Received: 7 October 2016 | Accepted: 15 June 2017

DOI: 10.1002/jcla.22300

RESEARCH ARTICLE

WILEY

Measurement of absolute copy number variation of Glutathione S-Transferase M1 gene by digital droplet PCR and association analysis in Tunisian Rheumatoid Arthritis population

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Funding information
Ministry of Higher Education and Scientific Research of the republic of Tunisia

Background: The investigation of copy number variations (CNVs) analysis of candidate genes is currently an important research area in modulating human diseases. We aimed to quantify CNVs in glutathione S-transferase M1 (*GSTM1*) gene and determine its genetic contribution in Tunisian rheumatoid arthritis (RA) and its subsets through an innovative technique for quantification.

Methods: A total of 165 RA cases and 102 healthy controls were included in the study. Using a recently powerful approach of digital droplet PCR (ddPCR), we quantified *GSTM1* gene to determine the presence of no, one, or multiple copy number (CN) at high levels of sensitivity and specificity. Odds ratio and Fisher exact test were performed to estimate the association risk for *GSTM1* CNVs in RA.

Results: Copy number identified by ddPCR was 0, 1, and 2 copies per diploid genome. A high frequency of '0' copy was revealed with 54% in RA patients. The deletion ('0' copy) of *GSTM1* was found to be a significant risk factor for anti-cyclic citrullinated peptide (anti-CCP) positive RA (OR=4.16, $CI_{95\%}$=[1.17-14.7]). In addition, a lack of association was found when comparing between the CNVs of RA patients and those of controls.

Conclusion: This study highlights the powerful accuracy of ddPCR for the quantification of CNVs and suggests that the variation in the CN of *GSTM1* is associated with anti-CCP positivity in RA. However, it does not indicate a specific role in the susceptibility to the disease in our Tunisian sample.

KEYWORDS

copy number variants, droplet digital PCR, *GSTM1*, rheumatoid arthritis

1 | INTRODUCTION

Rheumatoid arthritis (RA, OMIM# 180300) is a chronic autoimmune disease affecting approximately 1% of the adult population worldwide. It is characterized by the symmetrical inflammation of the synovial joints, increased cytokine production and pannus development, leading, in fact, to a progressive destruction of the cartilage and bones. Despite the fact that the exact etiology of RA remains unknown, population and family studies have indicated that genetic factors are major contributors to the liability of RA,[1] which are estimated to be as high as 60%.[2] Among the genetic factors, human leukocyte antigen (HLA) class II genes are depicted as being the most potent factor associated

with RA, but with a contribution of only one third of genetic susceptibility,[3] which indicates that additional genetic risk factors remain to be identified.[4,5] Apart from the previously mentioned features, tobacco smoking is considered as the most important environmental factor for modifying RA by mediating oxidative stress associated with both susceptibility and severity to RA.[6]

Inflammation is a central trait of RA occurring with the resulting reactive oxygen species (ROS), which cause oxidative damage to cellular molecules such as DNA and lipids, giving rise to a variety of cytotoxic products.[7] ROS are involved in RA as they are increasingly generated by phagocytes in the synovial fluid of inflamed joints during hypoxia-reperfusion events, and they cause DNA and lipid oxidation, leading to cartilage and bone destruction. The detoxification mechanism against ROS products is complex and requires several enzymes, namely, glutathione S-transferases (GST), which plays a key protective antioxidant role for cells against ROS aggression.[8] GSTs are multifunctional enzymes that play a pivotal function in the detoxification of varieties of both endogenous products of oxidative stress and exogenous carcinogens.

