The Association Between Interleukin-10 Gene Polymorphism And Il-10 Levels In Geohelminth Positive Adolescents And Adults In West Sumatera

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Abstract. Geohelminth infection can cause nutritional disorder and anemia. Behind these negative effect, worms turned out to provide protective effect on a number of diseases such as allergies and inflammations. This is related to its ability to modulate the host's immune response via Th-2, characterized by an increase production of Interleukin 10 (IL-10) which is an anti inflammatory cytokine. The genes encoding IL-10 are known to have single nucleotide polymorphism (SNP) in the promoter regions affecting IL-10 production. This study aimed to investigate the IL-10 polymorphisms in geohelminth infections and control and assess their effects on IL-10 production. This research was a comparative cross-sectional study. Samples were geohelminth-infected individuals aged >11 years old. Age, gender, and BMI category are matched between cases and controls. IL-10 levels were measured by using the ELISA method. IL-10 gene polymorphism was examined by using PCR and the Sanger sequencing method to detect SNP at rs1800896, rs1800871 and rs1800872. All subjects infected with geohelminth in this study were classified as mild infections. There was a significant increase in IL-10 levels in geohelminth-infected individuals when compared to controls ($p<0.05$). There was no association between the IL-10 gene polymorphism and the incidence of geohelminth infection ($p>0.05$), but there was a significant association between GA genotype in rs1800896 with elevated levels of IL-10 in geohelminth infections ($p <0.05$). We concluded that high levels of IL-10 in geohelminth infections were also influenced by IL-10 gene polymorphism, specially the GA genotype at rs1800896.

Keywords: Geohelminth, IL-10 gene polymorphism, IL-10 levels.

1 Introduction

Intestinal worm parasites are one of the most common causes of chronic infection in humans. Approximately 1.5 billion people are infected with soil-transmitted helminths worldwide by one or more types of intestinal worms, especially by worms that are transmitted through the soil, also called intestinal geohelminth or soil-transmitted helminths, such as *Ascaris lumbricoides*, *Necator americanus*, *Ancylostoma duodenale*, and *Trichuris trichiura* [1].

Geohelminth infection is more frequent and persistent in children and individuals living in endemic areas, due to exposure at all times of their lives, begin immediately after birth to

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adulthood. The highest prevalence is found in children of primary school age, but can also be related to adults who are influenced by the surrounding environment and work [1].

Geohelminth can cause nutritional disorders and anemia, especially in children with less nutritional intake [2]. Behind these negative effects, worms turned out to provide protective effects on some diseases such as allergies, which have been proven by many researchers [3,4,5]. This turned out to be related to the ability of worms to modulate the immune response of the host [6,7].

In chronic infections, worms trigger the differentiation of macrophages from classically activated macrophages (CAMs) to alternatively activated macrophages (AAMs), an increase in the number of T regulatory cells (Treg) which are characterized by the production of Interleukin-10 (IL-10) anti-inflammatory cytokines, and/or Transforming Growth Factor β (TGF-β) [8,9]. IL-10 is the most often discussed anti-inflammatory cytokine compared with other cytokines and is considered to be one of the key cytokines in neutralizing the effects of inflammatory responses that arise in several diseases.

Many studies have shown that in geohelminth infections there were higher levels of IL-10 when compared with individuals who were not infected with geohelminth [9,10,11,13]. Even in children aged 13-18 months whose mothers were infected with geohelminth, they also had higher levels of IL-10 than children whose mothers were not infected with geohelminth [12]. Nevertheless, some researchers reported that there was no difference in IL-10 levels between the people who were infected by geohelminth and the people who were not infected by geohelminth [13]. This is because IL-10 production is influenced by many factors, one of which is a variant of gene alleles (polymorphism) [14].

The IL-10 gene that is located on chromosome 1 in 1q21-32 has various genetic variants that are associated with variations in IL-10 production [15]. There are many genetic variants of the IL-10 gene, but the most widely studied are three single nucleotide polymorphism (SNPs), namely -1082 Guanine (G)> Adenine (A) (rs1800896), -819 Cytosine (C)> Thymine (T) (rs1800871) and -592 C> A (rs1800872), because they are located in the promoter region associated with transcription. A study of healthy volunteers reported that A alleles from A-1082G were associated with low IL-10 production in Peripheral Blood Mononuclear Cell (PBMC) cultures stimulated by concanavalin A [16]. Whereas G allele was associated with an increase in plasma IL-10 levels. SNP at -1082 is often studied about susceptibility and protective effects to certain diseases.

