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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 cytoxicity; 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 FcγRIIIA 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 allotypes demonstrate that antibody dependent cell-mediated cytoxicity (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*-158V allele with immune thrombocytopenic purpura (ITP) has been repeatedly reported.[12-15] The association of *FC*GR2A-H131R with ITP was also investigated in these studies with conflicting results. No association was found by Foster et al. in a small cohort[12] (n = 37) or by Breunis et al. in a larger cohort of Caucasian patients[15] (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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Table 1. FCGR2C genotypes in a Caucasian population

Number of FCGR2C-ORF	Number of FCGR2C-STOP	Genotype[a]	
0	0	nul/nul	2
	1	STOP	21
	2	STOP/STOP	108
	3	STOP/STOP/STOP	20
Total			**151 (79.9%)**
1	0	ORF	0
	1	ORF/STOP	27
	2	ORF/STOP/STOP	1
	5	ORF/(STOP)$_5$	1
2	0	ORF/ORF	6
	1	ORF/ORF/STOP	2
3	0	ORF/ORF/ORF	1
Total			**38 (20.1%)**

[a]Allele frequencies estimated from these genotypes are 12.6 and 87.4 respectively for FCGR2C-ORF and FCGR2C-STOP.

is supported by the fact that the *FCGR2A*-H131R polymorphism, which is not in LD with *FCGR3A*-V158F in the Asian population,[17] was not associated with ITP in a cohort of 104 Asian patients.[13] Breunis et al. also reported an association between the *FCGR2C*-ORF allele and ITP in their Caucasian cohort; however, the possibility that this might result from a LD between the *FCGR2C*-ORF/STOP and the *FCGR3A*-V158F polymorphisms was not reported. Overall, the issue of the LD between the *FCGR2C*-ORF/STOP polymorphism and the *FCGR3A*-V158F or *FCGR2A*-H131R polymorphisms has not yet been addressed. This probably results from the difficulty in *FCGR2C* genotyping, due to the fact that *FCGR2C* and *FCGR2B* are identical in this region and that *FCGR2C* is subject to copy number variations (CNV). To clarify this situation, we 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.

Results

***FCGR2C* genotyping of a Caucasian population using pyrosequencing.** Due to its chimeric nature resulting from an unequal crossover between *FCGR2B* and *FCGR2A,* analysis of *FCGR2C* is complex.[18] Consequently, in the region of *FCGR2C*-ORF/STOP polymorphism, *FCGR2C* is identical to *FCGR2B* and, except at the level of the *FCGR2C*-STOP codon, it is not possible to distinguish between *FCGR2C* and *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*.[19,20] The close relationship of both *FCGR2B* and *FCGR2A* paralogs with *FCGR2C* provides the opportunity to use a paralog ratio test[21] 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.[4,15] 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.[4] We also identified an unusual allele combination of *FCGRC*-ORF/STOP, with 5 copies of *FCGR2C*-STOP and one copy of *FCGR2C*-ORF (**Table 1**).

LD between FCGR2C-ORF/STOP, FCGR3A-V158F and FCGR2A-H131R. The *FCGR3A*-V158F and *FCGR2A*-H131R genotypes of the 189 individuals of our population were therefore analyzed together with the *FCGR2C*-ORF/STOP data. First, we did not find any association between *FCGR2C* CNV and *FCGR3A*-158V/F or *FCGR2A*-H131R genotypes (data not shown). Second, we analyzed the LD between *FCGR2C*-ORF/STOP and *FCGR3A*-V158F or *FCGR2A*-H131R, without taking the *FCGR2C* CNV into account. Our results demonstrated the high LD between *FCGR2C*-ORF/STOP and *FCGR3A*-V158F: the *FCGR2C-ORF* allele was strongly associated with the *FCGR3A*-158V allele (**Fig. 1**). Indeed, the frequency of the ORF allele was 53.5%, 20.2%, and 4.5% in VV, VF and FF donors, respectively. On the other hand, analysis of the LD between *FCGR2C*-ORF/STOP and *FCGR2A*-H131R revealed a lower LD, which tended to associate the *FCGR2C-ORF* allele with the *FCGR2A*-131H allele (**Fig. 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 CNV in *FCGR2A* and *FCGR2B.* Niederer HA et al. using a paralog ratio test to determine copy number variation identified a single genomic region (CNR2) containing CNV of *FCGR2C*, *FCGR3A* and *FCGR2A*.[22] 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 observed CNV of *FCGR2A* and *FCGR2B* in a large (> 600 individuals) control population.[19]