Glutathione S-transferase M1 (*GSTM1*) (OMIM 138350), a member of GST mu class cluster on the short arm of chromosome 1 (1p13.3), is highly polymorphic in human population.[9] The deletion of *GSTM1* emerges by separate equal or unequal recombination events involving crossing-over between two highly homologous repeat regions that flank the gene.[10] These recombination events produce deletion-junction regions of several kilobases, which have very high homology (98%) to the flanking repeat regions, resulting in a complete lack of the gene, and thus in a deficient conjugating activity.[11] This homozygous deletion has been observed in different ethnic populations such as Caucasians (48%-57%), African Americans (23%-41%), Asians (32%-53%), and Hispanics (40%-53%).[12] Although several studies have examined the contribution of null genotype of *GSTM1* (0 copy) to RA susceptibility, they have produced conflicting results.[13-21]

Copy number variations (CNVs) are large polymorphisms, such as deletions, insertions, translocations, and inversions of genomic material varying from 1 kilobase to several megabases.[22] CNVs are now recognized to be a prevalent form of common genetic variation and represent a substantial proportion of total genetic variability in human populations. Recent association studies have focused on the importance of CNVs as disease susceptibility variants to confer differential risk to common and autoimmune diseases such as RA. To the best of our knowledge, there have been very few studies on the association between CNVs of *GSTM1* and RA.[16] This is due to the unreliability of available techniques. For instance, PCR multiplex and real-time quantitative PCR (qPCR) do not measure the absolute number of copies, which is a prerequisite for the meaningful analyses of association between CNVs and RA. Indeed, previous studies pertaining to the genetic association of *GSTM1* have only focused on detecting "null" allele against "positive" allele. This study aims to investigate the association case-control study of *GSTM1* CNVs with the development of RA in Tunisian population taking advantage of a recent system of quantification for dPCR analysis, the digital droplet PCR (ddPCR),[23] which provides an absolute quantification of target DNA at high levels of sensitivity and specificity for more precision and reliability in the estimation of the exact copies number of gene of interest.

2 | MATERIALS AND METHODS

2.1 | Participants

A total of 165 unrelated Tunisian RA patients were recruited into the study. All patients fulfilled the 1987 American College of Rheumatology (ACR) criteria for RA.[24] The clinical and laboratory characteristics of the patients with RA are summarized in Table 1. The latter contains the following characteristics for each RA index: gender, presence of bone erosions at x-ray examination, rheumatoid nodules, other autoimmune diseases and *HLA-DRB1* shared epitope alleles, as well as seropositivity for rheumatoid factor (RF) and for anti-cyclic citrullinated peptide antibodies (Anti-CCP). One hundred and two ethnically matched healthy blood donors were used as controls. They were free of any autoimmune or inflammatory diseases at blood sampling and originating from the same area as the RA sample. Samples were obtained from subjects (patients and controls) after they provided written informed consent. The study was approved by the local ethics committee of the Hedi Chaker University Hospital of Sfax, Tunisia.

2.2 | Molecular genotyping and determination of *GSTM1* copy number

Genomic DNA from whole blood was obtained from peripheral blood leukocytes samples taken from each patient by standard methods.[25]

Copy number of *GSTM1* was quantified by digital PCR methods using the QX200™ Droplet Digital PCR (ddPCR; Bio-Rad Laboratories, Hercules, CA, USA) following the Minimum Information for the Publication of Digital PCR Experiment (dMIQE) guidelines.[23] Primers and specific fluorescent probe for *GSTM1* (OMIM: 138350) quantification were predesigned as described earlier (Hs03352485_cn, Applied Biosystems, Foster City, CA, USA) and used in this study. A Fluorescent

TABLE 1 Clinical and laboratory characteristics of Tunisian RA patients

	RA Tunisian patients	(n, %)
Female (n=157)	129	74.0
Rheumatoid Factor positive (n=139)	94	67.6
Anti-Cyclic Citrullinated Peptide Antibodies positive (n=126)	81	64.3
Presence of Erosion (n=145)	106	73.1
Presence of Nodules (n=81)	7	8.6
Carrying at least one HLA-DRB1 shared epitope allele (n=100)	62	62.0
Other autoimmune disease (AID) (n=144)	64	44.4

Other autoimmune diseases: Gougerot-Sjögren syndrome (SS), thyroid autoimmune diseases, and type 1 diabetes (TD1).

