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P2X7 1513A/C loss-of-function polymorphism and active tuberculosis disease in a cohort of Egyptian population: a pilot study

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Abstract

Background: Tuberculosis (TB) is a multifactorial disease, and increasing evidence shows that genetic variants in regulating genes of immune response confer susceptibility to active TB at the individual level. We aimed to identify the contribution of P2X7 receptor 1513A/C genetic polymorphisms to different clinical forms of active tuberculosis in a cohort of Egyptian population.

Methods: A case–control study that enrolled 25 newly diagnosed pulmonary TB (PTB) patients by positive sputum for AFB or positive culture, 25 extrapulmonary TB (EPTB) diagnosed by pathological/bacteriological/immunological studies and 25 healthy controls. A blood sample was taken before starting of therapy for P2X7 1513A/C polymorphism genotyping using PCR-restriction fragment length polymorphism.

Results: Fifty-two percent of the participants were in the third decade with equal gender distribution. P2X7 receptor 1513AA (homozygote wild), AC (heterozygote) and CC (homozygote mutant) genotypes were identified. AC and CC genotypes distribution were significantly more frequent in the active TB cases (either PTB or EPTB) rather than controls ($p < 0.05$). Further, P2X7 1513A/C genotypes' distribution did not associate with old TB or gender ($p > 0.05$), but significantly associated with history of smoking (x^2 trend analysis $p = 0.036$).

Conclusions: There is positive association between P2X7 receptor 1513A/C polymorphism and active tuberculosis in the Egyptians.

Keywords: Pulmonary tuberculosis, Extrapulmonary tuberculosis, Genotype, Smoking

Background

Tuberculosis (TB) is a communicable curable infectious disease caused by Mycobacterium tuberculosis (*MTB*) which is a highly host adapted intracellular bacterial pathogen of macrophages. TB generally affects the lungs (pulmonary tuberculosis "PTB"), but can also affects any organ of the body (extrapulmonary TB "EPTB") [1]. Although MTB has infected around a third of the world's population, only 3–10% develop active disease during their lifetime [2, 3]. This could be due to complex interactions of *MTB* with environmental (e.g., microbial), non-genetic host factors (e.g., acquired immunodeficiency) and host genetic factors that play a critical role in TB development [4].

Tuberculosis remains a public health problem in Egypt despite the efforts to decrease its spread in the community. According to WHO report in 2019, about 10 million globally fell ill with TB where 1.2 million died [5]. The current estimated incidence of TB in Egypt is 12 (11–14) cases/100,000 population, while mortality rate is 0.43 (0.39–0.48) cases/100,000 population [5].

Various genetic polymorphisms have been found in many studies to be associated with the development of

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active TB; however, the results are variable between the studies [3, 6–8]. Interestingly, the human P2X7 (purinergic receptor P2X, ligand-gated ion channel 7) gene, which encodes the P2X7 receptor, is highly expressed on macrophages. P2X7 receptor gene contains 13 exons and is localized on chromosome 12q24 [9]. The activation of P2X7 receptor by ATP opens cation-selective channel triggers the influx of Ca^{2+} and Na^{+} and the efflux of K^{+} to the macrophage, thus inducing caspase cascade with subsequent apoptosis as well as activating phospholipase D promoting phagosome–lysosome fusion and elimination of *MTB* [10].

P2X7 is a highly polymorphic gene with over 680 known single-nucleotide polymorphisms (SNPs), 11 of which have been documented as loss-of-function mutations. One of the most common SNPs is the substitution of adenine to cytosine in exon 13 in the position 1513 (A1513C, rs3751143) which changes glutamic acid at position 496 to alanine, resulting in the complete loss of function of the protein product in homozygotes and partial loss in heterozygotes [11]. Various studies throughout the world reported that P2X7 polymorphisms contributed to the susceptibility to TB [9, 12, 13], while others found that P2X7 polymorphism did not contribute to TB susceptibility [14]. However, the results are variable between the studies which could be attributed to various factors as the studied ethnic group, the variable genomic technologies used in each study, and the lack of proper study design in some studies that need more investigation and further studies [3]. To our knowledge, there are no similar studies done in the Egyptian population aiming to identify the genetic susceptibility of the development of TB as well as minimal data available from the Arabic area (Middle East and North Africa) with their different ethnic groups [3].

