รายงานวิจัยฉบับสมบูรณ์

โครงการ "การวิเคราะห์ความหลากหลายของลำดับเบสของอีพิโทปบนพื้นของสไตร์ตรงที่เป็น Th2R และ Th3R ของโปรตีนแอนติเจนชนิด circumsporozoite ของเชื้อมาลาเรียฟัลซิพารัมในกลุ่มคนไทย"

โดย

ศ.ดร.. ศรีสิน ดูสมิทธ์

นางสาวพุดิมา คำแขก และคณะ

ภาควิชาจุลชีววิทยาและอิมมิวโนโลยี

คณะเวชศาสตร์เขตร้อน มหาวิทยาลัยมหิดล

เมษายน ๒๕๔๖

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สัญญาเลขที่ BGJ/14/2544

รายงานวิจัยฉบับสมบูรณ์

โครงการ "การวิเคราะห์ความหลากหลายของลำดับเบสของอีพิโทปบนที่เซลล์ในส่วนที่เป็น Th2R และTh3R ของโปรตีนแอนติเจนชนิด circumsporozoite ของเชื้อมาลาเรียฟัลซิพารัม ในกลุ่มคนไทย"

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สนับสนุนโดยสำนักงานกองทุนสนับสนุนการวิจัย

สารบัญ

ABSTRACT

Allelic variation of the immunodominant T-cell epitopes, Th2R and Th3R on *Plasmodium falciparum* circumsporozoite protein (CSP) gene has been determined by sequencing in 40 malaria isolates from malaria endemic areas, west of Thailand. These malaria isolates contained the total of nine and seven allelic types of variants on Th2R and Th3R regions, respectively. Comparison of T-cell epitope sequences on Th2R and Th3R region of CSP with published sequences from endemic areas of different geographical locations, five new allelic types of Th2R regions (CSP-Th2R*16, CSP-Th2R*17, CSP-Th2R*18, CSP-Th2R*20, and CSP-Th2R*21) and three new allelic types of Th3R regions (CSP-Th3R*14, CSP-Th3R*15, and CSP-Th3R*16) were demonstrated. These new allelic types were not cluster together with those found previously in Thailand. The results also revealed that the nucleotide substitutions occurred predominantly in the first codon position and less frequently in the second codon position resulting in the amino-acid changes, which were non-synonymous mutation. Moreover, the genetic diversity in such regions were also analyzed using the combination of Polymerase Chain Reaction (PCR) and sequence specific oligonucleotide probes (PCR-SSOP) in 144 and 43 falciparum malaria isolates from falciparum malaria endemic areas, Ratchaburi and Kanchanaburi provinces along Thai-Myanmar border, respectively. Three (CSP-Th2R*05, CSP-Th2R*12 and CSP-Th2R*13) and five (CSP-Th2R*05, CSP-Th2R*12, CSP-Th2R*13, CSP-Th2R*14 and CSP-Th2R*15) allelic variants from 15 reported allelic types of CSP-Th2R region were identified in Ratchaburi and Kanchanaburi provinces, respectively. For CSP-Th3R regions, four allelic types were found in Ratchaburi province (CSP-Th3R*01, CSP-Th3R*04, CSP-Th3R*08 and CSP-Th3R*09) while three allelic types (CSP-Th3R*01, CSP-Th3R*04 and CSP-Th3R*10) were found in Kanchanaburi provinces. Interestingly, the patterns of hybridization of some isolates in both provinces did not correspond to any known alleles, suggesting new allelic types in CSP gene. The similar finding in both provinces was CSP-Th2R*05 and CSP-Th3R*01 which were the majority of CSP-Th2R and CSP-Th3R allelic types. In addition, the CSP-Th2R*05 and CSP-Th3R*01 were the most common allelic types in both all age groups and numbers of falciparum malaria exposure. Comparison of the CSP-Th2R and CSP-

Th3R allelic types from dot blot hybridization with DNA sequencing showed that most isolates gave similar results of CSP-Th2R and CSP-Th3R allelic types. These results revealed that the genetic diversity of CSP varied among *P. falciparum* isolates from different geographical areas. In the present study, the genotyping of *P. falciparum* isolates were also determined in the repeat regions of CSP gene and the three highly polymorphic regions encoding for merozoite surface protein-1 (MSP-1), -2 (MSP-2), and glutamine-rich protein (GLURP) by nested PCR. The results of amplification of the repeat regions in CSP gene showed three allelic sizes of variants (600, 700, and 900 bp). While the genetic diversity in MSP-1, MSP-2 and GLURP were large, with 12, 15 and 10 sizes of variants detected, respectively. The MSP-2 locus was usually highly polymorphic and FC27 and 3D7/IC families were both highly represented. These situations were similar to those found in Senegal and Trad province of Thailand. These results together with other similar studies are essential for further study to determine whether the CSP antigen polymorphism represents in fact a major obstacle for the development of anti-sporozoite vaccine against malaria parasite.

