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RESEARCH ARTICLE

Cytotoxic T-Lymphocyte Antigen-4 (CTLA-4) gene polymorphism is associated with pulmonary tuberculosis susceptibility in a Thai population

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ABSTRACT

Cytotoxic T lymphocyte antigen-4 (CTLA-4) is a T-cell inactivation receptor and has been found to be elevated in Tuberculosis (TB) patients. The functional polymorphisms in *CTLA-4* gene, including *CTLA-4*+49A/G (rs231775) and *CTLA*-4+6230A/G (rs3087243) have been reported to be associated with the risk for many diseases. The two aforementioned functional polymorphisms in the *CTLA-4* gene were genotyped using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) in order to investigate the possible susceptibility to pulmonary TB (PTB) in a Thai population. In this study, TB patients were grouped as 1) PTB with and without comorbidity of other diseases (PTB) 2) PTB without comorbidity of other diseases (PTBWO) 3) PTB comorbidity with other diseases (PTBD). We demonstrated that the allele frequency of *CTLA-4*+49A was higher in PTB, PTBWO, and PTBD groups than in healthy controls subjects (HCS), but no significant association of *CTLA-4*+49A/G polymorphisms with PTB, PTBWO and PTBD were seen. Whereas the *CTLA-4*+6230A allele was significantly higher in PTB, PTBWO and PTBD groups than in HCS, and the *CTLA-4*+6230A allele was found to be significantly associated with PTB, PTBWO and PTBD (*P*=0.007, OR 2.111, 95%CI(1.220-3.652); *P*=0.0218, OR 2, 95%CI(1.100-635); *P*=0.0439, OR 2.5, 95%CI(1.004-6.227) for PTB, PTBWO and PTBD respectively), as well as *CTLA-4*+6230AG genotype was found to be significantly associated with PTB and PTBWO (*P*=0.0432, OR 2.259, 95%CI(1.018-5.014); *P*=0.0392, OR 2.464, 95%CI(1.034-5.874) for PTB and PTBWO respectively). For the combination of *CTLA-4*+49A/G+6230A/G genotypes, +49AA+6230AA and +49AG+6230AG genotypes was more frequent in PTB and PTBWO groups. This study is the first to investigate *CTLA-4*+49A/G and +6230A/G polymorphisms in PTB patients in Thailand, The A allele and AG genotype of *CTLA-4*+6230A/G was significantly associated with PTB, suggesting a possible genetic influence on TB susceptibility. These findings indicate that *CTLA-4* polymorphisms, especially *CTLA-4*+6230A/G, may play a role in PTB risk in the Thai population.

Keywords: CTLA-4; *CTLA-4* polymorphisms; pulmonary tuberculosis; Thai population.

INTRODUCTION

Tuberculosis (TB) has been continuing to be a major healthcare problem worldwide. According to a World Health Organization (WHO) report, the Southeast Asia (SEA) region is home to around one-fourth of the world's population and accounts for more than 45% burden of the total annual incidence of TB. It is estimated that in 2022, more than 4.8 million people fell ill with TB and more than 600 000 died. This region also accounts for more than 38% of the estimated global incidence of multi-drug-resistant TB (MDR-TB)/ Rifampicin-resistant (RR) patients. Thailand is one of the six countries in the SEA with the highest TB burden (WHO, 2023). The outcome of pulmonary TB infection can range from complete pathogen clearance through asymptomatic latent infection to active TB disease. Only 5-15% of these people will develop clinically active TB (Arend *et al.,* 2001). This indicates that genetic differences between individuals may play an important role in susceptibility to TB infection (Möller & Hoal, 2010; Vannberg *et al*., 2011).

Cell-mediated immune response (CMI) and delayed-type hypersensitivity reactions play a major role in modulating the pathogenesis of TB (Aktas *et al*., 2009). Once *Mycobacterium tuberculosis (Mtb)* enters the body and reaches the lower respiratory tract via droplets and is engulfed by alveolar macrophages and dendritic cells. These antigen-presenting cells (APCs) then migrate to lymph nodes where they encounter naıve T-cells. T cell activation requires two signals: one signal triggered by MHC-peptide complex and another triggered by molecules on the macrophages, the B7.1 (CD80) and B7.2 (CD86) binding to the T-cells surface CD28 (Dyck & Mills, 2017). After activation, T-cells proliferate and differentiate into effector T-cells which subsequently migrate back to the lungs and interact with MHC/antigen complexes on the surface of *Mtb*-infected macrophages. These infected macrophages then release cytokines such as IFN-γ to activate other macrophages which can promote the killing of *Mtb* through the induction of autophagy, apoptosis and/or through increased expression of antimicrobial peptides (Lam *et al*., 2017). Lastly, granuloma will develop, which will subsequently become epitheloid cells (Sia & Rengarajan, 2019). Nevertheless, alteration in the balance in the cell-mediated immune response has been shown to be associated with the reduction in the protection and/or enhancement of the immunopathology of this disease (Dlugovitzky *et al*., 1997; Walker, 2017).