Until now, there have been no reports of the effect of IL-10 polymorphism on IL-10 levels in adult individuals infected with geohelminth. It is known that children's immunity is not developing as perfect as adults, and it is well known that age is associated with certain levels of cytokines. For this reason, it is necessary to conduct a study that examines IL-10's polymorphism in geohelminth infection and its effect on adolescent and adult IL-10 production.

2 Subjects and Methods

2.1. Location and Population of study

This research was conducted in West Sumatra, Indonesia. Subjects were geohelminth-positive children aged >11 years old from three elementary schools in Padang Pariaman and geohelminth-positive farmers from the outskirts of Padang. None of the subjects was using allergy or steroid medication. Cancer and tuberculosis sufferers were also eliminated from the

study. The total sample size was 41 individuals along with the age, gender and BMI category-matched control samples of uninfected individuals.

2.2 Ethical consent

Permission was obtained from the Ethics Commission of Faculty of Medicine of Andalas University in Padang. All subjects were briefed about the study including the objectives, risks, and benefits of the study and gave consent to be involved. For those younger than 18, consent forms were signed by their parents.

2.3. Stool sample collection and examination

Stool examinations were conducted at the Parasitology Laboratory of Faculty of Medicine of Andalas University by using a direct wet mount with iodine [17] and by the Kato Katz method [19] to calculate the number of geohelminth eggs.

2.4. Collection of blood samples

Blood was taken by using an aseptic procedure from the *mediana cubiti* vein by trained personnel, using 3 cc syringe. An amount of 2 cc blood was collected in an EDTA tube for DNA, and 1 cc was collected into tube marked by a yellow cap for ELISA examination. The samples were taken to the Biomedical Laboratory of Faculty of Medicine of Andalas University for analysis.

2.5. Measurement of levels of IL-10

The IL-10 levels were measured according to the manufacturer's instructions by using a Human IL-10 ELISA Kit (Sensitivity 2.59 pg/ml, assay range 5 pg/ml – 1500 pg/ml, Catalog Number E0102Hu, Bioassay Technology Laboratory, Shanghai) and read with an ELISA reader at 450 nm by using an automatic microplate reader (xMark, BioRad, USA).

2.6. Genotyping

Genomic DNA was isolated from blood samples by using the GF-1 Blood DNA isolation kit, the Vivantis brand (Catalog No. GF-BD-050 and GF-BD-100). DNA isolation was carried out according to the kit procedure, consisting of the sample preparation, cell lysis, DNA binding, washing, and DNA elution.

IL-10 promoter genotyping to detect SNP -1082 G/A (rs1800896), -819 C/T (rs1800871), and -592 C/A (rs1800872), was done by using PCR and sequencing method (Sanger method). The three SNPs were in an adjacent position so that they use one specific primer pair that has been designed with the Genious, 5'-TCTGAAGAAGTCCTGATGTC -3 '(forward), and 5'-ATGAATACCCAAGACTTCTCC -3' programs (reverse).

PCR was carried out at a final volume of 25 µL containing 100 ng DNA template, 12.5 ul My Taq HS Red Mix Bioline (Catalog No. BIO-25047), 300 nmol/ul primer IL10 promoter-F and IL10 promoter-R. The PCR stages were as follows: initial denaturation at 95°C for 3 minutes, followed by 35 cycles of a series of processes consisting of denaturation at 95 °C for 30 seconds, annealing at 63°C for 40 seconds, and elongation at 72°C for 1 minute. The PCR process ended with the final elongation step at 72 ºC for 5 minutes. PCR products were observed

by electrophoresis by using 1.5% agarose gel. After being dyed with DNA GelRed, it was observed under GelDoc. PCR products were measuring 813 bp. The remaining PCR products were purified, then 20 ul PCR products that have been pure would be sent for sequencing. Sequencing data were analyzed with the help of Geneious Bioinformatics software.

2.7 Data analysis

In the measurement of data normality, data from IL-10 levels were not normally distributed, although data transformation has been carried out. So that the difference in IL-10 polymorphism between geohelminth and control infections was analyzed by using chi-square/Kolmogorov Smirnov test. The relationship between IL-10 gene polymorphism and IL-10 levels was analyzed by using Mann-Whitney U/Kruskal Wallis test.

3 Results

3.1. Characteristics of Subjects

Out of a total of 41 geohelminth-infected individuals, *A. lumbricoides* was the most common cause (43.9%), followed by hookworm (29.3%), *T. trichiura* (14.6%), co-infection of *A. lumbricoides* and *T. trichiura* (9.8%), and co-infection of *Ascaris lumbricoides* and hookworm (2.4%). All subjects infected with geohelminth were classified as mild infections (Table 1).