บทคัดย่อ

การศึกษาความหลากหลายของลำดับเบสบนทีเซลล์อีพิโทปในส่วนที่เป็น CSP-Th2R และ CSP-Th3R ของโปรตีนแอนติเจนชนิด circumsporozoite ของเชื้อมาลาเรียชนิดฟัลซิพารัมที่เก็บได้จากผู้ป่วยในจังหวัดราชบุรีและกาญจนบุรีซึ่งอยู่บริเวณภาคตะวันตกของประเทศไทยที่มีชายแดนติดต่อกับประเทศพม่าและเป็นแหล่งที่มีการระบาดของไข้มาลาเรียค่อนข้างสูง จำนวน 40 ราย ด้วยเทคนิค DNA sequencing พบว่ามีความหลากหลายของลำดับเบสบนทีเซลล์อีพิโทปในส่วนที่เป็น CSP-Th2R จำนวน 9 ชนิด (CSP-Th2R*05, CSP-Th2R*12, CSP-Th2R*13, CSP-Th2R*16, CSP-Th2R*17, CSP-Th2R*18, CSP-Th2R*19, CSP-Th2R*20, CSP-Th2R*21) ซึ่งห้าชนิดความหลากหลาย ได้แก่ CSP-Th2R*16, CSP-Th2R*17, CSP-Th2R*18, CSP-Th2R*20, และ CSP-Th2R*21 เป็นความหลากหลายของลำดับเบสชนิดใหม่เมื่อเปรียบเทียบกับความหลากหลายของลำดับเบสชนิดต่างๆที่พบในแหล่งพบอัตราการติดเชื้อมาลาเรียค่อนข้างสูงในภูมิภาคต่างๆในโลก สำหรับความหลากหลายของลำดับเบสบนทีเซลล์อีพิโทปในส่วนที่เป็น CSP-Th3R พบว่ามีความหลากหลายของลำดับเบสเป็นจำนวน ๗ ชนิด (CSP-Th3R*01, CSP-Th3R*04, CSP-Th3R*08, CSP-Th3R*09, CSP-Th3R*14, CSP-Th3R*15, CSP-Th3R*16) โดย 3 ชนิด (CSP-Th3R*14, CSP-Th3R*15, CSP-Th3R*16) เป็นความหลากหลายของลำดับเบสชนิดใหม่เช่นเดียวกัน นอกจากนี้ยังพบว่าความหลากหลายของลำดับเบสเกิดจากการเปลี่ยนแปลงของเบสโดยเฉพาะตำแหน่งแรกซึ่งอาจพบบ้างในตำแหน่งที่สองและพบน้อย ในตำแหน่งที่สามโดยการเปลี่ยนแปลงดังกล่าวมีผลทำให้เกิดการเปลี่ยนแปลงของชนิดโปรตีน ซึ่งเรียกการเปลี่ยนแปลงนี้ว่าเป็นชนิดแบบ non-synonymous นอกจากนี้ยังทำการวิเคราะห์ความหลากหลายดังกล่าวด้วยเทคนิค PCR และ dot blot hybridization กับ DNA probe ที่มีความจำเพาะ ในผู้ป่วยติดเชื้อจากจังหวัดราชบุรีและกาญจนบุรี จำนวน 144 และ 43 รายตามลำดับ ผลจากการศึกษาพบว่ามีความหลากหลายในส่วนที่เป็น CSP-Th2R จำนวน 3 ชนิด (CSP-Th2R*05, CSP-Th2R*12, CSP-Th2R*13) และ 5 ชนิด (CSP-Th2R*05, CSP-Th2R*12, CSP-Th2R*13, CSP-Th2R*14, CSP-Th2R*15) สำหรับส่วนที่เป็น CSP-Th3Rพบว่ามีจำนวน 4 ชนิด (CSP-Th3R*01, CSP-Th3R*04, CSP-Th3R*08, CSP-Th3R*09) และ 3 ชนิด (CSP-Th3R*01, CSP-Th3R*04, CSP-Th2R*10) ในจังหวัดราชบุรีและกาญจนบุรีตามลำดับ นอกจากนี้ยังพบว่ามีเชื้อมาลาเรียบางสายพันธุ์ที่ไม่สามารถจำแนกชนิดได้ ซึ่งอาจจะเป็นความหลากหลายชนิดใหม่ เมื่อเปรียบเทียบผลการศึกษาโดยวิธี dot blot hybridization

และ DNA sequencing พบว่าทั้งสองวิธีให้ผลของลำดับเบสบนทีเซลล์อีพิโทปในส่วนที่เป็น CSP-Th2R และ CSP-Th3Rเหมือนกันเมื่อศึกษา ในเชื้อมาลาเรียของผู้ป่วยคนเดียวกัน นอกจากนี้ยังได้ทำการศึกษาว่าเชื้อมาลาเรียชนิดฟัลซิพารัมที่มีของลำดับเบสบนทีเซลล์อีพิโทปในส่วนที่เป็น CSP-Th2R และ CSP-Th3R ชนิดเดียวกันจะเป็นเชื้อมาลาเรียสายพันธุ์เดียวกันหรือไม่ โดยทำการวิเคราะห์แยกชนิดของยีน MSP-1, MSP-2, GLURP และ repeat region ของโปรตีนแอนติเจนชนิด circumsporozoite ด้วยวิธี nested PCR พบว่าเชื้อมาลาเรียดังกล่าวมีรูปแบบของยีน MSP-1, MSP-2, GLURP และ repeat region แตกต่างกันในเชื้อที่มีลำดับเบสบนทีเซลล์อีพิโทปในส่วนที่เป็น CSP-Th2R และ CSP-Th3R ชนิดเดียวกัน ซึ่งบ่งบอกว่าการติดเชื้ออาจเกิดจากเชื้อหลายสายพันธ์ จากผลการศึกษาทั้งหมดสามารถสรุปได้ว่าความหลากหลายของลำดับเบสขึ้นอยู่กับความแตกต่างของแหล่งที่ตั้งของพื้นที่ที่มีการระบาดของเชื้อมาลาเรียชนิดดังกล่าว เนื่องจากผลการศึกษาดังกล่าวพบว่าความหลากหลายของลำดับเบสบนทีเซลล์อีพิโทปในส่วนที่เป็น CSP-Th2R และ CSP-Th3R ของโปรตีนแอนติเจนชนิด circumsporozoite เหล่านี้มีผลต่อการตอบสนองทางภูมิคุ้มกันต่อเชื้อมาลาเรียชนิดฟัลซิพารัม ฉะนั้นความหลากหลายของลำดับเบสเหล่านี้จะมีผลกระทบต่อการผลิตและพัฒนาวัคซีนที่ใช้ป้องกันโรคมาลาเรียชนิดฟัลซิพารัมหากการศึกษาในลำดับต่อไปพบว่าความหลากหลายของลำดับเบสต่างเหล่านี้มีผลต่อการกระตุ้นภูมิคุ้มกันต่อเชื้อมาลาเรีย

EXECUTIVE SUMMARY

The genetic diversity displayed by *P. falciparum* field isolates, the occurrence of variant forms of the parasite at different frequencies in different geographic areas, and the complexity of the infections represent major obstacles for the development of effective malaria control measures such as the development of a vaccine against falciparum malaria. In the present study, we proposed to determine the genetic variation in T-helper cell epitopes (Th2R and Th3R) of *P. falciparum* CSP gene in parasite isolates obtained from malaria endemic areas with high rate of malaria transmission in Thailand using DNA sequencing and Polymerase Chain Reaction (PCR) and sequence specific oligonucleotide probes (PCR-SSOP). Thirty-one, four, four and one *P. falciparum* isolates obtained from Ratchaburi, Kanchanburi, Tak and Phetchaburi provinces, malaria endemic areas of Thailand were sequenced. These isolates contained the total of nine and seven allelic types of variants on Th2R and Th3R regions, respectively. Comparison of these sequences of T-cell epitopes on Th2R and Th3R region of CSP with published sequences from endemic areas of different geographical locations, five new allelic types of Th2R regions (CSP-Th2R*16, CSP-Th2R*17, CSP-Th2R*18, CSP-Th2R*20, and CSP-Th2R*21) and three new allelic types of Th3R regions (CSP-Th3R*14, CSP-Th3R*15, and CSP-Th3R*16) were demonstrated. These new allelic types were not clustered together with those found previously in Thailand. However, the predominant allelelic types of CSP-Th2R and CSP-Th3R regions were CSP-Th2R*05 and CSP-Th3R*01. The results revealed that the nucleotide substitutions occurred predominantly in the first codon position and less frequently in the second codon position resulting in the amino-acid changes, which were non-synonymous mutation. The genetic diversity was also analyzed using the combination of Polymerase Chain Reaction (PCR) and sequence specific oligonucleotide probes (PCR-SSOP) in 144 and 43 falciparum malaria isolates from falciparum malaria endemic areas, Ratchaburi and Kanchanaburi provinces along Thai-Myanmar border. Three (CSP-Th2R*05, CSP-Th2R*12 and CSP-Th2R*13) and five (CSP-Th2R*05, CSP-Th2R*12, CSP-Th2R*13, CSP-Th2R*14 and CSP-Th2R*15) allelic variants from 15 reported allelic types of CSP-Th2R region were identified in Ratchaburi and Kanchanaburi provinces, respectively. For CSP-Th3R regions, four (CSP-Th3R*01, CSP-Th3R*04, CSP-Th3R*08 and CSP-Th3R*09) and

