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Review Article

Single Nucleotide Polymorphisms in Plasmodium falciparum Genes: Their Roles in Antimalarial Drugs Resistance and Recent Detection Strategies

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Abstract: Introduction: Malaria is a serious tropical disease with *Plasmodium falciparum* as its most well-known causative parasite for producing higher levels of late stage parasites that leads to sequestration in vital organs which could lead to death. There is a growing trend of antimalarial drugs resistance against *Plasmodium falciparum*. Molecular assessment using polymerase chain reaction could trace the presence of mutation and also determine single-nucleotide polymorphism (SNP) in *Plasmodium falciparum* genes. This SNP can determine the particular population's response to antimalarial drugs. **Objectives:** This study aims to examine the relationship between SNP in *Plasmodium falciparum* genes and antimalarial drugs resistance. **Methods:** Literature searches were carried out through various databases which were then collected and analyzed. **Result:** We identified various SNPs from eleven known genes in *Plasmodium falciparum*, each SNPs causes a different mechanism which contributes to antimalarial drug resistance. Mechanisms varying from slower drug clearance to drug transport activity alteration. **Conclusion:** Results from most studies included in this review suggest that SNPs in *Plasmodium falciparum* genes participate in the resistance against various antimalarial drugs via several mechanisms and may be necessary for parasite survival when stressed.

Keywords: Single Nucleotide Polymorphism; SNP; *Plasmodium falciparum*; Sequencing; Antimalarial Drugs Resistance

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1. Background

Malaria is a disease caused by parasites from *Plasmodium* genus and being transmitted to people through *Anopheles* mosquitoes that is also known as the vector of the disease. Therefore, malaria is a vector-borne parasitic disease. There are currently five known species of *Plasmodium* infecting humans: *P. falciparum*, *P. malariae*, *P. vivax*, *P. knowlesi,* and *P. ovale*. However, *P. falciparum* is the most well-known agent that is able to produce higher levels of late stage parasites that leads to sequestration in vital organs such as liver, heart, lungs, kidney, and brain. This mechanism causes severe anemia which could lead into death [1].

P. falciparum itself accounted for more than 99% of malaria cases in the African region, half of the cases in the South-East Asia region, and 65% of cases in Western Pacific region in 2018. It was then estimated that there were 228 million malaria cases in over 90 countries worldwide [2]. In addition to that, *P. falciparum*, along with *P. vivax*, have also developed resistance to most antimalarial drugs. Even though the geographical distribution of drug resistance varies, this resistance is now posing as the primary threat for malaria control to reduce its morbidity and mortality [2], [3].

Antimalarial drugs consist of a limited number of drugs with the most widely used being quinine and its derivative, anti-folate combination drugs, artemisinin, and antibiotics (tetracycline and its derivatives). Drugs can be prescribed as single-agent therapy or combination therapy depending on the resistance of the agent [3]. The first drug resistance of antimalarial was reported back in 1957 when *P. falciparum* resistance toward chloroquine emerged in Thailand [4]. Today, *P. falciparum* has developed drug resistance to nearly all antimalarial drugs used to combat it such as chloroquine, quinine, piperaquine, mefloquine, and sulphadoxine-pyrimethamine [5].

Resistance to antimalarial drugs has been described as the ability of a parasite strain to survive or grow despite the drug being administered and absorbed in doses well tolerated by subjects and above the normally recommended dose. The drug in question must be able to reach the parasite or infected erythrocyte for the time it takes for it to function normally. Resistance can arise from mutation that is spontaneous. Resistance to other drugs can occur when only a single point mutation is required. Meanwhile, multiple mutations are required to generate resistance to other drugs [3].

In general, there are four basic methods to detect and measure antimalarial drugs resistance: *in vitro* test, *in vivo* test, animal model studies, and molecular tests. Molecular assessment uses polymerase chain reaction (PCR) could trace the presence of mutation encoding resistance to antimalarial drugs. This technique also able to determined single-nucleotide polymorphism (SNP) which becomes the main focus of this review [3].