Table 1. Characteristics of Subjects

Characteristics	*Geohelminth Infections*	Controls
	N= 41	N=41
Gender		
Man f (%)	13 (31,7)	13 (31,7)
Woman f (%)	28 (68,3)	28 (68,3)
Age (years) (median, range)	39 (11-65)	39 (11-65)
Weight (kg) (median, range)	46 (29-69)	50 (31-70)
Height (cm) (mean ± SD)	146,8 ± 8,8	148,8 ± 11,3
BMI (kg/m2) (mean ± SD)	20,7 ± 3,9	22 ± (3,5)
BMI category		
Underweight f (%)	15 (36,6%)	15 (36,6%)
Normal f (%)	16 (41,5%)	16 (41,5%)
Overweight f (%)	4 (9,8%)	4 (9,8%)
Obesity 1 f (%)	5 (12,2%)	5 (12,2%)
Geohelminth type		
A. lumbricoides f (%)	18 (43,9)	
T. trichiura f (%)	6 (14,6)	
Hook worm f (%)	12 (29,3)	
A.lumbricoides + *T. trichiura* f (%)	4 (9,8)	
A.lumbricoides + Hook worm f (%)	1(2,4)	
EPGS (median, range)	13 (2–1543)	
Intensity of infection		
Mild f (%)	41 (100%)	
Moderate and severe f (%)	0 (0%)	

N = population, f = frequency, BMI= Body mass index, SD =Standard Deviation,
EPGS = eggs per gram of stool

3.2 Comparison of IL-10 levels in geohelminth infections and controls

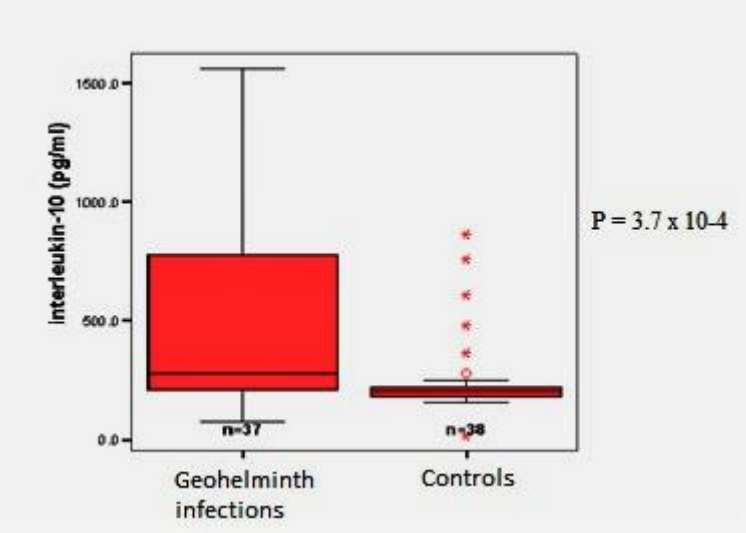

Fig. 1. Differences of IL-10 Levels in Geohelminth Infection and Control.

The plots box shows the median value (horizontal center line), interquartile range (edge box), and 95% confidence intervals (bars).

IL-10 levels in the study subjects ranged from 16 to 1564 pg/ml. As shown in Figure 1, there was an increase in IL-10 levels in geohelminth-infected subjects (median = 278 pg / ml) compared to controls (median = 204 pg / ml); with the Mann Whitney test, the difference was very significant, P = 3.7 x 10-4.

3.3 Relationship of IL-10 Regio Promoter Genes Polymorphism with IL- 10 Levels in Geohelminth Infection.

There are 2 types of genotypes at rs1800896, namely, AA is the highest frequency of 75% and GA with a frequency of 25%, whereas GG genotype is not found. At rs1800871 there are three types of genotypes, namely CC, TC, and TT, with a frequency of 6.6%, 50%, and 43.4%. At rs1800872 there are also three types of genotypes, namely CC, AC and AA with a frequency of 9.2%, 50%, and 40%. There are no significant differences in genotypic frequency between geohelminth infection and controls.

Geohelminth-infected subjects with AA genotypes at rs1800896 (-1082) have the lowest IL-10 levels (73 pg ml - 1067 pg/ml with a median of 255 pg/ml). IL-10 levels in the GA genotype ranged from 284 pg/ml - 1564 pg/ ml, with a median of 916 pg/ml. With the Mann Whitney statistical test, there were significant differences in IL-10 levels produced by geohelminth-infected subjects who have GA genotypes compared to AA genotypes at rs1800896 (-1082) with a value of P = 0.007, CI= 95% (Table 2). Whereas in control, there was no significant difference in IL-10 levels in both genotypes.

