



Genetic structure of apical membrane antigen-1 in *Plasmodium falciparum* isolates from Pakistan



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Abstract

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Plasmodium falciparum apical membrane antigen-1 (PfAMA-1) is a major candidate for the blood-stage malaria vaccine. Genetic polymorphisms of global *pfama-1* suggest that the genetic diversity of the gene can disturb effective vaccine development targeting this antigen. This study was conducted to explore the genetic diversity and gene structure of *pfama-1* among *P. falciparum* isolates collected in the Khyber Pakhtunkhwa (KP) province of Pakistan. A total of 19 full-length *pfama-1* sequences were obtained from KP-Pakistan *P. falciparum* isolates, and genetic polymorphism and natural selection were investigated. KP-Pakistan *pfama-1* exhibited genetic diversity, wherein 58 amino acid changes were identified, most of which were located in ectodomains, and domains I, II, and III. The amino acid changes commonly found in the ectodomain of global *pfama-1* were also detected in KP-Pakistan *pfama-1*. Interestingly, 13 novel amino acid changes not reported in the global population were identified in KP-Pakistan *pfama-1*. KP-Pakistan *pfama-1* shared similar levels of genetic diversity with global *pfama-1*. Evidence of natural selection and recombination events were also detected in KP-Pakistan *pfama-1*.

Keywords: *Plasmodium falciparum*, apical membrane antigen-1, genetic diversity, Pakistan

Introduction

Malaria caused by *Plasmodium* species is a major infectious disease in humans, causing a significant global public health burden. Irrespective of the massive control efforts to eliminate malaria over the past years, malaria is still prevalent in several endemic areas. The World Health Organization reported 247 million malaria cases and 619,000 deaths in 2021 [1]. Malaria control and elimination efforts have been challenged due to the spread of anti-malarial drug-resistant parasites and insecticide-resistant *Anopheles* mosquitoes. The lack of an effective vaccine creates a major obstacle in malaria control, indicating the indispensable need to develop an effective vaccine. Several plasmodial proteins such as circumsporozoite protein (CSP), Duffy binding protein, merozoite surface proteins, apical membrane antigen-1 (AMA-1), and thrombospondin-related anonymous protein have been considered promising vaccine candidates because of their antigenic properties and expression in either preerythrocytic or erythrocytic stages of malaria parasites [2,3]. However, the genetic polymorphisms in these antigens among clinical isolates are significant hurdles in the development of effective malaria vaccines [4,5].

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Conflict of interest

The authors declare no conflicts of interests.

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AMA-1 is a type I integral membrane protein commonly expressed on the surfaces of merozoites and sporozoites and plays important roles in parasite invasion into host cells [6,7]. It comprises a signal sequence, a cysteine-rich ectodomain, a conserved cytoplasmic region, and a transmembrane region [8]. The ectodomain of AMA-1 is further subdivided into 3 domains, viz., domains I (DI), II (DII), and III (DIII). The ectodomain is highly immunogenic and induces natural immune responses in individuals infected with *P. falciparum* [9,10]. Antibodies against AMA-1 prevent the invasion of erythrocytes by malaria parasites and establish a protective immune response [11,12]. This information suggests that the antigen is a feasible vaccine candidate. Similar to that in other major surface antigens of malaria parasites, substantial levels of genetic polymorphisms of *ama-1* have also been recognized in wild parasite populations; however, AMA-1 has been considered less variable than other potential vaccine candidate antigens such as CSP and merozoite surface proteins, supporting the notion that it is a promising blood-stage vaccine candidate [10,13,14]. Nevertheless, genetic diversities observed in global *ama-1* have emphasized the importance of continuous monitoring of genetic variations of *ama-1* among global malaria parasites for designing an effective vaccine targeting this antigen [15].

Pakistan is a malaria-endemic country, with reports of millions of cases per year [16]. *P. falciparum* and *P. vivax* are the prevalent species, accounting for 32% and 67% of malaria cases, respectively. Khyber Pakhtunkhwa (KP) and Balochistan provinces have been the most critical malaria hot spots in the country [16]. A study on the genetic analysis of the hypervariable DI of *P. falciparum* AMA-1 (*pfama-1*) in Pakistan *P. falciparum* isolated in Hazara division, KP, Pakistan, had been performed [17]. However, the sequences did not cover full-length gene sequences, rendering only limited information on the genetic nature of Pakistan *pfama-1*. This study was conducted to analyze the genetic nature of full-length *pfama-1* among *P. falciparum* isolates collected from the KP province of Pakistan.

