Research Article

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Association of P2X7 1513A/C Polymorphism with Susceptibility to Tuberculosis among Sudanese Patients

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ABSTRACT

Background: *Tuberculosis is a chronic, systemic infectious disease caused by M. tuberculosis mostly infecting the lung to cause pulmonary tuberculosis or localize in alternate body sites leading to extra-pulmonary tuberculosis (EPTB).The P2X7 receptor expressed in a wide variety of normal and disease-associated cell types, activated by extracellular adenosine 5'-triphosphate results in numerous events including the release of pro-inflammatory mediators, cell proliferation or death, and killing of intracellular pathogens. A deficiency of P2X7-mediated control of mycobacterial infection within macrophages in the lung may permit spread to extrapulmonary sites where the infection progresses to post–primary TB disease.*

Methods: *One hundred and twenty tuberculosis patients with 46 apparently healthy controls were included for genotyping of the P2X7 polymorphism using Polymerase chain reaction and restriction fragment length polymorphism (PCR–RFLP) and confirmed by sequencing a subset of samples.*

Results: *This study found that the P2X7 1513A/C polymorphism is significantly associated with tuberculosis infection (CC, AC OR=4.615, 2.058). The pulmonary tuberculosis was the most predominant in the study population but the CC, AC allele had statistical significant association with the Extra-pulmonary tuberculosis infections (OR=2.65). Polymorphisms rs2230912 was detected from sequencing results may be associated with TB infections.*

Conclusion: *The CC genotype is associated with susceptibility to TB infections among Sudanese patients and associated with the extrapulmonary TB.*

Keywords

TB, P2X7, Susceptibility, Pulmonary TB, Extra-pulmonary, PCR, RFLP.

Introduction

Tuberculosis is a chronic, systemic infectious disease that occurs when the M. tuberculosis is inhaled causing pulmonary tuberculosis and can localize in alternate sites leading to extra-pulmonary tuberculosis (EPTB) [1]. Extra-pulmonary TB accounts for approximately 10% of tuberculosis infections [2].

The incident new TB cases estimated by the WHO are 10.4 million worldwide, of which 5.9 million (90%) were adults, and 1.0 million (10%) among children till the year 2015 [3].

Candidate genetic variants and regions of the genome associated with tuberculosis risk have been identified from several linkage and association studies, but they have focused primarily on pulmonary disease. Because the pathophysiology of pulmonary and extra-pulmonary disease appears to differ, and because extra-pulmonary disease in particular may be associated with an underlying immune

defect, it is important to assess for genetic variants specifically associated with extra-pulmonary tuberculosis [4].

Purinergic receptor P2X, Ligand-gated ion channel, 7 (P2X7) receptors, in human is a trimeric ligand-gated cation channel coded by the P2RX7gene located at chromosome position 12q24. P2X7 is expressed in a wide variety of normal and disease-associated cell types. This receptor is activated by extracellular adenosine 5'-triphosphate and results in numerous events including the release of pro-inflammatory mediators, cell proliferation or death, and killing of intracellular pathogens. P2X7 plays important roles in inflammation, immunity, bone homeostasis, neurological function and neoplasia [5].

P2X7 is present on macrophages, dendritic cells, monocytes, natural killer cells, B-lymphocytes, T-lymphocytes and erythrocytes [6-9]. P2X7 is also present on human mast cells [10] microglia [11], osteoclasts [12] and eosinophils [13].

Activation of P2X7 on the macrophages causes an immediate opening of a cation selective channel, and the influx of Ca_{2-}, resulting in the induction of the caspase cascade, with resultant apoptosis, as well as the activation of phospholipase D (PLD). PLD promotes phagosome-lysosome fusion, leading to the death of mycobacteria. Several single nucleotide polymorphisms (SNPs) were identified in P2X7 in whites that impair ATP mediated mycobacterial killing. The most common of these, the 1513A→C, causes an amino acid change, from glutamic acid at amino acid position 496 to an alanine in the C-terminus of P2X7 [14,15].This polymorphism in the P2X7 gene increases susceptibility to extra pulmonary tuberculosis [16]. Killing of Mycobacteria resides in phagosomes within macrophages, and this requires fusion of the phagosome with a lysosome. ATP stimulation of P2X7 induces a range of cellular changes, including activation of PLD, phospholipase A2, and mitogen-activated protein kinase, which results in phagosome-lysosome fusion [17-19].

