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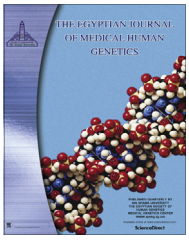

ORIGINAL ARTICLE

Association of −308G/A TNF-α gene polymorphism and spontaneous preterm birth in Acehnese ethnic group, Indonesia: This polymorphism is not associated with preterm birth

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Abstract *Background:* Single nucleotide polymorphism (SNP) within tumor necrosis factor alpha (TNF-α) gene promoter (−308G/A *TNFA*) is associated with higher gene expression. The role of this SNP as a risk factor for spontaneous preterm birth has been assessed in some regions and the findings were significantly different between race and ethnic groups.

Aim: To provide the scientific evidence whether allele A within SNP −308G/A *TNFA* promoter is a risk factor for spontaneous preterm birth among Acehnese ethnic or not.

Subjects and methods: In this case–control study, the genotypes of SNP −308G/A *TNFA* among 40 patients with spontaneous preterm birth and 40 patients with term birth were determined by real-time polymerase chain reaction (RT-PCR). The concentrations of TNF-α from blood were measured by enzyme-linked immunosorbent assay (ELISA). The differences in genotype distributions, dominant and recessive models, and allele frequencies between case and control groups were analyzed with Chi-squared test. Deviation of genotype frequencies from the Hardy–Weinberg equilibrium (HWE) was assessed by Fisher's exact test.

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Results: This study found that the concentration of TNF-α between preterm and control groups was not statistically different, 5.5 ± 2.9 mg/dL *vs.* 10.1 ± 17.9 mg/dL, $p = 0.112$. The level of TNF-α had no strong association with either genotype distribution or allele frequency of SNP −308G/A *TNFA*. Furthermore, there was no association between mutant genotypes and spontaneous preterm birth (OR: 0.32; 95%CI: 0.08–1.33, $p = 0.096$) and between mutant allele and spontaneous preterm birth (OR: 0.35; 95%CI: 0.09–1.37, $p = 0.105$).

Conclusion: SNP −308G/A *TNFA* is not associated with spontaneous preterm birth in Acehnese ethnic group.

1. Introduction

The preterm birth is defined as birth that occurs before 37 weeks of pregnancy [1]. The prevalence of preterm birth ranges from 5% in high income countries to 25% in low income countries [1]. Preterm birth is associated with massive economic burden and causes emotional distress to the parents [2]. Approximately 30% of preterm birth are caused by maternal or fetal complications such as preeclampsia or intrauterine growth restriction whereas 70% of them caused by spontaneous preterm labor [3].

Preterm is associated with multiple mechanisms of disease that could switch of the myometrium from a quiescent to a contractile state, mostly is accompanied by a shift in signaling from anti-inflammatory to pro-inflammatory pathways [4]. Increased expression of pro-inflammatory cytokines including tumor necrosis factor alpha (TNF-α), one of the most potent pro-inflammatory cytokines, and chemokines increases activity of proteases leading to extracellular matrix components dissolution [4].

TNF-α, a key pro-inflammatory cytokine, is an important regulatory molecule during pregnancy and its production is regulated both at the transcriptional and posttranscriptional levels [5]. Several single nucleotide polymorphisms (SNPs) have been identified within TNF-α gene (*TNFA*) [5] and one of the most intensively studied is SNP −308G/A that is located in *TNFA* promoter. In this SNP, homozygote AA and heterozygote GA are associated with increased *TNFA* gene expression compared to homozygous GG [6]. Several studies have been conducted to assess the association between this SNP and spontaneous preterm birth [7–11]. However, data revealed that the associations of SNP −308G/A *TNFA* and spontaneous preterm birth (and TNF-α level) differ significantly between race and ethnic groups [9,12,13]. Therefore, studies of the role of this SNP on spontaneous preterm birth in other race and ethnic groups are need. The objective of this present study was to determine the association between SNP −308G/A *TNFA* and spontaneous preterm birth among the Acehnese ethnic group.

2. Subjects and methods

2.1. Study setting

The approach of this case-control study was adopted from a previous study that was published by our group [14]. The cases were women with spontaneous preterm birth who came for treatment at Dr. Zainoel Abidin, Banda Aceh, Indonesia. Preterm birth cases were diagnosed using the criteria from The American Congress of Obstetricians and Gynecologists (ACOG). The controls were women with term birth based on ACOG criteria who were age-, and race-matched and not related to the cases. The preterm birth is defined as the birth occurring between 20 and 37 weeks of pregnancy, whereas term birth is defined as the birth after 37 weeks of gestation. Patients aged over 50 years and patients who had preeclampsia, signs and symptoms of infection, autoimmune disease, obesity, pregnancy with intrauterine growth restriction, twin baby, placental previa, fetal anomalies, gestational diabetes, poly- and oligohydramnios were excluded.

