Influence of oxidative stress-related genes on susceptibility to fibromyalgia

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Acknowledgment: We wish to thank AFIXA (Association of Fibromyalgia of Jaén, Spain) and AGRAFIM (Association of Fibromyalgia of Granada, Spain) for collaborating in this study. We also thank José Alberto Lopez-Sanchez and Marcos Manzaneque-Pradales for collaborating in laboratory tasks.

The authors have no conflicts of interest to report.

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Funded received: None.

Ethical Conduct of Research: The study was approved by the Ethical Committee of Research of Granada, Spain (reference: 0797-N-19). All participants provided a written informed consent.

Abstract

Background: Fibromyalgia (FM) is a complex syndrome to diagnose and treat due to its unknown etiology. Previously studies reported that patients with FM suffer from oxidative stress.

***Objectives*:** In the present study, we investigated single nucleotide polymorphisms (SNPs) in genes encoding enzymes involved in oxidative stress [superoxide dismutase 1 (SOD1), catalase (CAT), and NADPH oxidase (CYBA)] in patients with FM and in healthy subjects, as well as the possible relation with the demographic and clinical manifestations of FM.

***Methods*:** A total of 141 patients with FM and 73 healthy subjects participated in this case-control study. For DNA extraction, buccal swabs were collected from patients with FM and a peripheral blood sample was extracted from controls. We analyzed SNPs in genes related to oxidative stress (rs10432782 in SOD1, rs1001179 in CAT, and rs4673 in CYBA) using TaqMan™ probes. In FM patients, severity of FM, fatigue and pain were assessed by Fibromyalgia Impact Questionnaire (FIQ), Multidimensional Fatigue Inventory (MFI) and Visual Analogue Scale (VAS), respectively. The physical (PCS-12) and mental (MCS-12) health status were evaluated by the 12-Item Short Form Health Survey (SF-12).

***Results*:** The selected SNPs did not show significant differences between FM patients and controls. The rs10432782 (SOD1) was associated with FIQ score in patients with FM, while the rs4673 (CYBA) was associated with MFI score, MCS-12 score and duration of disease.

***Discussion*:** We have identified significant correlations between SOD1 and CYBA variants with clinical manifestations of FM. These results provide new insights into the pathogenesis of FM that could be useful for guiding future studies along the way to find the cause/s of this syndrome.

Keywords: fibromyalgia; oxidative stress; polymorphism.

Fibromyalgia (FM) is a chronic syndrome characterized by widespread musculoskeletal pain and other symptoms such as fatigue, sleep disturbances, headache, cognitive problems and psychiatric disorders. FM, which affects women in a greater proportion, is present in 2.10% of the world's population, in 2.31% of the European population and in 2.40% of the Spanish population (Cabo-Meseguer et al., 2017). Patients with FM report severe disability and incur high medical costs (Cabo-Meseguer et al., 2017). Despite the research in the last years, the pathogenesis of FM remains unclear. However, several factors have been proposed to be related to this syndrome. They include central sensitization (Bellato et al., 2012) and altered levels of both monoamine neurotransmitters (Rus et al., 2018) and enkephalinases (Martínez-Martos et al., 2019), inflammation (Molina et al., 2019; Ramírez-Tejero et al., 2018; Rus et al., 2016) and oxidative stress (La Rubia et al., 2013).

Oxidative stress is the result of an imbalance between the harmful reactive oxygen species (ROS) and the antioxidant defenses, which detoxify and neutralize ROS. Although the main source of ROS is the oxidative phosphorylation that occurs in the mitochondria, several enzymes are also capable of producing ROS, including the NADPH oxidases, responsible for the reduction of oxygen to superoxide anion. On the other hand, the antioxidant defenses include an array of antioxidant enzymes such as superoxide dismutases (SODs) and catalases (CATs). SODs catalyze the breakdown of the superoxide anion into oxygen and hydrogen peroxide, which is converted to water and oxygen by CATs (Ozgocmen, Ozyurt, Sogut, Akyol, et al., 2006). Although the mechanisms by which oxidative stress can cause FM or its clinical symptoms are still unclear, several works have suggested a relationship between oxidative stress and