Hence, we aimed to evaluate the allelic distribution of P2X7 receptor gene polymorphism 1513A/C among PTB and EPTB patients in a cohort of Egyptian population and to investigate the association between this genetic polymorphism and the susceptibility to develop active TB disease.

Methods

Study design

A case–control study that enrolled 50 patients newly diagnosed as tuberculosis (PTB and EPTB) presented to Main Alexandria University hospital, Maamora chest hospital or referred from primary care outpatients' clinics of ministry of health of Alexandria city of Egypt as well as 25 healthy control subjects. The study was conducted in Alexandria city of Egypt, and the patients were collected between over 12 months. The study was approved by the local ethical committee of Alexandria University according to Declaration of Helsinki standards, and a consent was signed by all participants.

Study population

The study included 50 patients with new diagnosis of TB who were divided into 2 groups: 25 patients with PTB diagnosed by positive smear using direct Ziel Neelsen (ZN) staining or positive culture for TB and 25 patients with EPTB diagnosed by pathological, histochemical, bacteriological and immunological studies according to the site of the disease. The patients with family history of TB, those receiving immunosuppressive medications as corticosteroids, and those with human immunodeficiency virus (HIV) positive were excluded from the study. Regarding the control group, 25 subjects were invited to participate in the study. The control subjects were healthy, with no history of TB or evidence of respiratory disease, had normal chest x-ray and normal laboratory testing.

Characteristics of the participants

All participants were subjected to detailed history taking (including age, gender, smoking history, respiratory symptoms, comorbidities, history of old TB, drug history and family history of TB), complete clinical examination, and routine laboratory investigations (including complete blood count, liver enzymes (ALT and AST), bilirubin (total and direct), renal function (urea and creatinine) and fasting blood glucose. Adenosine deaminase (ADA) measurement in the pleural fluid was used to assist the diagnosis of tuberculous pleural effusion [15]. Plain chest x-ray was performed to all participants. Computed Tomography (CT) scan of the chest was performed if needed following the physician recommendation. Magnetic resonance imaging of the vertebral column for selected cases with suspected TB of the vertebrae was requested. Fine needle aspiration from lymph nodes or tissue biopsies from pleura, genitalia, kidney and gastrointestinal tract were performed for selected cases of EPTB according to their clinical presentation as TB was diagnosed if caseating granuloma was identified.

Bacteriological evaluation

Early morning sputum sample obtained over 3 consecutive days was collected for bacteriological evaluation. In case of no spontaneous sputum, induced sputum or bronchoalveolar lavage was performed. In case of EPTB, urine specimen was collected in case of renal TB; pus specimen in tuberculous abscess; pleural fluid in pleural TB; and cerebrospinal fluid analysis in case of meningitis. The samples were examined microscopically after being stained by ZN stain according to the criteria defined by the American Thoracic Society (ATS) in order to detect

acid fast bacilli (AFB) [4]. ATS quantitation scale for AFB was considered [4]. TB culture was followed for all samples for isolation of *MTB* and drug sensitivity testing using BACTEC MGIT 960 system (BD life-science, New Jersey, USA). Further, samples were examined by GeneXpert MTB/RIF system (Cepheid smart-cycler® system, California, USA) in Maamora chest hospital for rapid diagnosis of cases and early identification of relevant Rifampicin resistance.

Genetic evaluation

Genomic DNA extraction

A blood sample was taken from all patients on admission to hospital before starting of therapy and from all healthy control subjects for P2X7 receptor 1513A/C polymorphism evaluation. All the samples were stored at − 18 °C for further analysis. DNA concentration was evaluated as µg/µl using spectrophotometer. DNA purity was measured as optical density at 260/280 nm. The readings should be greater than 1.8 for more accuracy. DNA quality and quantity of samples were assured through electrophoresis power supply EPS 500/400 (Pharmacia fine chemical, Uppsala, Sweden) on agarose gel 1.5% using 1 Kb DNA Ladder (Fermentas, EU) as standard.