At the admission time, clinical signs and symptoms and gynecology conditions were assessed and venous blood samples were collected under strictly standardized conditions and stored at −80 °C before being used. This study has been approved by the Ethics Committee of School of Medicine, Syiah Kuala University, Banda Aceh, Indonesia, No. 111/KE/FK/2012. The subject recruitment and sample collection were done only after obtaining written informed consent of the participants. The work was carried out in accordance with The Code of the World Medical Association (Declaration of Helsinki) for experiments involving humans. This study was conducted from 1 June 2012 to 30 July 2014.

2.2. Immunologic assay of TNF-α

The concentrations of TNF-α from blood sera were measured with a solid phase sandwich enzyme-linked immunosorbent assay (ELISA) using microtitre plates from Human TNF-alpha Quantikine ELISA Kit (R&D Systems, USA) per manufacture protocol. The ELISA has detection range 7.8–1.000 pg/mL and sensitivity 1.6 pg/mL.

2.3. Deoxyribonucleic acid (DNA) extraction

DNA isolation and real-time polymerase chain reaction (RT-PCR) were conducted in Prodia Laboratory, Jakarta. For DNA extraction purpose, 5 mL peripheral venous blood was collected from 80 subjects (40 cases and 40 controls). DNA was extracted from whole blood using the salting-out method as described previously [15]. DNA extraction and purification were completely performed according to high pure PCR template preparation kit instructions (Roche Applied Biosystem, USA).

2.4. SNP −308G/A TNFA genotyping

Genotyping of SNP −308G/A *TNFA* was performed by using a RT-PCR with specific fluorescence-labeled hybridization

probes that were published previously [16]. Briefly, to amplify gene target, the primers (sense, 5′-AAGGAAACAGACCAC AGACCTG; antisense, 5′-GGTCTTCTGGGCCACTGAC) were used at 0.5 μmol/liter. In addition, the detection probe specific for the G allele (5′-AACCCCGTCCCCATGCC) and the anchor probe (5′-CAAAACCTATTGCCTCCATTTCTT TTGGGGAC) were used at 0.2 μmol/liter. Amplification was performed with 60 cycles (denaturation at 95 °C for 1 min, annealing at 57 °C for 15 s, extension at 72 °C for 10 s) in a total 10-μL PCR mixture containing 1 μL of reaction buffer. PCR reagents from LightCyclerFastStart DNA MasterPLUSHybProbe (Roche Applied Biosystem, USA) were used. The thermocycler was a Light-Cycler 2.0 (Roche Applied Biosystem, USA). Finally, the genotypes of each sample were classified as AA, GA, and GG according to the derivative melting curves [16].

2.5. Statistical analysis

The data were analyzed using methods that have been published previously [17,18]. Collectively, demographic data and the level of TNF-α between case and control groups were analyzed with Anova or Student's *t*-test as appropriate with data. To assess the association between SNP −308G/A *TNFA* and the incidence of spontaneous preterm birth, the differences in genotype distributions, dominant and recessive models, and allele frequencies between case and control groups were analyzed with Chi-squared test. Deviation of the genotype frequencies from the Hardy–Weinberg equilibrium (HWE) was assessed by Fisher's exact test. Two sided of testing was used for all comparisons and $p < 0.05$ was considered as significant. The data were analyzed using Statistical Package for the Social Sciences (SPSS for Windows, Ver. 15, Chicago, IL).

3. Results

3.1. Sample characteristics and TNF-α concentration

A total of 80 participants, 40 cases (mean age was 30.6 ± 4.9 years old) and 40 controls (mean age was 29.9 ± 4.2 years old) were enrolled in this study. This study found that there was no significant difference for age between case and control groups (Table 1). In addition, there was no significant difference between the level of TNF-α in spontaneous preterm birth and the control group, 5.5 ± 2.9 mg/dL and 7.5 ± 7.1 mg/dL, respectively $p = 0.112$ (Fig. 1).

Table 1 Sample characteristics of cases ($n = 40$) and controls ($n = 40$).

