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IL4 Polymorphisms and IgE Levels on Malaria-Endemic Islands in Vanuatu

Mawuli DZODZOMENYO[1)2)], Akira KANEKO[1)3)], Mihoko KIKUCHI[4)], Ratawan UBALEE[4)], Hikota OSAWA[1)3)], Takahiro TSUKAHARA[1)], Takeo TANIHATA[5)], Hedvig PERLMANN[6)], Kenji HIRAYAMA[4)] and Takatoshi KOBAYAKAWA[1)]

[1)]Department of International Affairs and Tropical Medicine (Director: Prof. Takatoshi KOBAYAKAWA)
Tokyo Women's Medical University, School of Medicine
[2)]School of Public Health, University of Ghana
[3)]Malaria Research Laboratory, Department of Medicine, Karolinska Institutet
[4)]Department of Molecular Immunogenetics, Institute of Tropical Medicine, Nagasaki University
[5)]Department of Epidemiology, National Institute of Public Health
[6)]Department of Immunology, Stockholm University

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Recent findings suggest that susceptibility to malaria is associated with genetic variants in the IL4 promoter region, resulting in the up-regulation of serum IgE. In this study, using a population-based approach, we investigated the mutant allele frequencies at positions − 590 and + 33 of the IL4 gene and total and *Plasmodium* (*P.*) *falciparum*-specific IgE levels on 3 islands with variable malaria endemicities in Vanuatu: Malakula (meso-endemic), Aneityum (meso-endemic with intervention), and Futuna (non-endemic). A total of 878 and 750 samples were typed for −590 and +33 positions, respectively. Variant allele frequencies varied from 0.27 to 0.39 for C−590T and from 0.39 to 0.48 for C+33T among 3 islands. There was a strong linkage disequilibrium between the 2 alleles ($p<0.001$). For both mutant alleles higher frequencies were detected in Aneityum than in Futuna ($p<0.05$). In Aneityum there was a significant association between the carriage of C+33T allele and increased levels of *P. falciparum*-specific IgE ($p<0.05$). However these relations were not observed in Malakula. This is the first report on IL4 polymorphisms in Melanesian populations. The observed mutant allele frequencies lay between higher values in Asian populations and rather lower values in Caucasians. The data suggest that IL4 promoter polymorphisms may be one of the genetic factors that explain relations between malaria disease and IgE.

Key words: malaria, IL4, IgE, polymorphism, Vanuatu

Introduction

The outcome of malaria infection is a reflection of host-parasite interactions, which in turn are dependent on their diversities. Ideal places to study these questions are isolated areas in Melanesia (the Southwest Pacific) with great variety of malaria endemic situations and also different ethnic populations. Our study area is the Vanuatu archipelago, which consists of 80 islands with 120 languages, confirming long time isolation. The islands of Vanuatu were settled less than 4,000 years ago during a rapid population expansion from Island Southeast Asia, that continued into western Polynesia[1)]. Malaria is endemic in most populated islands in Vanuatu while some small islands are apparently malaria-free[2)]. Malaria is unstable with apparently low mortality in Vanuatu[2)3)], while stable with high mortality in Papua New Guinea[4)]. This study represents part of the project to search for host genetic factors to explain these differences.

There is accumulating evidence that host genetic factors control malaria disease by regulating anti-malarial immune responses[5)]. A number of host genes have been identified, which seem to contribute to the protection against or susceptibility to malaria infection and disease. The geographical distri-

bution of certain genes in human populations may vary due to different selection pressures.

As example, the protection from malaria due to red cell abnormalities was documented in the sickle cell trait[6)7)], thalassaemias[8)] and G6PD deficiency[9)].

A number of other host genes have also been identified which may be related to acquisition of protective immunity against malaria disease, including polymorphisms in MHC class I genes[10)], regulating the production or expression of the inflammatory cytokine TNF[11)]. The previous studies showed that the class I antigen HLA-Bw53 and the class II haplotype DRB1*1302-DQB1*0501, associated with significant protection against severe malaria, are present in higher frequencies in people in Sub-Saharan Africa[10)12)] but rare in Caucasians[13)], while absent in Pacific islanders[14)]. A sib-pair linkage between the chromosome region 5q31-q33 and *Plasmodium* (*P.*) *falciparum* blood infection levels has been reported[15)]. This region is also linked to production of the cytokine IL4[16)] and to elevated serum levels of IgE[17)], which in turn may be related to pathogenesis.