Materials and Methods

Ethics statement

The study protocol was approved by the Ethical Review Committee of Abdul Wali Khan University Mardan under the letter of AWKUM/ERC/578. Consent was obtained from all participants before conducting the study.

Parasite samples and DNA purification

Blood samples were collected from 19 patients infected with *P. falciparum*, which was confirmed by microscopy and rapid diagnostic tests in different hospitals and private sector laboratories in KP, Pakistan (Supplementary Fig. S1). During the 2 malaria seasons from March to May and August to November 2019, the area had an average annual rainfall of 384 mm. The mean temperature in the region was 20°C–40°C. The blood samples collected before treatment were spotted on filters, air-dried, and stored in individually sealed plastic bags at ambient temperature until use. Genomic DNA was extracted from the spotted blood samples using a QIAamp blood mini kit (Qiagen, Redwood City, CA, USA) according to the manufacturer's instructions and stored at -20°C.

PCR amplification and DNA sequencing

The full-length *pfama-1* was amplified by PCR using specific primer sets and amplification conditions described previously [18,19]. The PCR products were analyzed on a 1.5% agarose gel, purified, and cloned into the T&A vector (Real Biotech, Banqiao City, Taiwan). The ligation mixture was transformed into *Escherichia coli* DH5 α competent cells, and positive clones were selected by colony PCR. The nucleotide sequence of the cloned insert was analyzed by automatic DNA sequencing with M13 forward and M13 reverse primers (Genotech, Daejeon, Korea). Sequencing was also conducted using 2 additional internal primers (5'-CAGGGAAATGTCCAGTATTTGGTA-3' and 5'-TTCCATCGACCCATA-ATCCG-3') to obtain clear sequences for the central part of *pfama-1* [18]. To ensure accuracy, sequencing of at least 2 different clones from each isolate was performed. Raw data were filtered for quality assessment using the DNASTAR Lasergene software (DNASTAR, Madison, WI, USA). The 19 KP-Pakistan *pfama-1* nucleotide sequences were deposited in GenBank under accession numbers OM628702–OM628720.

Polymorphism analysis of *pfama-1*

The DNA sequence generated in this study was analyzed in comparison with a reference gene of *pfama-1* from the *P. falciparum* 3D7 strain (GenBank Accession No.: U65407). The following global *pfama-1* sequences deposited in GenBank were also included for analysis: Thailand (AB715735–AB715814), Myanmar (KU893276–KU893333), Philippines (AB715815–AB715869), Vietnam (MW938322–MW938452), Vanuatu (AB716010–AB716094), Solomon Islands (SI; AB715960–AB716009), Papua New Guinea (PNG; AB715870–AB715959), Ghana (AB715698–AB715734), and Tanzania (AB715636–AB715697) (Supplementary Table S1). Comparative sequence analyses were conducted to identify polymorphic loci using the MEGA6 program [20].

Statistical and population genetic analyses

The DnaSP v6.12 software package [21] was used to estimate parsimony informative sites, total number of mutations, pairwise nucleotide diversity (π), haplotype diversity, segregating sites, haplotypes, recombination between adjacent nucleotides per generation, and the minimum number of recombination events (R_m). Linkage disequilibrium was estimated between the various polymorphic sites based on the recombination events (R^2) index using the DnaSP v6.12 software package [21]. Tajima's D, Fu and Li's D, and F indices were calculated by a sliding window method using the DnaSP v6.12 software package [21]. Population genetics, including pairwise fixation index (F_{ST}) and haplotype frequencies, were evaluated using the analysis of molecular variance. The significance of the analysis of molecular variance was estimated by 1,000 per mutation, and the nucleotide diversity based on Nei's net distance was computed using Arlequin v3.5 [22]. The haplotype network plot was generated using the PopArt software [23].