SNP is substitution of a single base pair at a specific location in the genome that is present in a relatively large fraction of a population. This SNP can determine those particular population's susceptibility and response to antimalarial drugs. The most widely-studied SNP in antimalarial drugs resistance is polymorphisms in *kelch13* gene of *P. falciparum* which have been associated with slow parasite clearance to artemisinin-based combination therapy (ACT) along with increased in vitro parasites survival in response to derivatives of artemisinin (ART) [6], [7]. With the current available evidences, it can be determined that SNPs play important role in the development of antimalarial drugs resistance in *P. falciparum*. Therefore, this review will be discussing the relationship SNPs in *P. falciparum* and its effect to antimalarial drugs resistance.

2. Sequencing methods: Technologies used in sequencing DNA molecules

2.1 Sequencing Technologies

Sequencing technologies have evolved since its discovery by the Nobel Laureate Frederick Sanger in 1977. So far, there are three generations of sequencing methods available:

2.1.1 First generation

Chain termination method, also known as Sanger sequencing, is the first method to be discovered. In this method, four fluorescent dyes are used to label dideoxynucleotide triphosphates (ddNTPs), which terminate DNA polymerization. Using gel electrophoresis, the resulting marked oligonucleotides are then separated by size. Fluorescence emitted by each fragment is detected and base identification is performed. Sanger method is rarely used today due to its high cost and time-consuming process, although it still has a place in the validation of NGS studies [8], [9].

2.1.2 Second generation

Next generation sequencing or high-throughput screening belongs to the second-generation technology. NGS can create millions of short sequence-reads accurately in a short amount of time. It is fast, cost-effective, and is considered a more attractive method of sequencing. In recent years, genome projects have used this technology through platforms, namely Roche 454, ABI/SOLiD, and Illumina [10], [11].

2.1.3 Third generation

This generation sequencing directly sequence a single DNA molecule in a molecular level, so that DNA does not need to be denatured nor amplified. Although still under development, this method offers more advantages including the ability to identify longer reads in *de novo* assembly, with high precision and shorter duration [8], [12].

2.2 Next Generation Sequencing Platforms (NGS)

In recent years, NGS and bioinformatics analysis have become important tools in infectious disease research, including the identification and analysis of pathogens. Pathogen whole genome, exomes, or target genes sequenced can be analyzed to give a vast array of information, such as the detection of resistance genes and mutations, tracing pathogen spread or origins, and epidemic surveillance [6], [13]. There are copious NGS platforms available for use, each with its own advantages and disadvantages.

2.2.1 454/Roche FLX System

Roche 454 is the first commercially successful next generation sequencing technology. Found in 2005, this method infers nucleotide sequence by using an approach called pyrosequencing on solid support [9]. It detects chemiluminescence produced by inorganic pyrophosphates released during each nucleotide incorporation [9], [11]. Although speed and long read length is a huge advantage when using this platform, its relatively high error rate and expensive reagents is still a challenge. Roche 454 system runs 454 GS FLX Titanium Software which includes GS RunProcessor involved in producing standard flowgram format (SFF) files containing base calls and their quality. SFF files can then be converted to fastq format by using assembler, reference mapper, and amplicon variant analyzer provided by the software [11].

2.2.2 Illumina

Illumina, which platform was originally launched by Solexa in 2006, utilizes a method called sequencing by synthesis (SBS) with reversible terminators. Reversible dye-terminators enable base-by-base identification as they are incorporated into DNA strands [8]. Illumina offers a few series of sequencing systems, such as MiSeq systems to HiSeq and NovaSeq series whose usage can be adjusted to each laboratory setting. This platform, especially Illumina HiSeq 2000, is effective in producing a large output with cheap reagents [11]. Furthermore, it produces paired-end data by sequencing DNA molecules from the first end to the second and back. This creates a greater read depth and increases accuracy in genome mapping [8]. HiSeq software is used in this system to produce bcl files containing base calls and sequencing quality [11].