probe was used for the reference gene, *RPP30* (OMIM: 606115; Bio-Rad Laboratories), with two copies for normalization as described in a previous study.[26] A total of 20 μL of reaction volume was prepared with 20 ng of DNA template, primers, and probes with final concentrations of 900 and 250 nmol/L, respectively, and Droplet PCR Supermix (Bio-Rad) with final concentration of 1X. A volume of 70 μL of droplet generation oil (Bio-Rad) was then loaded into each of the corresponding oil wells. Thirteen thousand to sixteen thousand droplets were generated in 40 μL (with at least one copy of the gene per droplet) with the Droplet Generator, according to the manufacturer's instructions. Each water-in-oil emulsion was transferred by pipet to a separate well of a 96-well plate and amplified in a C1000 Touch™ Thermal Cycler (Bio-Rad) in the following conditions: 95°C (10 minutes), 40 cycles of 94°C (30 seconds) and 60°C (60 seconds), and 98°C (10 minutes). Discrimination between droplets that did not contain target (negatives) and those which did (positives) was achieved by applying a global fluorescence amplitude threshold in QuantaSoft™ (Bio-Rad) (version 1.4, Bio-Rad). This software package is endowed with the ddPCR system for data acquisition and analysis using droplet reader. Positive fluorescent events correspond to droplets containing at least one copy of *GSTM1* gene. Quantification assumes a random Poisson distribution of *GSTM1* copies into the droplets. Concentration (copies/μL) was calculated and normalized to the reference concentration using the same software. Copy number values for *GSTM1* gene were subsequently obtained from the ratio of these two concentrations. For samples with a copy number value far from an integer of more than 0.25, the quantification was preceded by a digestion with the restriction endonuclease enzyme *HaeIII* (New England Biolab, Hitchin, UK). The objective is twofold, to separate more efficiently possible linked copies of the gene ensuring proper random partitioning into droplets, and to achieve optimal accuracy in CN measurements. Digestion was done in a final volume of 10 μL with one unit of *HaeIII* enzyme and 40 ng of DNA, 1X of CutSmart™ buffer, and qspH2O. A quantity of 20 ng of digest DNA was used for the ddPCR protocol described above.

2.3 | Autoantibody analysis

The sera of RA patients obtained at the time of diagnosis were examined for RF by nephelometry and for Anti-CCP by enzyme-linked immunosorbent assay (ELISA) (Anti-CCP ELISA (IgG) EUROIMMUN®, Lübeck, Germany).

2.4 | Statistical analysis

The distribution of *GSTM1* CN between Tunisian RA patients and healthy controls was assigned by chi2 test (2x2) contingency tables for copies numbers frequencies and Fisher's exact test. The association of CNVs with RA and multivariable analysis by binary logistic regression was assessed using Odds Ratios (ORs) with 95% confidence intervals (CI) by the software Statistical Package for the Social Sciences (SPSS) version 17.0 for Windows (SPSS Inc., Chicago, IL, USA). Significant *P*-values were corrected (P_c) by the number of CN tested or subgroups analyzed according to Bonferroni's corrections (five independents tests). *P*-values ≤.05 were considered statistically significant.

3 | RESULTS

3.1 | Characterization of *GSTM1* CNVs in Tunisian population by digital droplet PCR

The analysis of 267 samples (165 patients with RA and 102 healthy controls) was carried out using the high-throughput digital droplet PCR for determining the absolute quantification of CNVs. Ten samples (8 patients and 2 controls) with missing data were excluded. The quantification of *GSTM1* CN identified in our Tunisian sample was 0, 1, and 2 copies per diploid genome. The mean number of droplets analyzed for all samples ranged between 13 000 and 16 000 droplets. A total of 2.55% of samples showed a *GSTM1* CN value higher than 2.25. These samples were analyzed a second time with a digestion step using *HaeIII* enzyme, leading to a CN value close to two, thus, declining the hypothesis for the presence of three copies in tandem conformation.

The distribution of *GSTM1* CN frequencies in cases is 54.1% for 0 copy, 37.6% for 1 copy and 8.3% for 2 copies; and in controls 52% for 0 copy, 41% for 1 copy and 7% for 2 copies. It is noteworthy to point that the deletion of *GSTM1* (0 copy) (54.1% and 52%) has a high frequency in our case-control Tunisian sample compared with the rest of copies (Table 2).