Assessments of natural selection signatures

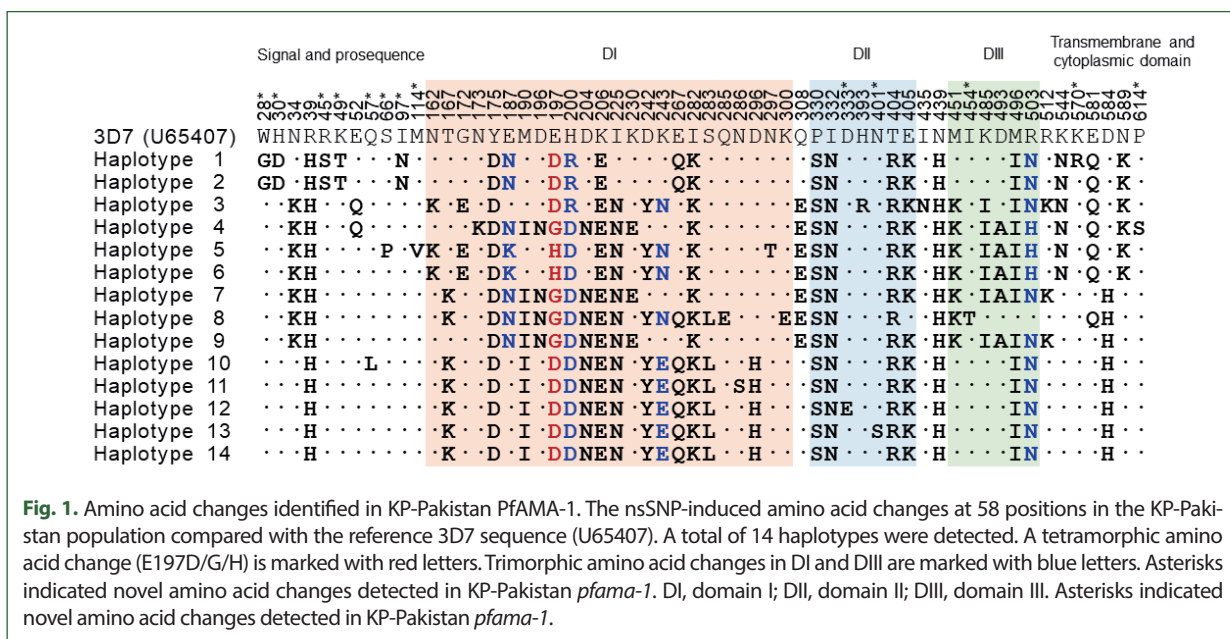
The global *pfama-1* sequences were aligned and filtered using the GUIDANCE server with the confidence score threshold (i.e., best score ~ 1) [24]. This low-quality alignment filtration is essential for the accuracy of the natural selection analysis [25]. The good-quality, re-

liable alignment was subjected to the Datamonkey server of the HYPHY package for the identification of selected loci with a default *P* value [26-28]. Individual sites underlying positive selection were inferred using the following 3 algorithms: fixed effects likelihood, internal branches fixed effects likelihood, and mixed effects model of evolution [28].

Results

Genetic polymorphic features of KP-Pakistan *pfama-1*

The 19 full-length *pfama-1* sequences were successfully amplified from the 19 KP-Pakistan *P. falciparum* isolates. The gene length was 1,869 bp, and no size polymorphism was identified in the sequences. Comparative analysis of the 19 KP-Pakistan *pfama-1* sequences with the 3D7 *pfama-1* reference sequence (U65407) revealed genetic polymorphisms in KP-Pakistan sequences. Across the sequences, 69 single nucleotide polymorphisms (SNPs) were identified, among which 58 were nonsynonymous SNPs (nsSNPs), resulting in amino acid substitutions at 58 positions and 14 distinct haplotypes. Most amino acid changes were found in DI (*n* = 24), DII (*n* = 7), and DIII (*n* = 6) (Fig. 1; Supplementary Table S2). A tetramorphic change (E197D/G/H) and 3 trimorphic changes (E187N/K, H200R/D, and K243E/N) were detected in DI. A trimorphic amino acid change (R503N/H) was identified in DIII. The other 53 amino acid changes throughout the sequences were dimorphic. We also comparatively analyzed KP-Pakistan *pfama-1* with previously reported global *pfama-1* and identified 113 nsSNPs causing amino acid substitutions (92 dimorphic, 17 trimorphic, 2 tetramorphic, and 2 pentamorphic) in global *pfama-1*, including KP-Pakistan *pfama-1*. Interestingly, 13 amino acid changes (W28D, H30D, R45S, K49T, Q57L, S66P, I97N, and M114V in the signal and prosequence region; D333E and N401S in DII, I454T in DIII; and K570K and P614S in the transmembrane and cytoplasmic domain) detected in KP-Pakistan *pfama-1* were novel that were not reported previously, although their fre-



