Investigation of candidate gene copy number identifies *FCGR3B* as a potential biomarker for rheumatoid arthritis

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Abstract

Objective

Copy number variants (CNVs) could explain a part of the missing heritability in rheumatoid arthritis (RA). Our goal is to investigate the association of RA with CNVs of three functional candidate genes, Glutathione S-transferase M1 (GSTM1), Glutathione S-transferase T1 (GSTT1) and Fcγ receptor type IIIAB (FCGR3B).

Methods

We quantified the absolute copy number of GSTM1, GSTT1 and FCGR3B genes using droplet digital PCR. Transmission of copy number alleles was investigated in trio families with RA using family-based association tests (Transmission Disequilibrium Test and Genotype Haplotype Relative Risk). Clinical, environmental and biological data on RA patients were also used to stratify patients sample in analysis.

Results

Copy numbers from zero to three were identified. Genotype combinations characterised in 182 trios allowed testing the association with RA. Genotypes without null allele of FCGR3B gene were significantly associated with RA ($3.41x10^{-7}$). Three copy numbers of this gene is observed only in cases of RA (n=14) and a protective effect of null allele was characterised (OR=0.3 (0.17-0.53)).

Conclusion

CNVs in FCGR3B are associated with RA in our set of samples. This gene may play a role in physiopathology of this disease.

Key words

rheumatoid arthritis, gene copy number, *FCGR3B*

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Introduction

Rheumatoid arthritis (RA) is a chronic autoimmune disease characterised by symmetrical inflammation of the peripheral joints resulting in a progressive destruction of cartilage and bone. This disease has a complex pathogenesis (1) with a multifactorial aetiology. The genetic component is estimated to be about 60% (2). Tobacco exposure (TE) is the strongest environmental risk ever described (3). Shared epitope (SE) alleles of *HLA-DRB1* gene play a prominent role (4, 5) in RA genetics while Genome Wide Association Studies (GWAS) using SNPs (6, 7) have identified about one hundred additional weak genetic risk factors. Altogether these genetic factors explain about 15% of RA heritability (7, 8). Identification of other disease-specific genomic variants defines one of the ways to explain missing heritability in RA.

Research findings in past few years indicate a strong association between copy number variants (CNVs) and several complex common diseases (9). Gene ontology analysis from several studies reveals that a substantial amount of genes with CNV are involved in immune responses, drug metabolism and responses to external biotic factors (9,10,11). Among them, the Glutathione S-transferase (GST) M1 gene (*GSTM1,* OMIM 138350) and the Glutathione S-transferase (GST) T1 gene (*GSTT1,* OMIM 600436) code phase II detoxification enzymes that are involved in the metabolism of xenobiotics and have an important role in cellular protection against oxidative stress (12). The *GSTM1* gene is located on chromosome 1p13.3 and exhibits a CNV that leads to the complete deletion of the gene in 53% of the Caucasian population (13). The *GSTT1* gene is located on chromosome 22q11.2 and the complete deletion of the gene has a frequency of 19% of the Caucasian population (13). For the *GSTM1* gene, this complete deletion is the result of a non-allelic homologue recombination (NAHR) between two high homologous box sequences on both sides of the gene, leading to deletion on one chromosome and duplication on the second one of the homologous pair (14, 15). A third gene, *FCGR3B* (OMIM 610665), which maps on chromosome 1q23.3 and codes the receptor IIIb of low affinity for Fc fragment of IgG is a good candidate in RA. This receptor is a glycosylphosphatidylinositol-anchored protein that is expressed constitutively by neutrophils and after gamma-interferon stimulation by eosinophils (16). Dysfunction of this receptor, important in phagocytosis and clearance of immune complexes, could result in an altered elimination of antigens and immune complex. A deletion of *FCGR* genes would lead to disruption in the presence of corresponding receptors on immune cells. The *FCGR3B* gene harbours CNVs with deletion and duplication, consequences of a NAHR mechanism (17). Deletion of the *FCGR3B* gene in the Caucasian population is recently estimated from 3 (17) to 10% (18).