perception of pain (Ibi et al., 2008; Kim et al., 2008; Schwartz et al., 2009), the main symptom of patients with FM. In this sense, a critical role for superoxide anion has been shown in the development of pain by peripheral and central nervous system sensitization (Wang et al., 2004). Moreover, enhanced production of hydrogen peroxide has been reported to contribute to pain development in FM patients (Close et al., 2005). ROS have also been involved in NMDA-receptor activation, an essential step in central sensitization, contributing to neuropathic pain (Gao et al., 2007). On the other hand, oxidative damage has been reported to interfere with the muscles by reducing local nociceptors, causing a diminution in the pain threshold (Fulle et al., 2000).

It is known the relevant role of genomic variants such as single nucleotide polymorphisms (SNPs) in several diseases. Recent genome-wide association studies (GWAS) investigated genes potentially involved in the pathogenesis of FM highlighting that genetic factors are possibly responsible for up to 50% of the disease susceptibility (D'Agnelli et al., 2019). A previous study analyzed some SNPs in genes encoding antioxidant enzymes (Ala9Val in MnSOD2 and Pro198Leu in GPX1) in patients with FM without finding differences between FM patients and controls (Akbas et al., 2014).

The lack of knowledge about the etiology of FM complicates both the diagnosis and treatment of this syndrome. Currently, there are no validated biological biomarkers that help the diagnosis of FM. Therefore, our aim was to identify candidate genes associated with FM susceptibility. For this, we investigated SNPs in genes encoding enzymes involved in oxidative stress [rs10432782 in SOD1, rs1001179 in CAT, and rs4673 in

Cytochrome B-245 Alpha Chain (CYBA)] in patients diagnosed with FM and in healthy controls, as well as the possible relation with the demographic and clinical characteristics of FM. To the best of our knowledge, this is the first study that analyzes these SNPs in patients with FM.

METHODS

Participants

This research has been carried out in accordance with the Declaration of Helsinki of the World Medical Association. The study was approved by the Ethical Committee of Research of XX (reference: XX) and all subjects provided written informed consent. One hundred and forty one patients with FM from XX (Association of Fibromyalgia of XX) and XX (Association of Fibromyalgia XX) participated in this case-control study. The patients with FM were selected by contacting two associations of Fibromyalgia patients (Association of Fibromyalgia of XX and Association of Fibromyalgia of XX). The FM participants had been previously diagnosed with FM by a professional rheumatologist and met the 1990 American College of Rheumatology (ACR) criteria for FM. In a single visit, the patients provided demographic and clinical data and completed the study questionnaires. Finally, we collected two buccal swabs for each of them. As controls, we used DNA samples from 73 healthy subjects, which were donated by XX.

DNA extraction and genotyping analysis

Buccal mucosa cells were collected from patients with FM and stored at room temperature. A non-organic DNA extraction procedure using stain extraction buffer, proteinase K, ammonium acetate (2M) and isopropyl alcohol was performed as described by Gomez-Martin et al. (Gómez-Martín et al., 2015). A peripheral blood sample was extracted from controls, put into EDTA coated tubes and stored at - 20 ºC until genomic DNA extraction. A standard organic extraction procedure by phenol/chloroform/isoamyl alcohol and proteinase K, followed by purificated with Microcon H 100 filters (Millipore, Germany) was used. To determine extracted DNA purity and concentration a NanoDrop 2000c (Thermo Scientific, USA) was used. Thereafter, DNA was stored at - 20 ºC until genotyping. Three SNPs in genes related to oxidative stress pathways were selected: rs10432782 (C__30038646_10) in SOD1 gene; rs1001179 (C__11468118_10) in CAT gene; and rs4673 (C_____2038_20) in CYBA gene. SNPs in these genes were selected using The National Center for Biotechnology Information and Ensembl genome browser websites (more information in S1 Table).