three allelic types (CSP-Th3R*01, CSP-Th3R* CSP-Th3R*04 and CSP-Th3R*10) were found in Ratchaburi and Kanchanaburi provinces, respectively. Furthermore, the patterns of hybridization of some isolates in both provinces did not correspond to any known alleles, suggesting new allelic types in CSP gene. The similar finding in both provinces was CSP-Th2R*05 and CSP-Th3R*01 which were the majority of CSP-Th2R and CSP-Th3R allelic types. Furthermore, the CSP-Th2R*05 and CSP-Th3R*01 were the most common allelic types in both all age groups and they did not change with the number of falciparum malaria attacks. Comparison of the CSP-Th2R and CSP-Th3R allelic types from dot blot hybridization with DNA sequencing had shown that most isolates gave similar results. These results revealed that geographic variation in genetic diversity of CSP. In addition, genotyping of *P. falciparum* isolates with the same Th types were also determined for the repeat region of the CSP gene and for three highly polymorphic regions of genes encoding for merozoite surface protein-1 (MSP-1), -2 (MSP-2), and glutamine-rich protein (GLURP) by nested PCR. The results of amplification of the repeat regions in CSP gene showed six allelic variants of different sizes while the MSP-1, MSP-2 and GLURP loci showed high polymorphism with 12, 15 and 10 sizes of variants detected, respectively. The MSP-2 locus was usually highly polymorphic and FC27 and 3D7/IC families were both highly represented. However, the RO33 family of MSP-1 was poorly polymorphic, with only one allele detected. These situations were similar to that of Senegal and Trad province of Thailand. These results and other similar studies are essential to determine whether the CSP antigen polymorphism represents in fact a major obstacle for the development of anti-sporozoite vaccine against the malaria parasite.

SIGNIFICANCE OF THE RESEARCH.

1. Two scientific papers will be published in international journal as follow below;

Publications:

1. Survey of Th2R and Th3R allelic variants in the circumsporozoite protein genes found in *Plasmodium falciparum* parasites from western Thailand (during submit in international journal)

2. Analysis of sequence polymorphism of T-cell epitope regions, Th2R and Th3R, on *Plasmodium falciparum* circumsporozoite proteins in Thai isolates

2. The study provides knowledge of the genetic diversity of T-cell epitopes on the *P. falciparum* CSP obtained from Thailand. This knowledge are exploited in the use of the CSP polymorphic regions as an epidemiological marker in studies of pre-erythrocytic immunity and for vaccine development.

FURTHER STUDY

To determine whether the malaria infected individuals could response to theses new allelic types of CSP-Th2R and CSP-Th3R regions by lymphoproliferative assays.

<u>หมายเหตุ</u> เนื่องหางานวิจัยนี้สามารถตีพิมพ์ในวารสารวิชาการระดับนานาชาติได้ ๒ เรื่อง จึงขอนำเสนอบทความวิจัยส่วนที่เพิ่มจากเงื่อนไขทุน คปก. โดยมีชื่อเรื่องว่า

"Survey of Th2R and Th3R allelic variants in the circumsporozoite protein genes found in *Plasmodium falciparum* parasites from western Thailand"

Survey of Th2R and Th3R allelic variants in the circumsporozoite protein genes found in *P. falciparum* parasites from western Thailand.

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(This paper is now being submitted for publication)

Abstract

Allelic variation in *P.falciparum* circumsporozoite protein (CSP) gene has been determined by sequencing the immunodominant T-cell epitopes, Th2R and Th3R in 40 isolates from malaria endemic area, west of Thailand. These isolates contained the total of nine and seven allelic types of variants on Th2R and Th3R regions, respectively. Comparison of these sequences of T-cell epitope on Th2R and Th3R region of CSP with published sequences from endemic areas of different geographical locations, five new allelic types of Th2R regions (CSP-Th2R*16, CSP-Th2R*17, CSP-Th2R*18, CSP-Th2R*20, and CSP-Th2R*21) and three new allelic types of Th3R regions (CSP-Th3R*14, CSP-Th3R*15, and CSP-Th3R*16) were demonstrated, suggesting geographical variation in genetic diversity of CSP. These new allelic types were not cluster together with those found previously in Thailand suggesting a considerable heterogeneity within some geographic area. Furthermore, the results revealed that the nucleotide substitutions occurred predominantly in the first codon position and less frequently in the second codon position resulting in the amino-acid changes, which were non-synonymous mutation. If the functional capacity in immune induction among these variants on Th2R and Th3R regions of CSP could be found, then a vaccine to stimulate CSP specific T-cell activity should be designed to include these variants for more effective universal vaccines.

Keywords: Circumsporozoite Protein; *Plasmodium falciparum*; Polymorphism; T-cell epitope

Abbreviations: CSP, circumsporozoite protein; HLA, human leukocyte antigen; CD4, cluster determinant 4; CD8, cluster determinant 8

Introduction

The circumsporozoite protein (CSP) is an antigen found at the surface of the sporozoite (Nussenzweig and Nussenzweig, 1985), the form of the malaria parasite injected by the mosquito. CSP has long been considered to be an important target of protective immune response against pre-erythrocytic parasites (sporozoites and the hepatic stages). The *P. falciparum* CSP gene, *pfcsp*, encodes a protein consisting of relatively conserved regions flanking a highly repetitive domain composed of tetrapeptide repeats, the sequence of the majority is NANP and that of the remainder is NVDP (Dame *et al*, 1984; Jongwutiwes *et al*, 1994). The immunodominant B-cell epitopes are confined to this repeat region, while T-cell epitopes are found in the C-terminal domain (Dame *et al*, 1984; Hill *et al*, 1997). These epitopes provide universal T-cell help for the production of anti-sporozoite antibody that inhibit hepatocyte invasion by sporozoite, and induce $CD4^+$ and $CD8^+$ cytotoxic T-effector cells that are thought to kill infected hepatocytes (Hill *et al*, 1997). The structure of the CSP from other parasite species is similar to that described above, though the sequences of the repetitive elements differed substantially between the species.

The CSP has been the focus of immunological studies since the 1970's and its gene was the first *Plasmodium* gene to be cloned and sequenced. Numerous experimental vaccines thus include or are based on CSP (Patarroyo *et al*, 1988). The most recent, RTS,S consists of 19 NANP repeats as well as the carboxyl terminus of the CSP fused to the hepatitis B surface antigen. To date, this formulation associated to the potent adjuvant (SBAS2) proved the most efficacious in protecting vaccinated human volunteers against an experimental challenge with a homologous strain. However, the protection observed in a Phase II trial of this vaccine in Gambian adults, proven to be of short duration.

