Accurate interrogation of *FCGR3A* rs396991 in European and Asian populations using a widely available TaqMan genotyping method

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A polymorphism in the receptor for the Fc region of IgG, Fc γ-receptor IIIa (FcγRIIIa, *FCGR3A* rs396991), has been inconsistently shown in the literature to have an effect on response to monoclonal antibody therapy in several indications. The rs396991 (T/G) polymorphism leads to an F176V substitution and increased affinity for IgG. This variant has proven difficult to genotype accurately, primarily because of extensive homology between the *FCGR3A* and *FCGR3B* genes. We have shown that rs396991 can be genotyped by PCR amplification, followed by direct Sanger sequencing of the product, without coamplification of *FCGR3B*, and that the rs396991 TaqMan assay (C__25815666_10) agrees with Sanger sequencing results in 100% of European and Asian samples tested, but it has a small error rate in African and American populations. C__25815666_10 is therefore suitable to interrogate rs396991 in studies involving Europeans and Asians; however for other populations, the default genotyping method should be PCR followed by Sanger sequencing. *Pharmacogenetics and Genomics* 25:569–572

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Fc γ-receptor IIIa (FcγRIIIa) is a low-affinity receptor for the Fc region of IgG in humans and is involved in antibody-dependent cell-mediated cytotoxicity, one of the mechanisms of action of therapeutic monoclonal antibodies such as rituximab, cetuximab and trastuzumab. It has been hypothesized that the efficacy of monoclonal antibodies is affected by polymorphisms in *FCGR* genes through an impact on their binding affinity to Fc receptors on effector cells. Specifically, for the *FCGR3A* rs396991 polymorphism (also described as F176V), the VV genotype has been shown to have an affinity for IgG that is at least two-fold greater than that of the FF genotype [1]. The presence of the V allele has been associated with an increased response to monoclonal antibody therapy in varying indications [2–4]. However, findings are inconsistent, with other reports demonstrating no association [5,6]. These discrepancies could be related to difficulties in genotyping the variant, resulting from significant homology between the *FCGR3A* and *FCGR3B* genes (Supplementary Figure 1, Supplemental digital content 1, *http://links.lww.com/FPC/A898*, alignment of *FCGR3A* with *FCGR3B*) or copy number variants (CNVs) in the *FCGR* locus [7]. Mellor *et al.* [8]. reviewed a range of publications that genotyped rs396991 in patients enrolled in clinical trials of therapeutic antibodies and indicated that, in several cases, the results deviate from Hardy–Weinberg equilibrium (HWE). They concluded that standardizing methodologies for accurate genotyping is required before definitive conclusions can be drawn with regard to the effect of rs396991 on response to monoclonal antibody therapy. van der Straaten *et al.* [9]. described a pyrosequencing assay that is specific to rs396991 and does not coamplify *FCGR3B*, and showed 100% concordance between this and a commercially available TaqMan genotyping assay for rs396991 from Life Technologies (C__25815666_10). However, they did not demonstrate specificity of C__25815666_10, as the cloned and sequenced TaqMan product was 100% homologous to both *FCGR3A* and *FCGR3B*. Here, we show that an assay for rs396991 involving PCR amplification followed by Sanger sequencing [3] is also *FCGR3A*-specific and does not coamplify *FCGR3B*; furthermore, we describe the specificity of C__25815666_10 in varying populations.

Genomic DNA samples from 1205 individuals who are part of the 1000 Genomes Project [10] or the International HapMap Project [11] (Supplementary text, Supplemental digital content 2, *http://links.lww.com/FPC/A899*, details of populations) were genotyped by PCR amplification of a region of *FCGR3A* around the rs396991 polymorphism, followed by direct Sanger sequencing of the product [3]