quencies were relatively low (Fig. 1; Supplementary Table S2). Meanwhile, the most amino acid changes in DI, DII, and DIII were commonly detected in global *pfama-1* (Supplementary Table S2). Global *pfama-1* exhibited similar patterns of amino acid changes, but the frequency of each amino acid change differed by country.

Nucleotide diversity and natural selection

We identified 69 segregating sites and 69 mutations in KP-Pakistan *pfama-1* isolates. Haplotype diversity and nucleotide diversity (π) were 0.982 ± 0.022 and 0.0109 , respectively (Table 1). Tajima's D, Fu and Li's D, and Fu and Li's F values were positive, suggesting that positive natural selection affected KP-Pakistan *pfama-1*. The overall nucleotide diversity (π) across global *pfama-1* ranged from 0.0043 ± 0.0006 (Vietnam) to 0.0141 ± 0.0003 (Ghana), suggesting mild levels of genetic diversity in global *pfama-1* populations. The π in KP-Pakistan *pfama-1* was lower than or similar to that in Asia and Pacific *pfama-1* populations but lower than that in Africa *pfama-1* populations (Table 1). All *pfama-1* sequences, except Vietnam *pfama-1* sequences, demonstrated positive Tajima's D values, indicating the role of balancing selection in global *pfama-1* (Table 1). The positive values of both Fu and Li's D and F also suggested evidence for the role of balancing selection in global *pfama-1*, except Vietnam *pfama-1*. A sliding window plot of π suggested that global *pfama-1* shared highly similar patterns of π across the sequences (Fig. 2). The highest peak of π was commonly identified at cluster 1 of the loop I (C1-L) region in DI of all global isolates. Similar profiles of Tajima's D across the gene were also identified in global *pfama-1*, except Vietnam *pfama-1* (Fig. 2). The F_{ST} analysis between KP-Pakistan and global *pfama-1* populations indicated genetic differentiation of KP-Pakistan isolates. KP-Pakistan *pfama-1* showed the low-

Table 1. The estimates of DNA sequence polymorphism and tests of neutrality in *pfama-1* among KP-Pakistan and global *pfama-1*

Isolates	Segregating sites (S)	Singleton variable sites	Parsimony informative sites	Total no. of mutations	K	H	Hd \pm SD	$\pi \pm$ SD	Tajima's D	Fu and Li's D	Fu and Li's F
KP-Pakistan (n=19)	69	18	51	69	20.409	16	0.982 ± 0.022	0.0109 ± 0.0022	0.1391 (P>0.1)	0.2370 (P>0.1)	0.2420 (P>0.1)
Vietnam ^a (n=131)	114	47	67	116	8.062	73	0.837 ± 0.034	0.0043 ± 0.0006	-2.0046 (P<0.05)	-3.1913 (P<0.05)	-3.1959 (P<0.05)
Myanmar ^b (n=58)	78	16	62	79	19.886	37	0.948 ± 0.019	0.0106 ± 0.0005	0.5719 (P>0.1)	0.1677 (P>0.1)	0.3851 (P>0.1)
Thailand ^b (n=80)	58	5	53	61	20.621	21	0.920 ± 0.013	0.0110 ± 0.0002	2.2222 (P<0.05)	0.9572 (P>0.1)	1.7516 (P<0.05)
Philippines ^b (n=55)	61	3	58	62	21.232	19	0.916 ± 0.019	0.0114 ± 0.0003	1.9545 (0.05<P<0.1)	1.6123 (P<0.05)	2.0796 (P<0.05)
PNG ^b (n=90)	68	2	66	75	22.923	28	0.954 ± 0.007	0.0123 ± 0.0002	1.8082 (0.05<P<0.1)	1.6759 (P<0.05)	2.0760 (P<0.05)
SI ^b (n=50)	56	3	53	57	22.044	9	0.862 ± 0.021	0.0118 ± 0.0003	2.5455 (P<0.05)	1.5447 (P<0.05)	2.2804 (P<0.05)
Vanuatu ^b (n=85)	50	6	44	50	18.905	5	0.633 ± 0.028	0.0101 ± 0.0003	2.8973 (P<0.1)	0.8642 (P>0.1)	2.0039 (P<0.05)
Ghana ^b (n=37)	75	7	68	85	26.261	32	0.992 ± 0.008	0.0141 ± 0.0003	1.0674 (P>0.1)	1.2179 (P>0.1)	1.3850 (P>0.1)
Tanzania ^b (n=62)	81	8	73	89	25.444	47	0.989 ± 0.005	0.0136 ± 0.0002	1.1819 (P>0.1)	1.1635 (P>0.1)	1.4020 (P>0.1)