Polymorphism in the vaccine candidate antigen sequence is a major concern, since it might adversely influence the induction of immunity as well as providing the parasite with a means to escape the protective responses induced. Sequencing of *pfcsp* genes or gene fragments from laboratory and field isolates, revealed that this antigen, like many others in *P. falciparum*, displays extensive genetic diversity. The number

and arrangement of the two repeated tetrapeptides varied with the parasite line, though this was not found to alter binding of antibodies. Variations were found in the pre- and post-repeat regions, but are thought to be immunologically neutral. Sporadic point mutations were also found in the N-terminal region. However, the most striking polymorphisms consisted of a series of non-synonymous point mutations which were centered on two important T-helper epitopes, Th2R and Th3R, found in the C-terminal domain of the molecule (Lockyer *et al*, 1989; Alloueche *et al*, 2000; Escalante *et al*, 2002). Allelic diversity in malaria parasites is widely assumed to be a mechanism for immune evasion (Day and Marsh, 1991; McCutchan *et al*, 1988). The variation within the CSP gene of *P.falciparum* was the result of selection by immune T-cell (Zevering *et al*, 1998). Correlation between human T-cell proliferations to the Th3R epitope with protection from falciparum malaria infection, was observed for a limited number of patient from a malaria endemic area (Hoffman *et al*, 1989). Recent studies further showed that human $CD4^+$ T-cell clones specific for a *P. falciparum* Th/Tc epitope that overlaps the Th2R epitope, recognized a large number of variant peptides that correspond to polymorphism detected in *P. falciparum* isolates from different geographical areas (Calvo-Calle *et al*, 1997).

In view of the concern that *pfscp* T-cell epitope polymorphisms might impinge on the efficacy of CSP-based vaccines, field isolates from diverse geographic locations were analyzed for the Th2R and Th3R diversity. Two trends emerged from these studies. The degree of variation observed though large did not fulfill the potential indicated by the number of positions where alternate residues were noted. The distribution of allelic variants varied with the geographical origin of the parasites. The lowest degree of variation was found in *P. falciparum* from Brazil, Papua New Guinea and Thailand, whilst the highest was observed in parasites of African origin.

Since sequencing was used to gather the above data, it was often limited to a relatively small number of field samples. A hybridisation-based method, developed for the analysis of samples from the Gambian RTS,S trial, provides an means to survey diversity in a larger number of samples (Alloueche *et al*, 2000). We wished to employ

this method to analyze diversity in *P. falciparum* parasites obtained from two regions located on the Thai-Myanmar border. However, the oligonucleotides developed for PCR-SSOP did not include some of the Th2R and Th3R allelic variants described after its inception. In order to establish whether this set of PCR-SSOP oligonucleotides can be meaningfully used for Thai samples, we surveyed the diversity of the CSP T-cell epitopes by sequencing the corresponding amplified region from a large number of samples obtained from patients infected with *P. falciparum* in two regions in western Thailand.

Materials and Methods

Blood sample collection.

Blood samples were obtained from consenting patients admitted to the Hospital for Tropical Diseases, Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand during June to September, 2001 with a diagnosed *P. falciparum infection*. Blood was collected in ethylene diamine tetraacetic acid (EDTA) tubes. Two hundred microlitres of blood were centrifuged and the red blood cell pellets were stored at – 20 °C.

DNA amplification, cloning and sequencing.

Amplification by the polymerase chain reaction (PCR) was carried out as described by Alloueche *et al.*, with minor modifications in the preparation of the template. Briefly, 5 µl of the lysed freeze-thawed infected red blood cell pellet were added to 150 µl phosphate buffered saline solution and mixed by brief vortexing. The released parasites were pelleted by centrifugation for 5 min at 5000 g, and the supernatant carefully discarded. Fifty µl of amplification reaction mixture, containing 1x PCR buffer, 200 nM of each primer, 250 µM dNTPs, 2.0 mM $MgCl_2$ and 2.5 U Taq DNA polymerase (Applied Biosystems, UK), were added directly to the pellet before initiating the amplification cycle. The primers were designed to hybridise to conserved regions spanning the Th2R and Th3R region (forward primer; 5'-

ACAATCAAGGTAATGGACAAGG-3' and reverse primer; 5'-ACGACATTAAAC ACACACTGGAAC-3'), and to result in the amplification of a 319 base pair fragment. The cycling conditions were 95°C for 5 min, 58°C for 2 min and 72°C for 2 min for 1 cycle, then 94°C for 1 min, 58°C for 2 min and 72°C for 1 min for 32 cycles followed by 10 min extension at 72°C. The PCR amplifications were performed using a PTC 200 (MJ Research, USA). The amplified products were electrophoresed on 1.5% agarose gels in Tris-borate-EDTA buffer, and visualization under UV illumination.

The unique PCR product obtained for each sample was purified using the QIAquick gel extraction kits (QIAGEN, Germany), and cloned using the TOPO TA Cloning kit (Invitrogen, U.S.A.). Plasmid DNA containing the CSP fragment was purified from positive bacterial colonies using the QIAquick Miniprep spin kit (QIAGEN, Germany). Sequencing was performed by automated sequencer at the Hopital Cochin, Paris, France. Sequence alignments were performed using the Gene Jockey II program (Biosoft, United Kingdom).

Results

The CSP gene was amplified from 83 samples. The genomic DNA was extracted from 40 *P. falciparum* infected individuals that gave strong PCR and then further cloned and sequenced. The results of CSP gene specific primers generated a single band of 319 bp PCR products form *P. falciparum* genomic DNA are shown in Fig. 1.

We had sequenced 48 clones of the CSP gene representing 40 Thai isolates of *P. falciparum*. By comparing these sequences of CSP-Th2R (amino acid residues 326-342) and CSP-Th3R regions (amino acid residue 367-378) of 7G8 clone previously published (Alloueche *et al*, 2000), nine (CSP-Th2R*05, CSP-Th2R*12, CSP-Th2R*13, and CSP-Th2R*16-21) and seven (CSP-Th3R*01, CSP-Th3R*04, CSP-Th3R*08, CSP-Th3R*09 and CSP-Th3R*14-16) allelic types of variants were found, respectively (Fig. 2 and Fig. 3). Among these variants, five new allelic types of CSP-Th2R regions designated as CSP-Th2R*16, CSP-Th2R*17, CSP-Th2R*18, CSP-Th2R*20 and CSP-Th2R*21 were described, while three new allelic type of CSP-Th3R regions designated as CSP-

Th3R*14, CSP-Th3R*15 and CSP-Th3R*16 were identified. The frequencies of CSP-Th2R and CSP-Th3R allelic variants are shown in Fig. 4. Some alleles such as CSP-Th2R*05 and CSP-Th3R*01 were frequent while others were rare or absent. We found that CSP-Th2R*16-21 were present at a frequency of 0.12. However, CSP-Th3R*14-16 were present at the frequency of only 0.06.