CTLA-4 belongs to the immunoglobulin superfamily and is expressed on the surface of activated T-cells. It is structurally similar to the T-cell co-stimulatory protein CD28 and functions as a competitive antagonist for B7 (CD80 and CD86). CTLA-4 has a higher affinity for B7 than CD28 and is responsible for T-cell inactivation. The CTLA-4 protein exists in two forms: a membrane bound and a soluble isoform (sCTLA-4) with immunoregulatory properties (Simone *et al*., 2014). A previous study reported that CTLA-4 on CD4 T-cells as well as regulatory T-cells were expressed higher in active TB patients than those with latent TB infection and healthy controls (Shu, 2019). The production of CTLA-4 is strongly influenced by genetic factors, and its higher expression may lead to the exhaustion of T-cells and their subsequent inability to eliminate *Mtb* (Mäurer *et al*., 2002). In humans, the *CTLA4* gene is located on chromosome 2q33 (Ueda *et al*., 2003). and can be found with several single nucleotide polymorphisms (SNPs). The two common functional *CTLA-4* gene polymorphisms, namely +49A/G (rs231775) on exon 1 and +6230A/G (rs3087243) in 3' untranslated region (3'UTR), are implicated many diseases such as cancer (Van Nguyen *et al*., 2021), autoimmune diseases (Kouki *et al*., 2000; Ueda *et al*., 2003), inflammatory bowel disease (Repnik & Potocnik, 2010) and various infectious diseases (Thio *et al*., 2004; Hikota *et al*., 2008). Previous studies have shown that the +49A/G polymorphism causes a Thr/Ala substitution in the leader peptide (Harper *et al*., 1991) and affects the inhibitory function of CTLA-4 (Mäurer *et al*., 2002). Another study showed that the +6230A/G polymorphism was associated with variations in the CTLA-4 protein expression on CD4 T-cells (Karabon *et al*., 2009). Treatment with CTLA-4 blocking antibodies has been shown to result in increased activation of T-cells and has led to immunotherapies for cancer such as melanoma (Zhao *et al*., 2018).

In TB, Thye *et al*. (2009) reported that the *CTLA-4*+6230G allele contributes to pathology of TB in the African population. The study in Southern Han Chinese showed that *CTLA4*+49AG genotype as well as haplotype +49A (rs231775) +6230G (rs3087243) 11430G (rs11571319) may reduce the risk of being infected with pulmonary tuberculosis, whereas the +49G +6230G 11430A haplotype was related to the pathogenesis of pulmonary tuberculosis (Wang *et al*., 2012). Conversely, +49GG genotype in Iran is associated with increased risk of tuberculosis, while +49GG genotype in Iraq may decrease risk of pulmonary tuberculosis (Paad *et al*., 2014; Enzi *et al*., 2020). The association variants in the *CTLA-4* gene might differ among the different ethnic groups. Presently, there were no previous studies conducted to investigate whether polymorphic sites in *CTLA-4* correlate with the susceptibility to pulmonary TB (PTB) in the Thai population. Therefore, we aim to investigate the occurrence of *CTLA-4*+49A/G, +6230A/G and their association with susceptibility to pulmonary PTB in The Thai population.