2.2.3 ABI/SOLiD

SOLiD or sequencing by oligo ligation detection was introduced by Applied Biosystems (now Life Technology) in 2006. In this method, base identification is carried out by massively parallel sequencing by ligation using DNA ligase based on the polony sequencing technique [8], [9]. The system uses a two-base coding system, which can be used to distinguish polymorphisms with sequencing errors, the latter only detected in one ligation reaction instead of two [9]. As errors are corrected by this system, SOLiD platform features a high accuracy in its sequence reads [14]. For data analysis, SOLiD uses Bioscope which offers resequencing framework and transcriptome analysis [11].

As of today, Illumina MiSeq is the most widely used platform in infectious disease studies and identification or surveillance of pathogens. Higher throughput platforms, such as Illumina NovaSeq and HiSeq amongst others, is preferable for single nucleotide polymorphism (SNP) research involving diverse organisms or large genomes [15]. This is reflected by the studies included in this review. Most of the studies disclosing their sequencing methods use Illumina to sequence the genome of these *Plasmodium falciparum*.

3. NGS Data Analysis: Identifying Single Nucleotide Polymorphisms (SNPs)

3.1 Definition

A region of a genome in a DNA sequence is remarkably similar among individuals across the globe. However, millions of single nucleotides differences have been identified. Common variation in the human genome consists of copy number variants, single nucleotide polymorphisms, insertion/deletions, and inversion. The simplest and the most common form of variation are single nucleotide polymorphisms (SNPs), commonly observed once every 1000 base pairs in the genome. A locus containing a SNP usually has two alleles that correspond to the two distinct bases occupying a certain location inside the genome.[16] Cancer and genetic susceptibility is associated with genes that regulate repair of DNA mismatch, metabolism, immunity, or cell cycle regulation that have SNPs. SNPs are generally located in various gene regions such as promoters, introns, exons, and UTR 5 'and 3'. Therefore, changes in gene expression and their effect on disease susceptibility vary depending on the location of the SNPs [17].

3.2 Types of SNP

SNPs are alleles that differ in a loci of a single base, with a percentage greater than 1% of population. For genes that codes protein, more than one hundred thousans SNPs on exons have been documented but 50% of them do not change the amino acid sequence of the encoded protein and are thus called synonymous, the other half changes the amino acid sequence and is said to be nonsynonymous. Other types of SNP change or initiate stop codons and others change the known splice site; SNPs with these characteristics are candidates for the class that produce significant functional consequences [16].

3.3 NGS data Analysis Workflow

Once the DNA library and samples were prepared using the NGS platform. An assessment of the quality of NGS reads is performed and the reads are compared to the reference genome. Then the identification of variants and annotations were carried out followed by visualization. The final stage involves prioritization and further review of the identified variations, accompanied by validation of the results obtained in the laboratory [18].

3.3.1 Quality Assessment

Sequencing platforms currently employed for example Illumina, ABI/SOLID, Roche FLX system, and Ion Torrent cannot be separated from errors [18]. Checking the quality of the sequenced read is mandatory. In this step, software such as FASTQC are used to run basic statistics, per base sequence quality check, per tile sequence quality check, per sequence quality scores, and so on. After running quality assessment, data undergo cleaning (pre-processing) which consists of trimming reads, excluding low quality reads, and getting rid of contaminations [19].

3.3.2 Sequences Alignment

Sequences that have been quality controlled are aligned to the reference genome usually obtained from Genome Reference Consortium (GRC) and University of Santa Cruz (UCSC) [3]. Alignment is an effective way to compare sequences of DNA or related proteins. Alignment can be used to capture a variety of information about the sequence being aligned, such as general structural functions or general evolutionary descent [5]. Two issues in choosing alignment software address the problem of ambiguity in assigning short reads to reference genomes and mutations resulting from readings with multiple mismatches should be removed from further analysis steps [18].