Compared to the 7G8 amino acid and nucleotide sequences, eight variants amino acid position were observed in the CSP-Th2R region of our isolates (Fig. 2) which were position 327, 332, 333, 337, 338, 339, 340 and 342 of the sequence. In addition, the variations at amino acid position 327 and 338 have never been reported. In such positions, the amino acid were substituted by G (amino acid position 327) and by M (amino acid position 338) which were found in Pf. 078 and Pf. 022/1 isolates, respectively. For the CSP-Th3R region, six variants amino acid positions were observed, which were at amino acid position 367, 368, 369, 370, 372 and 374 (Fig. 3). Only one of amino acid position (368) represented the variation in this region has never been reported in any known alleles so far. The amino acid in such position was substituted by N (Pf. 078 isolate) and by I (Pf. 022/1 isolate). In addition, the nucleotide substitutions occurring predominantly in the first codon position and less frequently in the second codon position were observed in both CSP-Th2R and CSP-Th3R regions. Furthermore, all of nucleotide substitutions in both regions created non-synonymous codon changes.

Discussion

In this study, we have determined the allelic variation of the immunodominant T-cell epitope regions, Th2R and Th3R, in *P. falciparum* CSP gene from field isolates obtaining from malaria endemic areas, west of Thailand and revealed nine allelic variants of CSP-Th2R epitopes (CSP-Th2R*05, CSP-Th2R*12, CSP-Th2R*13, CSP-Th2R*16-21), five of which were not identical to any allelic variants previously reported elsewhere and designated as CSP-Th2R*16, CSP-Th2R*17, CSP-Th2R*18, CSP-Th2R*20 and CSP-Th2R*21. It appears that three allelic types (CSP-Th2R*05, CSP-Th2R*12 and CSP-Th2R*13) were common as previously reported in Mae Sod, Tak province, North of Thailand (Jongwutiwes *et al*, 1994). However in such study,

the other allelic types, CSP-Th2R*14, CSP-Th2R*15 were reported which were not found in our study. The different profiles were reported in Gambian isolates which demonstrated the allelic type, CSP-Th2R*02, CSP-Th2R*03, CSP-Th2R*06-10, in one area (Lockyer *et al*, 1989) and CSP-Th2R*01-10 in another area (Alloueche *et al*, 2000). The studies in Papua New Guinea reported three allelic types (CSP-Th2R*01, CSP-Th2R*05 and unclassified CSP-Th2R) in one area (Shi *et al*, 1992) while only one allelic type (CSP-Th2R*05) was found in different area (Doolan *et al*, 1992). The results in Brazil showed one allelic variant (CSP-Th2R*01) in one study (Shi *et al*, 1992) and two allelic variants (CSP-Th2R*01 and unclassified CSP-Th2R) in another study.

Furthermore, it seems that the allelic type varied depend upon the geographical area by the findings that the CSP-Th2R*05 is the predominant type in Thailand with the frequency of 0.69 in our study and of 0.61 in previous report (Jongwutiwes *et al*, 1994). The studies in Papua New Guinea also showed that CSP-Th2R*05 is the predominant type with frequency of 0.87 (Shi *et al*, 1992) and of 1.0 (Doolan *et al*, 1992). However, these results in the CSP-Th2R allelic types were in contrast to those reported in other geographical areas, which showed their predominant types among Gambian isolates, CSP-Th2R*06 with frequency of 0.42 in one area (Lockyer *et al*, 1989), and CSP-Th2R*04 with frequency of 0.21 reported recently in another area. In contrast, the predominant type among Brazilian isolates was CSP-Th2R*01 (Yoshida *et al*, 1990).

Defining the polymorphism of CSP-Th3R epitopes among Thai isolates in endemic area, west of Thailand, seven allelic forms CSP-Th3R*01, CSP-Th3R*04 CSP-Th3R*08, CSP-Th3R*09, CSP-Th3R*14-16 were identified of which three have never been described elsewhere (CSP-Th3R*14, CSP-Th3R*15 and CSP-Th3R*16). By comparison of polymorphic Th3R CSP determinants from our study in endemic area, west of Thailand with the only one previous report in northern part of Thailand which reported seven allelic types (CSP-Th3R*01, CSP-Th3R*03, CSP-Th3R*04, CSP-Th3R*08-09, and CSP-Th3R*12-13) (Jongwutiwes *et al*, 1994). However, similar findings found that the predominant type was CSP-Th3R*01 with the frequency of 0.75 in the present study and of 0.52 in the previous study.

In contrast to the studies in other geographical areas, such as in Gambian isolates (Lockyer *et al*, 1989), it showed that six allelic type (CSP-Th3R*01-03, CSP-Th3R*05-07) while CSP-Th3R*05 was the predominant type with frequency of 0.3. Thirteen variants designated as Th2R*01-10 and CSP-Th3R*14-16 with CSP-Th3R*02 was the predominant type with the frequency of 0.33 (Alloueche *et al*, 2000). In addition, the studies in Papua New Guinea (Doolan *et al*, 1992) showed only one allelic type CSP-Th3R*04 with the frequency of 1.0 in one study while five allelic types type (CSP-Th3R*01, CSP-Th3R*04, CSP-Th3R*08-09 and CSP-Th3R*11) with the CSP-Th3R*04 as predominant type with frequency of 0.54 (Shi *et al*, 1992). Furthermore, the same studies in Brazil (Yoshida *et al*, 1990) showed that all their isolates displayed the same allelic type CSP-Th3R*01 with the frequency of 1.0 in one study and two allelic types (CSP-Th3R*01 and CSP-Th3R*09) were detected in the another study. In such study, the CSP-Th3R*01 was the predominant with frequency of 0.83 (Shi *et al*, 1992).

Thus, it seems likely that the distribution of the CSP-Th2R and CSP-Th3R alleles varies among different geographic areas. Similar geographically different allelic polymorphism has recently been reported in the CSP gene of *P. vivax* (Qari *et al*, 1992). In addition, the different studies have produced conflicting results regarding levels of diversity (Shi *et al*, 1992; Doolan *et al*, 1992; Malaria division, 2002-2003). The CSP gene of *P. falciparum* from high malaria-transmission region appeared more polymorphic than the CSP of parasites from relative low malaria-endemic regions. Transmission intensity is correlated with malaria prevalence and is a direct measure of the parasite reproduction; it is expected that higher transmission areas maintaining populations with higher effective population size and further diversifying (positive) natural selective could be a leading factor for sustaining the parasite polymorphisms (Escalante *et al*, 2002). However, the results obtained from the present study in Rajburi province, more new allelic types were found than those reported previously in 1994 in Tak province where malaria endemicity is quite higher (Malaria division, 2002-2003) in the present study, we also included four isolates from Tak province which showed the same allelic types as previously described. These new allelic types were not cluster together with those found previously in Thailand suggesting a

considerable heterogeneity within some geographic area. However, more CSP gene of *P. falciparum* isolates need to be investigated in order to understand levels of the gene diversity in any population. The antigenic diversity on the CSP is an important factor in the epidemiology of malaria as well as implications for the development of protective immunity especially anti-sporozoite vaccine if the functional capacity of these variation epitopes in immune induction could be demonstrated. These CSP variations may contribute to the widespread lack of natural sporozoite immunity (Khusmith *et al*, 1998; Zevering *et al*, 1994).