MATERIALS AND METHODS

Samples

Anonymized genomic DNA samples were studied from fifty pulmonary TB (PTB) patients and eighty healthy control subjects. These samples were stored at –20°C and are the same set of the samples previously reported (Kulpraneet *et al*., 2015, 2019). Briefly, the patients were recruited from HRH princess Maha Chakri Sirindhorn Medical Center, Faculty of Medicine, Srinakharinwirot University, Ongkharak, Nakornnayok, Thailand. The fifty patients in this study were newly diagnosed pulmonary tuberculosis (PTB). All patients were either smear/culture-positive or with clinical–radiological and histological diagnosis for PTB. Their mean age was 47 (ranging 18-82) years for men and 48 (ranging 24-84) for women, and the male/female ratio was 31/19. Patients who had a positive HIV serology were excluded. In these 50 PTB patients, 12 had medical records (retrospective) of comorbidity with other diseases, as, diabetes mellitus (5), asthma (1), on corticosteroids (3), lymphadenopathy (1), alpha-phetoprotein positive (1), Dithionite Induced Calcium Precipitation (DCIP) test positive (1), the rest 38 had no medical records (retrospective) of comorbidity with other diseases. Those fifty pulmonary TB patients in this study were grouped according to patient retrospective medical records of comorbidity with and/or without other diseases as the followings: 1) pulmonary TB patients with and without comorbidity with other diseases (PTB) (50); 2) pulmonary TB patients without comorbidity with other diseases (PTBWO) (38); and 3) pulmonary TB patients comorbid with other diseases (PTBD) (12). The healthy control subjects (HCS) were students and laboratory personnel who were clinically in good health and were willing to participate in the study. Among the HCS, 29 were males (mean age 34.65, ranging 21-59) and 59 females (mean age 34.49, ranging 19-75). This study was approved by the Ethical Committee of the Faculty of Medicine, Srinakharinwirot University (SWUEC/E-108/2563). It was conducted according to the principles established in the Declaration of Helsinki.

DNA purification

Anonymized genomic DNA from patients with PTB and HCS were stored at –20°C for the study. The DNA were extracted as previously described (Kulpraneet *et al*., 2019).

Genotyping of *CTLA-4* gene polymorphisms

The *CTLA-4*+49A/G (rs231775) and +6230A/G (rs3087243) single nucleotide polymorphisms (SNPs) were investigated using PCR-restriction fragment length polymorphism (RFLP) analysis as described previously with minor modifications (Heward *et al*., 1999; Torres *et al*., 2004). Briefly, the PCR amplification was performed in 10 µl reactions using 1 µl of genomic DNA, 5 µl of 2× Ready-to-Use PCR Master Mix (iNtRON Biotechnology, Korea) and 5 pmoles of primers. The PCR was carried out in an Eppendorf (Corning, USA) using a 2 min denaturation at 94°C followed by 34 cycles with 94°C for 20 sec, 65°C for 10 sec and 72°C for 30 sec for *CTLA-4*+49 and by 34 cycles with 94°C for 20 sec, 56.5°C for 10 sec and 72°C for 30 sec for *CTLA-4*+2630. The final extension was at 72°C for 5 min. Negative controls not containing the DNA template were included in each experiment. After amplification, the PCR products were then digested with *Bbv*I and *Nco*1 restriction enzymes (New England Biolabs, UK) for *CTLA-4*+49A/G and *CTLA-4*+6230A/G respectively. The digested products were analyzed on 2.5% agarose gel containing RedSafe Nucleic Acid Staining Solution (iNtRON biotechnology, Korea) and visualized under UV light (UVITEC Cambridge). The sequence with a A at position 49 was not cut by *Bbv*1 and the sequence with a G was digested. For *CTLA-4*+6230 the following

fragments were obtained: GG 216 bp, AA 196 and 20 bp. The samples were tested in duplicate by different persons. The genotypes were scored without knowledge of the phenotypes by two observers, and the results were 100 percent concordant (Figure 1).

Statistical analysis

The data was analyzed using SPSS software. Hardy-Weinberg equilibrium was assessed by using the chi-square test for each group. Allele and genotype distribution of the *CTLA-4*+49A/G, +6230A/G polymorphisms between PTB patients and HCS were analyzed statistically using the Chi-square ($\chi 2$) test, while n<5 were not calculated. Odds ratios (OR) and 95% confidence intervals (95% CI) were calculated for each analysis. A *P*-value of less than 0.05 was considered to be significant.

RESULTS

The distribution of genotype and allele frequencies of *CTLA-4+*49A/G and *CTLA-4+*6230A/G polymorphisms in pulmonary TB patients (PTB), pulmonary TB without comorbidity with other diseases (PTBWO), pulmonary TB with comorbidity with other diseases (PTBD) and healthy control subjects group (HCS) in the present study were in Hardy–Weinberg equilibrium when compared with the observed and expected genotype frequencies of each SNP (p>0.05).