Due to their importance in detoxification or in immune complex presentation, CNVs of *GSTM1*, *GSTT1* and *FCGR3B* genes are considered as a potential risk for RA. Many case/control association studies using various methodologies for the characterisation of CNV have been published for these genes. Heterogeneous results regarding the association of CNV with RA were reported and they could be due to mistyping of copy number and/or small sample size. In this study, we use a methodology of digital PCR (19) which gives an absolute quantification of copy numbers. Using a familial sample allows us to better characterise CNV genotypes, leading to the investigation of CNV's association and linkage with RA.

Materials and methods

Patients

200 trio families, consisting in one RA patient and his/her two parents from French European origin, were included in this study. The Ethics Committees of Hôpital Bicêtre and Hôpital Saint Louis (Paris, France) approved the study (CPPRB 94-40) and all subjects provided informed written consent. RA satisfied the revised criteria of the American College of Rheumatology (ACR) (20). For each RA index, sex, age at RA onset, presence of bone erosions at x-ray examination, presence of rheumatoid

nodules, as well as seropositivity for rheumatoid factor (RF) and for anti-cyclic citrullinated peptide antibodies (ACPA), presence of HLA-DRB1 shared epitope allele and tobacco exposure (TE, (21)) were collected. Genomic DNA was extracted from whole blood samples by salting out or phenol/chloroform method.

Characterisation of CNVs by ddPCR

We used QX200™ Droplet Digital PCR (ddPCR) (Bio-Rad Laboratories, California, USA), following the Minimum Information for the Publication of Digital PCR Experiment (dMIQE) guidelines (22) with target probes (Applied Biosystems, Foster City, CA, USA) for *GSTM1* (Hs03352485_cn), *GSTT1* (Hs00767125_cn) and *FCGR3B* (Hs04211858_cn) genes. For the reference gene with two copies, we used primers and probe for *RPP30* gene (OMIM 606115) as described in a previous study (19). DNA was restriction digested with either *HaeIII* or *TaqI* (New England Biolab, England), in order to separate possible tandem duplication of genes. Digestion was done with one unit of enzyme, 25ng of DNA and 1X of *CutSmart*™ buffer. We then prepared 20µl of reaction volume with 16ng of DNA template, target probe (with a final concentration of 1X), reference primers and reference probes (with final concentrations of, respectively, 900 and 250 nmol/L), and Droplet PCR Supermix (Bio-Rad) with final concentration of 1X. Then, we added 70µl of droplet generation oil and 13,000 to 16,000 droplets were generated in 40µl (with at least one copy of the gene per droplet) with the Droplet Generator, according to the manufacturer's instructions. Next, we performed an amplification in a C1000 Touch™ Thermal Cycler (Bio-Rad) in the following conditions: 95°C (10 min), 40 cycles of 94°C (30 sec) and 60°C (60 sec), and 98°C (10 min). We used QuantaSoft™ (v. 1.4, Bio-Rad) software to estimate CNVs by counting the positive and negative fluorescence signal events. In addition, concentration (copies/µl) by Poisson law were calculated and normalised to the reference concentration using the same software. We used no-template reaction for the negative control and samples with known CN as positive controls (NA10851/NA10852/NA10865/NA11893/NA11894, http://ccr.coriell.org/, New Jersey, USA). In order to confirm data, 10% of the samples were randomly repeated at least twice in independent experiments.

Table I. Characteristics of 182 RA index cases.

	Number[1]	Percentage
Female	163/182	89.56 %
Age at onset less than 40 years	137/182	75.27 %
RF positive	142/182	78.02 %
ACPA positive	135/176	76.70 %
Presence of erosion	156/182	85.71 %
Presence of nodules	45/182	24.73 %
Tobacco exposure[2]	90/170	52.94 %
Carrying at least one HLA-DRB1 shared epitope allele	142/182	78.02 %

[1]number of index cases/number of index cases with data. [2]previous and/or actual tobacco exposure (smokers and ex-smokers, (20)).
RF: rheumatoid factor; ACPA: anti-cyclic citrullinated peptide antibodies.