IL4 is a multifunctional cytokine which serves as an important regulator in isotype switching from IgM/IgG to IgE[18)19)]. IL4 also regulates the differentiation of precursor T-helper cells into the Th2 subset that regulates humoral immunity and specific antibody production[20)]. Polymorphisms in the IL4 gene are known to play a significant role in allergic diseases such as atopic asthma[21)22)]. Recent investigations have revealed that genetic variants of the IL4 promoter regions lead to different transcription efficiency or dysregulation[23)], thereby causing variations in cytokine production.

In the IL4 gene, a single nucleotide polymorphism at 590 bp upstream of the transcriptional start site (C−590T) is known to have a higher luciferace reporter activity[24)], and this mutant allele has been found to modulate total serum IgE levels in asthma[22)]. An association between the C+33T mutant allele of the IL4 gene and increased levels of IgE was also documented in Japanese asthmatic patients[25)]. In this population, a strong linkage disequilibrium was found between the +33T allele and the −590T allele[26)], suggesting that both alleles may be acting on enhancement of the transcription and regulation of IgE production.

In *P. falciparum* infections, elevated levels of both total and malaria-specific IgE in sera of populations from malaria-endemic regions were documented[27)28)] and though the role of these IgEs are not particularly clear, IL4 seems to be involved in their excessive production. A recent study in Burkina Faso suggested that the mutant allele IL4 −590T is associated with elevated levels of anti-malarial antibody in the Fulani, thereby protecting the members of this ethnic group against malaria[29)]. Other studies also showed an association between genetic variants of the IL4 gene with increased total IgE levels and susceptibility to malaria[30)31)].

Sequestration of parasitized red blood cells in the endothelium of various organ vessels is an important mechanism in severe forms of malaria and this has been hypothesized to result from the up-regulation of TNF-α[32)]. A recent study also indicate the involvement of IL4 in inducing the high expression of the adhesion molecule V-CAM on endothelial cells[33)], a receptor for the *P. falciparum* erythrocyte membrane protein 1, a molecule implicated in sequestration.

In the IL4 gene, while high frequencies of the T mutant alleles (C−590T/C+33T) were found in Asian populations[25)34)], rather low frequencies were reported in Caucasians and populations from Sub-Saharan Africa[30)31)35)] except for the Fulani ethnic group where high frequencies were found[29)]. In this study, using a population-based approach, we investigated the mutant allele frequencies of the IL4 gene at positions −590 and +33 in Melanesian populations in Vanuatu and whether these polymorphisms were influenced by variable malaria endemicities. We also investigated the association between these polymorphisms and total and malaria-specific IgE production.

Since most of previous studies on the relation between IL4 polymorphism and malaria were conducted in Sub-Saharan Africa, this study in Melanesia provides a unique opportunity to compare results from this region with previous results from Sub-Saharan Africa.

Materials and Methods

Study areas

This study was designed as a component of malariometric surveys conducted on 3 islands of Vanuatu from 1997 to 1998. The details of these surveys were already reported elsewhere[36]. Briefly, Malakula is a meso-endemic island without any effective control measure. Aneityum is also potentially meso-endemic with a malaria-free situation since 1991, when an effective control measure was taken. Futuna is a non-endemic island due to the absence of vector *Anopheles* mosquito.

Filter paper blood sampling

During the above-mentioned surveys, finger-prick blood samples were also drawn into a 75 μl heparinized capillary tube (Drummond Scientific Company, USA) and transferred unto chromatographic filter paper (ET31CHR, Whatman Limited, England). After drying, the labeled filter paper samples were stored in separate small plastic bags at −20℃ until analyses performed on them.

DNA extraction and PCR

From each of randomly selected sub-samples, DNA was extracted from a quarter (19 μl) of one blood spot dried on filter paper using the method as previously described[37]. The IL4 +33 was typed by PCR amplification of a 200 bp fragment (forward primer 5'-TGCATCGTTAGCTTCTCCTG-3' and reverse primer 5'-biotin-GCTCTGTGAGGCTGTTCAAA-3') under the following conditions: denaturing at 95℃ for 10 min, amplification for 30 cycles at 95℃ for 15 sec, 60℃ for 15 sec and 72℃ for 30 sec and finally 72℃ for 10 min. The IL4 −590 position was typed by amplifying a 252 bp fragment (forward primers 5'-biotin-GCCTCACCTGATACGACCTG-3' and reverse primers 5'-GGGGCTCCTTCTCTGCAT-3') under the following conditions; denaturing at 95℃ for 10 min, amplification for 32 cycles at 95℃ for 15 sec, 62℃ for 15 sec, 72℃ for 30 sec and then finally extension at 72℃ for 10 min. All PCR products were run on 1% agarose gel to detect amplified fragments and visualized with UV illumination after staining with ethidium bromide. Template-free controls were included in each experiment.

