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LETTER TO THE EDITOR

Response to Bortolotti et al. 2012 – a re-evaluation of our polymerase chain reaction-restriction fragment length polymorphism genotyping method

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Recently, Bortolotti et al. described a real time single nucleotide polymorphism (SNP) genotyping assay for the HLA-G +3142 C>G polymorphism (1). In their study, 221 DNA samples from healthy subjects were genotyped by three different methods, including polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP), real-time PCR and DNA sequencing. The PCR-RFLP protocol was originally described by our research group and has been applied in different studies (2, 3). To our surprise, Bortolotti et al. reported that 19% of the individuals genotyped as heterozygous by PCR-RFLP were identified as homozygous for the G allele (GG) by real-time PCR and sequencing methods. The authors explained this discrepancy by high rates of partial digestion in the PCR-RFLP assay and, therefore, suggested that the PCR-RFLP technique was unreliable. In order to check for the reliability of the PCR-RFLP method designed by our group, the replicability of genotyping results for the HLA-G +3142 C>G polymorphism between this same PCR-RFLP methodology and DNA sequencing was verified. For that, we analyzed 289 DNA samples from critically ill subjects who were previously sequenced for the whole 3′ UTR (untranslated region) of the *HLA-G* gene (4). These patients were genotyped in this study by the PCR-RFLP method described by our group and the genotyping results from the two methods were compared.

The amplification of the HLA-G +3142 C>G polymorphism was performed as described in the study from Cordero et al. (2): 50 ηg–250 ηg of genomic DNA were added to a final volume of 25 µl, with final concentrations as follows: PCR buffer 1×, 2.0 mM $MgCl_2$, 0.2 mM of each dNTP, 1.0 U of Taq DNA polymerase and 10 pmol of each primer (GmiRNA_F 5′-CATGCTGAACTGCATTCCTTCC-3′ and GmiRNA_R 5′-CTGGTGGGACAAGGTTCTACTG-3′). PCR was performed in a Techne TC-412 thermal cycler (Bibby Scientific Ltd., Stone, UK). Samples were submitted to 94°C for 5 min, 32 cycles of 30 s at 94°C, 30 s at 65.5°C and 60 s at 72°C, followed by a final extension step of 5 min at 72°C. The amplified products of 406 bp were then submitted to digestion with 3 U of the *Bae*GI restriction enzyme. Specifically, 8.7 µl of amplified PCR products were mixed with 0.3 µl of *Bae*GI (10,000 U/ml) and 1.0 µl of NEBuffer 1 10× (New England Biolabs Inc., Ipswich, MA), with no addition of water, yielding a 10 µl mix. This mix was digested for 3 h at 37°C, producing 316 and 90 bp fragments for the G allele or a single undigested 406 bp fragment for the C allele, which was visualized under UV irradiation in a 2% agarose gel stained with ethidium bromide.

In order to check for the reliability of our PCR-RFLP protocol, 140 samples were selected (48 CC, 45 CG and 47 GG by sequencing) to be blindly genotyped by a different researcher. After that, another 149 samples (12 CC, 15 CG and 122 GG by sequencing) were genotyped in a nonblind way. Genotyping by PCR-RFLP was performed only once for all samples, reproducing what is normally done in an association study with a large cohort. The results from these analyses are displayed in Table 1. Among all analyzed samples, only one sample presented disagreement in genotypes

Table 1 Comparison between 289 DNA samples from individuals genotyped for HLA-G +3142 C>G polymorphism through sequencing and polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) methods

+3142 C>G genotypes	Sequencing *N*	PCR-RFLP *N*
CC	60	60
CG	60	61
GG	169	168
Total	289	289

+3142 C>G genotypes	Sequencing *N*	PCR-RFLP not blind *N*
CC	12	12
CG	15	16
GG	122	121
Total	149	149

+3142 C>G genotypes	Sequencing *N*	PCR-RFLP blind *N*
CC	48	48
CG	45	45
GG	47	47
Total	140	140

obtained by PCR-RFLP and sequencing (PCR-RFLP = CG, sequencing = GG). This genotyping error frequency represents a 0.35% error rate associated to the PCR-RFLP method. Considering the GG genotype alone (169 individuals), the analysis of our protocol yielded an error rate of 0.6% (only one individual with a wrongly asserted genotype). These results are in strong disagreement with those from Bortolotti et al., where one third of the subjects with the GG genotype (25 of 75 individuals) were mistakenly genotyped as heterozygotes by their PCR-RFLP protocol (1). Two points that are worth mentioning to be taken into consideration in this assay are digestion time and dilution of the sample. Although the *Bae*GI enzyme is classified as a 'Time-Saver Qualified enzyme', we always respected a 3 h digestion time and shorter digestion times were never tested. Also, dilution of the PCR product with water before digestion was never conducted in our samples. From a total reaction volume of 10 µl, 8.7 µl corresponded to the original PCR product solution. Finally, as in all scientific methodologies, positive and negative controls were always added in each experiment in order to control enzyme digestion conditions. The results of this study confirm that the restriction enzyme assay, if conducted in accordance with the steps described above, is effective and reliable in genotyping the HLA-G +3142 C>G polymorphism and encourage its use in future studies.

Conflict of Interest

The authors have declared no conflicting interests.

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