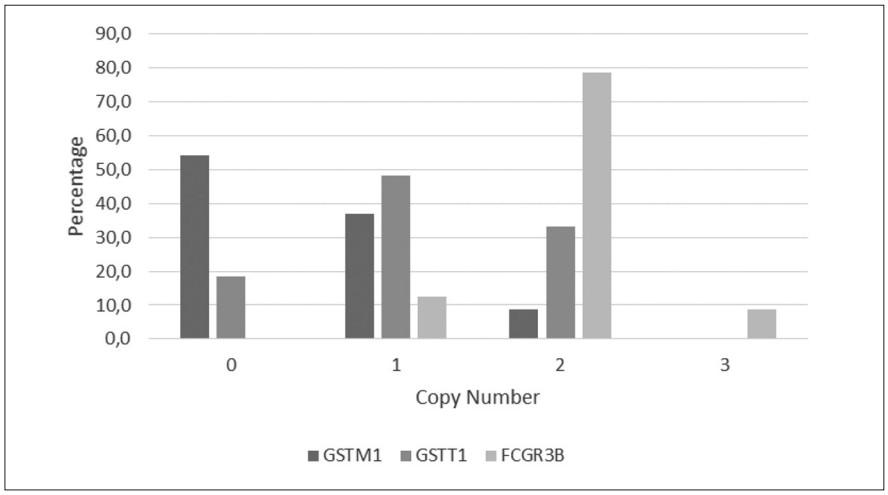

Fig. 1. Percentage of copy numbers for *GSTM1*, *GSTT1* and *FCGR3B* genes in parents of 182 trios (364 subjects).

Family-based association tests with CNV genotyping data

CN characterisation in trio families led to genotype combinations. Then we first based the association analyses on the transmission disequilibrium test (TDT), done with FBAT software (23), which compares for a given allele, its transmission from heterozygous parents to RA patients with the transmission expected from Mendel's law (*i.e.* 50%). A multiallelic mode was used for *FCGR3B* gene.

We also computed the genotype haplotype relative risk (GHRR) (24), in order to compare the frequency of genotypes between RA cases and pseudo-controls for which the genotypes derived from non-transmitted parental alleles (25). Odds ratios and 95% confidence intervals were computed by considering the 1/1 genotype as the reference.

We applied these methods to the whole sample and to subgroups defined according to RA cases' characteristics, namely: more or less than the age of 40 years old at RA onset, presence or absence of erosion, nodules, rheumatoid factor (RF), anti-cyclic citrullinated peptide antibodies (ACPA), *HLA-DRB1*-"shared epitope" (SE), or tobacco exposure (TE, smokers/ex-smokers or non-smokers (21)). In order to correct for multiple testing, we considered a *p*-value threshold of 5.56×10^{-4} after applying Bonferroni correction for multiple testing.

Results

Copy numbers characterisation

Absence of interference between target assays and primers and probes for

reference gene was verified (data not shown). Among 200 trios, 182 families fully genotyped for the three genes have been studied and the characteristics of the corresponding RA patients are described in Table I. For *GSTM1* gene, copy number counting in parents (364 subjects) showed a frequency of 54.1% for 0 copy, 37.1% for 1 copy, and 8.8% for 2 copies. Absence of gene copy was less frequent for *GSTT1* gene with 18.4%, whereas we observed 48.4% of 1 copy and 33.2% for 2 copies. The copy number frequencies for *FCGR3B* were really different than for the two other genes, with 0.3% of 0 copy, 12.4% of 1 copy, 78.6% for 2 copies and 8.8% for 3 copies (Fig. 1). Distribution of copy numbers for each gene was then investigated regarding subgroups stratifying related to age at onset and presence or absence of ACPA, RF, erosion, nodules, tobacco exposure and ≥1 *HLA-DRB1* SE allele. For the three genes, the percentages of copy numbers were homogeneous between the subgroups tested (data not shown).