K, average number of pairwise nucleotide differences; H, number of haplotypes; Hd, haplotype diversity; π , observed average pairwise nucleotide diversity.

^aCited from [19].

^bCited from [18].

est F_{ST} values against *pfama-1* from the Philippines, PNG and SI, but it showed higher F_{ST} values against *pfama-1* from Myanmar, Thailand, and Vietnam (Table 2).

Recombination and linkage disequilibrium

The R_m of KP-Pakistan *pfama-1* was estimated to be 9. The values between adjacent sites

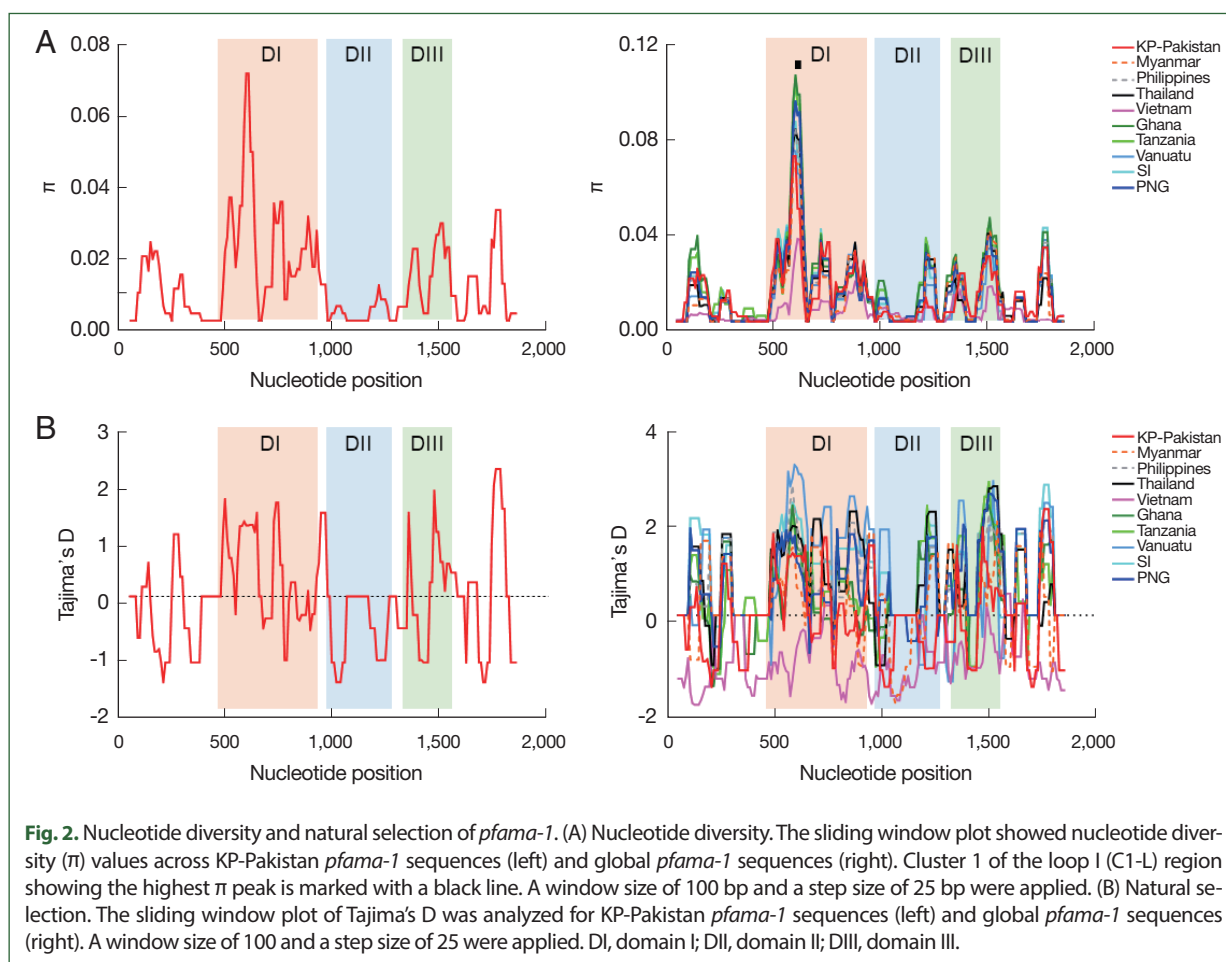


Table 2. Estimation of genetic differentiation between global *pfama-1* population

	KP-Pakistan	Myanmar	Thailand	Vietnam	Philippines	PNG	SI	Vanuatu	Ghana	Tanzania
KP-Pakistan	-									
Myanmar	0.19025	-								
Thailand	0.13256	0.06743	-							
Vietnam	0.57838	0.45520	0.40917	-						
Philippines	0.07009	0.09227	0.04578	0.46879	-					
PNG	0.08690	0.05991	0.04382	0.40086	0.02891	-				
SI	0.10522	0.10515	0.07468	0.48316	0.04465	0.02829	-			
Vanuatu	0.18820	0.10437	0.11096	0.43044	0.07502	0.06868	0.05845	-		
Ghana	0.11194	0.09206	0.05441	0.47405	0.05433	0.04658	0.06201	0.14206	-	
Tanzania	0.11193	0.08872	0.06651	0.45434	0.06556	0.04287	0.06663	0.15077	0.01126	-