Characteristic	Group		*p*-Value
	Preterm, *n* (%)	Term, *n* (%)	
Age (year ± SD)*	30.6 ± 4.9	29.9 ± 4.2	0.448
Age group**			0.941
20–25 year	6 (15.0)	7 (17.5)	
26–30 year	14 (35.0)	16 (40.0)	
31–35 year	15 (37.5)	14 (35.0)	
36–40 year	3 (7.5)	2 (5.0)	
41–45 year	2 (5)	1 (2.5)	

SD: standard deviation.
* *p*-Value measured by Student *t*-test analysis.
** *p*-Value measured by Anova test.

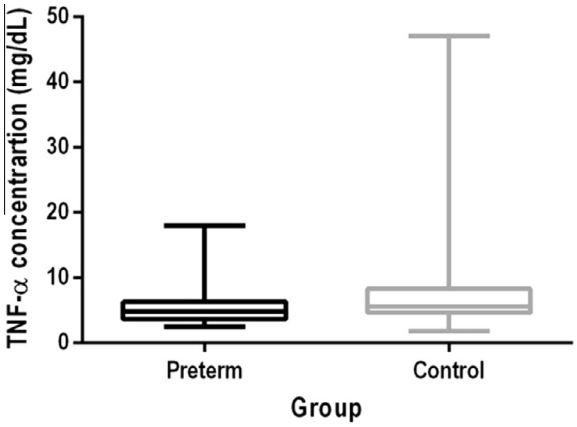

Figure 1 The comparison of TNF-α level between spontaneous preterm birth and term birth group.

Table 2 The association of SNP −308G/A *TNFA* and TNF-α level ($n = 80$).

Genotype and allele	Frequency, *n* (%)	TNF-α level (mean ± SD, mg/dL)	*p*-Value*
Genotype			0.612
GG	69 (86.2)	8.1 ± 13.9	
GA	11 (13.8)	5.9 ± 2.7	
AA	0	–	
Dominant model			0.612
GA + AA	11 (13.8)	5.9 ± 2.7	
GG	69 (86.2)	8.1 ± 13.9	
Allele			0.105
G	149 (93.1)	5.9 ± 2.7	
A	11 (6.9)	7.9 ± 13.4	

SD: standard deviation.
* *p*-Value measured by Anova test.

Table 3 The association of SNP −308G/A *TNFA* and spontaneous preterm birth.

Genotype and allele	Group		OR (CI 95%)	*p*-Value*
	Preterm, *n* (%)	Term, *n* (%)		
Genotype			0.32 (0.08–1.33)	0.096
GG	37 (92.5)	32 (80)		
GA	3 (7.5)	8 (20)		
AA	0	0		
Dominant model			0.32 (0.08–1.33)	0.096
GA + AA	3 (7.5)	8 (20)		
GG	37 (92.5)	32 (80)		
Allele			0.35 (0.09–1.37)	0.105
A	3 (3.7)	8 (10)		
G	77 (96.3)	72 (90)		

OR: odd ratio.
* *p*-value measured by Chi-squared test.