Association of CTLA-4 exon 1 position 49 A/G polymorphism with pulmonary TB susceptibility

The distribution of *CTLA-4*+49 G allele was higher than A allele in PTB (57 vs 43), PTBWO (57.9 vs 42.1), PTBD (54.2 vs 45.8) and HCS group (64.37 vs 35.63). When the allele and genotype frequencies of +49A/G between PTB and HCS, between PTBWO and HCS, as well as between PTBD and HCS were compared, there were no statistically significant differences (*P* >0.05). Similarly, there were no significant differences in the dominant, recessive, and over-dominant model when comparing between PTB and HCS, between PTBWO and HCS as well as between PTBD and HCS (*P*>0.05). There were no associations that could be drawn between *CTLA-4*+49A/G and the risk of PTB, PTBWO and/or PTBD (Table 1).

Association of CTLA-4 3'UTR +6230 A/G polymorphism with pulmonary TB susceptibility

The distribution of the genotypes of *CTLA-4 3'UTR +6230 A/G* is shown in Table 2. In PTB, there were more patients with the AA genotype (17.39% *vs* 7.5% of HCS) or AG (47.83% *vs* 35.0% HCS), and significantly fewer patients with GG (34.78% *vs* 57.5% HCS) (*P*<0.05). The gene frequencies of A was also significantly higher in PTB patients than in HCS (41.30% vs 25%). A significant association of A allele of *CTLA-4*+6230 with pulmonary TB susceptibility (*P*=0.007, odd ratio (OR)=2.11, 95% CI=1.22-3.65) was observed. The effect of this allele was similar to the autosomal dominant model of inheritance. The presence of one A allele (AG+AA) caused significant OR of 2.54 (95% CI=1.20-5.38, *P*=0.014). Similarly, in PTBWO, there were more patients with the AA genotype (14.3% *vs* 7.5% of HCS) or AG (51.4% *vs* 35.0% HCS) and significantly fewer patients with GG (34.3% *vs* 57.5% HCS). PTBWO patients possessing the allele of *CTLA-4* +6230A/G were observed to be significantly associated with pulmonary TB susceptibility (*P*=0.0218, OR=2.0, 95% CI=1.10-3.63,). The presence of one A allele (AG+AA) resulted in the significant OR of 2.59 (95% CI=1.13-5.93, *P*=0.002). In PTBD, similarly, there were more patients with the AA genotype (27.3% *vs* 7.5% of HCS) or AG (36.4% *vs* 35.0% HCS), and fewer patients with GG (36.4% *vs* 57.5% HCS). The gene frequencies of A were also significantly higher in PTBD than in HCS (45.5% vs 25%). These results also showed a significant association of A allele of *CTLA-4* +6230 with pulmonary TB susceptibility (*P*=0.0439, OR=2.5, 95% CI=1.004-6.277).

Association of combination of CTLA-4 exon 1 position 49 A/G and CTLA-4 3'UTR +6230 A/G polymorphism with pulmonary TB susceptibility

Distribution of *CTLA-4*+49A/G**/**+6230A/G genotype combination frequencies between pulmonary tuberculosis (PTB) patients and healthy control subjects (HCS) is shown in Table 3. In PTB, there were more patients with the +49AA/+6230AA genotype (15.22% *vs* 7.59% of HCS) or +49AG/+6230AG (41.3% *vs* 30.38% HCS), and fewer patients with +49GG/+6230GG (21.74% *vs* 37.97% HCS). It is likely that there was an association of +49GG/+6230GG genotype of *CTLA-4*+49/+6230 with a lower risk of pulmonary TB, however