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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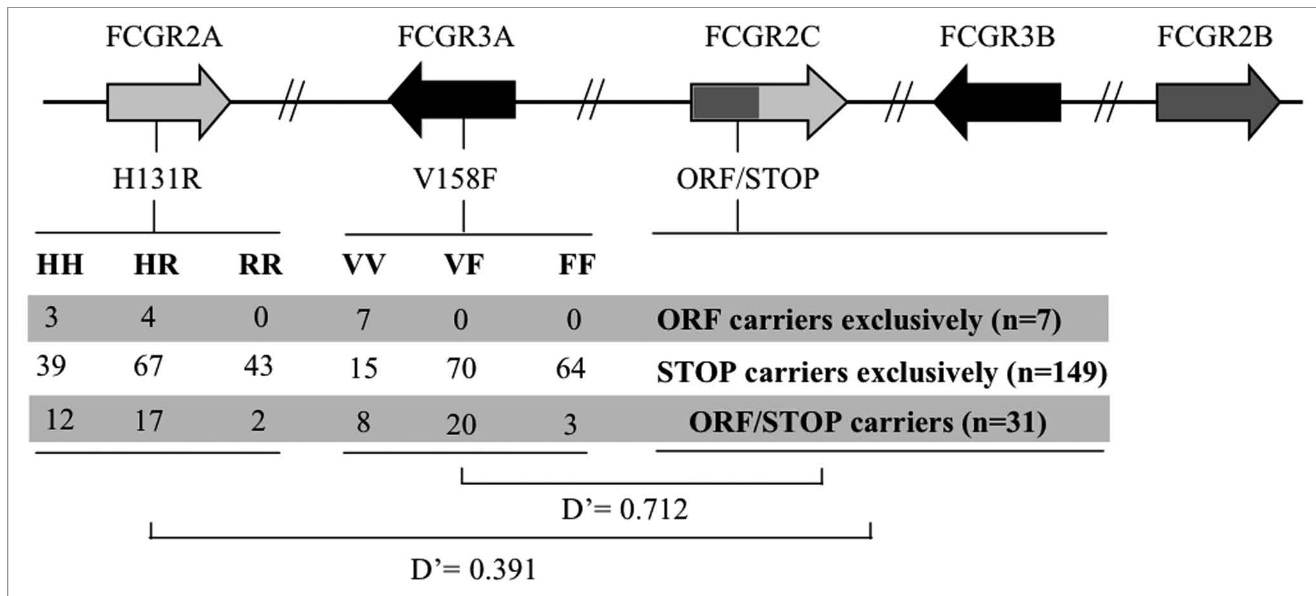

Figure 1. LD between *FCGR2C*-ORF/STOP, *FCGR3A*-V158F and *FCGR2A*-H131R. Polymorphisms of interest were localized on the genomic cluster using gene order and orientation annotated in build 37.2 of NCBI. For *FCGR3A*-V158F and *FCGR2A*-H131R, number of individual for each genotype is indicated depending 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 *FCGR2B* + 0 *FCGR2C*-ORF)/3 *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.[19] 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).[23] 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.[4] However, Breunis et al.[15] 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 $CD32^{neg}$ rheumatoid arthritis patients as compared with $CD32^{pos}$ patients, suggesting a LD between *FCGR3A*-158F and *FCGR2C*-STOP alleles.[22] 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.[15] 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.

The importance of *FCGR3A*-V158F, *FCGR2A*-H131R and *FCGR2C*-ORF/STOP polymorphisms is well established in several autoimmune diseases[11] and in response to therapeutic antibodies.[5-10] In accordance with our previous report,[17] this study strongly supports the notion that the different LD between these polymorphisms should be systematically taken into account in these types of study.

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 Biot-2abci2e3-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

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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 1μL 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 reagents (**Fig. S2**).

***FCGR3A*-V158F and *FCGR2A*-H131R genotyping.** *FCGR3A*-V158F (rs 396991) and *FCGR2*A-H131R (rs 1801274) polymorphisms were genotyped as previously described.[5]

Statistical analyses. LD between polymorphisms was estimated using Haploview.[24] The association between *FCGR2C* CNV and *FCGR3A*-158V/F or *FCGR2A*-H131R genotypes was tested using a CHI 2 test.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Supplemental Material

Supplemental material may be found here:
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