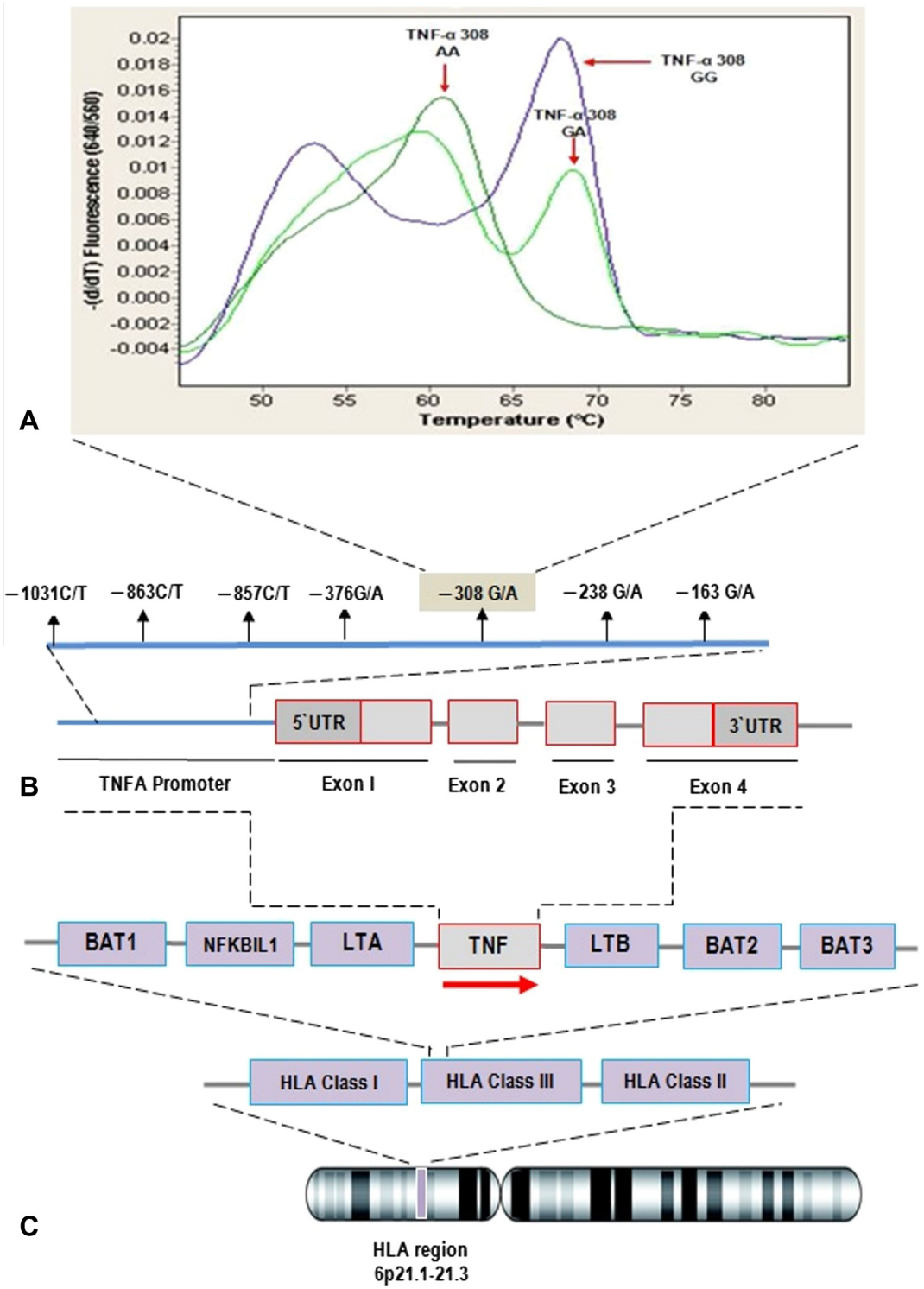

Figure 2 (A) The melting curve of RT-PCR of SNP −308G/A *TNFA*. The melting temperature for GG homozygote is 68 °C, GA heterozygote is 68 °C and AA homozygote is 59 °C. (B) The structure of *TNFA* and the location of SNP −308G/A *TNFA*. *TNFA* consists of four exons: exon 1 (338 bp) encodes the 5′ untranslated region (5′UTR) and a majority of the signal sequence, exon 2 (46 bp) encodes the rest of the signal sequence, exon 3 (48 bp) encodes the mature TNF-α protein and exon 4 (1210 bp) encodes the mature TNF-α protein and the 3′UTR. SNP −308G/A *TNFA* is located on *TNFA* promoter [19]. (C) *TNFA*, flanked by lymphotoxin alpha (LTA) and lymphotoxin beta (LTB), is encoded in the region of the human major histocompatibility complex (HLA) class III on chromosome 6 (6p21.3). The red arrow indicates gene orientation.

Table 4 The list of studies that conducted to assess the association between SNP −308G/A *TNFA* and spontaneous preterm birth.

