**FCGR2C genotyping by pyrosequencing reveals linkage disequilibrium with FCGR3A V158F and FCGR2A H131R polymorphisms in a Caucasian population**

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Abbreviations: ADCC, antibody-dependent cell-mediated cytotoxicity; CNV, copy number variations; FcγR, Receptor for the Fc portion of IgG; ITP, immune thrombocytopenic purpura; LD, linkage disequilibrium; NK, natural killer

The FCGR3A-V158F and FCGR2A-H131R polymorphisms are associated with clinical responses to therapeutic mAbs and with immune thrombocytopenic purpura (ITP). The FCGR2C-ORF/STOP polymorphism, controlling FcγRIIC expression on natural killer cells and therefore FcγRIIC-mediated antibody dependent cell-mediated cytotoxicity, is also associated with ITP. Using a new pyrosequencing assay to determine this polymorphism in a control population, we observed the expected allele frequencies (ORF:12.6%) and percentages of individuals with a single copy (10.0%) or 3 copies (12.1%) of FCGR2C, or with at least one FCGR2C-ORF allele (20.1%). No association of FCGR2C copy number variations with the FCGR3A-V158F or FCGR2A-H131R genotype was detected. More importantly, our results demonstrate a strong and a weaker linkage disequilibrium associating the FCGR2C-ORF allele with the FCGR3A-158V and the FCGR2A-131H allele, respectively.

**Introduction**

The low-affinity receptors for the Fc portion of IgG (FcγRs) are encoded by a 200 kb gene cluster located on chromosome 1 and include FCGR2A, FCGR3A, FCGR2C, FCGR3B and FCGR2B (in following order from the centromere). The FCGR3A and FCGR2A genes display a functional allelic dimorphism resulting in a valine to phenylalanine substitution at position 158 (V158F) of FcγRIIA and a histidine to arginine substitution at position 131 (H131R) of FcγRIIA. The FcγRIIIA-158V and FcγRIIA-131H allotypes have greater affinity for IgG1 and IgG2, respectively.1,2 On the other hand, the FCGR2C-ORF/STOP polymorphism in exon 3 of the gene controls the expression (ORF allele) or the absence (STOP allele) of FcγRIIC on natural killer (NK) cells.3 Moreover, it has been recently reported that an additional mutation at the splice sites of intron 7 in their FCGR2C-ORF alleles results in another stop codon in ≈20% of the FCGR2C-ORF donors and, therefore, the absence of FcγRIIC expression in these donors.4 We and others have shown that the FCGR3A-V158F and FCGR2A-H131R polymorphisms are associated with clinical responses to therapeutic mAbs such as rituximab,5-7 cetuximab,8 or trastuzumab.9 The better responses observed in patients expressing the FcγRIIIA-158V and FcγRIIA-131H allelotypes demonstrate that antibody dependent cell-mediated cytotoxicity (ADCC) or other functions exerted by cells expressing these receptors play a critical role in mediating the clinical effects.10 It has been shown that FcγRIIC-expressing NK cells can mediate ADCC to antibody-coated targets,3,4 suggesting that this receptor might also be involved in the anti-tumor responses mediated by cytolytic mAbs.

These polymorphisms of FCGR2A, FCGR3A and FCGR2C have also been reported to be associated with immune-complex mediated auto-inflammation.11 For instance, an association of the FCGR3A-V158F allele with immune thrombocytopenic purpura (ITP) has been repeatedly reported.12-15 The association of FCGR2A-H131R with ITP was also investigated in these studies with conflicting results. No association was found by Foster et al. in a small cohort15 (n = 37) or by Breunis et al. in a larger cohort of Caucasian patients15 (n = 116), but Carcao et al. reported that FCGR2A-131H was associated with ITP in a cohort of 98 Caucasian children.14 The latter result might be explained at least partially by the linkage disequilibrium (LD) between the FCGR3A-V158F and FCGR2A-H131R polymorphisms that we and others reported in Caucasian populations.16,17 This hypothesis

