EUROPEAN ACADEMIC RESEARCH
ISSN 2286-4822
www.euacademic.org

EUROPEAN ACADEMIC RESEARCH
Vol. X, Issue 11/ February 2023

Impact Factor: 3.4546 (UIF)
DRJI Value: 5.9 (B+)

Association between β-Chemokine Gene Polymorphisms and Tuberculosis Susceptibility in Sudanese Population in Sinnar State

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Abstract

Chemokines play a major role in leukocyte recruitment during the formation of tuberculous granulomas. A prospective, cross sectional, descriptive case - control study was carried out during the period from 2017 to 2022 at Sennar Teaching Hospital in Sennar State, Sudan.

***Objective**: To determine the association between polymorphisms in CCL2/MCP-1, and CCL5/ RANTES genes and TB in Sudanese population from Sennar state.*

***Methods:** A prospective, cross sectional, descriptive case - control study was carried out during the period from 2017to 2022 at Sennar Teaching Hospital in Sennar State, Sudan. Case study of 160 patients with tuberculosis and 220 healthy matched controls from Sudan. In the study population, three single nucleotide polymorphisms (SNPs) in the promoter regions of CCL5 and one single nucleotide polymorphisms (SNPs) in the promoter regions of CCL2 were genotyped using polymerase chain reaction and restriction fragment length polymorphism (PCR-RFLP). These four SNPs influence the expression of these genes.*

***Results:** It appeared that in the tuberculosis population the MCP-1(2518A/G) was significantly more often encountered than in the control population. For the other polymorphisms tested, no association with tuberculosis was found in the population tested. Therefore only the MCP-1(2518A/G) allele was associated with tuberculosis in the Sudanese population.*

***Conclusion:** This indicates that the genotypes obtained for CCL5 are associated with an increased risk of developing active TB.*

Keywords: β-Chemokine Gene Polymorphisms, Tuberculosis Susceptibility, Sudanese Population in Sinnar State

INTRODUCTION

Tuberculosis (TB) is an infectious disease caused by *Mycobacterium tuberculosis (M. tuberculosis)* kills approximately 2 million people annually, Worldwide, TB remains a leading cause of death [1]. TB is likely to have affected humans for most of their history and remains a major cause of death worldwide despite the discovery of effective and affordable chemotherapy more than 50 years ago. With 1.3 million TB deaths (including TB deaths in HIV-positive individuals) in 2012 [2].

Protective immune response against *M. tuberculosis* requires the formation of discrete granulomatous lesions to contain the mycobacterial, and a complex interaction between activated T cells, macrophages and polymorph nuclear leucocytes .The

granuloma creates a microenvironment where infected macrophages, dendritic cells and different T-cell populations exist in close proximity and limit *M. tuberculosis* growth and spread [3,4]. Th1 response, and in particular interferon γ (IFN-γ) production, are essential for the activation of macrophages and the control of infection .Tumor necrosis factor (TNF)-α -and IFN-γ activate the production of pro-inflammatory cytokines, including TNF-α and in particular macrophage inflammatory protein (MIP)-1α and MIP-1β, which have been shown to play a role in recruitment and activation of macrophages and leucocytes at the sites of infection [3,4]. The interaction of macrophages with other effecter cells in the formation of granulomas occurs in the milieu of both cytokines and Chemokines (CKs). These molecules serve to attract and activate other inflammatory effecter cells such as lymphocytes [5].

Several studies have established that *M.tuberculosis* can induce the expression of CKs, and certain CKs can be detected in clinical samples from people with active TB. *RANTES*, *MCP-1/CCL3*, MIP-1α, and Interleukin-8 (IL-8) are released by human Alveolar macrophages (AMs) upon infection with *M. tuberculosis in vitro* and monocytes and Lymph nodes (LN) cells [6].

MATERIALS AND METHODS:

Study population:

A prospective, cross-sectional, observational, descriptive case study was conducted from 2017 to 2023 in Sennar Teaching Hospital. 160 patients with active pulmonary TB and 220 healthy controls were included. EDTA blood samples were taken from all patients and healthy controls. All tuberculosis patients had microbiological (by culture and/or smear) or radiological evidence of *M. tuberculosis* disease (table 1). None of the patients reported infection with other infectious diseases and that included hepatitis B, hepatitis C, HIV. and Corona Virus Disease-2019 (COVID-19). The healthy controls had no evidence of tuberculosis disease by clinical examination, and were matched on age, gender and BCG status.