Genotyping of the 1513 A/C polymorphism

The 1513A → C SNP was genotyped by PCR-restriction fragment length polymorphism (RFLP) with the following primers: 5′-AGA CCT GCG ATG GAC TTC ACA G-3′ (forward) and 5′-AGC GCC AGC AAG GG CTC-3′ (reverse) [16]. The PCR device used was Peqlab-Primus-25 (LabMakelaar Benelux B.V., Zevenhuizen, The Netherlands). The PCR conditions were: initial denaturation at 95 °C for 5 min; 36 cycles of 95 °C for 30 s, 36 cycles of 66.3 °C for 30 s, 36 cycles of 72 °C for 40 s, and final elongation at 72 °C for 7 min. The PCR products were digested at 37 °C for 4 h with 5.0 U of HaeII (Promega). The digested products were run on a 3% agarose gel and visualized with 10 ng/ml ethidium bromide. The PCR products were separated by electrophoresis using 15 µl of PCR product and 10 µl of Molecular marker (O´ Gene Ruler ™ 1 Kb; Fermantas, EU) as standard. The run was performed at 55 V in (Wealtac-Corp) submarine (20 × 20 cm) for 2 h. Bands were detected on ultraviolet Transilluminator, photographed by digital camera (Panasonic).

Statistical analysis

The data were expressed as mean ± standard deviation (SD) or numbers and percentage (%) for quantitative and qualitative data, respectively. Student-*t* test, Fisher's exact test, and Monte Carlo test were used as appropriate. Hardy–Weinberg equation [17] was used to calculate the expected common homozygotes, expected heterozygotes, expected rare homozygotes and the frequency range of the alleles from the observed genotypes. The equation is an expression of the principle known as Hardy–Weinberg equilibrium, which states that the amount of genetic variation in a population will remain constant from one generation to the next in the absence of disturbing factors. Two-tailed p value ≤ 0.05 was considered as significant. All the statistical analysis was done using SPSS statistics (version 17.0. Chicago: SPSS Inc.).

Results

Study population

Table 1 describes the frequencies of the sites affected in EPTB patients included in the current study and their tools of diagnosis. The majority of EPTB patients had tuberculous pleural effusion (32%) followed by tuberculous lymphadenitis (20%). Table 2 shows the demographic and clinical characteristics of the participants of the studied population. The male: female ratio was 1.03:1, whereas 52% of the participants were in the 3rd decade of age. There was no statistically significant difference between the 3 groups (PTB, EPTB, and controls) regarding age, gender, and smoking history ($p > 0.05$, Table 2). Sixteen percent of EPTB had old TB history; 16% of EPTB and 28% of PTB patients had associated comorbidities as diabetes mellitus, hypertension, and/or chronic obstructive pulmonary disease (COPD) ($p = 0.017$, Table 2).

Genetic evaluation and related correlations

Three genotypes of P2X7 1513A/C were detected: heterozygote AC genotype, homozygote wild type AA genotype and homozygote mutant type CC genotype (Fig. 1). The frequency of P2X7 1513AA genotype was

Table 1 The frequencies of various EPTB diseases among the studied population

EPTB ($n = 25$)	N	%	Diagnostic tool
Tuberculous pleural effusion	8	32	ADA level Pleural biopsy
Tuberculous lymphadenitis	5	20	Biopsy
Renal tuberculosis	3	12	Urine culture for TB
Pott's disease	3	12	MRI imaging
Tuberculous abscess	2	8	Pus culture for TB
Ocular tuberculosis	2	8	Ocular ultra sound
GIT tuberculosis	1	4	Biopsy Gene-expert
Tuberculous meningitis	1	4	CSF analysis Gene-expert

TB tuberculosis, *EPTB* extrapulmonary tuberculosis, *GIT* gastro-intestinal tract, *ADA* adenosine deaminase, *CSF* cerebrospinal fluid, *MRI* magnetic resonance

