Accurate interrogation of FCGR3A rs396991 in European and Asian populations using a widely available TaqMan genotyping method
Kay E. Murphy a, Heather A. Niederer b, Karen S. King c, Elizabeth C. Harris c, Sarah M. Glass c and Charles J. Cox a

A polymorphism in the receptor for the Fc region of IgG, Fcγ-receptor IIIa (FcyRIIIa, 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
Copyright © 2015 Wolters Kluwer Health, Inc. All rights reserved.
Pharmacogenetics and Genomics 2015, 25:569–572
Keywords: F176V, Fc γ-receptor IIIa, TaqMan, FCGR3A, FCGR3B, genotyping, rs396991
*Genetics, aBiopharm Discovery, GlaxoSmithKline, Stevenage, UK and *Genetics, GlaxoSmithKline, Research Triangle Park, North Carolina, USA
Correspondence to Kay E. Murphy, PhD, Genetics, GSK Medicines Research Centre, Gunnels Wood Road, Stevenage, Hertfordshire SG1 2NY, UK
Tel: + 44 143 876 6619; fax: + 44 143 876 2798; e-mail: kay.e.murphy@gsk.com
Received 4 June 2015 Accepted 14 August 2015

Fc γ-receptor IIIa (FcyRIIIa) 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].
Pharmacogenetics and Genomics

(FCGR3A-Sanger). Supplementary Figure 2 (Supplemental digital content 3, http://links.lww.com/FPC/A901) 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 genespecific 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/A899 (alignment of FCGR3A with FCGR3B) and Supplemental digital content 4, http://links.lww.com/FPC/A902] are within HWE 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 (11%) having 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/gene-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/A901, 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.
| Population | Number compared | Number discordant (%) | Number compared | Number discordant (%) | Number compared | Number discordant (%) | Number compared | Number discordant (%) |
|------------|----------------|-----------------------|----------------|-----------------------|----------------|-----------------------|----------------|-----------------------|
| African (ASW, LWK, YRI) | 295 | 23 (7.8) | 246 | 78 (32) | 34 | 38 | 22 | 20 |
| American (CLM, MXL, PUR) | 180 | 2 (1.1) | 180 | 22 (12) | 25 | 26 | 20 | 20 |
| Asian (CHB, CHS, JPT) | 291 | 0 | 284 | 51 (18) | 35 | 35 | 27 | 27 |
| European (CEU, FIN, GBR, IBS, TSI) | 428 | 0 | 375 | 80 (21) | 38 | 38 | 27 | 27 |
| Total number (%) in all populations | 1194 | 25 (2.1) | 1085 | 231 (21) | 34 | 35 | 25 | 25 |

MAF, minor allele frequency.

*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 Tuscans in Italy (TSI).
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.

Acknowledgements
The authors would like to thank Eddie Carden, Dana Fraser and Andrew Slater for their assistance with data submission to dbSNP. Genotyping was carried out at BioProcessing Solutions Alliance, RUCDR, Piscataway, New Jersey, USA. This work was funded by GlaxoSmithKline.

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

References
1 Koene HR, Kleijer M, Algra J, Roos D. von dem Borne AE, de HM. Fc gammaRIIIa-158 V/F polymorphism influences the binding of IgG by natural killer cell Fc gammaRIIIa, independently of the Fc gammaRIIIa-48 L/R/H phenotype. Blood 1997; 90:1109–1114.
2 Musolino A, Naldi N, Bortesi B, Pezzuolo D, Capelletti M, Missale G, et al. Immunoglobulin G fragment C receptor polymorphisms and clinical efficacy of trastuzumab-based therapy in patients with HER-2/neu-positive metastatic breast cancer. J Clin Oncol 2008; 26:1789–1796.
3 Quartuccio L, Fabris M, Pontarrini E, Salvin S, Zabotti A, Benucci M, et al. The 158VV Fcgamma receptor 3 A genotype is associated with response to rituximab in rheumatoid arthritis: results of an Italian multicentre study. Ann Rheum Dis 2014; 73:716–721.
4 Weng WK, Levy R. Two immunoglobulin G fragment C receptor polymorphisms independently predict response to rituximab in patients with follicular lymphoma. J Clin Oncol 2003; 21:3940–3947.
5 Dornan D, Spiess O, Yeh RF, Duchateau-Nguyen G, Dutour A, Zhi J, et al. Effect of FCGR2A and FCGR3A variants on CLL outcome. Blood 2010; 116:4212–4222.
6 Hurvitz SA, Betting DJ, Stern HM, Stinson J, Seshagiri S, et al. Analysis of Fcgamma receptor IIa and IIa polymorphisms: lack of correlation with outcome in trastuzumab-treated breast cancer patients. Clin Cancer Res 2012; 18:3478–3486.
7 Thabet MM, Huizinga TW, Marques RB, Stoeken-Rijssbergen G, Bakker AM, Kureemee FA, et al. Contribution of Fcgamma receptor IIa gene 158 V/F polymorphism and copy number variation to the risk of ACPA-positive rheumatoid arthritis. Ann Rheum Dis 2009; 68:1775–1780.
8 Mellor JD, Brown MP, Irving HR, Zalcberg JR, Dobrovic A. A critical review of the role of Fc gamma receptor polymorphisms in the response to monoclonal antibodies in cancer. J Hematol Oncol 2013; 6:1.
9 van der Straaten T, Martijn R, et al. A critical map of genetic variation from 1,092 human genomes. Nature 2012; 491:56–65.
10 Altshuler DM, Gibbs RA, Peltonen L, Altshuler DM, Gibbs RA, Peltonen L, et al. Integrating common and rare genetic variation in diverse human populations. Nature 2010; 467:52–58.
11 Haldorsen T, Martijn R, et al. An integrated map of genetic variation from 1,092 human genomes. Nature 2012; 491:56–65.
12 Koene HR, Kleijer M, Algra J, Roos D. von dem Borne AE, de HM. Fc gammaRIIIa-158 V/F polymorphism influences the binding of IgG by natural killer cell Fc gammaRIIIa, independently of the Fc gammaRIIIa-48 L/R/H phenotype. Blood 1997; 90:1109–1114.
13 Sareneva I, Koskinen LL, Korponay-Szabo IR, Kaukinen K, Kurppa K, Zibern F, et al. Linkage and association study of FcgammaR polymorphisms in celiac disease. Tissue Antigens 2009; 73:54–58.
14 Cunningham F, Amode MR, Barrett D, Beal K, Billis K, Brent S, et al. Ensembl 2015. Nucleic Acids Res 2015; 43(Database issue):D662–D666.
15 Breunis WB, van Mirre E, Bruin M, Geissler J, de Boer M, Peters M, et al. Copy number variation of the activating FCGR2C gene predisposes to idiopathic thrombocytopenic purpura. Blood 2009; 113:1029–1038.
16 Niederer HA, Wilcocks LC, Rayner TF, Yang W, Lau YL, Williams TN, et al. Copy number variation of the activating FCGR2C gene predisposes to idiopathic thrombocytopenic purpura. Blood 2008; 112:1029–1038.
17 Holloix EJ, Detering JC, Dehugura T. An integrated approach for measuring copy number variation at the FCGR3 (CD16) locus. Hum Mutat 2009; 30:477–484.