ATP mediated killing of mycobacteria by macrophages is independent of a range of other anti-mycobacterial mechanisms, such as ROI, RNI, SCL11A1, Fas ligation, and complement-mediated lysis [20]. The source of ATP in-vivo is unclear, but infection of macrophages with M. tuberculosis does result in increased ATP levels within the extracellular medium [21]. The1513A→C polymorphism causes an amino acid change, from glutamic acid at amino acid position 496 to an alanine in the C-terminus of P2X7 that leads to reduced receptor function on the macrophage surface, which reduces the response of P2X7 to ATP, and reduces ATP-mediated mycobacterial killing.

After inhalation of M. tuberculosis, initial control of infection in alveolar macrophages may rely in part on the activation of P2X7 receptors. A deficiency of P2X7-mediated control of mycobacterial infection within macrophages in the lung may permit spread to extra pulmonary sites where the infection either progresses to post–primary TB disease, or is controlled by the emerging specific T-cell response. With later waning of T-cell immunity, reactivation of LTBI may result in increased frequency of extra pulmonary TB in subjects with non-functioning SNPs in P2X7 [22,23].

Material and Methods

Hospital based case control study conducted at the National Center for Neurological Sciences (NCNS) and Faculty of Medical Laboratory Sciences and Al-Neelain University from 2014 to 2017 in Khartoum, Sudan. All patients diagnosed clinically and radiologically having pulmonary or extra-pulmonary tuberculosis and verified with positive PCR for M.tuberculosis from blood specimens were included in this study. Ethical clearance was obtained from the Ethical Review Board of National Center for Neurological Sciences and verbal consent was obtained from each patient. PCR-RFLP was used for genotyping for the DNA extracted from five mL of venous blood that was collected into K3EDTA anticoagulant.

The DNA was extracted using saturated salt methods then a reaction mix of 23 µl for the PCR was prepared using 4 µl of 5x PCR buffer (master mix ready to use from Solis BioDyne, (ESTONIA), from the primers 0.75µl of each forward and reverse (10 pmol each). 3 µl the DNA sample was completed to 23 µl by distilled water. For the amplification DNA was denatured for 3 min at 94°C; 35 amplification cycles were performed with an automated thermal cycler (ESCO HEALTHCARE). Each cycle consisted of denaturation at 94°C for 30 sec, annealing of primers at 63°C for 30 sec, extension at 72°C for 45 sec and primer final extension at 72°C for 5 min finally holding temperature 4°C. The product band size is 319bp. The restriction Enzyme used was HaeII (New England Biolabs (UK) Ltd) and applied as instructed. Five µl of the digested mixture was loaded into 2% (agarose gel) containing ethidium bromide and visualized using gel documentation system UV transilluminator (Syngene). The sizes were determined using 100-bp ladder (SOLIS BIODYNE, ESTONIA).

Primers used for the amplification of P2X7 1513A→C

Forward	5′CAACCAAGACTACAAGTACCGCGTCAGTGA 3′
Reverse	5′-AACCAGCGGAAGAGGTCAAGGG-3

When the restriction enzyme cuts the product to 200 &119bp then the genotype is homozygous for the mutant CC allele, if No cut so still 319 it is AA and if there was 319, 200 &119 it is heterozygous AC.

Statistical analysis

For statistical analysis IBM SPSS Statistics version 19 was used [24,25]. P value of 0.05 was considered significant and Odds ratio (OR) assessment with 95% confidence limits were calculated by logistic regression.

Cycle sequencing

The genotyping results for RFLP-PCR were further confirmed by sequencing a subset of samples (no=14\86) 16% of the studied group. Standard sequencing was performed for both

strands of P2X7 gene by Macrogen Company (Seoul, Korea). Chromatograms were showen by FinchTV programs. Sequences were submitted and given accession numbers from GenBank at NCBI database.

Bioinformatics Analysis

The chromatogram sequences were visualized through Finch TV program version 1.4.0 [26]. The nucleotides sequences of the P2X7 gene were searched for sequences similarity using nucleotide BLAST [27]. NCBI (https://www.ncbi.nlm.nih.gov/) and subjected to multiple sequence alignment using BioEdit software version 7.2.5 [28].