Table 2 Demographic and clinical characteristics of studied population

Characteristics	Groups			Sig. (*p* value)
	Control (*n* = 25)	PTB (*n* = 25)	EPTB (*n* = 25)	
Age (years); *n* (%)				0.615
< 30	11 (44)	6 (24)	8 (32)	
30–39	9 (36)	13 (52)	10 (40)	
40+	5 (20)	6 (24)	7 (28)	
Gender; *n* (%)				0.948
Male	13 (52)	13 (52)	12 (48)	
Female	12 (48)	12 (48)	13 (52)	
Medical history; *n* (%)				0.017*
Free	23 (92)	18 (72)	68)	
Old TB	0 (0)	0 (0)	4 (16)	
Comorbidities[a]	2 (8)	7 (28)	4 (16)	
Smoking status; *n* (%)				0.100
Smoker	3 (12)	12 (48)	(32)	
Ex-smoker	3 (12)	1 (4)	(8)	
Non-smoker	19 (76)	12 (48)	15 (60)	
Smoking index (mean ± SD)	1.7 ± 4.9	8.2 ± 10.9	9.3 ± 17.1	0.086

TB tuberculosis, *PTB* pulmonary tuberculosis, *EPTB* extrapulmonary tuberculosis, *n* number, *SD* standard deviation

[a] Including COPD, hypertension, diabetes mellitus

*Significant $p < 0.05$

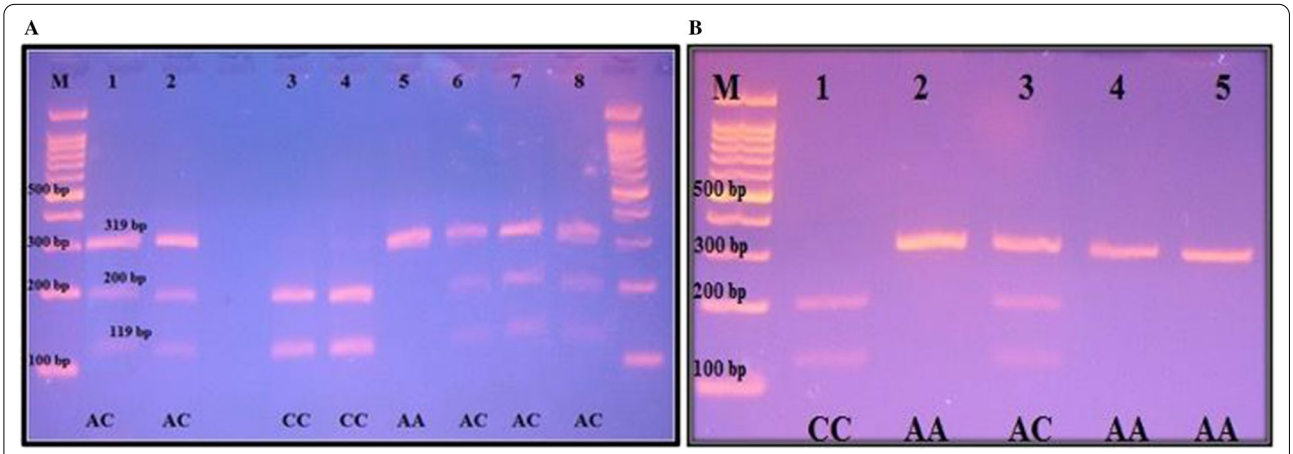

Fig. 1 1513A/C polymorphism in the P2X7 gene was detected by RFLP analysis. Heterozygote AC genotype produced products of 319, 200 and 119 base pair (bp), homozygote wild AA genotype produced digested products of 319 bp and homozygote mutant CC genotype produced digested products of 200 and 119 bp. Lane M, 100 bp marker

significantly more prevalent in the control subjects versus all TB cases (17/ 25 patients (68%) versus 20 / 50 patients (40%), $p = 0.036$; Table 3). On the other hand, the frequency of P2X7 1513AC and CC genotypes was significantly higher in the all TB patients (48% and 12%, respectively) when compared to healthy control subjects (32% and 0%, respectively, $p = 0.036$; Table 3). Further, P2X7 1513AC genotype was significantly more frequent in EPTB (56%), while the 1513CC genotype was significantly more frequent among PTB group (16%, $p = 0.048$, Table 3).