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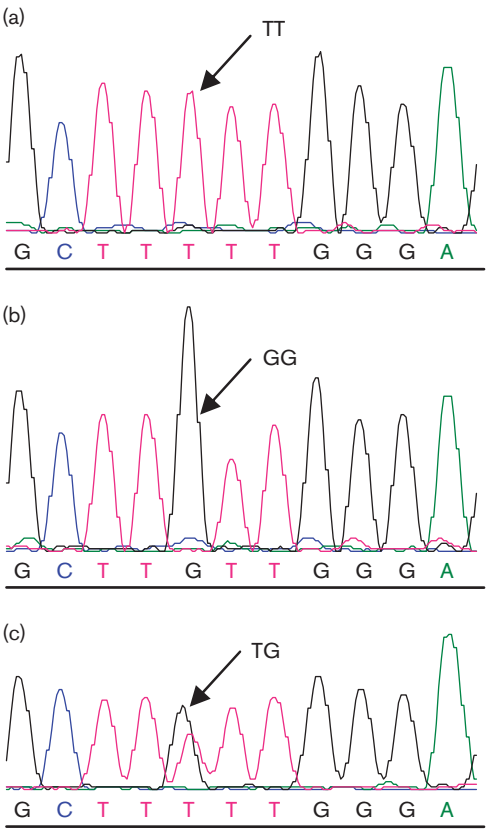
(FCGR3A-Sanger). Supplementary Figure 2 (Supplemental digital content 3, *http://links.lww.com/FPC/A900*) depicts primer positions. To show that *FCGR3B* is not coamplified by FCGR3A-Sanger, the same samples were amplified using PCR primers designed to be specific to *FCGR3B* around rs200215055 (*FCGR3B* polymorphism in the equivalent position to *FCGR3A* rs396991), followed by Sanger sequencing (FCGR3B-Sanger). A total of 149 samples were genotyped in duplicate for both assays. *FCGR3B* and *FCGR3A* are over 98% homologous in the area depicted in Supplementary Figure 1 (Supplemental digital content 1, *http://links.lww.com/FPC/A898*), making design of gene-specific primers challenging. To assess the effect of non-specific primers on rs396991 genotyping, a subset of 91 genomic DNA samples was also genotyped using PCR primers, which amplify both *FCGR3A* and *FCGR3B*, followed by Sanger sequencing [FCGR3(A + B)-Sanger]. Primer sequences are detailed in Supplementary Figure 1 and Supplementary Table 1 [Supplemental digital content 1, *http://links.lww.com/FPC/A898* (alignment of *FCGR3A* with *FCGR3B*) and Supplemental digital content 4, *http://links.lww.com/FPC/A901* (primer sequence table), respectively], and details of the FCGR3A-Sanger, FCGR3B-Sanger and FCGR3(A + B)-Sanger assays, including primer design, are detailed in Supplementary text (Supplemental digital content 2, *http://links.lww.com/FPC/A899*). Electropherograms were examined for all three Sanger assays described, and no evidence of nonspecific amplification was observed for the FCGR3A-Sanger or FCGR3B-Sanger assay. However, the FCGR3(A + B)-Sanger assay showed a 'heterozygous' TG result at rs396991 for 34 samples, which had a homozygous TT result with FCGR3A-Sanger and a homozygous GG result with FCGR3B-Sanger at the same position (Fig. 1). Results for the 91 samples genotyped using all three Sanger assays (Supplementary Table 2, Supplemental digital content 5, *http://links.lww.com/FPC/A902*) are within HWE for the FCGR3A-Sanger and FCGR3B-Sanger assays but deviate from HWE for the FCGR3(A + B)-Sanger assay.

Although the FCGR3A-Sanger assay accurately genotypes rs396991, the TaqMan assay format is a widely used platform [12,13] because of its ease of use and quick turnaround time. To investigate the specificity of the C__25815666_10 TaqMan assay, it was used (Supplementary text, Supplemental digital content 2, *http://links.lww.com/FPC/A899*, description of the method) to retype the 1205 individuals already typed with FCGR3A-Sanger, and the results were compared. A total of 250 samples were assayed in duplicate. The results for the FCGR3A-Sanger and C__25815666_10 assays agreed in 1169 out of the 1194 samples with results for both assays (Table 1), there being 11 samples out of the total 1205 genotyped that failed to give a result with either FCGR3A-Sanger or C__25815666_10. Results of the two assays agreed in 100% of the Asian and European samples compared; however, there was some disagreement in the African and American populations, with 23 African participants (7.8%) and two American participants (1.1%) having