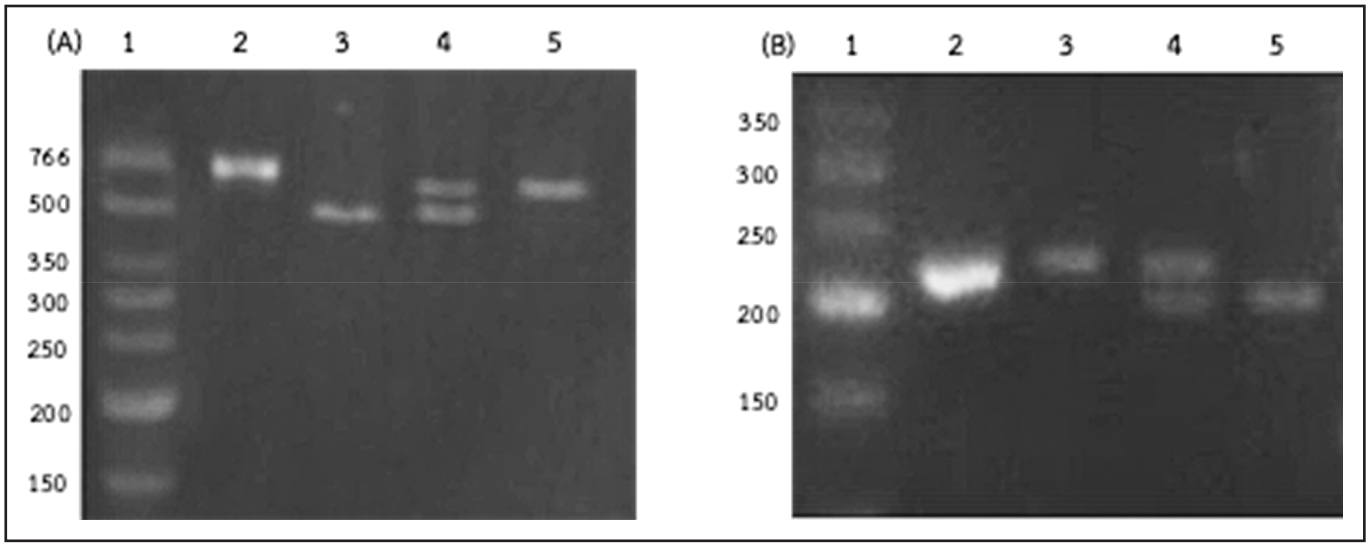

Figure 1. Representative genotypes on a 2.5% agarose gel electrophoresis.
(A) **CTLA-4 +49A/G polymorphism**: lane 1, molecular size marker (bp); lane 2, sample with undigested PCR product; lanes 3, 4, and 5, samples with GG, AG, and AA genotypes, respectively.
(B) **CTLA-4 +6230A/G polymorphism**: lane 1, molecular size marker (bp); lane 2, sample with undigested PCR product; lanes 3, 4, and 5, samples with GG, AG, and AA genotypes, respectively.

Table 1. Distribution of *CTLA-4*+49A/G genotype and allele frequencies in pulmonary TB (PTB) patients, pulmonary TB without comorbidities (PTBWO), pulmonary tuberculosis with comorbidity (PTBD) and healthy control subjects (HCS)

SNP	Genotype	PTB 50	PTBWO 38	PTBD 12	HCS 87	PTB vs HCS	PTBWO vs HCS	PTBD vs HCS
		N (%)	N (%)	N (%)	N (%)	*P* value OR (95% CI)	*P* value OR (95% CI)	*P* value OR (95% CI)
+49 A/G(rs231775)	GG (Ref)	16 (32)	12 (31.6)	4 (33.3)	34 (39.08)	1	1	1
	AG	25 (50)	20 (52.6)	5 (41.7)	44 (50.57)	0.6316 1.207 (0.5585–2.610)	0.5565 1.288 (0.5536–2.996)	NA
	AA	9 (18)	6 (15.8)	3 (25)	9 (10.34)	0.1744 2.125 (0.7082–6.376)	0.3049 1.889 (0.5548–6.431)	NA
HWE P-value		0.841	0.823	1	0.543			
Allele model	G (Ref)	57 (57)	44 (57.9)	13 (54.2)	112 (64.37)	1	1	1
	A	43 (43)	32 (42.1)	11 (45.8)	62 (35. 63)	0.2272 1.363 (0.8240–2.254)	0.3311 1.314 (0.7571–2.280)	0.3315 1.529 (0.6462–3.616)
Dominant model	GG (Ref)	16 (32)	12 (31.6)	4 (33.3)	34 (39.08)	1	1	1
	AG+AA	34 (68)	26 (68.42)	8 (66.67)	53 (60.92)	0.4072 1.363 (0.6543–2.840)	0.4237 1.39 (0.6193–3.120)	NA
Recessive model	AA (Ref)	9 (18)	6 (15.8)	3 (25)	9 (10.34)	1	1	1
	AG+GG	41 (82)	32 (84.21)	9 (75)	78 (89.66)	0.2017 0.5256 (0.1937–1.427)	0.3889 0.6154 (0.2024–1.871)	NA
Over-dominant model	AG (Ref)	25 (50)	20 (52.6)	5 (41.7)	44 (50.57)	1	1	1
	GG+AA	25 (50)	18 (47.37)	7 (58.33)	43 (49.43)	1 1.0233 (0.5103–2.0517)	0.8414 0.9209 (0.4294–1.9751)	0.5656 1.4326 (0.422–4.863)