Furthermore, all participants were subjected to a questionnaire interview to elicit age, sex, occupation, home, habits, past illness, history of having tuberculosis, X-ray and other clinical information. The study was approved by the Ethics Committee of Soba University Hospital, Khartoum, Sudan.

Table 1: Characteristics of the Study population

		Patients	**Controls**	**p-value**
Total number		160	220	
Mean age /yrs (range)		26 (11-70)	30 (11-70)	
Gender (male/female)		111/49	65/155	0.0041
Occupation	Governmental employee	22 (13.8)	21 (9.55%)	
	Workers	63 (39.4%)	44 (20%)	
	Other job	40 (25%)	40 (18.3%)	
	Jobless	3 (1.8%)	13 (5.9%)	
	Housewife	13 (8.1%)	17 (7.7%)	
	Student	19 (11.9%)	85 (38.6%)	
BCG vaccination		112 (70.6%)	212 (96.8%)	0.923
Definite tuberculosis	Presence of MTB in sputum based on both smear and culture	92(57.5%)	0 (0%)	
	Presence of MTB in sputum	109(68.1%)	0 (0%)	

	specimen only by smear			
	Presence of MTB in sputum specimen only by culture	47 (29.4%)	0 (0%)	
Hepatitis C test		Negative	Negative	
Hepatitis B Ag test		Negative	Negative	
HIV1, 2 test		Negative	Negative	
Nasopharyngeal swab for (COVID-19)		Negative	Negative	

DNA isolation

DNA was extracted from the blood using the Guanidine chloroform method as fellow; 2 ml blood samples were transferred into 15ml falcon tubes. Prior to digestion, blood samples were washed using the red blood cells lysis buffer to break down RBCs using Vortexing for 1 minute followed by centrifugation for 10 minutes at 3000 rpm. Washing process was repeated for 3 times to ensure complete removal of RBCs. Following the washing process, pellet samples were then subjected to digestion by adding 1ml White blood cells lysis buffer,10µl of 20mg/µl proteinase K, 300 µl of 7.5M Ammonium Acetate, and 1ml of 6MGuanidine Chloride then samples were incubated at 37˚C overnight. On the following day samples were cooled down on the bench to room temperature and transferred into another 15ml falcon tubes containing 1ml pre-chilled Chloroform and centrifuged at 3000rpm for 20 minutes. Three layers were separated. The supernatant was collected to a new labeled falcon tube .10 ml of pre-chilled absolute Ethanol was added and mixed gently by moving the falcon tube back and forth quickly. Samples were put into a -20˚c freezer for24 hours. Then, samples were subjected to quick vortex for one minute. Samples were centrifuged for 15 minutes at 3000 rpm and the supernatant were discarded. Washing twice with 4ml 70% ethanol was performed and after each wash the supernatant was drained with much care to avoid losing of the DNA pellet at the bottom of the falcon tube. The falcon tube was inverted upside down on a tissue paper leaving the pellet to dry from alcohol for at least two hours. Finally, the DNA pellet was re-suspended in200µl of deionized water and was put into 4˚C for one day to insure total dissolving of the pellet. Vortexing was applied gently and the DNA was transferred into a new 1.5 Eppendorf tube.

Genotyping

Genomic variants of chemokine genes were detected by PCR followed by restriction enzyme fragment analysis (PCR-RFLP). All PCR primers and restriction enzymes are stated in Table 2. Each of the PCRs consisted of a pre-denaturation step of 4 minutes at 94°C and 40 cycles each of 30 seconds denaturation at 94°C, 30 seconds annealing at 55°C and 30 seconds elongation at 72°C. This was followed by a post-elongation step of 7 minutes at 72°C. Restriction endonucleases were obtained from Fermentas (st. Leon-rot, Germany), and Roche (Penzberg, Germany) and were used as described by the manufacturer. Restriction fragments were visualized by electrophoresis on 2% agarose gels (Hispanagar, Sphaero Q, Leiden, The Netherlands).