Considering the allelic frequency distribution analysis (using Hardy–Weinberg equilibrium), the 1513C loss-of-function allele among the TB groups (PTB and EPTB) was significantly more frequent than the controls (36% (36 alleles) versus 16% (8 alleles); $p = 0.029$);

Table 3 Association between P2X7 receptor 1513A/C polymorphism genotypes and the studied groups

P2X7 receptor gene polymorphism	Groups			*p* value
	Control ($n=25$)	All TB cases ($n=50$)		
AA[a]; *n* (%)	17 (68)	20 (40)		0.036*
AC; *n* (%)	8 (32)	24 (48)		
CC; *n* (%)	0 (0)	6 (12)		
	Control ($n=25$)	PTB ($n=25$)	EPTB ($n=25$)	
AA[a]; *n* (%)	17 (68)	11 (44)	9 (36)	0.048*
AC; *n* (%)	8 (32)	10 (40)	14 (56)	
CC; *n* (%)	0 (0)	4 (16)	2 (8)	

TB tuberculosis, *PTB* pulmonary tuberculosis, *EPTB* extrapulmonary tuberculosis

[a] The AA genotype served as the reference category, CC or AC versus AA

*Significant $p<0.05$

while the frequency of the 1513A allele was significantly higher in the control group when compared to the TB groups (84% (42 alleles) versus 64% (64 alleles); $p=0.029$).

There was no statistically significant association between the 1513A/C genotypes and gender or history of old TB ($p>0.05$, Fig. 2A). However, there was a statistically significant association between the P2X7 1513A/C genotypes and smoking status in x^2 trend analysis ($p=0.036$, Fig. 2B) as P2X7 receptor 1513AC and CC genotypes were more frequently observed in the smokers (active or former smoker) rather than the non-smokers (50% vs. 38.3% and 14.3% vs. 4.3%, respectively).

Discussion

In the current study, P2X7 1513AC and CC genotypes associated with partial and complete loss-of-function, respectively, were significantly frequent in active TB cases either PTB or EPTB. This polymorphism was significantly associated with active smoking status but not with history of old TB or gender. To our knowledge, this is the first study investigating the association between P2X7 genetic polymorphisms and susceptibility to different clinical forms of active tuberculosis in a cohort of Egyptian population.

Singla et al. [18], who studied two genetic polymorphisms of P2X7 gene (−762T/C and 1513A/C) in an Indian population, found a significantly higher frequency