CN characterisation in each trio family led to different genotypes combinations for each gene (Table II). These combinations have been established regarding the alleles transmission in family without *de novo* deletion event assumption. Eleven combinations were described for each gene, ten are shared between *GSTM1* and *GSTT1* genes and five are shared between the three genes. *FCGR3B* specific combinations are mostly due to subjects with 3 copies.

Table II. Copy number transmission in 182 trios for *GSTM1*, *GSTT1* and *FCGR3* genes.

CN in trios[a] parent 1 x parent 2=>child	(ID) Genotypes in trios[b] parent 1 x parent 2=>child	*GSTM1*[c] n (%)	*GSTM1*[c] n (%)	*FCGR3B*[c] n (%)
0x0=>0	(A) 0/0x0/0=>0/0	55 (30.2)	7 (3.8)	0
0x1=>0	(B) 0/0x0/1=>0/0	33 (18.1)	14 (7.7)	0
0x1=>1	(C) 0/0x0/1=>0/1	35 (19.2)	16 (8.8)	0
0x2=>0	(D) 0/0x0/2=>0/0	0	1 (0.5)	0
0x2=>1	(E) 0/0x1/1=>0/1	18 (9.9)	23 (12.6)	1 (0.55)
0x2=>2	(F) 0/0x0/2=>0/2	1 (0.5)	0	0
1x1=>0	(G) 0/1x0/1=>0/0	8 (4.4)	12 (6.6)	0
1x1=>2	(H) 0/1x0/1=>1/1	10 (5.5)	8 (4.4)	0
2x2=>2	(I) 1/1x1/1=>1/1	1 (0.5)	15 (8.2)	111 (61)
1x2=>2	(J) 0/1x1/1=>1/1	5 (2.7)	25 (13.7)	19 (10.4)
1x2=>1	(K) 0/1x1/1=>0/1	6 (3.3)	42 (23.1)	15 (8.2)
1x1=>1	(L) 0/1x0/1=>0/1	10 (5.5)	19 (10.4)	2 (1.1)
2x2=>1	(M) 0/2x1/1=>0/1	0	0	2 (1.1)
1x3=>3	(N) 0/1x1/2=>1/2	0	0	2 (1.1)
3x3=>2	(O) 1/2x1/2=>1/1	0	0	1 (0.55)
2x3=>3	(P) 1/1x1/2=>1/2	0	0	12 (6.6)
2x3=>2	(Q) 1/1x1/2=>1/1	0	0	11 (6)
1x3=>2	(R) 0/1x1/2=>1/1	0	0	6 (3.3)

[a]18 Copy number variants combinations described in trios families ('0' means zero copy of the gene, '1' means one copy...). [b]18 genotypes combinations derived from CNV data in trios families ('0/0' means genotype homozygous for null allele…). [c]number and percentage of trio families with the combination described, for each gene.

Table III. Transmission disequilibrium test (TDT) in RA trios families for *GSTM1*, *GSTT1* and *FCGR3B* CNV alleles.

Tested allele	Informative families[a]	Frequency of tested allele[b]	P_{TDT}[c]
GSTM1, null allele	107	0.728	0.67
GSTT1, null allele	138	0.423	0.051
FCGR3B, multi-allelic	69	-	0.15

[a]Families with at least one heterozygous parent, used for TDT. [b]TDT has tested null allele for GSTM1 and GSTT1 genes, and all alleles for FCGR3B. [c]*p*-value for transmission disequilibrium test (TDT) using an additive model.