TABLE 2 Distribution and association analysis of *GSTM1* CNVs with susceptibility to RA

GSTM1 copy number	Cases (n, 157)	Controls (n, 100)	*P*-value	OR	CI 95%
0	85 (54%)	52 (52%)	.87	-	-
1	59 (38%)	41 (41%)			
2	13 (8%)	7 (7%)			
0 copy vs 2 copies	54% vs 8%	52% vs 7%	1	0.9	[0.27-2.56]
1 copy vs 2 copies	38% vs 8%	41% vs 7%	.8	0.8	[0.24-2.31]
2 copies vs 0+1 copy	8% vs 92%	7% vs93%	.81	1.19	[0.46-3.11]

OR, Odds ratio and 95% CI, confidence interval.

3.2 | *GSTM1* CNVs association analysis with RA

We performed the analysis of association between the identified CNVs of *GSTM1* and RA susceptibility. No significant difference was observed in the distribution of *GSTM1* CN between RA patients and healthy controls (*P*=.87). Furthermore, no significant association was shown when we compared individuals with complete deletion (0 copy) and 1 copy to individuals harboring 2 copies (OR=0.9, $CI_{95\%}$=[0.28-2.56], *P*=1 and OR=0.8, $CI_{95\%}$=[0.24-2.31], *P*=.8; respectively). The same negative result was found when we compared individuals with 2 copies to individuals with ≤1 copies (OR=1.19, $CI_{95\%}$=[0.46-3.11], *P*=.81) (Table 2).

We subsequently examined the association of *GSTM1* CNVs with RA stratified according to clinical and immunological characteristics. The obtained results showed a trend of association of *GSTM1* CNVs with the subgroup of anti-CCP$^+$, which lost significance after adjustment with Bonferroni correction (*P*=.05, P_c=.25). Otherwise, no significant difference was revealed considering RF (*P*>.05). In addition, no significant association was detected considering AIDs, hand erosions, or *HLA-DRB1**04 alleles (*P*>.05) (Table 3).

Moreover, the comparison of patients carrying deletion of *GSTM1* (0 and 1 copy) to those with 2 copies identified a significant association of deletion of *GSTM1* with anti-CCP positivity (OR=4.16, $CI_{95\%}$=[1.17-14.7], *P*=.01, P_c=.05) (Table 3). In addition, we applied multivariable analysis using the binary logistic regression. Our results confirmed the significant risk for anti-CCP subgroup adjusted and unadjusted with the rest of subgroups (OR=11.7, $CI_{95\%}$ =[1.52-91.48], *P*=.018 and OR=5.05, $CI_{95\%}$ =[1.14-22.29], *P*=.032, respectively).

4 | DISCUSSION

The characterization of CNVs of candidate genes is an important area in the discovery of new genomic factors of complex diseases such as RA. The present study investigated the contribution of CNVs of *GSTM1* gene in the susceptibility to RA and its characteristics in Tunisian population quantified by digital droplet PCR (ddPCR), the highest scalable and robust methodology for absolute measure and quantification of CNVs.

Previous studies have enumerated the technical difficulties and inaccuracy in the quantification of CNVs by qPCR.[27] In fact, by using standard real-time assays, the different events of CNVs (deletion or duplication) can be difficult for quantification, which can make systematic errors, leading to equivocal results with noninteger CN, while normalizing DNA concentrations used in standard samples and in taking cycle threshold (Ct) measurements.[28] The reproducibility and accuracy of the dPCR in CNV analysis were proven when comparing qPCR and dPCR.[29,30] Furthermore, the measurement of CNVs by ddPCR is more precise given that nucleic acid concentrations are obtained without using standard samples, but rather determined by counting positive droplets instead of determining cycle thresholds by qPCR, which is inherently error prone.