PNG, Papua New Guinea; SI, Solomon Islands.

(Ra) and per gene (Rb) were 0.0238 and 44.4, respectively (Table 3). Possible recombination events were also identified in global *pfama-1*. The Rm values of Africa *pfama-1* were greater than those of Asia and Pacific *pfama-1*. The increasing distance across the gene with the decreased linkage disequilibrium index (R^2) in global *pfama-1* suggests that recombination could be a major force contributing to the genetic diversity of *pfama-1* (Supplementary Fig. S2).

Haplotype network analysis

The haplotype network analysis of 667 global *pfama-1* and 3D7 reference sequences re-

Table 3. Comparison of recombination events among global *pfama-1*

	<i>n</i>	Ra ^a	Rb ^b	Rm ^c
KP-Pakistan	19	0.0238	44.4	9
Vietnam	131	0.0000	0.001	20
Myanmar	78	0.0141	26.3	24
Thailand	38	0.0243	45.3	20
Philippines	61	0.0218	40.8	17
PNG ^d	68	0.0500	93.4	28
SI ^e	56	0.0120	22.5	14
Vanuatu	50	0.0010	1.8	9
Ghana	75	0.0921	172	27
Tanzania	81	0.0915	171	28

^aRa, recombination parameter between adjacent sites.

^bRb, recombination parameter for entire gene.

^cRm, minimum number of recombination events between adjacent sites.

^dPNG, Papua New Guinea.

^eSI, Solomon Islands.