Family-based association tests with CNV genotyping data

According to familial genotypic configuration, the absence of *GSTM1* or *GSTT1* was tested by the TDT in combinations A to L (except D for *GSTT1* and F for *GSTM1*, Table II). For the *FCGR3B* gene, all the combinations were used for family-based association tests.

No disequilibrium of null allele was detected in 138 informative families for *GSTT1* gene, as well as observed in 107 informative families for the *GSTM1* gene. For the *FCGR3B* gene, less than 50% of families were informative and no disequilibrium of transmission was observed as well (Table III). When stratifying the whole familial sample regarding clinical, biological, environmental and genetic factors for the index cases, as described in methods, results were also not significant (data not shown).

In order to increase the number of tested families, we estimated then GRR for genotypes with null allele genes and evaluated risk associated with these genotypes compared to the reference one (1/1). The distribution of CNV genotypes between cases and pseudo-controls was only significantly different for *FCGR3B* gene ($P_{GRR}=3.41x10^{-7}$) after Bonferroni correction for multiple testing. For this later gene, a protective effect of the 0/1 genotype compared to the 1/1 genotype is observed ($OR_{0/1\ versus\ 1/1}=0.3$; IC95%=(0.17-0.53)). Even if the GRR test is not significant for *GSTT1* and *GSTM1*, an increase in risk is found for the 0/1 genotype compared to the reference one for *GSTT1* ($OR_{0/1\ versus\ 1/1}=1.83$; IC95%=(1.14-2.93)).

For the three genes, no significant results were found by stratifying for RA cases characteristics (data not shown).

Discussion

In the current study, we used ddPCR technology to identify CNVs for *GSTM1*, *GSTT1* and *FCGR3B* genes and to describe genotypes in order to analyse their transmission in RA families.

For the *GSTM1* gene, the homozygous genotype for null allele in trios parents has a frequency (53.8%) similar as previously described in Caucasian population (52%, (13)). One family (Table II) presents two subjects with a 0/2 genotype, resulting of a duplication of the gene on the same chromosome. This duplication event has been demonstrated to oc-

cur through inter chromosome NAHR, leading to the simultaneous deletion of *GSTM1* on one chromosome and a duplication on the second one (14). As the homozygous for null allele genotype of *GSTM1* observed in our study is much more frequent than 0/2 genotype, we are able to confirm the assumption that the null allele was positively selected, thus highlighting the purifying selection of *GSTM1* described previously (26). Analysis of *GSTM1* CNVs in trio families did not show a transmission disequilibrium of null allele to RA patients and distribution of genotypes are similar in RA cases and in pseudo-controls from our trios. Deletion of the *GSTM1* gene was previously associated in cases/controls studies with severity of the disease, independently of *HLA-DRB1 SE* (27, 28) or with ACPA-positive RA (29). Association between smoking and RA in patients carrying *GSTM1* deletion was also described with a higher risk in a group of smoker patients carrying the homozygous genotype for null allele than in non-smoker patients, suggesting that *GSTM1* could influence the relationship between smoking and disease severity (30). However, other studies observed no significant association between RA and *GSTM1* not even in smokers or in other sub-groups of patients (31-33). Moreover, when *GSTM1* CNVs was analysed by qPCR in a case/control study, no association between RA and the gene of interest was found, but a significant association with the severity of RA in ACPA-positive patients was highlighted (34). These heterogeneous results can be explained by the differences between the studies regarding populations and sample sizes. Our analysis investigating the transmission of *GSTM1* CNVs in families, using accurate genotyping data, showed that the null allele was not associated with RA. Performing the same methodology of CNV characterisation in a large case/control association study could help to discover the association of *GSTM1* gene deletion with RA.