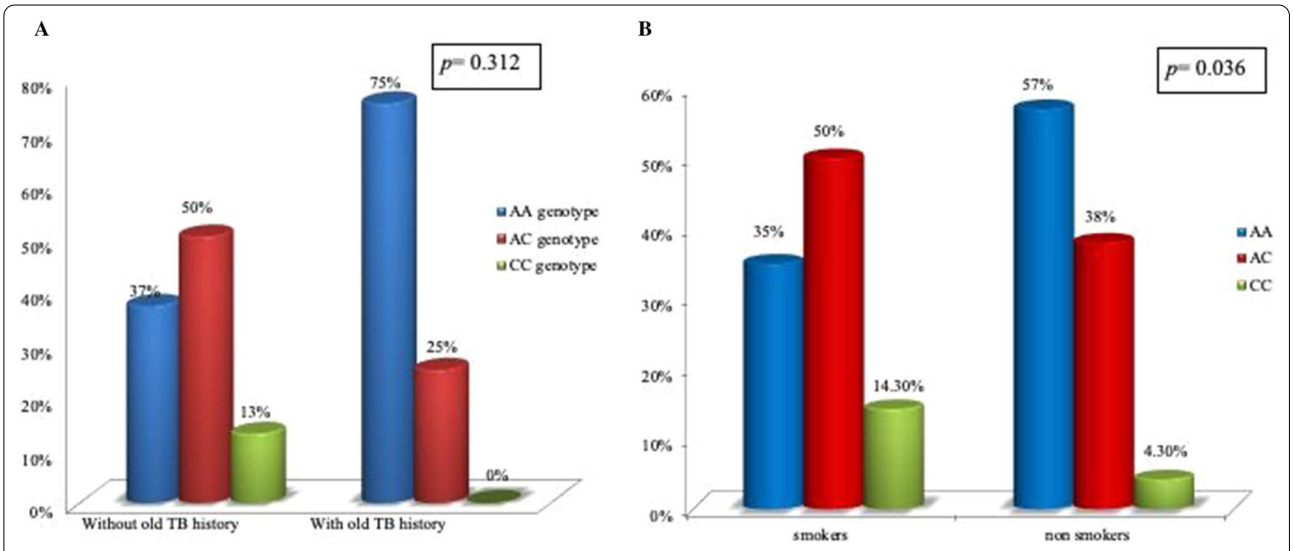

Fig. 2 **A** P2X7 receptor 1513A/C genotypes frequency distribution among studied population regarding previous history of old TB; **B** P2X7 receptor 1513A/C genotypes distribution among smoker (either active or former smokers) and non-smoker subjects included in the study

of C allele in the disease groups than in the control group with a significant difference in the PTB versus EPTB. Similarly, Nino-Moreno et al. [16] found a significant association between P2X7 gene 1513A/C polymorphism rather than −762T/C and NRAMP1/SLC11 A1 gene polymorphisms and the susceptibility of PTB in a Mexican population. Wu et al. [19] and Mokrousov et al. [20] found that the P2X7 gene 1513A/C polymorphism was significantly higher in PTB than the control group in Chinese and Slavic Russian populations, respectively. Further, Ben-Selma et al. [21] found that the frequency of 1513CC and AC genotypes were significantly more frequent in the EPTB group than in the control and PTB groups in Tunisian patients. Similarly, Fernando et al. [22] and Sharma et al. [23] found a significant association between the 1513C allele and active EPTB rather than PTB in Asian population. These results are in accordance with our current results and support the association between P2X7 gene 1513A/C polymorphism and various forms of active TB infection in different ethnic groups.

TB patients with P2X7 gene 1513AC heterozygous genotype had significant impairment in macrophages' ability to kill *Mycobacterium Bovis* BCG after ATP stimulation [22], while this ATP-mediated mycobacterial killing ability was totally ablated in macrophages obtained from P2X7 gene 1513CC homozygous patients with TB emphasize the complete loss of P2X7 function in leukocytes [24, 25]. Further, a deficiency of P2X7-mediated control of *MTB* infection within pulmonary macrophages may permit spread to extrapulmonary 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 latent TB may result in increased frequency of EPTB in subjects with nonfunctioning SNPs in P2X7 [9, 22]. Additionally, signaling through P2X7 plays a more important role in the control of reactivated TB infection than in the initial containment of *MTB* within the lung. After reactivation of dormant bacilli, the absence of P2X7-mediated killing of mycobacteria in pulmonary macrophages could then result in spread to extrapulmonary sites [26].

On the other hand, Ozdemir et al. [27], Xiao et al. [28] and Li et al. [29] did not find significant associations between the P2X7 receptor 1513A/C polymorphism and different clinical forms of TB in Eastern Turkish population, Chinese Han population and Gambian population, respectively. This could be explained on the basis of various factors. Firstly, the ethnic origin of patients; it has been reported that ethnic-specific genetic variations may influence host immunity to TB, causing different tuberculosis susceptibilities [2] as we found low allele frequency of the 1513C allele in our Egyptian cohort as an example of African population. Secondly, sample size which may affect statistical calculations. Lastly, as genetic susceptibility to tuberculosis is polygenic, the other functional SNPs occurring in the P2X7 gene (as −1269T/G, −1140 C/T, −838 C/T, −762T/C and −298 G/A) may be associated with susceptibility to active tuberculosis [29, 30]. In addition, interactions with other polymorphisms may be of great importance, at least in some populations, as variations in NRAMP1, MBL, VDR, P2X7 receptor, IL-10 and IL-1 receptor antagonist (IL-1Ra) are all significantly associated with TB susceptibility [2, 31, 32].