Pyrosequencing

For both positions (IL4 +33, −590), the PCR products were immobilized on streptavidin-sepharose paramagnetic beads (Amersham Bioscience, Sweden) and the strands separated using 0.2 M NaOH. After separating the supernatant, 10 pmol of sequencing primer was added and annealed to the captured strands. The pyrosequencing primer sequences were 5'-TGTCCACGGACACA-3' and 5'-CCCAGCACTGGGG-3' for +33 and −590 positions respectively. The reactions were performed in a 96-well plate. The primed single-stranded DNA templates were then transferred to the microtitre plate-based PSQ™ Pyrosequencer, where real time sequencing of the IL4 gene was performed. Prior to this, an optimum dispensation order was determined and selected positions with mutations in the entire codon were detected after running the samples.

IgE antibody measurements

Levels of total IgE and *P. falciparum*-specific IgE were determined in study subjects from Aneityum and Malakula using ELISA as previously described[27]. A quarter (19 μl) of one blood spot dried on filter paper was extracted in phosphate-buffered saline with 0.5% bovine serum albumin (Fraction V, Boehringer Mannheim, Mannheim, Germany). For specific IgE, antigen was prepared from mature stages of the *P. falciparum* laboratory strain F32.

Statistical analysis

Allelic frequency distribution was tested according to the Hardy-Weinberg equilibrium by χ^2 test. The χ^2 test was also used to test for linkage disequilibrium of polymorphisms using observed and expected allele frequencies. The t-test was used to compare log-transformed IgE levels in different groups. The SPSS ver. 11 statistical package was used for the statistical analysis. P values less than 0.05 were considered significant.

Ethical considerations

The study was approved by the Department of Health, Vanuatu and the ethics committee of Tokyo Women's Medical University (No. 69). Study subjects were briefed on purposes and procedures of

Table 1 Polymorphisms in IL4 gene on 3 islands in Vanuatu: Malakula (meso-endemic), Aneityum (meso-endemic with intervention), and Futuna (non-endemic)

	IL4 −590					IL4 +33				
	n	CC	CT	TT	T allele frequency	n	CC	CT	TT	T allele frequency
Malakula	269	150 (56)	95 (35)	24 (9)	0.27	260	87 (34)	144 (55)	29 (11)	0.39
Aneityum	472	177 (37)	220 (47)	75 (16)	0.39	385	97 (25)	210 (55)	78 (20)	0.48
Futuna	137	74 (54)	48 (35)	15 (11)	0.28	105	44 (42)	41 (39)	20 (19)	0.39
Total	878	401 (45)	363 (42)	114 (13)	0.34	750	228 (30)	395 (53)	127 (17)	0.43

Percentages are in parentheses.

Table 2 Age-specific geometric mean IgE levels on Aneityum and Malakula

Age group (years old)	Aneityum			Malakula		
	n	Total IgE (ng/ml)	*P. falciparum*-specific IgE (ng/ml)	n	Total IgE (ng/ml)	*P. falciparum*-specific IgE (ng/ml)
0-5	48	508	0.99	36	424	3.35
6-15	41	1,720	0.97	87	2,208	3.03
16-30	34	1,882	0.91	36	2,752	3.60
> 30	37	1,772	0.93	−	−	−

Table 3 Association between IL4 polymorphisms and IgE levels in subjects aged older than 5 years on Malakula and Aneityum

IgE (mean log ng/ml)	IL4 −590						IL4 +33					
	Malakula			Aneityum			Malakula			Aneityum		
	CC	CT/TT	p	CC	CT/TT	p	CC	CT/TT	p	CC	CT/TT	p
(n)	(45)	(31)		(18)	(36)		(19)	(57)		(15)	(39)	
Total	3.46	3.36	0.498	3.18	3.24	0.702	3.52	3.38	0.315	3.07	3.28	0.171
P. falciparum-specific	0.51	0.52	0.925	− 0.1	0	0.111	0.57	0.5	0.209	− 0.14	0	0.028*

*significant.

the survey and a written informed consent was obtained from each adult subject while in the case of children by their guardians who determined the child's participation.