Furthermore, confirming previous observations (Jongwutiwes *et al*, 1994; Lockyer *et al*, 1989; Allouche *et al*, 2000; Shi *et al*, 1992; Doolan *et al*, 1992; Yoshida *et al*, 1990), we also found that the nucleotide substitutions occurred predominantly at the first codon position, less frequently in the second position and were always non-synonymous. The non-synonymous mutations of the imunodominant T-cell epitope domains of the *P. falciparum* CSP gene in our finding strongly supports the co-intention that the mutations arose as a result of selection pressure from human T-cells (De la Cruz *et al*, 1987). It may be important to extend the study to assess the functional capacity of these CSP-Th2R and CSP-Th3R variants in T-cell activation.

The variants of both the CSP-Th2R and CSP-Th3R regions affect human T-cell recognition for $CD4^+$ and $CD8^+$ T-cell responses (Guttinger *et al*, 1988; Hill *et al*, 1992). This result is consistent with the proposal that this variation has evolved through immune selection. Murine and human T-cells primed with one sequence usually fail to respond to variants of CSP-Th2R and CSP-Th3R regions (Guttinger *et al*, 1988; de la Cruz *et al*, 1988 and 1989). A study on human T-cell proliferation in immune subjects in Gambia suggested the cross-reactivity of T-cells from some subjects to variant Th2R and Th3R peptides corresponding to three laboratory isolates. However, 40% of subjects responded to only one variant and 25% failed to respond to any variant. In contrast to the another study it has been demonstrated that T-cells did not cross-react with any variant sequences of CSP-Th2R and CSP-Th3R regions (de la Cruz *et al*, 1988). If there is such significant cross-reactivity in humans, polyvalent vaccine may be approachable.

The comparison of polymorphic CSP determinants from several malaria endemic regions lends support to the hypothesis of host pressure-induced selection of polymorphic protein-bearing parasites. It appears that the primary function of the surface protein determinants are to ensure successful host-parasite interaction. Non-silent mutations in the regions of the CSP that nullify parasite interaction with host cellular receptors will be rapidly selected against and the representative parasites would disappear from the population of the parasites (Shi *et al*, 1992). From an immunologic perspective, parasite protein sequences and/or conformations predicate the immune dominance or immunogenicity of a region and the epitopes that interface with the host immune system would preferentially accumulate changes to escape the pressures of immunity. In the case of purely immunodominant determinants, however, non-silent changes can be positively selected, particularly if the mutation results in an amino acid change that helps the parasite evade host immune pressures. In view of these considerations, the T-helper cell epitope regions, Th2R and Th3R, would fit the definition of an immunogenic site.

In conclusion, comparison of our *P. falciparum* isolates with widely separated geographical regions in the world revealed that this polymorphism appears to be limited and certain CSP alleles might be characteristic of particular geographic areas. These results and other similar studies are essential for further study to determine whether the CSP antigen polymorphism represents in fact a major obstacle for the development of anti-sporozoite vaccine against the malaria parasite.

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References

Alloueche A, Silveira H, Conway DJ, *et al.* High-throughput sequence typing of T-cell epitope polymorphisms in *Plasmodium falciparum* circumsporozoite protein. *Mol Biochem Parasitol* 2000; 106: 273-82.

Calvo-Calle JM, Hammer J, Sinigaglia F, *et al.* Binding of malaria T cell epitopes to DR and DQ molecules *in vitro* correlates with immunogenicity *in vivo*. *J Immunol* 1997; 159: 1362-73.

Dame JB, Williams JL, Mc Cutchan TF, *et al.* S tructure o f t he g ene e ncoding t he immunodominant surface antigen on the sporozoite of the human malaria parasite *Plasmodium falciparum*. *Science* 1984; 225: 593-9.

Day K and Marsh K. Naturally acquired immunity to *Plasmodium falciparum*. *Parasitol Today* 1991; 7: 68-71.

de la Cruz VF, Lal AA, McCutchan TF. Sequence variation in putative functional domains of circumsporozoite protein of *Plasmodium falciparum*. *J Biol Chem* 1987; 262: 11935-9.

de la Cruz VF, Good MF, Miller GL, Maloy WL, Lal AA, McCutchan TF. Lack of cross-reactivity between variant T cell determinants from malaria circumsporozoite protein. *J Immunol* 1988; 141: 2456-60.

de la Cruz VF, Maloy WL, Miller LH, *et al.* The immunological significance of variation within malaria cicumsporozoite protein sequences. *J Immunol* 1989; 142: 3568-75.

Doolan DL, Sual AJ, Good MF. Geographically restricted heterogeneity of the *Plasmodium falciparum* circumsporozoite protein: relevance for vaccine development. *Infect Immun* 1992; 60: 675-82.

Escalante A A, Grebert H M, Isea R, *et al.* A study of genetic diversity in the gene encoding the circumsporozoite protein (CSP) of *Plasmodium falciparum* from different transmission areas-XVI. Asembo Bay Cohort Project. *Mol Biochem Parasitol* 2002; 125: 83-90.

Guttinger M, Caspers P, Takacs B, Trzeciak A, Gillessen D, Pink RJ, Sinigaglia F. Human T cells recognize polymorphic and non-polymorphic regions of the *Plasmodium falciparum* circumsporozoite protein. *EMBO J* 1988; 7: 2555-7.

Hill AVS, Allsopp CEM, Kwiatkowski D, *et al.* Extensive genetic diversity in the HLA class II region of Africans with a focally predominant allele, DRB1* 1304. *Proc Natl Acad Sci USA* 1992; 89: 2277-81.

Hill AVS, Jepson A, Plebanski M, Gilbert S. Genetic analysis for host-parasite coevolution in human malaria. *Phi Trans R Soc Lond* 1997; 352: 1317-25.

Hoffman SL, Oster CN, Mason C, *et al.* Human lymphocyte proliferative response to a sporozoite T cell epitope correlates with resistance to falciparum malaria. *J Immunol* 1989; 142: 1299-303.

Jongwutiwes S, Tanabe K, Hughes MK, *et al.* Allelic variation in the circumsporozoite protein of *Plasmodium falciparum* from Thai field isolates. *Am J Trop Med Hyg* 1994; 51: 659-68.

Khusmith S, Tapchaisri P, Tharavanij S, *et al.* Antigenic diversity of *Plasmodium vivax* and their geographic distribution in Thailand. *Southeast Asian J Trop Med Public Health* 1998; 29: 512-8.

Lockyer MJ, Marsh K, Newbold CI. Wild isolates of *Plasmodium falciparum* show extensive polymorphism in T cell epitopes of the circumsporozoite protein. *Mol Biochem Parasitol* 1989; 37: 275-80.

Malaria division. Annual Report, Department of Communicable Disease Control, Ministry of Public Health 2002-2003.

McCutchan TF, de la Cruz VF, Good MF, Wellems TE. Antigenic diversity in *Plasmodium falciparum*. *Prog Allergy* 1988; 41: 173-92.

Nussenzweig V, Nussezweig RS. Circumsporozoite proteins of malaria parasites. *Cell* 1985; 42: 401-3.