Fig. 1



Representative Sanger sequencing electropherograms for the (a) FCGR3A-Sanger, (b) FCGR3B-Sanger and (c) FCGR3(A + B)-Sanger assays in rs396991 TT homozygotes. (a), (b) Specific amplification of the *FCGR3A* and *FCGR3B* genes, respectively. (c) Nonspecific amplification by the FCGR3(A + B)-Sanger assay, yielding, what appears to be, a 'heterozygous' TG result. Arrows show the position of *FCGR3A* rs396991/*FCGR3B* rs200215055.

C__25815666_10 results that were discordant with the FCGR3A-Sanger genotype. In 22 out of the 23 African discordant samples, the rs396991 genotype was TG by FCGR3A-Sanger and TT by C__25815666_10, which resulted in an under-representation of the minor (G) allele in the C__25815666_10 dataset (Table 1). As noted on the Life Technologies website (*https://www.lifetechnologies.com/order/genome-database/details/genotyping/C__25815666_10*), a primer sequence for C__25815666_10 is located over the *FCGR3A* rs449443 polymorphism. There is an *FCGR3B* equivalent of *FCGR3A* rs449443, namely *FCGR3B* rs71632957 (Supplementary Figure 2, Supplemental digital content 3, *http://links.lww.com/FPC/A900*, shows polymorphism positions). The alleles of *FCGR3A* rs449443 and *FCGR3B* rs71632957 were also typed by the FCGR3A-Sanger and FCGR3B-Sanger assays, and all the samples with discordant results for rs396991 between FCGR3A-Sanger and C__25815666_10 had a polymorphism present at either rs449443 or rs71632957. The minor allele frequencies of rs449443 and rs71632957 in different populations are shown in Table 1.

Table 1 Comparison of rs396991 genotype data generated by FCGR3A-Sanger, C__25815666_10 and the 1000 Genomes Project, and minor allele frequencies of rs396991, rs449443, rs71632957 and rs200215055

	rs396991 genotype by FCGR3A-Sanger assay and C__25815666_10		rs396991 genotype by FCGR3A-Sanger assay and 1000 Genomes Project		rs396991 MAF (%)			rs449443 MAF (%)	rs71632957 MAF (%)	rs200215055 MAF (%)
Population[a]	Number compared	Number discordant (%)	Number compared	Number discordant (%)	C__25815666_10	FCGR3A-Sanger assay	1000 Genomes Project	FCGR3A-Sanger assay	FCGR3B-Sanger assay	FCGR3B-Sanger assay
African (ASW, LWK, YRI)	295	23 (7.8)	246	78 (32)	34	38	22	5.9	6.4	0.3
American (CLM, MXL, PUR)	180	2 (1.1)	180	22 (12)	25	26	20	1.4	0.6	0
Asian (CHB, CHS, JPT)	291	0	284	51 (18)	35	35	27	0	0	0
European (CEU, FIN, GBR, IBS, TSI)	428	0	375	80 (21)	38	38	27	0	0.2	0.5
Total number (%) or MAF in all populations	1194	25 (2.1)	1085	231 (21)	34	35	25	1.7	1.7	0.3

MAF, minor allele frequency.
[a]Populations are defined as follows: 'African': African ancestry in Southwest USA (ASW), Luhya in Webuye, Kenya (LWK) and Yoruban in Ibadan, Nigeria (YRI). 'American': Colombian in Medellin, Colombia (CLM), Mexican ancestry in Los Angeles, California (MXL) and Puerto Rican in Puerto Rico (PUR). 'Asian': Han Chinese in Beijing, China (CHB), Han Chinese South (CHS) and Japanese in Tokyo, Japan (JPT). 'European': Utah residents with Northern and Western European Ancestry from the CEPH collection (CEU), Finnish in Finland (FIN), British from England and Scotland, (GBR), Iberian populations in Spain (IBS) and Toscans in Italy (TSI).

