fairly common, with allele frequencies of 13.2% in controls and 8.6% in HHC referrals in our cohort (see table, http://genetics.nature.com/supplementary_info/), comparable with those previously observed[1,4]. We did not observe IVS4+48G/A on the C282Y allele (176 C282Y homozygotes tested), consistent with previous observations that IVS4+48G/A was not detected on the C282Y allele[1,2]. IVS4+48G/A was also absent from all of 9 H63D (187 C→G) homozygotes and all 36 H63D/C282Y compound heterozygotes, indicating that it is not present in C282Y or H63D founder haplotypes.

We represent 11 laboratories in which C282Y genotyping is routinely practised. That standard PCR conditions in our laboratories have not been subject to mistyping strongly suggests that the prevalence of the C282Y mutation is unlikely to have been widely overestimated as proposed[1,2]. Incorrect assignation of C282Y heterozygotes as homozygotes is improbable in Italy, where non-expressing C282Y homozygotes are not observed[5,6]. Similar C282Y allele frequencies are seen in the United Kingdom whether the detection method uses the Feder reverse primer[7] or not[8]. Furthermore, of 10,000 blood donors from South Wales tested using PCR-SSP (ref. 9), which does not use this primer, 1 in 140 is homozygous for C282Y. To eliminate the possibility of mistyping, we have replaced our antisense primer[1] in PCR-RFLP, and most of about 40 UK laboratories participating in the pilot UK National External Quality Assessment Scheme (*HFE* testing organizer, C. Darke) use PCR-SSP (ref. 9). Finally, we have demonstrated that the validity of our previous publications[5–8,10–17] is not compromised by recent findings[1,2].

Acknowledgements

We thank P. Adams and M. Somerville for pre-publication manuscripts.

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The C282Y mutation in HFE is the major mutation responsible for hereditary haemochromatosis. The accurate determination of this mutation is important in diagnosis and risk assessment for this disease[1]. Jeffrey *et al.*[2] recently reported that 15 of 31 (48%) patients originally genotyped as C282Y homozygotes, using PCR primers widely used in the clinical molecular genetics community[1], were in fact C282Y heterozygotes. The misdiagnosis occurred because these individuals harboured a single-base polymorphism (5569A) in the C282 wild-type allele at the location