DNA genotyping was performed using TaqMan® Genotyping Master Mix (Applied Biosystems, USA) that included all essential components (except probes, templates and water) for polymerase chain reaction (PCR). Allelic discrimination assays were carried out in a 7900HT Fast Real-Time PCR System (Applied Biosystems, USA). Results were read using SDS software v.2.4 (Applied Biosystems, USA).

Clinical characteristics of patients with FM

The demographic and clinical data were obtained from FM patient interview and questionnaires. The questionnaires to determine the clinical characteristics were only

179 completed by FM patients, not by controls. Functional capacity in daily living activities
180 was evaluated by the Spanish version of the Fibromyalgia Impact Questionnaire (FIQ),
181 which shows high internal consistency, with a Cronbach's alpha coefficient of 0.82
182 (Rivera & González, 2004). The FIQ is the instrument most often used to estimate FM
183 severity, and the score ranges from 0 to 100. Fatigue was assessed by the Spanish
184 version of the Multidimensional Fatigue Inventory (MFI) (Munguía-Izquierdo et al.,
185 2012), which has a potential score range from 20 to 100. The Spanish version of the
186 MFI has high internal consistency, with a Cronbach's alpha of 0.93 (Soriano-
187 Maldonado et al., 2015). Musculoskeletal pain was scored according to a Visual
188 Analogue Scale (VAS) of 10 cm. The VAS shows a high sensibility (80%), specificity
189 (80%), and area under the curve (0.864) in the FM population (Marques et al., 2008).
190 For all these questionnaires, higher values reflect worse symptomatology. The physical
191 (Physical Component Summary, PCS-12) and mental (Mental Component Summary,
192 MCS-12) health status was assessed by the Spanish version of the 12-Item Short Form
193 Health Survey (SF-12) (Alonso et al., 1995). The score ranges from 0 to 100 with lower
194 values reflecting worse health status. The PCS-12 and the MCS-12 show a Cronbach's
195 alpha of 0.85 and 0.78, respectively (Vilagut et al., 2008).

196

197 **Statistical analysis**

198 The Hardy-Weinberg equilibrium (HWE) was tested for the selected SNPs using
199 SNPstats software (Sole et al., 2006). Statistical analyses were performed using the
200 statistical package IBM SPSS Statistics 24 for Windows (SPSS Inc, Chicago, IL, USA).
201 Binary logistic regression was used to test association of each SNP (using codominant,
202 dominant and recessive genetic models) with FM risk and clinical manifestations. The

degree of statistical significance for the variable "age" was established by applying the Mann-Whitney U-test. The level of statistical significance was set at $P < 0.05$. ILIBRIO

To assess the relation between SNPs, demographic and clinical features of patients with FM, we categorized the variables. We categorized the age in two groups of age, <50 years and ≥50 years, and the duration of the disease in duration < 10 years and duration ≥ 10 years. According to the FIQ score, FM was classified as mild (below 50), moderate (50-70) and severe (70-100) (Castaño et al., 2018). The level of pain, determined by the VAS score, was classified as low (below 5), medium (5-8) and high (8-10). The physical and mental health status, determined by the PCS-12 and the MCS-12 respectively, were categorized as severe (below 30), moderate (30-50) and mild (50-100). The level of fatigue, determined by the MFI score, was classified as mild (below 50), moderate (50-70) and severe (70-100).

RESULTS

Study subjects

In Table 1 are summarized the demographic and clinical characteristics of the participants. One hundred and forty one patients with FM participated in the present study, of whom 130 (92.20%) were women and 11 were men (7.80%). Of the 73 healthy controls included in the study, 69 (94.52%) were women and 4 were men (5.48%). There were significant differences in age between cases and controls ($p = 0.002$). The duration of disease in patients with FM was 11.83 ± 9.07 (mean ± standard deviation). Not all FM patients completed correctly all the questionnaires, so questionnaires that were not correctly completed or the incomplete questionnaires were

discarded for the analysis. Table 1 includes the number of patients who did not correctly complete each of the questionnaires.

