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No genetic association between EPHX1 and Crohn's disease

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Dear Editor

In a case-control study on the associations between functional genetic polymorphisms in biotransformation enzymes and Crohn's disease, we found a strong association of the Tyr113His (348T>C) polymorphism in exon 3 of the microsomal epoxide hydrolase (EPHX1) gene and Crohn's disease.[1] The three referees all agreed that the study was interesting and should be published so that other groups can attempt to replicate the results in independent study cohorts. This was done recently by Cuthbert *et al*,[2] who investigated 344 controls and 307 patients with Crohn's disease, and who were unable to reproduce our results. In addition, they reported that our data for the EPHX1 exon 3 polymorphism in the control group were not in Hardy-Weinberg equilibrium (HWE), as also noticed earlier by Györffy *et al*.[3] Our data on EPHX1 exon 3 genotyping were obtained by restricted fragment length polymorphism (RFLP) analyses by applying the method as described by Lancaster *et al*.[4]

However, recently it was reported that a silent substitution polymorphism (G to A) at codon 119 of the EPHX1 gene may exist, which may flaw the PCR- RFLP method applied by us, since the presence of this polymorphism may disturb the proper binding of the reverse primer, covering the 119 G>A area, resulting in an over-classification of His113 alleles.[5,6] Therefore, we developed a dual-colour allele-specific discrimination assay for genotyping the polymorphism at codon 113 of the EPHX1 gene. EPHX1 genotypes were detected using the iCycler iQ Multicolour Real-Time Detection System (Bio-Rad Laboratories) using molecular beacons. PCR was performed with the forward primer 5′-CAA CTC CAA CTA CCT GAA G-3′ and the reverse primer 5′-TGA CAT ACA TCC CTC TCT G-3′ in the presence of the FAM- labeled wild-type beacon (5′-CGC GAT GAT TCT CAA CAG ATA CCC TCA CTT CAA TCG CG-3′) and the HEX-labeled mutant beacon (5′-CGC GAT ATT CTC AAC AGA CAC CCT CAC TTC AAT CGC G-3′). The 25 microliter reaction mixture contained 200 ng of genomic DNA, 10 mM Tris/HCl (pH 9.0), 50 mM KCl, 0.1% Triton X-100, 4 mM MgCl2, 0.25 mM dNTPs, 50 ng of each primer, 200 nM of each beacon and 2.5 U Taq-DNA-polymerase. The PCR conditions were 3 min at 95°C, then 40 cycles of 30 s at 95°C, 30 s at 59°C and 30 s at 72°C. Fluorescent signals were measured at 59°C. Genotypes were assigned using the iCycler iQ Optical System Software version 3.1. At each PCR run (in 96 wells plates) in several wells sterile H2O instead of genomic DNA was added as negative controls for amplification. Since the PCR-RFLP analyses were performed in the first half of 1999, only part of the samples was still available (125 of 149 controls and 149 of 151 cases) and these were re-evaluated by the i-Cycler method.

Genotype distribution of the EPHX1 Tyr113His polymorphism in patients with Crohn's disease and controls was now in Hardy-Weinberg equilibrium (chi2 = 2.47, p = 0.12 and chi2 = 0.82, p = 0.37, respectively) and genotype distribution was not significantly different between cases and controls (chi2 = 3.5, p = 0.17). The Tyr allele frequencies of 0.70 and 0.68 obtained for cases and controls, respectively, are very similar to the corresponding values of 0.71 and 0.70, as reported by Cuthbert *et al*.[2]

Thus, in answer to the question as posed by Cuthbert *et al*: "Genetic association between EPHX1 and Crohn's disease: population stratification, genotyping error, or random chance?", we can conclude that a genotyping error was responsible for our earlier published association between the EPHX1 Tyr113His polymorphism and Crohn's disease.[1] We now clearly state, that our revised data do not support an association between this EPHX1 polymorphism and Crohn's disease. Similar genotyping errors may also be present in several other studies on the EPHX1 exon 3 polymorphism in association with a variety of diseases, since many studies are based on methods using a reverse primer covering the "119 silent mutation area" of the EPHX1 gene.[4,7-10] This may also have consequences for interpretation of results in the cited papers. However, a rapid literature search by Pubmed reveals more than 100 papers on EPHX1

polymorphisms during the last 10 years, suggesting that many more papers may deal with genotyping problems as outlined above.

In addition, Cuthbert *et al* also reported that another polymorphism tested in our study 1, the CYP1A1 exon 7 Ile/Val polymorphism, was not in HWE in the control group.[2] This is correct, however this deviation from HWE may be attributed to random chance, due to rarity of the Val allele in our population, which makes the chi2 test inappropriate under such conditions. For instance genotype distribution is according to the HWE, when only two individuals less would have been classified as Val/Val homozygotes.

We thank Cuthbert and co-workers[2] and Györffy and co-workers[3] for their interest in our work. In addition, we conclude that (interpretation of) data in many other published studies on EPHX1 Tyr113His (exon 3) polymorphism should be critically re-evaluated.

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