Results

One hundred and twenty patients were diagnosed having tuberculosis, pulmonary TB were 66 patients (55%) and the remaining 54 patients were extra pulmonary TB, including 39 patients (32.5%) Pott's disease, 13 (10.8%) with brain tuberculomata and 2 (1.7%) with other types of TB. Fifty-two patients (60.5%) of the patients had the genotype AA, while 8 (9.3%) had the mutant genotype CC and 26 (30.2%) were heterozygous with the genotype AC. The control group included 46 apparently healthy subjects. Thirty-seven patients (80%) had the genotype AA, 1 (2.2%) had the mutant genotype CC and 8 (17.4%) were heterozygous with the genotype AC (Table 2), (Figures 1, 2 and 3)

	Type of tuberculosis		
Type	Pulmonary Tuberculosis	66	55.0%
	Pott's disease	39	32.5%
	Brain tuberculomata	13	10.8%
	Others TB	2	1.7%

Table 1: Distribution of tuberculosis type among the studied group.

Gene	Genotype	Pulmonary Tuberculosis	Pott's disease	Brain tuberculomata	Others TB	P-value
P2X7	AA	29	20	3	0	.034
	CC	3	2	2	1	
	AC	14	6	6	0	

Table 2: Association of P2X7 genotype with type of TB.

Table 3

P2X7 1513A/C	Case				
	Patients (n:61)		Control (n:46)		
	Count (86)	Column N %	Count (46)	Column N%	P-value
AA	52	60.5%	37	80.4%	0.064
CC	8	9.3%	1	2.2%	
AC	26	30.2%	8	17.4%	

The mutant genotype CC and CA are associated with TB infection Odd Ratio OR= (4.615) & (2.058) respectively at the gene P2X7 There is an association between the studied polymorphisms P2X7 and the type of TB infection P-value (.034) as shown in table (2), Genotype CC is associated with extra pulmonary TB OR= (2.65) (Tables 4 and 5).

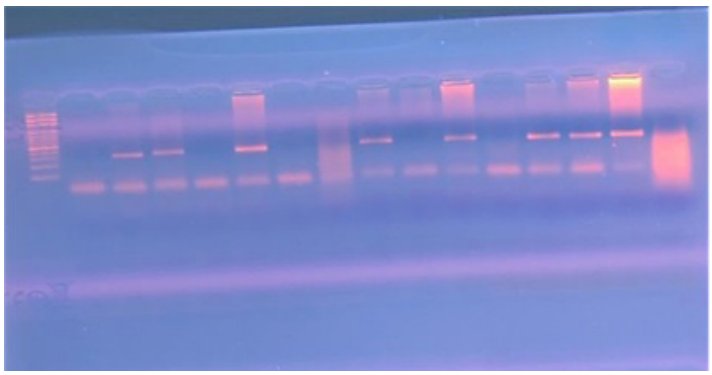


Figure 1: The product band size is 319bp for P2X7 polymorphism 1513 A/C among the studied patients group ((NCNS) 2014-2017, n: 120).

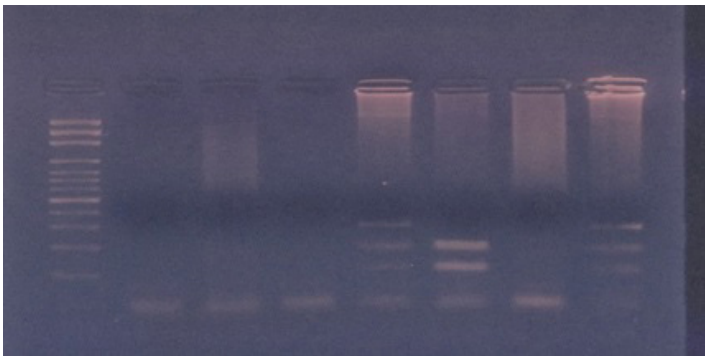


Figure 2: The restriction Enzyme used for P2X7 SNP was HaeII.
Lane 1: DNA ladder 100bp
Lane 5 & 8: Show 319, 200 &119 it is heterozygous AC allele.
Lane 6: Shows 200 & 119bp CC homozygous mutant allele.