Publicly available individual reference data for rs396991 consist of ~200 Asian samples from the International HapMap Project (*http://hapmap.org*, HapMart), 50% of which failed genotyping. In addition, data for 1092 samples from the 1000 Genomes Project were included in Ensembl release 79 [14]; however, these data are not present in Ensembl release 80, which was made available immediately before submission of this manuscript. The 1000 Genomes Project sample data from Ensembl release 79 (*http://mar2015.archive.ensembl.org/Homo_sapiens/Variation/Individual?db=core;r=1:161544252–161545252;v=rs396991;vdb=variation;vf=266434*) were compared with the rs396991 genotypes generated here using the FCGR3A-Sanger assay, and the results are summarized in Table 1. There is significant discordance between the two datasets, which is driven by under-representation of the G allele in the 1000 Genomes Project data. This may be explained by the high sequence similarity between *FCGR3A* and *FCGR3B* in this region. Over 99% of the 1205 individuals genotyped have a homozygous GG genotype at rs200215055, which is the *FCGR3B* polymorphism in the equivalent position to *FCGR3A* rs396991 (Table 1 shows minor allele frequencies and Supplementary Figure 2, Supplemental digital content 3, *http://links.lww.com/FPC/A900*, shows polymorphism positions). The 1000 Genomes Project Phase 1 next-generation deep sequencing reads where *FCGR3A* has the minor (G) allele at rs396991 may have been incorrectly mapped to identical *FCGR3B* consensus sequences, or not mapped at all, which could have led to under-representation of mapped *FCGR3A* reads containing the rs396991 G allele. Genotype data generated by the FCGR3A-Sanger assay for rs396991 and rs200215055 and by the FCGR3B-Sanger assay for rs449443 and rs71632957, on 1205 genomic DNA samples from the 1000 Genomes and International HapMap Projects, have been submitted to dbSNP and will be publicly available in dbSNP build B145.

The Mendelian inheritance patterns in the FCGR3A-Sanger rs396991 data were checked for 48 trios, and there was one anomaly – that is, in the trio containing samples NA12750 (father, TT), NA12751 (mother, TG) and NA12740 (child, GG). FCGR3A-Sanger assay electropherograms showing the rs396991 genotype for these three samples are shown in Supplementary Figure 3 (Supplemental digital content 6, *http://links.lww.com/FPC/A903*). This anomaly could be explained by CNVs, which have been described at the *FCGR* locus [15,16]. Hollox *et al.* [17]. carried out a CNV analysis of the *FCGR3* locus in 485 Centre d'Etudes du Polymorphisme Humain and International HapMap Project samples and demonstrated the Mendelian inheritance. Approximately 4% of the samples were found to have only one copy of *FCGR3A*, and these included NA12750 and NA12740. The true genotype of NA12750 is likely to be *FCGR3A* 'T-', with NA12740 having inherited *FCGR3A* 'G' from NA12751 and *FCGR3A* '-' from NA12750, giving it a true *FCGR3A*

genotype of 'G-', which is shown as 'GG' by the rs396991 FCGR3A-Sanger and C__25815666_10 assays.

We have demonstrated that the FCGR3A-Sanger assay has specificity for *FCGR3A* and does not coamplify *FCGR3B*, and it is therefore a suitable method for accurate interrogation of rs396991. The FCGR3A-Sanger assay is a robust assay that is more amenable to high throughput than pyrosequencing. We have also shown that the C__25815666_10 assay is 100% concordant with the FCGR3A-Sanger assay in tested Asian and European populations, but it has an error rate of 7.8% in Africans and 1.1% in Americans. Our assessment would indicate that this is because of the presence of *FCGR3A* (rs449443) and *FCGR3B* (rs71632957) polymorphisms under the C__25815666_10 primer sequence. The challenge in accurately genotyping rs396991 is highlighted by the lack of accurate, publicly available reference data. Our submission of FCGR3A-Sanger-generated rs396991 reference data to dbSNP will allow confident assessment of rs396991 genotyping data validity in future studies.

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Conflicts of interest

All authors were employees of GlaxoSmithKline at the time of writing the manuscript and held or currently hold stock in GlaxoSmithKline.

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