Further, Shemon et al. [33] described an association between the loss P2X7 receptor function in Thr357Ser homozygous polymorphism (located in gene 1096C −>G) in Caucasian population which led to impairment of ATP-induced apoptosis and *MTB* killing in macrophages. This Finding provides robust support for the concept of low P2X7 function in mediating TB susceptibility other than 1513C P2X7 polymorphism. Fairbairn et al. [34] also highlighted the role of P2X7 in lysosome–phagosome fusion in macrophages and *TB* bacteria killing through ATP stimulation; however, blocking of macrophage-phosphodiesterase D activity resulted in macrophage death but not TB bacteria.

Interestingly, P2X7 receptor 1513AC and CC genotypes were significantly frequent observed in the smokers rather than the non-smokers, suggesting that P2X7 receptor gene polymorphism could be associated with smoking. Tobacco smoke was shown to reduce alveolar macrophage activity, induce immune-depression of pulmonary lymphocytes, reduce the cytotoxic activity of natural killer cells, induce alteration of the activity of the pulmonary dendritic cells and mucociliary clearance [35], and may cause changes in chromosomes that affect gene activity and expression [36]. Further, active and passive smoking have been found to increase the risk of latent TB infection in previous study [37], increase PTB severity and relapse of both PTB and EPTB after treatment [38].

On the other hand, we found no statistically significant association between P2X7 receptor 1513A/C polymorphism and previous history of old TB. However, Fernando et al. [22] found that 35% of latent TB patients developed active EPTB disease and had a strong association with the P2X7 receptor 1513C allele.

We believed that the current results could be translated in the clinical care of TB especially among those with latent TB whom represent the majority of the infected TB patients [39]. Further, we thought that effective smoking cessation programs could be helpful in decreasing the genetic polymorphism and guard against development of active tuberculosis disease.

The current study has some limitations. Firstly, we did not analysis other genetic polymorphisms that could be linked also to disease susceptibility and affected with

associated P2X7 receptor gene polymorphism. Secondly, the sample size was relatively small and coffined to one geographical area of Egypt. However, this is an early pilot study with promising results that could be a step for further investigation. Further, a meta-analysis is also still recommended to compare the current results with previous studies in the literature.

Conclusion

P2X7 gene receptor 1513A/C polymorphism positively associated with tuberculosis susceptibility in a cohort of Egyptian population. This polymorphism could be linked to ethnicity and exposure to smoking rather than previous history of TB; however, further studies with large scale are still required to confirm this association.

Abbreviations

ADA: Adenosine deaminase; AFB: Acid fast bacilli; ALT: Alanine transaminase; AST: Adenosine transaminase; ATS: American Thoracic Society; CT: Computed tomography; EPTB: Extrapulmonary tuberculosis; MTB: Mycobacterium tuberculosis; PTB: Pulmonary tuberculosis; SD: Standard deviation; SNPs: Single-nucleotide polymorphisms; TB: Tuberculosis; ZN: Ziel Neelsen stain.

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Author contributions

YK contributed to concept of the work, protocol design, supervision, interpretation of the data, critical review of the manuscript; AE contributed to full genetic analysis, analysis and interpretation of the data, drafting of the manuscript; HS contributed to protocol design, review and interpretation of the data, statistical analysis, validation, drafting of the manuscript; AS contributed to collection of the cases, data collection, microbiological analysis, statistical analysis, drafting of the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article. But still the datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate

The study has been approved by the ethical committee of Faculty of medicine—Alexandria University—Egypt. Informed consent was obtained from all individual participants included in the study.

Consent for publication

All authors read and approved the final manuscript. And we justify that the current manuscript does not contain any individual person's data in any form.

Competing interests

The authors have no conflicts of interest to declare that are relevant to the content of this article.

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