3.3.3 Variants Identification

In variant identification step, the main parameter is sequence coverage , as identified mutations need several reads. There are four categories of variant identification tools: somatic callers, copy Number Variants (CNV) identification, germ line callers, and Structural Variants (SV) identification [3]. In rare diseases, detection of germ line mutations is the main part of finding the etiology. Also, identification of somatic mutations by differentiating sequences between normal or tumor pairs from a single subject is the focus of cancer studies [6]. Examples of variant identification tools are Galaxy platform, SanGeniX platform, ExomeCNV, and GASVPro (GASVPro-HQ) [18].

3.3.4 Variants Annotation

Variants annotation provides prediction of functional impact caused by variants and computer-aided annotation allows researchers to screen and prioritize potential disease-causing mutations for further analysis [21]. Examples of variant annotation tools are SanGeniX platform, snpEff, AnnTools, Galaxy platform, and SeattleSeq [3]. Most of these tools focus on SNP annotations, as they can be easily analyzed and identified. INDELs (insertion and/or deletion) identification is also identified by several tools, whereas structural variant annotation is limited to CNVs only. Generally, the annotation form provides database links to various public variant databases such as dbSNP [21].

3.3.5 NGS Data Visualization

Variants annotation provides prediction of functional impact caused by variants and computer-aided annotation allows researchers to screen and prioritize potential disease-causing mutations for further analysis [21]. Examples of variant annotation tools are SanGeniX platform, snpEff, AnnTools, Galaxy platform, and SeattleSeq [3]. Most of these tools focus on SNP annotations, as they can be easily analyzed and identified. INDELs (insertion and/or deletion) identification is also identified by several tools, whereas structural variant annotation is limited to CNVs only. Generally, the annotation form provides database links to various public variant databases such as dbSNP [21].

3.3.6 Filtering/Prioritization and Validation

Because NGS uses whole-exome and whole-genome sequencing, NGS faced challenges in narrowing down the list of variants and interpreting the remaining ones in a biological context. To reduce the number of candidate variants, it is common practice to exclude variants that are commonly found in public SNP databases, published studies, or in-house databases under the assumption that such variants represent benign variations. Another method of narrowing the search for genomics is to use pedigree information, that is, to sequence distant relatives with phenotype of interest to identify the cause of the mutation [21]. Sanger sequencing is one example of methods for NGS result validation. Sanger sequencing is a part of first-generation sequencing technologies that was invented in 1977. But, it is considered a time consuming process and it takes a week to process only few thousands of nucleotide [18].

4. Plasmodium falciparum Antimalarial Resistance and The Role of SNPs

Malaria is a common and life-threatening disease in many subtropical and tropical areas [23]. This disease is caused by the protozoan parasite *Plasmodium*. The parasite that causes malaria is female mosquitoes *Anopheles*, which usually bite humans between dusk and dawn. Malaria in humans is caused by these five known species of *Plasmodium*: *P. falciparum*, *P. malariae*, *P. vivax*, *P. knowlesi*, and *P. ovale*. Of the five species, *P. vivax and P. falciparum* are the most common causes and pose the greatest threat, and *P. falciparum* is the most dangerous [23], [24].

Currently, there are more than 91 countries and territories where there is a risk of malaria transmission [23]. *P. falciparum* itself accounted for more than 99% of malaria cases in the African region, half of the cases in the South-East Asia region, and 65% of cases in Western Pacific region in 2018. It was then estimated that there were 228 million malaria

cases in over 90 countries worldwide [2]. Meanwhile *P. vivax* dominates both North and South America, representing about 75% of malaria cases [24]. Still in the same year, it was estimated that 228 million cases of malaria occurred in the world with a death rate of 405,000, mostly children in the African region [24], [25].

Success in the prevention, control and management of malaria depends on the continued efficacy of the antimalarial drug artemisinin-combination therapy (ACT). A significant hazard comes from the development and spread of antimalarial medication resistance. Around 100,000 more deaths per year are anticipated as a result of the rise of ACT resistance in areas with high rates of malaria. Knowing how antimalarial drug resistance develops can be based on information about the molecular and genetic makeup of Plasmodium [26].