INSERT TABLE 1

Correlations between SNPs and susceptibility to FM

Firstly, we performed a case-control analysis. All SNPs were successfully genotyped in the present population. The rs1001179 (CAT) was discarded for following analysis because it did not meet the HWE criterion ($p < 0.0001$). Table 2 shows the genotypes frequencies for rs10432782 (SOD1) and rs4673 (CYBA) in patients with FM and controls. The GG genotype for rs10432782 (SOD1) was more prevalent in FM patients (4.3%) than in controls (2.7%), although without reaching statistical significance. The AA genotype for rs4673 (CYBA) was more common among controls (26.0%) than among patients with FM (19.9%), but without reaching statistical significance.

INSERT TABLE 2

Associations between SNPs and demographic and clinical manifestations in patients with FM

Significant associations between genetic variants and FM clinical manifestations are presented in Table 3. The rs10432782 was significantly associated with the FIQ score. G allele carriers showed lower FIQ score than T carriers ($p < 0.001$; OR 0.085 [0.022-0.327]). Duration of disease is lower in A carriers for rs4673 than in G carriers ($p =$

0.014; OR 3.233 [1.274-8.206]). Moreover, A carriers for rs4673 presented lower MCS-12 score than G carriers (p = 0.027; OR 2.719 [1.122-6.585]), as well as moderate fatigue in comparison to G carriers, which presented severe fatigue (p = 0.017; OR 0.152 [0.032-0.710]). There were no significant associations among the SNPs examined and the age and the VAS and PCS-12 scores.

INSERT TABLE 3

DISCUSSION

The main goal of the present study was to identify candidate genes associated with FM susceptibility. Our results have not shown significant differences between FM patients and controls for the rs10432782 SNP in the SOD1 gene or for the rs4673 SNP in the CYBA gene, although we can predict a tendency of some genotypes among the FM population. Given the role that oxidative stress may play in the pathogenesis of FM, we hypothesize that we have not found statistically significant differences between groups for the SNPs analyzed probably due to the limitations of candidate-gene studies, which are discussed later. In contrast, a previous study identified associations of SNPs in guanosine triphosphate cyclohydrolase 1 (GCH1) gene, catechol-*O*-methyltransferase (COMT) gene and opioid receptor μ1 (OPRM1) gene with higher risk of FM susceptibility (Estévez-López et al., 2018).

In agree with our result for rs10432782, a previous study that examined SNPs in genes encoding two antioxidant enzymes failed to find significant differences for MnSOD2 and glutathione peroxidase 1 (GPX1) between FM patients and controls (Akbas et al., 2014). The same authors reported higher SOD enzyme activity in FM patients compared

271 to controls (Akbas et al., 2014). Nevertheless, results regarding SOD enzyme activity in
272 patients with FM are controversial, because other authors reported lower SOD activity
273 (Bagis et al., 2005; La Rubia et al., 2013) and even unchanged SOD activity in patients
274 with FM in comparison to healthy controls (Koca et al., 2018; Toker et al., 2014). These
275 conflicting results may be due to the characteristics of the study participants. Each study
276 uses different inclusion and exclusion criteria to select participants, and patients with
277 FM usually have many comorbidities, which could explain the diversity of results
278 obtained in these studies.

279 The CYBA gene encodes the Cytochrome B-245 Alpha Chain (also known as p22-
280 phox) subunit of the enzyme NADPH oxidase. There are no studies available that
281 analyze NADPH oxidase activity or CYBA variants in patients with FM. However,
282 association studies have revealed significant associations between CYBA gene
283 polymorphisms and cardiovascular diseases, such as hypertension, coronary artery
284 disease, myocardial infarction, cerebrovascular disease, and diabetic and non-diabetic
285 nephropathy (San José et al., 2008) Along this lines, recent study described a
286 prothrombotic state in patients with FM that may increase the risk of cardiovascular
287 disease in these patients (Molina et al., 2019).