Table 2. Distribution of *CTLA-4*+6230A/G genotype and allele frequencies in pulmonary TB (PTB) patients, pulmonary TB without comorbidities (PTBWO), pulmonary tuberculosis with comorbidity (PTBD) and healthy control subjects (HCS)

SNP	Genotype	PTB 46	PTBWO 35	PTBD 11	HCS 80	PTB vs HCS	PTBWO vs HCS	PTBD vs HCS
		N (%)	N (%)	N (%)	N (%)	*P* value OR (95% CI)	*P* value OR (95% CI)	*P* value OR (95% CI)
+6230 A/G (rs3087243)	GG (Ref)	16 (34.78)	12 (34.3)	4 (36.4)	46 (57.5)	1	1	1
	AG	22 (47.83)	18 (51.4)	4 (36.4)	28 (35)	0.0432 2.259 (1.018–5.014)	0.0392 2.464 (1.034–5.874)	NA
	AA	8 (17.39)	5 (14.3)	3 (27.3)	6 (7.5)	0.0227 3.833 (1.152–12.75)	0.0805 3.194 (0.8310–12.28)	NA
HWE P-value		1	0.806	0.779	0.740			
Allele model	G (Ref)	54 (58.70)	42 (60)	12 (54.5)	120 (75)	1	1	1
	A	38 (41.30)	28 (40)	10 (45.5)	40 (25)	0.007 2.111 (1.220–3.652)	0.0218 2 (1.100–3.635)	0.0439 2.5 (1.004–6.227)
Dominant model	GG (Ref)	16 (34.78)	12 (34.3)	4 (36.4)	46 (57.5)	1	1	1
	AG+AA	30 (65.21)	23 (65.72)	7 (63.64)	34 (42.5)	0.0141 2.537 (1.196–5.379)	0.0022 2.593 (1.134–5.929)	NA
Recessive model	AA (Ref)	8 (17.39)	5 (14.3)	3 (27.3)	6 (7.5)	1	1	1
	AG+GG	38 (82.61)	30 (85.71)	8 (72.72)	74 (92.5)	0.089 0.3851 (0.1246–1.191)	0.2549 0.4865 (0.1379–1.716)	NA
Overdominant model	AG (Ref)	22 (47.83)	18 (51.4)	4 (36.4)	28 (35)	1	1	1
	GG+AA	24 (52.17)	17 (48.57)	7 (63.64)	52 (65)	0.1562 0.5874 (0.2806–1.2296)	0.0978 0.5085 0.227-1.1392)	NA

Table 3. Distribution of *CTLA-4*+49A/G/*CTLA-4*+6230A/G genotype combination frequencies in pulmonary TB (PTB) patients, pulmonary TB without comorbidities (PTBWO), pulmonary tuberculosis with comorbidity (PTBD) and healthy control subjects (HCS)

CTLA-4+49/ +6230 Genotype combinations	PTB 46	PTBWO 35	HCS 79	PTB vs HCS	PTBWO vs HCS
	N (%)	N (%)	N (%)	*P* value OR (95% CI)	*P* value OR (95% CI)
GG/GG (Ref)	10 (21.74)	7 (20)	30 (37.97)	1	1
GG/AG	2 (4.35)	1 (2.86)	0 (0)	NA	NA
GG/AA	1 (1.27)	1 (2.86)	0 (0)	NA	NA
AG/GG	5 (10.87)	4 (11.43)	16 (20.25)	0.920344 1.0667 (0.3108–3.6608)	NA
AG/AG	19 (41.3)	16 (45.71)	24 (30.38)	0.066798 0.4211 (0.1653–1.0727)	0.043394 0.35 (0.124-0.988)
AG/AA	0 (0)	0 (0)	0 (0)	NA	NA
AA/GG	1 (2.17)	1 (2.86)	0 (0)	NA	NA
AA/AG	1 (2.17)	1 (2.86)	3 (3.80)	NA	NA
AA/AA	7 (15.22)	4 (11.43)	6 (7.59)	0.0858 0.2857 (0.0775–1.0529)	NA
Other combination	36 (78.26)	28 (80)	49 (62.03)	0.0606 0.4537 (0.1968–1.0458)	0.0588 0.4083 (0.1588–1.0502)
AG/GG (Ref)	5 (10.87)	4 (11.43)	16 (20.25)	1	1
Other combination	41 (89.13)	31 (88.57)	63 (79.75)	0.1761 0.4802 (0.1633–1.4118)	NA
AG/AG (Ref)	19 (41.3)	16 (45.71)	24 (30.38)	1	1
Other combination	27 (58.70)	19 (54.29)	55 (69.62)	0.2146 1.6127 (0.7558–3.441)	0.1138 1.9298 (0.8501–4.3811)
AA/AA (Ref)	7 (15.22)	4 (11.43)	6 (7.59)	1	1
Other combination	39 (84.78)	31 (88.57)	73 (92.41)	0.2269 2.1838 (0.6862–6.9498)	NA