Patarroyo ME, Amador LR, Clavijo P, *et al*. A synthetic vaccine protects humans against challenge with asexual blood stages of *Plasmodium falciparum* malaria. *Nature* 1988; 332: 158-61.

Qari SH, Goldman IF, Povoa MM, Santi S, Alpers MP, Lal AA. Polymorphism in the circumsporozoite protein of the human malaria parasite *Plasmodium vivax*. *Mol Biochem Parasitol* 1992; 55: 105-13.

Shi Y, Alpers MP, Povoa MM, Lal AA. Diversity in the immunodominant determinants of the circumsporozoite protein of *Plasmodium falciparum* parasites from malaria-endemic regions of Papua New Guinea and Brazil. *Am J Trop Med Hyg* 1992; 47: 844-51.

Yoshida N, Di Santi SM, Dutra AP, Nussenzweig RS, Nussenzweig V, Enea V. *Plasmodium falciparum*: Restricted polymorphism of T cell epitopes of the circumsporozoite protein in Brazil. *Exp Parasitol* 1990; 71: 386-92.

Zevering Y, Khamboonruang C and Good MF. Natural amino acid polymorphisms of the circumsporozoite protein of *Plasmodium falciparum* abrogate specific human $CD4^+$ T cell responsiveness. *Eur J Immunol* 1994; 24: 1418-25.

Zevering Y, Khamboonruang C and Good MF. Human and murine T-cell responses to allelic forms of a malaria circumsporozoite protein epitope support a polyvalent vaccine strategy. *Immunology* 1998; 94: 445-54.

Table 1. Summary of CSP-Th2R and Th3R allelic types of 48 recombinant clones using automated DNA sequencer

Province	CSP-Th2R		CSP-Th3R		CSP-Th2R & CSP-Th3R	
	Type	No.clones	Type	No.clones	Type	No.clones
Ratchaburi	***05**	25 (52.1%)	***01**	28 (58.3%)	***05 & *01**	25 (52.1%)
	***12**	5	*08	1	*12 & *09	5
	*16®	1	*09	5	*16® & *01	1
	*17®	1	*16®	1	*17® & *01	1
	*18®	1			*18® & *01	1
	*20®	1			*20® & *08	1
	*21®	1			*21®&*16®	1
Kanchaburi	***05**	6	***01**	5	***05 & *01**	5
	***13**	1	*04	1	*05 & *14®	1
	***19**	1	*14®	1	*13 & *15®	1
			*15®	1	*19 & *04	1
Tak	***05**	1	***01**	2	*05 & *01	1
	***12**	3	*09	2	*12 & *01	1
					*12 & *09	2
Petchaburi	***05**	1	***01**	1	*05 & *01	1
	Total	**48 (100%)**	**Total**	**48 (100%)**	**Total**	**48 (100%)**

® New allelic type and the most common allelic variant is in bold.

Figure legend

Fig. 1. Schematic representation of agarose gel electrophoresis of PCR products from purified DNA samples using primers binding to conserved sequences flanking the CSP-Th2R and Th3R regions: lane M, 100 bp ladder; lane 1, negative control (no template); lane 2, 7G8 positive control; lane 3, 3D7 negative control; lane 4-8, purified DNA from blood of falciparum malaria patients.

Fig. 2. CSP-Th2R variation in field and laboratory *P. falciparum* isolates. Nucleotide and deduced amino acid numbering is according to the 7G8 sequence. Identity with the 7G8 sequence is indicated by a dash. DNA mutations are shown below that position where corresponding amino acid substitution is listed. The codes Pf. 003, 005, 078/1, 022, 025/1, 056, 078, 074, and 022/1 refer to the patients from whom the *P. falciparum* infected blood were obtained.

Fig. 3. CSP-Th3R variation in field and laboratory *P. falciparum* isolates. Nucleotide and deduced amino acid numbering is according to the 7G8 sequence. Identity with the 7G8 sequence is indicated by a dash. DNA mutations are shown below that position where corresponding amino acid substitution is listed. The codes Pf. 003, 005, 019/1, 022, 074,and 078 refer to the patients from whom the *P. falciparum* infected blood were obtained.

Fig. 4. Allelic frequency of CSP-Th2R and CSP-Th3R variants in Thai *P. falciparum* isolates using DNA sequencing.