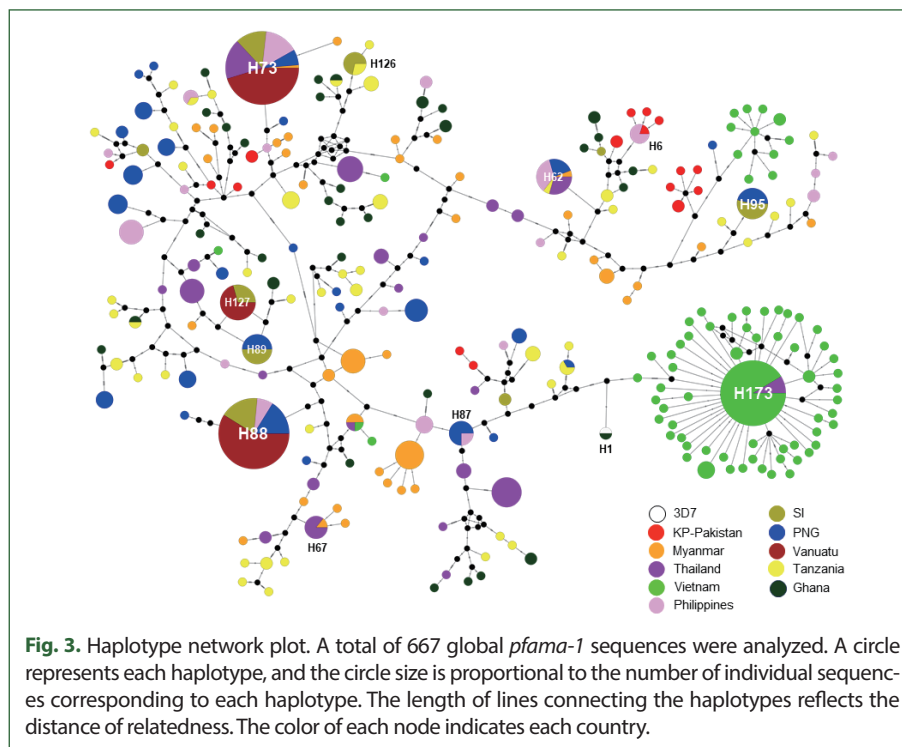


Table 4. The *pfama-1* codons predicted to underline natural selection in KP-Pakistan and global samples

Domain	Amino acid position	Episodic positive selection by MEME	Pervasive positive selection	
			FEL	IFEL
Signal and prosequence	34 ^a		*	*
	35		*	
	52 ^a		*	*
DI	167 ^a	T-K	*	*
	172 ^a	G-E	*	*
	187 ^a		*	*
	197 ^a	E-Q/G/D/R/H	*	*
	200 ^a	H-D/L/R		
	242 ^a	D-Y	*	*
	267 ^a		*	*
	283 ^a	S-L	*	*
	308 ^a	Q-E/K	*	*
DII	330 ^a	P-S	*	*
	395	K-R	*	*
	404 ^a	T-R	*	
	405 ^a		*	
	435 ^a	I-N/T	*	*
DIII	439 ^a	N-H/D	*	*
	451 ^a	M-K	*	*
	485 ^a	K-I	*	*
	493 ^a	D-A	*	*
Transmembrane and cytoplasmic	503 ^a	R-N/H	*	*
	512 ^a	R-K	*	*
	544 ^a	K-N	*	*

^aPositions of amino acid changes that were commonly found in global *pfama-1*.

vealed a complicated network of 260 distinct haplotypes (Fig. 3). Most haplotypes were singletons. Haplotype 73 (H73) was the most predominant with a frequency of 10.6% and was shared by *pfama-1* from different countries, including Myanmar, Thailand, the Philippines, Vanuatu, PNG, and SI. Haplotype 88 (H88) was the second major haplotype with a frequency of 10.2% and was shared by *pfama-1* from Vanuatu, SI, PNG, and the Philippines. KP-Pakistan *pfama-1* constructed 16 haplotypes that were scattered in the network. Only one sequence from Ghana shared a haplotype (H1) with 3D7.

Assessments of natural selection signatures

We analyzed the pattern of natural selection signatures across global *pfama-1*. The episodic positive selection analysis using the mixed effects model of evolution method suggested that 18 amino acid changes were under natural selection ($P \leq 0.05$) (Table 4). The pervasive positive selection signatures analyzed using fixed effects likelihood and internal branches fixed effects likelihood methods suggested that 20 amino acid changes were under natural selection (Table 4). All amino acid changes predicted to be under positive natural selection matched the amino acid changes commonly detected in global *pfama-1*.