5. SNP in *Plasmodium falciparum* and its relation with antimalarial drug-resistant

No.	Authors, year	Gene	Point Mutation	Phenotype	Mechanism
1	Breglio KF, 2018	PF3D7_1012900	Nonsynonymous SNP encoding T38I amino acid substitution (threonine to isoleucine)	Poor rates of parasite clearance in ART-treated patients (p = 5.89E - 7). Not significantly associated with chloroquine resistance (p = 0.0002)	Isoleucine cannot be phosphorylated
2	Miotto O, et al 2019	*Pfkelch13* (PF3D7_1343700)	C580Y	Day 3 parasite positivity associated with ART-R, ring-stage resistance to artemisinin	Slower medication clearance increases drug exposure, causes mutations to build up, and makes it easier for multi-drug resistant lineages to develop.
3	Wang Z, et al. 2016	PF3D7_1012700	490648	Increased IC50 to dihydroartemisinin and artemether	NIF4 (NLI interacting factor-like phosphatase)
4		PF3D7_1148800	1941896	Reduced lumefantrine sensitivity	Plasmodium exported protein (hyp11)
5	Miotto, et al	Ferredoxin (PF3D7_1318100)	D193Y	A delayed rate of parasite removal following therapy with artemisinin-derived drugs.	Ferredoxin gene mutation possibly affects parasite's protection against oxidative stress induced by artemisinin.
6	Briolant, et al	*Pfmdr2* (PF3D7_1447900)	F423Y	Pyrimethamine resistance in vitro	Alterations in pyrimethamine efflux from outside the parasite.
7	Afoakwah, et al	*Pfcrt* (PF3D7_0709000)	K76T	Twenty times increase in chloroquine treatment failure	The loss of positive charge from Lys residue allows chloroquine efflux through Pfcrt

8	Veiga MI, et al	*Pfmdr1* (PF3D7_0523000)	N86Y Y184F	N86Y Increases resistance to CQ and AD	The P-glycoprotein homologue 1 (Pgh1) protein inside Plasmodium's digestive vacuole is encoded by the *Pfmdr1* gene, which is found on chromosome 5. The mutation seems to affect ATPase-coupled transmembrane transporter activity which functions as primary transporter of a substance such as drugs.
9	Emilia AE, et al.	*Pfmdr1* (PF3D7_0523000)	N86Y Y184F	Co-mutation of N86Y and Y184F increases resistance to AL.	
10	Jiang T, et al.	*Pfdhps* (PF3D7_0810800)	A437G S436A/F K540E/A	One of the defining characteristics of sulfadoxine resistance is the A437G substitution, which is also a part of the "quintuple Pfdhps-Pfdhfr mutations" linked to SP resistance.	The molecular targets of pyrimethamine are the thymidylate synthase (TS) enzyme. The 7, 8-dihydro-6-hydroxymethylpterin pyrophosphokinase (PPPK) enzyme is the molecular target of sulfadoxine. The production pathway for folic acid depends on these two enzymes. SP resistance is linked to specific point mutations in both genes.
11	Siddiqui FA, et al.	Falcipain-2a (PF3D7_1115700)	T343P D345G A353T V393I A400P Q414E Found in domain of cysteine protease	FP2a SNP-carrying parasites showed reduced FP2a activity and artemisinin sensitivity.	Reduce hemoglobin breakdown, which is necessary for artemisinin efficacy, by interfering with the activity of cysteine protease inhibitors by interfering with the inhibitor's binding to FP2a.

12	Salem MS, et al.	*Pfdhfr* (PF3D7_0417200)	N51I S108N C59R	Pdhfr polymorphisms associated with sulfadoxine-pyrimethamine resistance	Parasite-killing activity of sulfadoxine-pyrimethamine is inhibited because of the alteration in dihydrofolate reductase gene

4.1 Autophagy-related protein 18 (PF3D7_1012900)

In the PF3D7_1012900 gene encoding autophagy-related protein 18, the prolonged parasite clearance half-life seen in individuals following artemisinin combination therapy is correlated with an SNP. Autophagy is an internal mechanism used to recycle and destroy damaged organelles. Autophagy-related proteins have shown co-location with apicoplast and are associated with apicoplast inheritance. There is an association between autophagy and the increase in phosphatidylinositol 3-phosphate (PI3P) that occurs in ART-resistant isolates. In this gene, the change of the 38th amino acid from threonine to leucine causes phosphorylation not to occur because isoleucine cannot be phosphorylated like threonine [27].