Results

Genotypes and allele frequencies

Table 1 summarizes the distribution of genotypes and allele frequencies of the two IL4 polymorphic positions in the study populations. A total of 878 and 750 samples were typed for IL4 −590 and +33, respectively. In Malakula, Aneityum and Futuna, T allele frequencies were 0.27, 0.39 and 0.28 for IL4 −590 and 0.39, 0.48 and 0.39 for IL4 +33, respectively. Genotype distributions of both −590 and +33 positions were in agreement with the Hardy-Weinberg equilibrium ($p<0.05$). In all islands, the frequency of the homozygous TT allele was the lowest. Comparisons between islands for the genotype CC alone vs CT/TT revealed significantly higher frequencies of CT/TT genotypes in Aneityum than in Futuna for both −590 ($p<0.001$) and +33 positions ($p<0.01$). However there was no significant difference between Malakula and Futuna in these frequencies. There was a significant linkage disequilibrium between the −590 and +33 positions ($p<0.001$).

IgE levels

Table 2 summarizes age-specific distribution of geometric mean total and *P. falciparum*-specific IgE in subjects from Aneityum and Malakula. In both islands total IgE values are significantly higher in subjects >5 years old than those ≤5 years old.

Among age groups >5 years old, IgE levels were not significantly different. Hence the subsequent analysis on association between IL4 polymorphisms and IgE levels was conducted for subjects >5 years old. Age effect was not observed for specific IgE levels in both islands. In each age group mean specific IgE value was significantly higher in Malakula than in Aneityum.

Associations between IL4 polymorphisms and IgE levels

Table 3 summarizes associations between carriage of a T allele (CC vs CT/TT) and serum IgE levels (total and *P. falciparum*-specific) at the two polymorphic sites. We detected a significantly higher *P. falciparum*-specific IgE level in IL4 +33T allele carriers (CT/TT) than non carriers (CC) in Aneityum ($p<0.05$). This relation was not observed in subjects from Malakula.

Discussion

This is the first report to describe the degree of IL4 polymorphisms in Melanesian population and also to demonstrate an association between carriage of the mutant T allele and elevated levels of malaria-specific IgE.

The IL4 promoter mutant allele frequencies observed in Vanuatu were rather unique and different from those previously reported in other geographical populations. The observed mutant allele frequencies lay between higher values in Asian populations and rather lower values in Caucasian and Sub-Saharan populations. A study in Japanese subjects revealed that the frequency of the IL4 +33 mutant allele was 0.7 in asthmatic patients[25]. Another study in Taiwan depicted a similar high frequency of 0.78 of the IL4 −590T in patients with systemic lupus erythematosus[34]. In Caucasians lower frequencies (0.13 and 0.15) of the −590T allele were reported in asthmatic patients in Germany[35] and UK[38], respectively. Relatively low frequencies were also reported in populations from Sub-Saharan Africa with frequencies ranging from 0.22[36] to 0.28[31], except for the Fulani ethnic group where 0.45 mutant allele frequency was found[29].

As an example of ethnically specific genetic profiles of Vanuatu populations, we previously reported an unprecedentedly high prevalence of cytochrome P450 (CYP) 2C19-related poor metabolizer genotype individuals on the islands of Vanuatu[39]. In addition, there was substantial variation among populations of Vanuatu. Comparisons of genetic, linguistic and geographic patterns among populations suggest a strong geographic component to the current distribution of CYP2C19 alleles in Vanuatu[39]. Previous studies of patterns of malaria incidence and frequencies of alpha-thalassaemia and G6PD deficiency in the Papua New Guinea [8,40] and Vanuatu[2] have indicated selection on these loci by malaria.

The observed linkage disequilibrium between C−590T and C+33T in our study is consistent with the previous finding in a Japanese population[26]. The genetic association between single nucleotide polymorphisms (SNPs) in the IL4 promoter region and elevated levels of total serum IgE or IgG have been documented by several workers[25,29,35]. In addition, the clinical significance of IL4 polymorphisms in disease sequels has been reported in malaria[30,32,41] and in asthma[42].

Our study found a significant difference in frequencies of T allele carriers for both mutant alleles between meso-endemic Aneityum and non-endemic Futuna. Furthermore in Aneityum there was a significant association between the carriage of IL4 +33T allele and mean concentrations of *P. falciparum*-specific IgE. The observations in Aneityum suggest the role of IL4 promoter polymorphisms in up-regulating *P. falciparum*-specific IgE serum levels in the study subjects. A similar relation but with different repertoires was observed in a case-control study in West Africa, where the IL4 −590T allele frequency of 0.22 in one ethnic group[30] and a rather high frequency of 0.45 in another group (the Fulani) were found with association of elevated *P. falciparum*-specific IgG levels[29]. In the same region, the severity of malaria in children was also found to be associated with elevated levels of *P. falciparum*-specific IgE[43].