this was not statistically significant (*P*=0.066, OR=0.4211, 95% CI=0.1653-1.072; *P*=0.0858, OR=0.2857, 95% CI=0.0775-1.0529; *P*= 0.0606, OR=0.4537, 95% CI=0.1968-1.0458, respectively when compared +49GG/+6230GG to +49AG/+6230AG, +49AA/+6230AA, and other combination genotype). In PTBWO, there were more patients with the +49AA/+6230AA genotype (11.43% *vs* 7.59% of HCS) or +49AG/+6230AG (45.71% *vs* 30.38% HCS), and fewer patients with +49GG/+6230GG (20.0% *vs* 37.97% HCS) (*P*<0.1). There was a statistically significant association of +49GG/+6230GG genotype of *CTLA-4*+49/+6230 with pulmonary TB low risk when compared +49GG/+6230GG to the +49AG/+6230AG genotype (*P*=0.0433, OR=0.35, 95% CI= 0.124-0.988).

DISCUSSION

Tuberculosis (TB) has affected humanity and is associated with immunosuppression (Besen *et al*., 2011). In this study, the T-cell inhibitory molecule expressed by *CTLA-4* gene variants were determined in patients infected with pulmonary tuberculosis (PTB) who were serologically HIV-negative. The results showed that *CTLA-4* +49A/G, +6230A/G may play a role in influencing tuberculosis susceptibility.

Functional polymorphisms of *CTLA-4* have been reported to be implicated in the pathogenesis of many diseases. A prior study in Taiwanese women and Southern Han Chinese patients showed that the *CTLA-4* +49AG genotype may reduce the risk of being infected with pulmonary TB (Wang *et al*., 2012; Liu *et al*., 2024). Conversely, +49GG genotype in Iranians were shown to be associated with an increased risk of TB, but the +49GG genotype in Iraqis may decrease risk of pulmonary TB (Paad *et al*., 2014; Enzi *et al*., 2020). In contrast our results showed that there were no statistically significant differences of allele and genotype frequencies as well as dominant, recessive, and over-dominant models when comparing between PTB and HCS, between PTBWO and HCS as well as between PTBD and HCS. Despite, the genotype AA and allele A frequencies were higher while genotype GG and allele G frequencies were fewer in PTB, PTBWO, and PTBD patients than in HCS. (Table 1). Thus, *CTLA-4* +49A in this study might play a role in susceptibility to PTB. Early findings in meta-analysis studies in patients with Hepatitis B (HBV) infection suggested that A at position +49 of the *CTLA4* gene may significantly increase the risk of persistent HBV infection, whereas G at position +49 may positively influence virus clearance (Thio *et al*., 2004; Xu *et al*., 2013).

This study showed a significant association of *CTLA-4* +6230AG and *CTLA-4* +6230AA with pulmonary TB susceptibility. When the allele frequencies were analyzed, a significantly higher number of patients possessing +6230A than G allele was observed among all three groups of pulmonary TB patients when compared to HCS. Furthermore, in the dominant model analysis, patients presenting with at least one A (GA or AA genotype) showed a significantly higher frequency of having TB than patients with GG genotype (Table 2). Accordingly, an earlier study in the USA showed that *CTLA-4*+6230A was associated with HBV persistence (Thio *et al*., 2004). Another Swedish study revealed that *CTLA-4*+6230A was

associated with increased colorectal cancer risk (Van Nguyen *et al*., 2021). On the other hand, a study in a Ghanaian population showed a significant influence of the +6230G variant on severe pathology in pulmonary TB (Thye *et al*., 2009). *CTLA-4*+6230 representing the substitution of A to G has been reported to correlate with lower mRNA levels of soluble alternative splicing forms of CTLA-4, leading to the augmentation of T-cell activation and proliferation and may further reduce *Mycobacterium* growth (Ueda *et al*., 2003). Contrarily, the over-activation of T-cells may lead to the increased level of TB pathogenesis as shown in Ghanaian population. Alternatively, *CTLA-4*+6230A exhibits CTLA-4-mediated inhibition of T-cell responses, which may render the immune system unable in clearing the bacterial infection (Walunas *et al*., 1994). Our study suggested that individuals carrying *CTLA-4*+6230A may increase their susceptibility in developing TB, whereas individuals carrying *CTLA-4*+6230G may show a decrease in risk.