4.2 Kelch protein K13 (PF3D7_1343700)

The PF3D7_1343700 gene encoding the K13 kelch protein has various SNPs, one of which is the C580Y allele which has been confirmed as the cause of parasite resistance to artemisinin. In Southeast Asia, the mutation at codon position C580Y has evolved to become the most prevalent and is linked to the longest half-life of parasite clearance. This SNP's phenotype is day 3 parasite positive related with ART-R and ring-stage artemisinin resistance. The increased exposure to drugs, accumulation of mutations, and facilitation of multidrug resistant lineages are all caused by the decreased drug clearance mechanism [28], [29].

4.3 Protein-serine/threonine phosphatase (PF3D7_1012700)

Based on research conducted by Wang Z, et al, it was found that SNP at position PF3D7_1012700, NIF4 (NLI interacting factor-like phosphatase) has shown a strong correlation with an increase in antimalarial IC50 both in dihydroartemisinin and artemether in various analysis methods. It should be noted that with large scale GWAS it was found that NIF4 was related to half-life delayed parasite clearance (DPC) or called $PCt^{1}/_{2}$ in patients given artemisinin derivatives such as artemether (lipid soluble methyl ether form of dihydroartemisinin) in Southeast Asia. The parasite clearance half-life of more than 5 hours was used to characterize the DPC phenotype, where it was postulated that this was an effect of the parasite's dormant ring-stage stage. This stage is said to be resistant to high concentrations of artemisinin and its derivatives over a short period [30].

4.4 Plasmodium exported protein (hyp11) (PF3D7_1148800)

The polymorphism at position 1941896 in the PF3D7_1148800 gene is said to be associated with reduced sensitivity of parasites to the drug lumefantrine. The gene encodes a protein that Plasmodium exports (hyp11), but its function is uncertain. Non-canonical PEXEL / HT, which is localized to the RBC membrane, exports with the hyp11 protein. The cytoadherence of infected red blood cells against different endothelial receptors in blood arteries is made possible by the parasite-induced alteration of infected red blood cells, which contributes to P. falciparum's pathogenicity. Several different proteins that are exported to the cytoplasm of infected red blood cells and are encoded by the parasites modify the infected red blood cells. Once exported, the proteins are linked to structures

produced by the parasite, such as Maurer's clefts, or they stay soluble in the cytoplasm of red blood cells [30], [31].

4.5 Ferredoxin (PF3D7_1318100)

After taking artemisinin, the D193Y mutation-causing SNP in the ferredoxin gene is linked to a slower parasite clearance. The ferredoxin mutation may impact the parasite's capacity to withstand the oxidative stress brought on by artemisinin, conferring resistance. Ferredoxin is a component of the electron transport chain. Halofantrine-resistant malaria is also linked to SNP of the ferredoxin gene [32].

4.6 Pfmdr2 (PF3D7_1460900)

Pfmdr2 is a *Plasmodium falciparum* gene encoding a P-glycoprotein which is mostly involved in translocation of heavy metals. A study by Briolant et al found that F432Y mutation in pfmdr2 gene induces pyrimethamine resistance in *Plasmodium falciparum* isolates *in vitro*. A SNP in the gene causes an amino acid change in MDR2 protein from phenylalanine to tyrosine. The resistance is thought to have originated from alterations in pyrimethamine efflux from outside the parasite. The change in amino acid does not alter protein size nor charge characteristics, because both residues are hydrophobic. It is speculated that the resulting tyrosine residue acts as a phosphorylation site which might change the structure and activity of MDR2 protein [33].

4.7 Pfcrt (PF3D7_0709000)

Chloroquine resistance transporter is a transporter found in the *Plasmodium falciparum* digestive vacuole membrane. Its physiological function hasn't been extensively studied, but is thought to play a role in peptide transport from the vacuole to cytosol for nutrient provision [34].