Table 2: PCR primers and restriction enzymes for genotyping the different Single Nucleotide Polymorphisms

Gene(s)	Rs number	Primer sequence(s) (5'→-3')	Restriction endonuclease	Allele	Length (bp[1])	Reference
MCP-1 (CCL2)						
-2518 G/A	rs1024611	F 5'-CCG AGA TGT TCC CAG CAC AG-3'	PvuII	G	708 + 222	[62]
		R 5'-CTG CTT TGC TTG TGC CTC TT-3'		A	930	
RANTES (CCL5)						
-403G/A	rs2107538	R403fw CACAAGAGGACTCATTCCAACTCA	RsaI	G	180+26	[63]
		R403rv GTTCCTGCTTATTCATTACAGATCGTA		A	206	
-28C/G	rs2280788	R28fw ACTCCCCTTAGGGGATGCCCGT	HincII	C	152+23	[63]
		R28rv GCGCAGAGGGCAGTAGCAAT		G	175	
Int1.1 T/C	rs2280789	RIfw CCTGGTCTTGACCACCACA	MboII	C	225+118	[64]
		RIrv GCTGACAGGCATGAGTCAGA		T	343	

[1] bp: number of base pairs

Statistical analysis

The mean age of the patient population and the control population were compared by the unpaired t-test. Gender, occupation and BCG-vaccination status between the patient and control population were compared with the Fisher exact test. Verification of Hardy-Weinberg equilibrium (HWE) was performed in the control population with Pearson's $\chi 2$ test. The effect of the *MCP*-1 2518A/G, *CCL5/ RANTES -403 G/A, -28 C/G* and *In 1.1 T/C* polymorphisms on susceptibility to tuberculosis were assessed with the Fisher exact test. P-value of <0.05 was deemed statistically significant All statistical analyses were performed using SPSS for Windows v11.0 statistical analysis software.

RESULTS:

Characteristics of tuberculosis patients and healthy control subjects

One hundred and sixty Sudanese tuberculosis patients were included into the study (table 1).The control population comprised 220 healthy unrelated people from the same endemic area in Sudan; they were matched on gender and BCG-status (table 1) and showed no signs of any lung disease. Unfortunately the occupation of the control population differed from that of the patient population.

Distribution of MCP-1 2518A/G, CCL5*/ RANTES -403 G/A, -28 C/G* and *In 1.1 T/C* gene polymorphisms:

To detect the possible deficiencies in MCP-1 2518A/G, *CCL5/ RANTES -403 G/A, -28 C/G* and *In 1.1 T/C* production among tuberculosis patients, genotype (table 3) and allele frequencies (table 4) in the promoters of the genes encoding these chemokine were determined. To determine if the SNPs reached Hardy-Weinberg equilibrium (HWE), the Pearson's $\chi 2$ test was performed. It appeared that in the control population, the genotype distributions for the MCP-1 2518A/G, *CCL5/ RANTES -403 G/A, -28 C/G* and *In 1.1 T/C* SNPs reached Hardy-Weinberg equilibrium (HWE) (table 3). In the patient population all genotype distributions were in Hardy-Weinberg disequilibrium.

Table 3: Genotype distributions and Hardy Weinberg Equilibrium in Sudanese tuberculosis patients and healthy controls

Genotype	Tuberculosis patients N=160(%)	HWE of patient population*	Control N=220(%)	HWE of control population*
MCP-1-2518 G/A				
GG	152(65.52)		100(48.54)	<0.01
AG	58(25.00)	<0.01	68(33.01)	
AA	22(9.48)		38(18.44)	
CCL5 *-403 G/A*				
GG	58(25.00)	<0.01	70(33.98)	<0.01
AG	145(62.5)		73(35.44)	
AA	29(12.5)		63(30.58)	
CCL5 *-28 C/G*				
CC	150(93.8)	<0.01	216(98.2)	<0.01
CG	10(6.2)		4(1.8)	
GG	0(0.0)		0(0.0)	
CCL5 *In1.1 T/C*				
TT	218(93.97)	<0.01	183(88.83)	<0.01
TC	10(4.31)		23 (11.16)	
CC	4(1.72)		0(0.00	

To determine if there was an association between any of the studied SNPs and tuberculosis, the allele frequencies between the control population and the patient population were compared with the Fisher Exact test. It appeared that in the tuberculosis population the *MCP*-1(2518A/G) was significantly more often encountered (p<0.0001) (Table 4) than in the control population. For the other polymorphisms tested, no association with tuberculosis was found in the population tested (Table 4). Therefore only the *MCP*-1(2518A/G) allele was associated with tuberculosis in the Sudanese population (Table 4).