288 Although we could not prove any significant association between FM and the
289 rs1001179 SNP in CAT gene, previous studies showed lower CAT enzyme activity in
290 patients with FM in comparison to healthy subjects (Cordero et al., 2012; Fatima et al.,
291 2017; La Rubia et al., 2013; Sendur et al., 2009).

292 We have identified, for the first time, significant associations among rs10432782
293 (SOD1) and rs4673 (CYBA) and the clinical data of FM patients. The rs10432782
294 (SOD1) was associated with the severity of FM, determined by the FIQ. In addition, the

rs4673 (CYBA) was associated with the mental health status (determined by the MCS-12), the fatigue (assessed by the MFI) and the duration of disease in FM patients. The identification of these correlations among SOD1 and CYBA variants and FM clinical parameters contributes to increase the knowledge about the pathophysiology of this complex syndrome. To the best of our knowledge, there are no data available on associations between these SNPs and FM clinical characteristics. However, it has been reported that the SOD2 Ala16Val polymorphism was associated with migraine (Palmirotta et al., 2015). To this regard, migraine and headache are frequently associated with FM (Evans & de Tommaso, 2011). Previous studies investigated the relationship among CAT and SOD enzyme activities and clinical manifestations of FM. While a significant negative correlation was reported between CAT activity and headache, determined using the Headache Impact Test (HIT-6) (Cordero et al., 2012), other authors did not find relationships among CAT activity and several clinical parameters such as fatigue, pain, severity of FM (FIQ), depression or anxiety (Sendur et al., 2009). No correlations were previously found between SOD activity and the FIQ score (Ozgocmen, Ozyurt, Sogut, & Akyol, 2006) or the VAS score (Bagis et al., 2005) in patients with FM. Although we have found a significant association between duration of disease and rs4673, other authors reported that duration of disease did not affect oxidative stress parameters in patients with FM (Bagis et al., 2005). On the other hand, we failed to find significant associations between age and the SNPs analyzed, suggesting that age may not influence variants of the oxidative stress-related genes studied in the present work. Similarly, a previous study reported that age did not affect oxidative stress parameters in patients with FM (Bagis et al., 2005). Regarding age, our results have shown significant differences between cases and controls. However, these differences might not affect the results obtained, since our data have shown that age

may not influence the SNPs analyzed. Moreover, the statistical analyzes between cases and controls performed in the present study were adjusted for age. On the other hand, the age of both study groups was around the most frequent age range for the appearance of FM, which is between 40 and 49 years (Cabo-Meseguer et al., 2017).

Candidate-gene studies have several limitations. The main limitation of the present study is the sample size of both FM and control groups, which limits the statistical power. Therefore, our findings need to be verified by additional studies in other cohorts of FM patients. Second, in candidate-gene analysis we only analyze the selected genes without knowing what happens in the rest of the genome, which may be interesting in complex diseases such as FM.

Conflict of interest

Authors declare no conflicts of interest.

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TABLE 1

Summary of demographic and clinical characteristics of the participants

	FM patients (Total n=141) M (SD) or n	Controls (Total n=73) M (SD) or n
Age (years)	54.40 (7.40)	51.48 (7.44)
<50	35	31
≥50	103	42
Missing	3	-
Duration of FM (years)	11.83 (9.07)	-
<10	53	-
≥10	63	-
Missing	25	-
FIQ	69.68 (15.28)	-
mild (<50)	14	-
moderate (50-70)	43	-
severe (70-100)	72	-
Missing	12	-
MFI	81.46 (12.12)	-
mild (<50)	0	-
moderate (50-70)	26	-
severe (70-100)	107	-
Missing	8	-
VAS	7.00 (2.01)	-
low (<5)	20	-
medium (5-8)	66	-
high (8-10)	44	-
Missing	11	-
PCS-12	28.83 (5.65)	-
Severe (< 30)	80	-
moderate (30-50)	49	-
mild (50-100)	0	-
Missing	12	-
MCS-12	32.99 (11.14)	-
severe (< 30)	60	-
moderate (30-50)	53	-
mild (50-100)	16	-
Missing	12	-