Table 2. Relationship of the IL-10 Regio Promoter Polymorphism with IL-10 Levels in Subjects Infected

Markers	Genotype	IL-10 level (pg/ml)[a]	P
rs1800896 A[b]/G	AA	255 (73-1067)	0,007 [e]
	GA	916 (284-1564)	
rs1800871 C[c]/T	CC	946 (798-1094)	0.065
	TC	364 (175-1564)	
	TT	248 (73-1067)	
rs1800872 C[d]/A	CC	501 (73-1094)	0,490
	AC	364 (175-1564)	
	AA	262 (174-1067)	

[a] median (range) [b,c,d] ancestral allele [e] significantly different

4 Discussion

In this study, the geohelminth-infected group had higher IL-10 levels than the control group. The results of this study were in line with the research of Fugueirido et al. (2010), which found that IL-10 was increased in people infected with geohelminth, although the method they used was different from this study.

A study conducted by Sanchez (2015) did not find any association between the levels of IL-10 and the geohelminth infection in Honduran children, although the principle of measuring IL-10 used was almost the same as this study. This may be due to differences in the age of the research subjects. In this study, the age of subjects ranged from 11 years old to 65 years old, where the immune organs have been fully developed, while the research subjects at Sanchez were aged 7-15 years old. At the age of 11 years and above, the intensity of helminths decreases and the levels of Th2 cytokines increase (IL-9, IL-10, and IL-13), which suggests that the Th2 response is associated with increased age [16].

High levels of IL-10 in geohelminth infections are caused by the ability of the worms to modify the immune response towards Th2, increasing type-2 cytokines, such as IL-4, IL-5, and IL-10. Worms also can trigger regulator responses through the T regulator to produce IL-10 and TGF β [8,9].

In this study, almost all study subjects examined had SNP in the promoter region of IL-10 gene (94.7%). This was consistent with the result of a previous study [19], which stated that polymorphism in IL-10 was very high.

In rs1800896 there were only two genotypes, namely AA and GA. Most subjects had AA genotypes (75%). No GG genotype at rs1800896 was found in the study subject. This was in line with the results of previously reported studies, that AA genotypes were very specific to the Asian population, even reaching 97.5%, while the GG genotype was not found in Asian populations, but was more common in Caucasia populations [20,21].

The genotype frequency of AA and GA at rs1800896 is almost the same between geohelminth infection and control. The frequency of TC genotypes at rs1800871 and AC genotypes at rs1800872 was seen to be slightly higher in controls than in cases, after statistical tests there was no significant difference, indicating that there is no association between the genotype frequency and the incidence of geohelminth infection as was reported by Fugueirido et al., in 2013.

IL-10 levels, like other cytokines, are influenced by certain diseases; their secretion is also influenced by the gene polymorphisms that encode them, especially polymorphisms located in the promoter region. About 50-70% variation of IL-10 synthesis is influenced by genetic factors [22]. Other factors that influence cytokine secretion are smoking, body mass index and age. In this study, the influence of BMI and age has been abolished.

In vitro IL-10 expression is mainly determined by a polymorphism in the position of -1082 G/A (rs1800896). Several studies have shown that the genotypic sequences of AA, AG, and GG had low, medium and high IL-10 levels (Stanczuk et al., 2001). In this study, geohelminth-infected subjects with AA genotypes at rs1800896 (-1082) had the lowest IL-10 levels, whereas GA genotypes had high IL-10 levels. In this study, geohelminth-infected subjects with AA genotype at rs1800896 had the lowest IL-10 levels, whereas GA genotypes had high levels of IL-10. There was a significant difference in the levels of IL-10 produced by geohelminth-infected subjects who had GA genotypes versus the AA genotype at rs1800896, whereas, in the controls, there was no significant difference in the levels of IL-10 in both genotypes of the marker. These findings indicated that high levels of IL-10 in geohelminth infections were also influenced by IL-10 gene polymorphism, especially the GA genotype at rs1800896.

We acknowledge the limitations of this cross-sectional study, which prevent the conclusion on the causal relationship between IL-10 gene polymorphism and IL-10 1evel. In this study we did not know whether the subjects studied had been infected for a long time (chronically) or had just been infected, but by looking at the living environment, the work that has been occupied for a long time, and the history of not getting deworming drugs in the past years, either intestinal worm medicine or elephantiasis medication, it can be assumed that the research subjects have been chronically infected.

It is clear that further and specifically design investigations are needed to clarify the relationship between IL-10 levels, GA genotype at 1800896, and protective effect in geohelminth infection.

5 Conclusion

This study has shown that geohelminth infection significantly increased IL-10 levels, but a significant increase in IL-10 levels was only found in geohelminth infected individuals who had the GA genotype at rs 1800896. This shows that apart from the influence of geohelminth infection, there is also the role of IL-10 gene polymorphism in increasing IL-10 levels in individuals infected with geohelminth, especially the GA genotype at 1800896.

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