Discussion

The complex biological properties of parasites and vectors and the large genetic and anti-

genic variations in prospective vaccine candidate antigens have hindered the development of an effective malaria vaccine despite extensive attempts over the past few decades. Although the RTS, S/A01, the first malaria vaccine endorsed by the World Health Organization for routine immunization of children in transmission areas, has developed based on CSP [29], there are controversies on its efficacy due to modest and short-lived protection efficiency and insufficient effectiveness against parasites with different alleles of CSP [30,31]. To address these limitations, a multistage vaccine combining different candidate antigens such as Duffy binding protein and AMA-1 was proposed [32,33]. The biological significance and immunological functions of PfAMA-1 suggest that this antigen is an attractive vaccine candidate. Nonetheless, the genetic polymorphisms observed in global *pfama-1* also emphasize the importance of continuous surveillance of the genetic diversity of the gene in the global parasite population [18,19].

Similar to that in *pfama-1* from other geographical areas [18,19], KP-Pakistan *pfama-1* also exhibited genetic polymorphisms causing amino acid changes. The most common amino acid changes in global *pfama-1*, especially those in DI, DII, and DIII, were also identified in KP-Pakistan *pfama-1*. These common amino acid changes observed in DI and DIII of global *pfama-1* matched with B-cell epitopes 3, 4, 5, 9, and 10, supporting the notion that these are major regions under natural selection and contribute to host immune escape [18,19,34]. Meanwhile, 13 novel amino acid changes not reported in global *pfama-1* were identified in KP-Pakistan *pfama-1*, most of which were distributed in the signal and prosequence region. Among these amino acid changes, Q57L, S66P, I97N, I454T, and K570R were located in the intrinsically unstructured/disordered region, and D333E was mapped in red blood cell-binding sites. The amino acid changes I97N, M114V, D333E, N401S, and I454T were also located in the intrinsically unstructured/disordered region or B-cell epitope. These findings suggest the potential roles of these amino acids in the modulation of host immune responses; however, further studies would be required to clarify the biological significance of these amino acid changes.

KP-Pakistan *pfama-1* demonstrated similar patterns of genetic diversity and natural selection with those of *pfama-1* from other geographical regions. The π value for the global *pfama-1* population varied, with the π values of Asia and Pacific *pfama-1* populations being relatively lower than that of the Africa *pfama-1* population. Although the π value of global *pfama-1* differed by country, similar patterns of π across *pfama-1* were identified in global *pfama-1*, including KP-Pakistan *pfama-1*. The sliding window plot revealed that the high levels of π were similarly observed in DI and DIII of global *pfama-1*, supporting that the domains are the central regions contributing to the genetic heterogeneity of *pfama-1* [18,19]. The positive values of Tajima's D, Fu and Li's D, and F of KP-Pakistan *pfama-1* suggested that the gene was under balancing selection. Similar patterns of natural selection were also detected in global *pfama-1*, except Vietnam *pfama-1* [18,19,35]. Meiotic recombination is also a driving force generating the genetic diversity of *pfama-1* [18,19]. Potential recombination events were also detected in KP-Pakistan *pfama-1*, suggesting that interallelic recombination is a force causing the genetic diversity of KP-Pakistan *pfama-1*. F_{ST} is a measure of population substructure and is the most common statistic to analyze the overall genetic differentiation among populations as follows: no differentiation (0), low genetic differentiation (0–0.05), moderate differentiation (0.05–0.15), or high differentiation (0.15–

0.25) [36]. Global *pfama-1* displayed low or moderate levels of F_{ST} between and among populations originating from different continents or countries. The only exception was Vietnam *pfama-1* [19]. Although global *pfama-1* demonstrated a complicated haplotype diversity with 260 distinct haplotypes, low or moderate levels of F_{ST} values between and among populations suggest that global *pfama-1* has a relatively stable genetic structure in the global population.

This study has some drawbacks due to the limited number of global *pfama-1* sequences obtained from parasites collected at different time points, which could not reflect the genetic structure and evolutionary aspect of the current global *pfama-1* population. Further in-depth analysis using a greater significant number of global *P. falciparum* populations is required to understand the genetic structure of *pfama-1*.

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