TABLE 3 CNVs frequencies of *GSTM1* gene in Tunisian patients with RA subgroups stratified by clinical and immunological data

CNVs Subsets	CN=0 (%)	CN=1 (%)	CN=2 (%)	*P*-value	*P* corrected (5 tests)	CN≤1 vs CN =2 (%)	*P*-value	*P* corrected (5 tests)	OR 95% CI
Anti-CCP$^+$/anti-CCP$^-$ (n=126)	38/16.6	23/12.6	3/6	**.05**	.25	61/29.3	**.01**	**.05**	4.16 [1.17-14.7]
RF$^+$/RF$^-$ (n=139)	34.5/18	28/10.8	5/3.5	.57	NS	62.5/28.7	.47	NS	-
ER$^+$/ER$^-$ (n=145)	40.7/13	27/11	5.5/2.8	.72	NS	67.6/24	.60	NS	-
AID$^+$/AID$^-$ (n=144)	27/26.3	13.2/25	4.2/4.2	.17	NS	40.3/51.4	.68	NS	-
*HLA-DRB1**04$^+$/*HLA-DRB1**04$^-$ (n=94)	22.3/34	14.9/19	4.2/5.3	.91	NS	37.2/53	.85	NS	-

Anti-CCP, anti-citrullinated peptide antibodies; RF, rheumatoid factor; AID, other autoimmune diseases; ER, erosions; OR, odds ratios, and 95% CI, confidence interval. Bold indicates significant *P*-value.

The quantified CN of *GSTM1* gene was 0, 1, and 2 copies in our case-control Tunisian sample with a high frequency (54%) of the complete deletion (0 copy) of the gene. This finding is consistent with previous studies in Swedish and Caucasians populations.[16,31,32] With our preliminary study on a limited sample size compared to other studies, we did not detect a genetic association of *GSTM1* CNVs with susceptibility to RA. Several previous meta-analyses have confirmed this finding in US Caucasians, UK Caucasians, UK Asians, and Sweden Caucasians as well as in US Africans. Besides, when stratifying by ethnicity, such meta-analysis study revealed the absence of association between the *GSTM1* CN (0 copy) and RA susceptibility in Caucasians or all Asians.[33]

Interestingly, the major finding in our study is the characterization of *GSTM1* CNVs association with patients seropositive for Anti-CCP. The deletion of *GSTM1* (0 copy or 1 copy) was associated with increasing risk for Anti-CCP positive RA (P_c=.05). This was confirmed when we performed multivariable analysis using the binary logistic regression by pooling all subgroups tested ($P_{anti\text{-}CCP}$=.032). Our observation does confirm earlier studies in Swedish EIRA (2426 incident RA cases and 1257 controls) and US cohorts.[16,34] This result is important as these antibodies are specific for the disease and have a prognostic and pathogenic significance. We suggest that CNVs of *GSTM1* gene had no effect in the susceptibility to RA, but may influence its severity as the deletion of the gene is associated with seropositivity for Anti-CCP antibodies in RA Tunisian patients.

The heterogeneity of results between our study and some others in the involvement of CN of *GSTM1* in the pathogenesis of RA may be explained by the genetic background and/or environmental differences existing among populations as well as the low number of samples analyzed.

In conclusion, the use of ddPCR method allowed us to determine CN accurately and permits to precisely explore the role of CNVs of *GSTM1* in RA. The performance of this method can allow the characterization of genotypes of CNVs and its transmission in familial-based studies of complex diseases. For future research works, it can be said that our findings should be replicated with large case-control and familial samples.

ACKNOWLEDGMENTS

This work was supported by the Ministry of Higher Education and Scientific Research of Tunisia, Genopole®, and Evry-Val d'Essonne University. We are grateful to RA patients and rheumatologists for their participation in this study.

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How to cite this article: Achour Y, Ben Kilani MS, Ben Hamad M, et al. Measurement of absolute copy number variation of Glutathione S-Transferase M1 gene by digital droplet PCR and association analysis in Tunisian Rheumatoid Arthritis population. *J Clin Lab Anal*. 2018;32:e22300. https://doi.org/10.1002/jcla.22300