P2X7 1513A/C		Case					Odd Ratio
		Patients n:120		Control n:48			
		Count	Column N%	Count	Column N%	P-value	
AA	Present	52	60.5%	37	80.4%	.020	.372
	Absent	34	39.5%	9	19.6%		
CC	Present	8	9.3%	1	2.2%	.161	4.615
	Absent	78	90.7%	45	97.8%		
AC	Present	26	30.2%	8	17.4%	.108	2.058
	Absent	60	69.8%	38	82.6%		

Table 4: Distribution of P2X7 1513A/C genotypes in patients and controls ((NCNS) 2014-2017, n: 120).

P2X7 1513A/C		TB					Odd Ratio
		Pulmonary Tuberculosis		Extra-pulmonary Tuberculosis			
		Count	Column N%	Count	Column N%	P-value	
AA	Present	29	63.0%	23	57.5%	.600	1.261
	Absent	17	37.0%	17	42.5%		
CC	Present	3	6.5%	5	12.5%	.464	.488
	Absent	43	93.5%	35	87.5%		
AC	Present	14	30.4%	12	30.0%	.965	1.021
	Absent	32	69.6%	28	70.0%		

Table 5: Frequency distribution binary P2X7 Genotypes in types of TB ((NCNS) 2014-2017, n: 120).

		Odd Ratio				Odd Ratio
		Extra pulmonary Tuberculosis		Pulmonary Tuberculosis		
		Count	Column N%	Count	Column N%	
P2X7 AA	Present	23	57.5%	29	63.0%	0.65
	Absent	17	42.5%	17	37.0%	
CC	Present	5	12.5%	3	6.5%	2.65
	Absent	35	87.5%	43	93.5%	
AC	Present	14	30.0%	12	30.4%	0.98
	Absent	28	70.0%	32	70.0% 69.6%	

Table 6: Association of P2X7 genotype with type of Extra-pulmonary.

Bioinformatics analysis

Substitution from A>C rs3751143 was confirmed as detected by RFLP in tuberculosis patients (Figure 3), in the position 12:121184501 of the gene sequence from gene bank (Refseq. NC_000012.12).

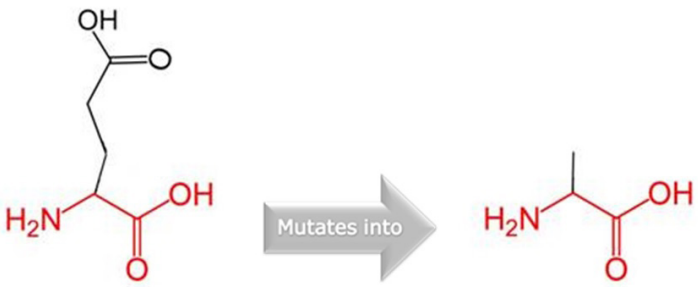
Substitution from A>G in the position 121184393 of the same gene rs2230912 was detected in 21% (3/14) of the samples sent for sequencing (Figure 4). Alignments by BioEdit software version 7.0.9.0 were carried out among the nucleotides of the P2X7 gene in patients, gene sequence from gene bank (Refseq. NC_000012.12).

Nucleotide Sequence Accession Numbers

The nucleotide sequences of the P2X7 gene deposited in the GenBank database (National Center for Biotechnology Information; (https://www.ncbi.nlm.nih.gov/) under the following accession numbers: MG367653, MG367654, MG367655, MG367656, MG367657, MG367658, MG367659, MG367660, MG367661, MG367662, MG367663, MG367664, MG367665.

The detected mutations were analyzed using bioinformatics tools HOPE (www.cmbi.ru.nl/hope/method/), the information regarding the detected SNPs were obtained from National Center for Biological Information (NCBI).

The figure below shows the schematic structures of the original (left) and the mutant (right) amino acid for the mutation of a glutamic acid into an alanine at position 496 detected in this study. The backbone, which is the same for each amino acid, is colored red. The side chain, unique for each amino acid, is colored black.



Each amino acid has its own specific size, charge, and hydrophobicity-value. The original wild-type residue and newly introduced mutant residue often differ in these properties. The mutant residue is smaller than the wild-type residue, the wild-type residue was negatively charged, the mutant residue is neutral, and the mutant residue is more hydrophobic than the wild-type residue.

The mutated residue is located in a domain that is important for binding of other molecules. Mutation of the residue might disturb this function.

SIFT software for Predicting damaging amino acid substitutions, Polyphen-2 software for Prediction of functional modification was used (PolyPhen-2, v.2.2.2; http://genetics.bwh.harvard.edu/pph) and Provean software (http://provean.jcvi.org) was also used.