However in our study the mutant allele frequencies in another meso-endemic Malakula were similar to those in Futuna, suggesting selection towards IL4 mutation due to malaria endemicity is not uni-

versal.

Although we could not detect any significant association between the IL4 T allele carriage and total IgE, in earlier studies in Sub-Saharan Africa this association was found significant[30)31)]. Discrepancy between our study in Melanesia and the previous studies in Sub-Saharan Africa can be explained mainly by regional differences of environmental factors which contribute to total IgE levels, including repertoires of helminthes infections. There are also differences of degree of malaria disease severity in study subjects. While the study in Sub-Saharan Africa was conducted mainly on symptomatic cases including cerebral malaria, our study in Vanuatu was on asymptomatic parasite carriers.

The association between IL4 polymorphisms and specific IgE levels detected in Aneityum was not observed in Malakula, although both islands are potentially meso-endemic. This discrepancy may be explained by differences of malaria situations in the previous years. In Aneityum an integrated malaria control program was introduced in 1991 and a nearly malaria-free situation was maintained at the time of the survey, while in Malakula malaria transmission was continuous without any effective control measure. The observed difference of *P. falciparum*-specific IgE levels represents difference of transmission between two islands, while similar levels of total IgE are due to other common infections including intestinal helminthes. The difference of transmission might result in different immune response and persistence between Aneityum and Malakula, including *P. falciparum*-specific IgE levels.

In Aneityum we could not observe any difference of specific IgE level between subjects older than 5 years and those under 5 years, who have never been exposed to malaria, suggesting cross reactivity and limited role of specific IgE in asymptomatic subjects in our studies. It is obviously interesting to investigate relation between IL4 polymorphisms and IgE levels in severe malaria patients on these islands in future. In conclusion, this is the first report on the IL4 promoter polymorphisms in Melanesian populations. The observed mutant allele frequencies lay between higher values in Asian populations and rather lower values in Caucasians. The data suggest that IL4 promoter polymorphisms may be one of the genetic factors that explain association between malaria disease and IgE. However, further investigations are necessary to clarify the implications of these polymorphisms on malaria disease manifestations in Melanesia.

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ヴァヌアツのマラリア流行島嶼における IL4 多型と IgE 濃度

[1]東京女子医科大学　医学部　国際環境・熱帯医学（主任：小早川隆敏教授）
[2]ガーナ大学　公衆衛生学部
[3]カロリンスカ大学病院　マラリア研究所
[4]長崎大学熱帯医学研究所　分子免疫遺伝学
[5]国立保健医療科学院　疫学
[6]ストックホルム大学　免疫学

Mawuli DZODZOMENYO[1)2)]・金子　明[1)3)]・菊池三穂子[4)]・Ratawan UBALEE[4)]・大澤　彦太[1)3)]
塚原　高広[1)]・谷畑　健生[5)]・Hedvig PERLMAN[6)]・平山　謙二[4)]・小早川隆敏[1)]

血中 IgE 上昇に帰結する IL4 プロモーターの遺伝的変異が，マラリアに対する感受性と相関することが最近の研究によって示唆されてきている．本研究においては集団遺伝学的方法を用いヴァヌアツにおけるマラリア流行度が異なる 3 島嶼において，IL4 −590 および +33 塩基における変異対立遺伝子頻度，血中総 IgE および熱帯熱マラリア原虫特異的 IgE 濃度を調べた．3 島嶼は中等度の流行が続く Malakula，中等度の流行だが対策が功を奏している Aneityum およびマラリア流行がない Futuna である．これらの島嶼住民より採取した血液サンプルより IL4 −590 および +33 についてそれぞれ計 878 および 750 サンプルの解析を行った．変異対立遺伝子頻度はこれら 3 島嶼間において C−590T が 0.27～0.39，C+33T が 0.39～0.48 の範囲で変動した．両対立遺伝子間には顕著な連鎖不均衡が認められた（$p<0.001$）．これら両変異対立遺伝子とも Aneityum においては Futuna より高い頻度で認められた（$p<0.05$）．Aneityum においては IL4 +33 位における変異対立遺伝子の存在する群での血中熱帯熱マラリア原虫特異的 IgE 濃度は有意に上昇していた（$p<0.05$）．しかしながら，これらの関係は Malakula においては認められなかった．本研究はメラネシア住民集団において当該変異遺伝子頻度に関する最初の報告である．見出された変異対立遺伝子頻度はこれまで報告されている，より高いアジア住民集団とより低いヨーロッパ住民集団の中間の値であった．さらに IL4 多型が特異的 IgE とマラリア病形の関係に関る遺伝的因子の一つであることが示唆された．