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This probably results from the difficulty in genotyping, due to the fact that the region and that is subject to copy number variation, in contrast to FCGR2B (see Supplemental Material). An additional level of complexity arises from the fact that FCGR2C is subject to CNV, in contrast to FCGR2A and FCGR2B. The close relationship of both FCGR2B and FCGR2A paralogs with FCGR2C provides the opportunity to use a paralog ratio test to evaluate both polymorphisms and the CNV of FCGR2C in a pyrosequencing approach (see Supplemental Material). In our control Caucasian population (Table 1), the percentages of individuals with a single copy (11.1%; n = 21) or 3 copies (12.2%; n = 23) of FCGR2C were close to those reported in the previous studies using multiplex ligation-dependent probe amplification. It is of note that we identified two individuals with two null alleles corresponding to a total lack of FCGR2C. In our control population, 79.9% of individuals did not express FcγRIIC because they only carry the FCGR2C-STOP allele (149/151) or lack the FCGR2C gene (2/151), whereas 20.1% of them possessed at least one FCGR2C-ORF allele (Table 1) leading to FcγRIIC expression in those who do not have the additional mutation at the splice sites of intron 7. We also identified an unusual allele combination of FCGR2C-ORF/STOP, with 5 copies of FCGR2C-STOP and one copy of FCGR2C-ORF (Table 1).

**Discussion**

We have developed an approach based on both a paralog ratio test and a pyrosequencing assay for the determination of the FCGR2C-ORF/STOP polymorphism in a control population. This paralog ratio test is based on the premise that there are no probes specific for coding regions, Breunis et al. did not observe CNV of FCGR2A and FCGR2B. Niederer HA et al. used a paralog ratio test to determine copy number variation as a single genomic region (CNR2) containing CNV of FCGR2C, FCGR3A and FCGR2A. Frequencies of CNV for this region were respectively 1.3% for 1 copy and 4.5% for 3 copies. It is important that primer pair used to amplify a 279 bp fragment of FCGR2A were localized in 3’-UTR sequence. On the other hand, using multiplex ligation-dependent probe amplification with probes specific for coding regions, Breunis et al. did not observe CNV of FCGR2A and FCGR2B in a large (≥ 600 individuals) control population.

In our pyrosequencing approach, we have also used primer pair localized in coding region of FCGR2A and FCGR2B where no CNV has been reported so far. Moreover, if the CNR2 described by Niederer et al. expanded to the coding region of FCGR2A, it will be expected that 4.5% of individuals would carry 3 copies of FCGR2A. In these individuals, those with the frequent

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**Table 1. FCGR2C genotypes in a Caucasian population**

| Number of FCGR2C-ORF | Number of FCGR2C-STOP | Genotype* |
|----------------------|------------------------|-----------|
| 0                    | 0                      | nul/nul 2 |
| 1                    | 1 STOP                 | 21        |
| 2                    | 2 STOP/STOP            | 108       |
| 3                    | 3 STOP/STOP/STOP       | 20        |
| **Total**            | **38 (20.1%)**         |           |

*Allele frequencies estimated from these genotypes are 12.6 and 87.4 respectively for FCGR2C-ORF and FCGR2C-STOP.
**Figure 1.** LD between FCGR2C-ORF/STOP, FCGR3A-V158F and FCGR2A-H131R. Polymorphisms of interest were localized on the genomic cluster using gene order and orientation annotated in build 37.2 of NCBI. For FCGR3A-V158F and FCGR2A-H131R, number of individual for each genotype is indicated depending on FCGR2C-ORF/STOP polymorphism.

FCGR2C-STOP/STOP genotype and carrying two copies of FCGR2B should have a ratio between FCGR2B/FCGR2C-ORF and FCGR2A < to 1 \([(2 \times FCGR2B + 0 \times FCGR2C-ORF)/3 \times FCGR2A = 0.66]\). Nevertheless, we did not observed such a result in our cohort (all the ratios were > to 1). Therefore, our results in accordance with those of Breunis et al. do not substantiate the hypothesis of CNV in the coding region of FCGR2A.