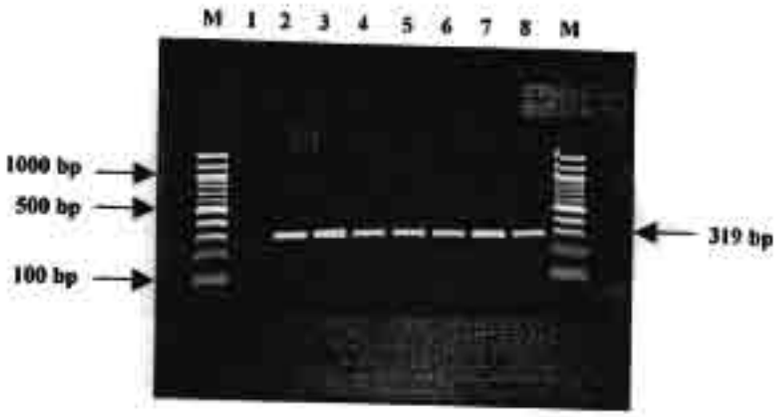

Fig. 1. Kumkhaek *et al.*

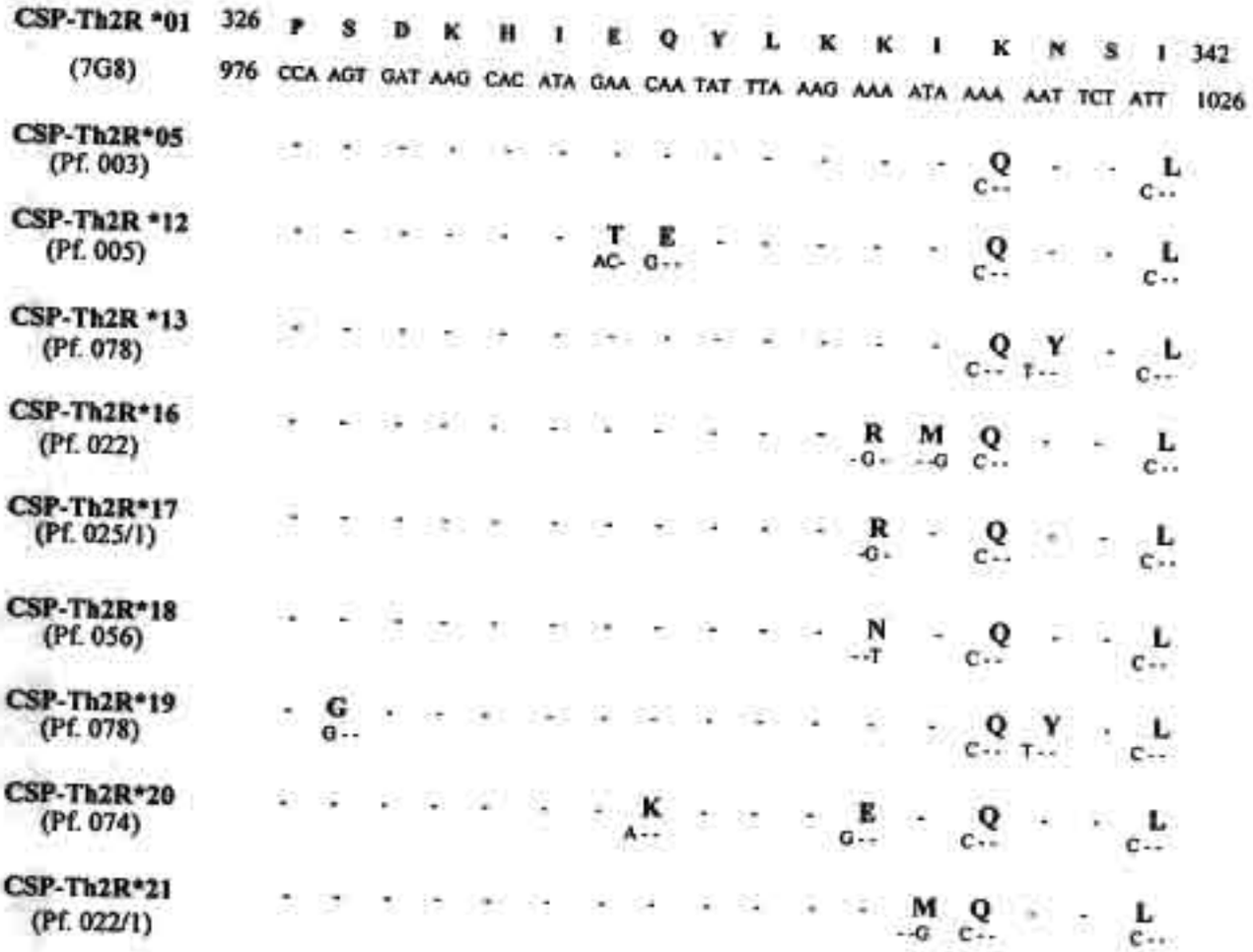

Fig. 2. Kumkhaek *et al.*

CSP-Th3R*01 (7G8)	367	N	K	P	K	D	E	L	D	Y	E	N	D	378
	1097	AAT	AAA	CCT	AAA	GAC	GAA	TTA	GAT	TAT	GAA	AAT	GAT	1133
CSP-Th3R*01 (Pf. 003)		-	-	-	-	-	-	-	-	-	-	-	-	
CSP-Th3R*04 (Pf. 078)		D G--	-	-	-	-	Q C--	-	-	-	-	-	-	
CSP-Th3R*08 (Pf. 074)		-	-	-	-	-	Q C--	-	-	-	-	-	-	
CSP-Th3R*09 (Pf. 005)		G GG-	-	S T--	-	-	-	-	-	-	-	-	-	
CSP-Th3R*14 (Pf. 019/1)		-	-	-	N --T	-	-	-	-	-	-	-	-	
CSP-Th3R*15 (Pf. 078)		D G--	N --C	-	-	-	Q C--	-	N A--	-	-	-	-	
CSP-Th3R*16 (Pf. 022/1)		-	I -T-	-	-	-	-	-	-	-	-	-	-	

Fig. 3. Kumkhaek *et al.*

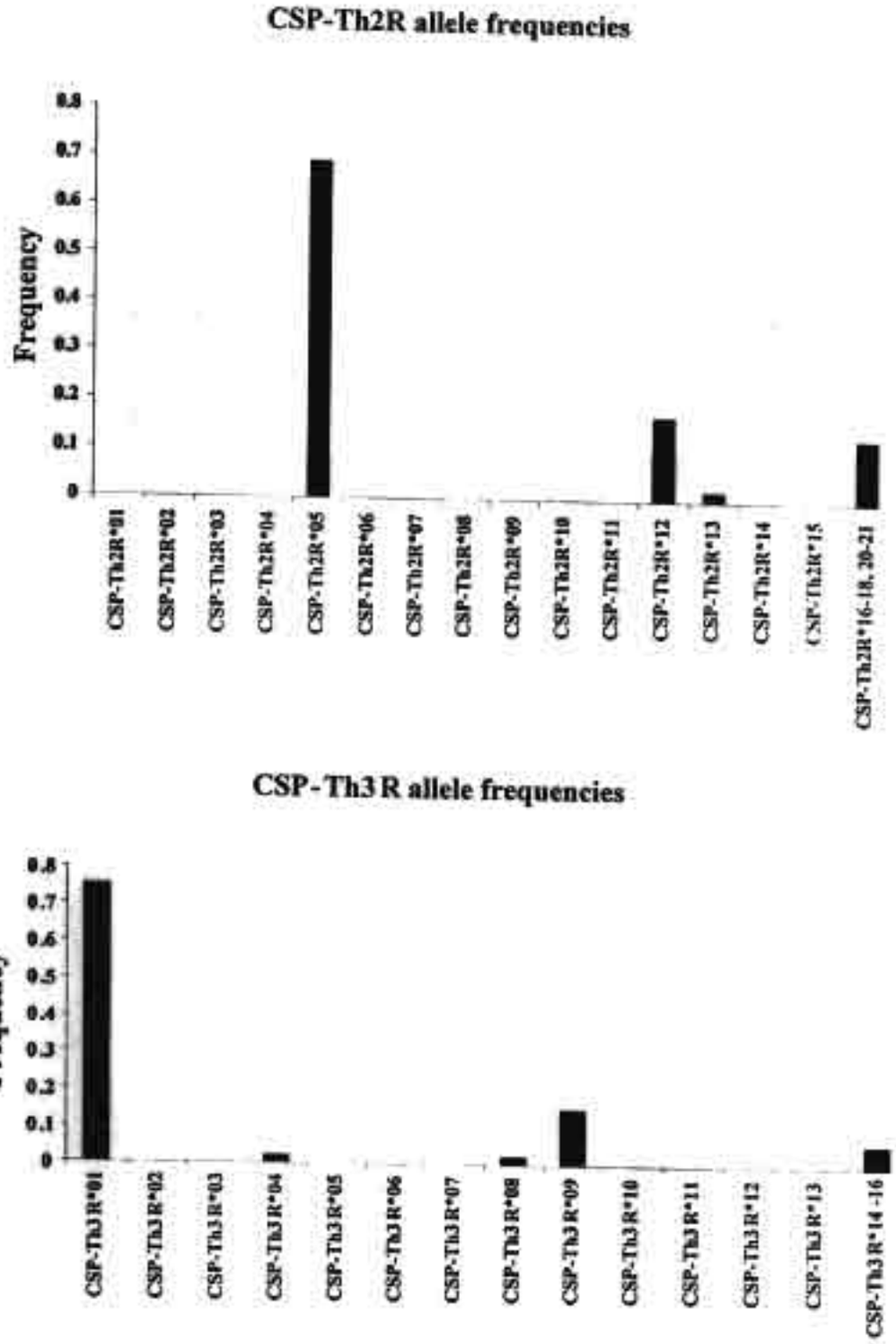

Fig. 4. Kumkhaek *et al*