Mutation	Nucleotid/ Amino acid	SIFT	Polyphen score/ prediction	Provean score	Prediction (cutoff= -2.5)
rs3751143	A > C, E496A	Damaging	0.995/ Probably Damaging	-2.117	Neutral
rs2230912	A> G, Q460R	Tolerated	0.003/ Benign	-0.855	Neutral

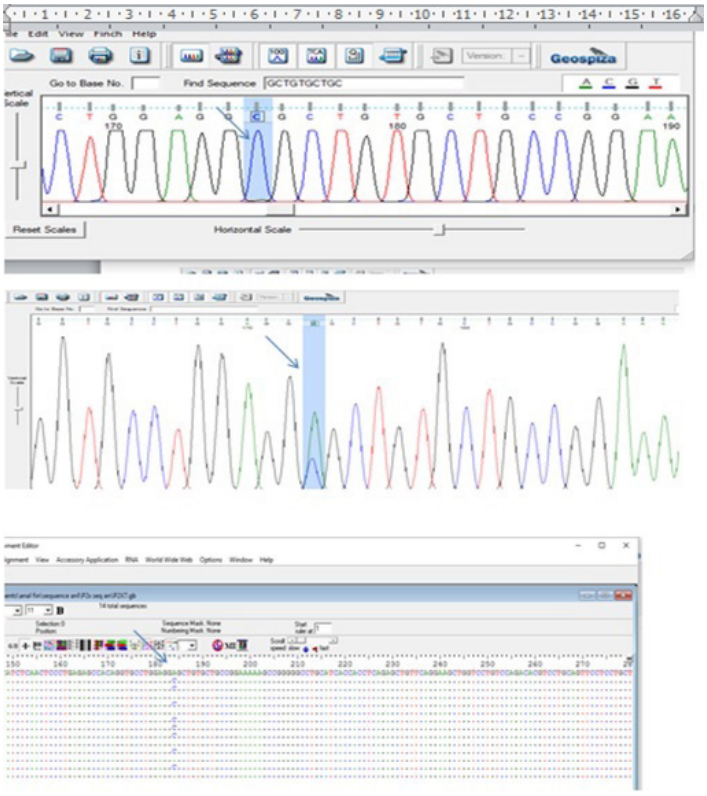


Figure 3: A>C substitution sequence chromatogram rs3751143 in Tuberculosis patients, was shown by FinchTV program.
B. BioEdit multiple sequence alignment showed: First the gene sequence from gene bank (Refseq. NC_000012.12).

Figure 4: A > G substitution sequence chromatogram rs2230912 in Tuberculosis patients, was shown by FinchTV program. B. BioEdit multiple sequence alignment showed: First the gene sequence from gene bank (Refseq. NC_000012.12).

Discussion

In this study statistical significant association for the mutant CC with tuberculosis infection was confirmed. Within the types of tuberculosis, it is statistically associated with extra pulmonary tuberculosis 2.65 Odd ratio. To the best of our knowledge this is the first report in Sudan to detect the P2X7 1513A→C polymorphism among tuberculosis patients. Several studies linked the 1513A→C polymorphism with extra pulmonary. Since Macrophages play an important role in the immunopathogenesis of tuberculosis and extracellular ATP induces macrophage bactericidal activity through activation of the purinergic P2X7 receptor [5-9,17,18]. In support of our results, Tekin et al. found 1513A→C polymorphism is associated with extra pulmonary tuberculosis in children, Also Fernando et al that allele associated with reduced killing of Mycobacterium tuberculosis and strongly associated with extra pulmonary, but not pulmonary TB (odds ratio, 3.8) [29]. Jiangdong Wu et al investigated the association between single nucleotide polymorphisms (SNPs) in P2X7 gene and serum immunoglobulin G (IgG) responses to Mycobacterium tuberculosis in TB patients they found P2X7 SNPs, 1513A>C, may be associated with the susceptibility to tuberculosis [30]. Sambasivan et al assessed the association of −762 T/C, 1513A/C and 1729T/A P2X7 polymorphisms in patients with PTB and healthy controls to establish association if any with risk of developing the disease but they found the 1513A/C polymorphism did not show any significant association in Asian Indians [31].

From the sequencing result the rs2230912 A/G was detected in (3/14) 21% of cases a finding that calls for further studies to assess the association of the SNP with the tuberculosis.

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