Using a pyrosequencing approach, we evaluated frequencies of FCGR2C-ORF/STOP alleles in a control Caucasian population. These frequencies are concordant with previous results showing that the FCGR2C-ORF allele is present in only half of the 40% of individuals with detectable CD32⁺ NK cells (i.e., expressing one of the FcγRII). The different genotype frequencies observed in our study (Table 1) were mostly close to those previously reported in the Caucasian population by Breunis et al. and by van der Heijden et al. However, Breunis et al. did not identify individuals with at least 2 copies of FCGR2C-ORF in their control group, whereas 4.3% and 0.5% had two or three copies of the FCGR2C-ORF allele in our population, respectively. Given the allele and CNV frequencies, all the ORF/STOP combinations expected were observed.

Our investigations showed a high LD between FCGR2C-ORF/STOP and FCGR3A-V158F. Despite the reported association of FCGR3A, FCGR2A and FCGR2C polymorphisms with several autoimmune diseases, their LD has not been extensively studied. Using flow cytometry, Steward-Akers et al. showed that the FcγRIIIA-158F/F genotype was over-represented in CD32⁻ rheumatoid arthritis patients as compared with CD32⁺ patients, suggesting a LD between FCGR3A-158F and FCGR2C-STOP alleles. On the other hand, Breunis et al. reported that they did not observe an absolute linkage when the FcγRIIIA-158V data were combined with the FCGR2C-ORF allele and FCGR2C-386C/-120T promoter haplotype. Second, we found a lower LD between FCGR2C-ORF allele and the FCGR2A-131H allele. This low LD between FCGR2C-ORF/STOP and FCGR2A-H131R could explain why Breunis et al. did not find an association between ITP and FCGR2A-H131R, whereas they found an association of ITP with both the V158F and the ORF/STOP polymorphisms. Moreover, the conclusion that the FCGR2C ORF/STOP polymorphism predisposes to ITP, as proposed by Breunis et al., should be re-evaluated in light of its LD with FCGR3A-V158F.

**Materials and Methods**

**DNA samples.** A total of 189 Caucasian DNA samples (CHRU of Tours, regular collection approved by the Ministry of Health, DC-2008–308) were initially extracted using the QIAamp DNA Blood Mini Kit (Qiagen) from peripheral blood of healthy donors.

**PCR amplification.** A 110 pb region common to FCGR2A, FCGR2B and FCGR2C and spanning the site of the FCGR2C-ORF/STOP polymorphism was amplified by polymerase chain reaction (PCR) (Fig. S1). The primer sequences used were Biot2abi2e3-S 5’-CTC TGC CCC TCA G-3’ and 2abce3-AS 5’-TGT CAG AGT CAC ACA GT-3’. The forward primer was 5’-biotinylated to allow single-strand DNA template isolation.
for the pyrosequencing reaction. Each PCR mix contained 50 ng genomic DNA, 10 pmol of each primer, and 0.3 μL Taq polymerase (Eurobio) in a total volume of 55 μL. Cycling was performed in a Bio-Rad iCycler as follows: 94°C for 5 min, 35 cycles of 94°C for 1 min, 62°C for 30 sec, 72°C for 30 sec and a final extension at 72°C for 2 min. Successful and specific amplification of the region of interest was verified by visualizing 5 μL of the PCR product in an 8% acrylamide gel.

Pyrosequencing. Preparation of the single stranded DNA template for pyrosequencing was performed using the PSQ Vacuum Prep Tool (Biotage) according to the manufacturer's instructions: 45 μL of biotinylated PCR product was immobilized on streptavidin-coated Sepharose high-performance beads and processed to obtain single stranded DNA using the PSQ 96 Sample Preparation kit (Biotage). The template was incubated with 1 μL of 10 pmol sequencing primer (pyro2-AS: 5'-TGG AGC ACG TTG ATC CAC-3') at 80°C for 2 min on a PSQ 96 plate. The sequencing reaction of the complementary strand was automatically performed on a PSQ 96MA instrument (Biotage) at room temperature using PyroGold (Fig. S2).

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