Year	Analysis	Ethnic/country	Sample (case/control)	Association	Reference
1997	Maternal	Not mentioned/USA	203/41	No association	[32]
1997	Fetal	Not mentioned/USA	44/41	No association	[32]
1999	Maternal	African American/USA [a]	55/110	No association	[23]
1999	Maternal	African American/USA [b]	26/110	OR: 3.1 (1.33–7.83), $p = 0.008$ [GA + AA *vs.* GG] OR: 2.1 (1.04–4.27), $p = 0.03$ [G *vs.* A]	[23]
2001	Fetal	African/Kenya	34/961	RR: 7.3 (2.85–18.90), $p = 0.002$ [GG *vs. AA*] RR: 6.7 (2.00–23.00), $p = 0.008$ [*AG vs. AA*]	[25]
2003	Maternal	Asian/China	54/79	OR: 12.1 (1.14–128.75), $p = 0.03$ [GG *vs.* AA]	[26]
2004	Maternal	African-American & Caucasian/USA [c]	125/250	OR: 2.7 (1.7–4.5) [G *vs.* A]	[13]
2004	Maternal	Caucasian/Australia	202/185	No association	[33]
2004	Maternal	Mixed population/UK	48/82	No association OR: 3.9 (1.38–11.05), $p = 0.004$ [G *vs.* A][†]	[24]
2006	Maternal	Mexican/Mexico	86/174	No association	[34]
2006	Maternal	African-American/USA	49/224	No association	[9]
2006	Fetal	African-American/USA	45/222	No association	[9]
2006	Maternal	Euro-American/USA	78/184	No association	[9]
2006	Fetal	Euro-American/USA	86/191	No association	[9]
2006	Maternal	African-American & Caucasian/USA	79/80	No association	[35]
2006	Fetal	African-American & Caucasian/USA	78/77	No association	[35]
2007	Fetal	Caucasian/Italy [d]	176/164	No association	[7]
2007	Fetal	Caucasian/Italy [e]	112/164	No association	[7]
2007	Fetal	Caucasian/Italy [f]	64 /164	No association	[7]
2007	Maternal	Caucasian/Austria [g]	30/1384	No association	[11]
2007	Maternal	Caucasian/Austria [h]	80/1384	No association	[11]
2009	Maternal	Mulatto/Brazil	113/97	No association	[10]
2009	Maternal	White & Mulatto/Brazil	79/104	No association	[10]
2010	Maternal	Asian/China	250/247	No association	[36]
2010	Fetal	Asian/China	250/247	No association	[36]
2010	Maternal	African American/USA[c]	81/366	No association	[8]
2010	Maternal	Non-Hispanic White/USA[c]	149/411	No association	[8]
2010	Maternal	Caucasian/Poland	32/63	No association	[27]
2011	Maternal	Mixed/USA [j]	33/830	OR: 7.5 (1.84–30.72), $p = 0.005$ [AG + GG *vs.* AA]	[37]
2012	Maternal	Caucasian/Austria [k]	76/197	No association	[30]
2012	Fetal	Caucasian/Austria [k]	74/198	No association	[30]
2012	Maternal	Caucasian/Austria [l]	29/197	No association	[30]
2012	Fetal	Caucasian/Austria [l]	36/198	No association	[30]
2013	Maternal	Arab/Iran	64/71	No association	[31]
2013	Fetal	Caucasian/Australia [m]	46/582	No association	[29]
2013	Fetal	Caucasian/Australia [n]	120/582	No association	[29]
2013	Fetal	Caucasian/Australia [o]	166/582	No association	[29]
2014	Maternal	African/South Africa	34/58	No association	[38]
2014	Maternal	Caucasian/Poland	150/150	No association	[39]
2015	Maternal	Asian/Indonesia	40/40	No association	This study

OR: odd ratio.
RR: relative risk.
[a] Idiopathic preterm and preterm with rupture of membrane.
[b] Preterm with rupture of membrane only.
[c] Preterm with and without bacterial vaginosis.
[d] Preterm with and without respiratory distress syndrome.
[e] Preterm without respiratory distress syndrome.
[f] Preterm with respiratory distress syndrome.
[g] Preterm < 34 weeks.
[h] Preterm > 34 weeks.
[k] Preterm without periventricular leucomalacia.
[l] Preterm with periventricular leucomalacia.
[m] Preterm without cerebral palsy.
[n] Preterm with cerebral palsy.
[o] Preterm with and without cerebral palsy.
[j] Preterm < 28 weeks.
[†] Preterm among non-smoking.

3.2. Hardy–Weinberg equilibrium (HWE) analysis

Global minor allele frequency of SNP −308G/A *TNFA* is 0.0903 (based on NCBI data). Based on HWE calculation, the genotype distribution among cases and overall sample was consistent within HWE, with $p = 0.80$ and $p = 0.51$, respectively. It indicates that the genotype distribution of sample from this study had no HWE deviation.

3.3. Association of SNP −308G/A TNFA and TNF-α concentration

Within total study samples (cases and controls), the level of TNF-α between individuals with G and A allele was not significantly different (5.9 ± 2.7 mg/dL *vs.* 7.9 ± 13.4 mg/dL, $p = 0.105$) (Table 2). In addition, there is no association between SNP −308G/A *TNFA* and TNF-α level in genotype distribution and dominant model.