Table 4: **Allele frequencies of tuberculosis patients in comparison to a matching healthy control population**

Genotype	Tuberculosis patients N=160(%)	Control N=220(%)	P-value for Association	OR (95% CI)
MCP-1-2518 G/A				
G-allele	362 (78.0)	268 (65.0)	**<0.0001**	1.91 (1.41-2.57)
A-allele	102 (22.0)	144 (35.0)		
CCL5 *-403 G/A*				
G-allele	261 (56.2)	213 (51.7)	0.20	1.20 (0.92-1.57)
A-allele	203 (43.8)	199 (48.3)		
CCL5 *-28 C/G*				
C-allele	310(96.8)	436 (99.0)	0.3	1.49 (0.76-2.74)
G-allele	10(3.2)	4 (1.0)		
CCL5 *In1.1 T/C*				1.47 (0.78-2.76)
T-allele	446 (96.1)	389 (94.4)	0.26	
C-allele	18 (3.8)	23 (5.6)		

P-values are calculated with the Fisher exact test.

DISCUSSION

It is clearly known that TB susceptibility is determined not only by *M. tuberculosis* infection and environmental factors, host genetic factors also play an important role in the pathogenesis of this infectious disease [7].

Many polymorphic genes have been identified as TB candidate genes [8].

The Association between *CCL2/MCP-1*, and *CCL5/ RANTES* gene polymorphisms and tuberculosis as in many association studies, it is important to describe the patient and control population clearly.

A large number of studies have shown that polymorphisms in the *CCL5* and *CCL2* genes were implicated in susceptibility to tuberculosis in several populations [9].

Chu et al. were the first to investigate the association between the incidence of TB and the *CCL5* -28 C>G polymorphism [10]. Subsequent studies demonstrated inconsistent and contradictory results, with some studies failing to find evidence of an association between the *CCL5* -28 C>G polymorphism and susceptibility of TB.

In our study, we genotyped the four expression SNPs *CCL5* -403 G/A, *CCL5* -28 C/G, *CCL5* In1.1T/C, *MCP*-1-2518 G/A in the Sudanese tuberculosis population from Sinnar state.

The assays used here were also used by others to find out the allele frequencies of these selected SNPs, and no variation was found in these studies, which makes genotyping errors less plausible [11].

One of the genes studied that was in HWE in the control population was the *CCL5 -28 C/G* polymorphism. The allele frequencies of this polymorphism differed between the patients with tuberculosis and the control population.

The *CCL5* -28G allele was more regularly found in the patient population than in the healthy control population. As in other studies [12].The association of the *CCL5 -28G* allele with tuberculosis was also found in other populations created from Tunisia [11] and Spain [13],but not in populations created from India [14] and China [10].

No association for the *CCL5* -403 G/A and In1.1 T/C polymorphisms was found in our Sudanese population. The *CCL5 -28G* variant was previously reported to elevate promoter activity and increases *CCL5* protein expression [15].

Therefore, these population studies appear that the ability to produce a more elevated CCL5 level increases the risk of developing tuberculosis.

This seems in contrast with the results obtained from an animal study published by Cardona [16].

Although the immune system from the mouse can differ from that from the human, most data are still generated from immune na€ıve animal models. In the study of Cardona et al. (2003) it was shown that lower *CCL5* expression levels were found in the more susceptible DBA/2 mice than in C57BL/6 mice. But as both mice strains were able to cause tuberculosis and did express high levels of *CCL5*, this difference could still be an artifact [16].The role of the high levels of *CCL5* in the generation of tuberculosis in mice seems to be related to the granuloma formation. In vivo intraperitoneal *CCL5* administration resulted in a trend to larger granuloma sizes, while anti-CCL5 treatment resulted in reduced granuloma sizes without altering the specific granuloma composition [17].

CONCLUSION:

This study has been done in Sennar Teaching Hospital on 160 patients with active pulmonary TB and 220 healthy controls. Their ages are around 11 – 70 years old.

The goal of this research is to determine the association between polymorphisms in *CCL2/MCP-1*,and *CCL5/ RANTES* genes and TB in Sudanese population from Sennar state.

It appeared that in the tuberculosis population the MCP-1(2518A/G) was significantly more often encountered (p<0.0001) (Table 4) than in the control population. For the other polymorphisms tested, no association with tuberculosis was found in the population tested (Table 4). Therefore only the MCP-1(2518A/G) allele was associated with tuberculosis in the Sudanese population in Sennar state (Table 4).

Recommendation:

After the enumeration of the results that are related to the following thesis, there are some ideas which could help further in the field of the research and are better to be recommended as follow:

- We need further studies on this SNPs and tuberculosis.
- We need to popularize this study in further state.

Acknowledgement

I thank Dr Hisham Noor and Dr Rayan Abubaker Musa for their help.

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