As the *CTLA4*+49 and *CTLA4*+6230 SNPs have been shown to be in strong linkage disequilibrium (Amundsen *et al*., 2004; Munthe-Kaas *et al*., 2004), the combinations of *CTLA-4*+49A/G and *CTLA-4*+6230A/G genotypes were explored. There were more patients carrying the +49AA/+6230AA genotype or +49AG/+6230AG and fewer patients carrying +49GG/+6230GG in both PTB and PTBWO groups when compared with HCS. There was a noticeable but insignificant association of +49GG/+6230GG genotype of *CTLA-4* with decreased susceptibility to pulmonary TB (P<0.1) (Table 3). In contrast to a study in south Han Chinese, the GGA haplotype of *CTLA4*+49A/G (rs231775), +623A/G (rs3087243), and 11430G/A (rs11571319) was associated with the pathogenesis of pulmonary TB while the haplotype AGG was associated with healthy controls (Wang *et al*., 2012). The functional of the protein expressed via the translation of *CTLA-4*+49A/G is similar to that shown by *CTLA-4*+6230A/G polymorphism, in which the change of +49A for allele G causes threonine to alanine conversion in the CTLA-4 protein. This results in inefficient CTLA-4 glycosylation, reduced cell surface expression and decease affinity of CTLA-4 for B7-1 binding, which in turn leads to a lower inhibition of T- lymphocyte activity (Amundsen *et al*., 2004; Munthe-Kaas *et al*., 2004). The lower frequency of GG genotype of *CTLA-4*+49A/G polymorphism in PTB. PTBWO, PTBD patients compared to HCS showed in this study was consistent with a study in an Iraqis population, which revealed that the +49GG genotype may decrease while +49AA may increase the risk of pulmonary TB (Enzi *et al*., 2020). Therefore, these results suggested that the combination of +49GG/+6230GG may enhance T-cell activation and further reduction of bacterium growth, while the combination +49AA/+6230AA or +49AG/+6230AG may cause an opposite effect.

In the fifty pulmonary TB patients who participated in this study, 38 patients did not have medical records of comorbidities (PTBWO) while 12 were comorbid with diseases (PTBD) such as diabetes mellitus, asthma, or are being treated on a regimen of corticosteroids. The comorbidity may result in inefficient immune responses and may alter the efficiency in damage to the Mycobacterium (Dooley & Chaisson, 2009). However, when comparing *CTLA-4*+49A/G, +6230A/G genotype, allele, dominant, recessive, and overdominant models as well as the combination of these two genotypes between the PTB and HCS groups, between PTBWO and HCS groups as well as between PTBD and HCS groups, similar associations were observed. These results may imply that in terms of *CTLA-4*+49A/G+6230A/G polymorphisms, the comorbidities with other diseases do not influence the development of TB susceptibility. Due to the low number of sample sizes, neither sex nor severity dependent association were determined. The difference of the results in this study from the previous reports may due to the different interaction and combination of different base changes at several sites of several genes, which lead to alteration of pulmonary TB susceptibility in various ethnic groups (Delgado *et al*., 2002).

In conclusion, this study demonstrated that the *CTLA-4* +49A/G and +6230A/G polymorphisms were participated with altered pulmonary TB susceptibility. Individuals carrying the *CTLA-4*+6230A allele may have an increased risk of developing pulmonary TB, while those carrying the *CTLA-4*+6230G allele may have a decreased risk. Additionally, the combination of +49AA/+6230AA or +49AG/+6230AG may further increase susceptibility, whereas the *CTLA-4* +49GG/+6230GG genotype may significantly reduce susceptibility to pulmonary TB. These findings suggested that *CTLA-4* gene variants may influence susceptibility to pulmonary TB by regulating CTLA-4 expression and further regulating T-lymphocyte responses. This is the first report that shows a significant association of *CTLA-4* gene polymorphism with pulmonary TB susceptibility in Thai population, especially *CTLA-4*+6230A/G. Therefore, *CTLA-4*+6230A/G gene polymorphisms could serve as a prognostic marker for Thai patients who may be susceptible in developing pulmonary TB. However, further confirmation and validation of these results require a larger study cohort.

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DISCLOSURE

The authors declare having no conflict of interest.

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