3.4. Association of SNP −308G/A TNFA and preterm birth

This study found that there was no significant difference in the genotype distributions and allele frequencies of SNP −308G/A *TNFA* between case and control groups (Table 3). There was no association between mutant genotype (GA and AA) and spontaneous preterm birth (OR: 0.32; 95%CI: 0.08–1.33, $p = 0.096$) and between mutant allele (A allele) and preterm birth (OR: 0.35; 95%CI: 0.09–1.37, $p = 0.105$).

4. Discussion

TNFA is located on the region of the human major histocompatibility complex (HLA) class III and consisting four exons (Fig. 2) [19]. SNP −308G/A *TNFA* is located on *TNFA* promoter and the genotypes of this SNP were determined by RT-PCR using specific fluorescence-label probe and melting curve (Fig. 2). AA and GA genotype of SNP −308 *TNFA* have higher gene expression rate compared to GG [6]. Therefore, in theory, individual with one of those genotypes will produce higher level of TNF-α cytokine.

It is clear that cytokines, including TNF-α, are important during pregnancy and preterm labor [20]. An animal study demonstrated that the increased TNF-α was an important factor responsible for preterm birth [21]. In humans, the concentration of TNF-α in amniotic fluid concentration was associated with preterm birth among African-American race but not Caucasian [12]. In this present study, we found that there was no association between TNF-α concentration from serum and spontaneous preterm birth. Interestingly, a large study including 101,042 Danish women also revealed that there was no association between an elevated of TNF-α from serum during mid-pregnancy and spontaneous preterm birth [22].

Several studies have been conducted to assess the role of SNP −308G/A *TNFA* as a risk factor for spontaneous preterm birth (Table 4). In short, it indicates that single SNP −308G/A *TNFA* has no strong association with spontaneous preterm birth. Previously, stratified analysis has been applied to find robust association. For example, a study found that the association exists between SNP −308G/A *TNFA* and preterm birth with rupture of membrane, but the association did not exist if the analysis included both preterm birth with rupture of membrane and idiopathic preterm [23]. In the same scenario, another study found an association between this SNP with spontaneous preterm birth among non-smoking women, but there was no association among both smoking and non-smoking women [24]. The association of this SNP with spontaneous preterm birth was also different between women with and without bacterial vaginosis [13]. It might indicate that the role of SNP −308G/A *TNFA* is easily modified by certain environmental factors.

However, it should be noted that from 1997 to 2015, only 6 out of 23 studies revealed positive association between SNP −308G/A *TNFA* and preterm birth [13,23–27] (Table 4). Some of these studies found that the association exist within specific structures only such as among non-smoking women [24], among preterm birth with rupture of membrane [23] and when using < 28 weeks as a preterm cut point instead of 37 weeks [27]. In addition, meta-analysis study in 2006 found that there was no strong association between SNP −308G/A *TNFA* and preterm birth (OR: 1.41; 95%CI: 0.90–2.19) [28] and these data were supported by another meta-analysis that was published in 2013 (OR: 0.82; 95%CI: 0.65–1.03) [29]. Taking together, these data indicate that there is no strong association between SNP −308G/A *TNFA* and spontaneous preterm birth.

There are some limitations of our present study. The number of cases and the controls was relatively small. In addition, this study did not obtain prospective data examining the effect of SNP −308G/A *TNFA* on spontaneous preterm birth. However, cross-sectional study is an acceptable study design to determine the risk factor or protective effect of particular SNP with any disease.

There are some recommendations that should be followed when assessing the association between SNP and particular disease. First, the investigator should include the race or ethnic group of the study sample. Previous studies found that the association of a particular SNP was associated with race or ethnicity [9,40,41]. Second, to assess the association, the odd ratio (OR) and 95% of confidence interval (95%CI) with *p*-value should be provided as some previous studies expressed the association in *p*-value only [7,9,30,31]. In addition, at least, the association of the genotype distributions, dominant model and allele frequency to particular interested disease should be revealed. Finally, the deviation of sample genotype frequencies from the Hardy–Weinberg equilibrium (HWE) should be calculated.

5. Conclusion

This study demonstrated that the levels of TNF-α are not significantly different between women with spontaneous preterm birth and term birth. In addition, there is no association between the levels of TNF-α among women (spontaneous preterm birth and term birth) and G/A transition at position −308 of *TNFA* promoter. Finally, there is no significant association between genotype distributions and allele frequency of SNP −308G/A *TNFA* and spontaneous preterm birth in the Acehnese ethnic group.

Conflict of interest

None declared.

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