Note: Abbreviations: FM (Fibromyalgia), FIQ (Fibromyalgia Impact Questionnaire), M (mean), MCS-12 (mental health status), MFI (Multidimensional Fatigue Inventory), n (number of subjects), PCS-12 (physical health status), SD (standard derivation) and VAS (Visual Analogue Scale).

TABLE 2

Genotypes frequencies in patients with fibromyalgia (FM) and controls

SNP	Genotype	FM patients **(n=141)**	Controls (**n=73)**	OR [95% CI]	P[a]
	Codominant				
	TT	117 (83.0%)	61 (83.6%)	1.00 [reference]	0.781
	TG	18 (12.8%)	10 (13.7%)	0.871 [0.369-2.056]	
	GG	6 (4.3%)	2 (2.7%)	1.661 [0.321-8.578]	
rs10432782	**Dominant**				
(SOD1)	TT	117 (83.0%)	61 (83.6%)	1.00 [reference]	0.995
	TG/GG	24 (17.0%)	12 (16.4%)	1.003 [0.461-2.181]	
	Recessive				
	GG	6 (4.3%)	2 (2.7%)	1.691 [0.329-8.699]	
	TG/TT	135 (95.7%)	71 (97.3%)	1.00 [reference]	0.530
	Codominant				
	GG	44 (31.2%)	23 (31.5%)	1.00 [reference]	0.534
	GA	69 (48.9%)	31 (42.5%)	1.165 [0.593-2.288]	
	AA	28 (19.9%)	19 (26.0%)	0.764 [0.345-1.689]	
rs4673	**Dominant**				
(CYBA)	GG	44 (31.2%)	23 (31.5%)	1.00 [reference]	0.966
	GA/AA	97 (68.8%)	50 (68.5%)	1.014 [0.542-1.895]	
	Recessive				
	AA	28 (19.9%)	19 (26.0%)	0.697 [0.351-1.383]	
	GA/GG	113 (80.1%)	54 (74.0%)	1.00 [reference]	0.302

Note: [a] Binary Logistic Regression adjusted by sex and age.

TABLE 3

Significant associations between genetic variants and fibromyalgia (FM) clinical manifestations

SNP	Genotype	Duration of FM		FIQ score		MFI score[c]		MCS-12 score	
		OR [95% CI]	P[a]	OR [95% CI]	P[b]	OR [95% CI]	P[b]	OR [95% CI]	P[b]
rs10432782 (SOD1)	TT	-	-	1.00 [reference]	**<0.001**	-	-	-	-
	TG/GG	-		0.085 [0.022-0.327]		-		-	
rs4673 (CYBA)	GG	1.00 [reference]	**0.014**	-	-	1.00 [reference]	**0.017**	1.00 [reference]	**0.027**
	GA/AA	3.233 [1.274-8.206]		-		0.152 [0.032-0.710]		2.719 [1.122-6.585]	

Notes: [a] Binary Logistic Regression adjusted by sex and age. [b] Binary Logistic Regression adjusted by sex, age and duration of FM. For the Binary Logistic Regression, patients with FM were dichotomized into two groups as follows: Duration of FM (< 10 years *vs.* ≥ 10 years), FIQ score (mild *vs.* moderate-severe severity of FM), MCS-12 score (severe *vs.* moderate-mild mental health status) and MFI score (moderate vs. severe fatigue). [c] There were no FM patients with mild levels of fatigue.