Regarding *GSTT1* CNVs, we described a frequency of homozygous genotype for null allele (18.1%) similar to the one described in Caucasian population (19%, (13)). Among the families with at least one heterozygous parent, null allele is preferentially transmitted to RA cases although not significant result (*p*-value for TDT test = 0.051, Table III). Distribution of genotypes also revealed a difference between cases and controls (*p*-value for GRR test = 0.014, Table IV), but this result did not reach the significant threshold after correction for multiple testing ($5.56x10^{-4}$). Several studies in different populations realised between 2005 and 2011 were combined and revealed absence of association with RA, whereas increased risk of null allele was identified in ACPA positive RA and in heavy smoker patients (35). Our analysis in such subgroups did not reveal, however, significant results.

Frequency of *FCGR3B* homozygous genotype for null allele in our sample (0.3%) is lower than described in other studies on Caucasian populations (17, 18). Regarding presence of 3 copies in genome, our frequency of 8.8% is similar to the frequency of CN superior to 2 described in (18). The majority of RA patients of our study have two copies of FCGR3B gene. This result is concordant with a recent study (17). The presence of two copies is less frequent in controls than in RA, which is concordant with a strong protective effect of null allele. We were also able to identify in our sample a genotype distribution significantly associated with RA, with a genotype 1/2 strictly associated with RA cases. Low *FCGR3B* gene copy numbers were associated with RA in some studies (36, 37, 17) but not in others (38-40). *FCGR3B* copy number superior to 2 was related to ACPA-negative RA (41) but our sample did not replicate this observation (only 4 cases among 14 harbouring 3 copies of *FCGR3B* are ACPA-negative RA). The *FCGR3B* gene presents 95% of sequence homology with *FCGR3A* gene. Antibodies that recognise CD16 generally recognise both gene products. However, *FCGR3B* cannot mediate antibody-dependent cell-mediated cytotoxicity and should have the role of a trap for immune complexes (42). In our sample, over representation of copy number >2 in RA cases could indicate that an overexpression of *FCGR3B* gene induces a dysregulation of mechanisms leading to pathophysiological effects. Further functional studies are needed to prove it.

In conclusion, digital PCR offers an appropriate methodology to accurately

Table IV. Genotype relative risk (GRR) in RA trio families.

CNV *GSTM1* genotypes	0/0	0/1	1/1
Cases (n,%)	96 (53)	69 (38)	16 (9)
Controls[a] (n,%)	100 (55)	66 (36)	15 (8)
OR[b] (CI 95%)	0.9 (0.42-1.92)	0.98 (0.45-2.14)	–
	0.93 (0.44-1.94)		–
P_{GRR}[b]	0.853		
CNV *GSTT1* genotypes	0/0	0/1	1/1
Cases (n,%)	34 (19)	99 (55)	48 (27)
Controls[a] (n,%)	30 (17)	80 (44)	71 (39)
OR[b] (CI 95%)	1.67 (0.91-3.09)	1.83 (1.14-2.93)	–
	1.79 (1.15-2.79)		–
P_{GRR}[c]	0.014		
CNV *FCGR3B* genotypes	0/1	1/1	1/2
Cases (n,%)	20 (11)	148 (81)	14 (8)
Controls[a] (n,%)	56 (31)	126 (69)	0 (0)
OR[b] (CI 95%)	0.3 (0.17-0.53)	–	
P_{GRR}[c]	3.41×10^{-7}		

[a]Controls: pseudo-controls which the genotypes derived from non-transmitted parental alleles in trio families. [b]Evaluation of risk for one genotype (0/0 or 0/1 for GSTM1 and GSTT1 genes; 0/1 for FCGR3B gene) or two pooled genotypes (0/0 and 0/1 for GSTM1 and GSTT1 genes) against the reference one (1/1). [c]*p*-value for genotypes relative risk (GRR) according to McNemar chi-square.

genotype CNVs. Analysis of familial samples leads to the identification of duplication events and to the characterisation of genotypes, essential for candidate gene studies of complex diseases. As copy numbers of genes were described as biomarkers in the clinical diagnosis of several cancers (43, 44), the ddPCR protocol we described here could be used for this important issue and for the identification of such biomarkers in RA.

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