A SNP in *pfcrt* gene (PF3D7_0709000) causing a change in amino acid lysine to threonine is commonly found in *Plasmodium falciparum* lines displaying chloroquine resistance [35]. Pfcrt K76T mutation is associated with 20 times increase in the failure of chloroquine treatment. The proposed mechanism for this resistance lies in the change of ionic charge at the 76th amino acid position. Lysine residue provides positive charge which prevents chloroquine to cross the transporter and allows chloroquine accumulation in the digestive vacuole. In K76T mutant, threonine replaces lysine thus the positive charge is no longer there. This allows chloroquine efflux from the digestive vacuole, reducing its concentration, and producing resistance [36].

4.8 Pfmdr1 (PF3D7_0523000)

Five commonly occurring amino acid mutations have been found in pfmdr1 during this study. The amino-terminal alterations, including N86Y and Y184F, are more prevalent in parasites from Asia and Africa. In contrast, South American isolates are more likely to have the three carboxy-terminal mutations S1034C, N1042D, and D1246Y (D1246Y is present in about 3% of the 1,502 African genomes in some recently sequenced genomes). With the aid of these recently sequenced genomes, it is now possible to conduct a thorough analysis of pfmdr1 haplotypes in malaria-prone areas like Southeast Asia and Africa. Unfortunately, due to a lack of data, this review omits strains from South America and the Western Pacific. N86Y alone significantly reduces amodiaquine (AQ) and chloroquine (CQ) susceptibility in this investigation (AD). Compared to N86Y, the Y184F mutation has a much smaller effect on drug responsiveness in vivo. However, a different study found that the co-mutation of N86Y and Y184F dramatically increased resistance to artemether-lumefantrine (AL) [38].

Sanger pf3k data collection, which included 2512 genomes, was used to construct the data for genome analysis. The annotated pf3d7 genome version 9.2 was used to extract

SNPs, and a customized Perl script was used to call each base, allelic balance, and sample depth. A MySQL database with pf3k metadata was used to store the output. The database's Pfmdr1 (PF3D7 0523000) entry was obtained. To establish a match for the isolates, the study analysis was carried out interactively against a few reference genomes [37].

4.9 Pfdhps (PF3D7_0810800)

In *pfdhps* gene, the predominant SNP found at codon A437G in comparison with the other SNPs such as S436A, K540E, A581G and A613S mutations. Therefore, A437G substitution is considered important in sulfadoxine resistance. On the other hand, A347G also has a strong linkage with N51I which is one of the most common SNP in *pfdhfr* gene. These SNPs show that *pfdhfr* and *pfdhps* have a strong linkage despite not being in the same chromosome. One of the quintuple pfdhps-pfdhfr mutations thought to contribute to sulfadoxine-pyrimethamine (SP) resistance is A437G [39].

In this study, dried filtered blood spots (DBS) were processed using the Chelex-100 extraction method to obtain their genomes. Using nested PCR, the *pfdhfr* and *pfdhps* genes were amplified. The amplified nested PCR products were purified, and the Sanger sequencing method was used to detect the *pfdhfr* and *pfdhps* gene alterations (Genewiz, Soochow, China). Using DNAstar, all of the sequences were examined (DNASTAR Inc., Madison, WI, USA) [39].

4.10 Falcipain-2a (PF3D7_1115700)

The FP2a gene sequences from 140 clinical isolates of P. falciparum on the China-Myanmar border were analyzed in this study. Comparing the examined strains to the reference strain 3D7, 97% of them possessed FP2a SNPs [40]. When compared to African strains, it was discovered that these SNPs were much more diversified [41]. Artemisinin sensitivity and FP2a activity were also reduced in parasites with FP2a SNPs. The findings of this study suggest that the usage of ACT altered FP2a in Southeast Asia, which lowered hemoglobin digestion and contributed to the development of artemisinin resistance. Up to six of the many SNPs detected on FP2a were in the protease domain and may have an impact on the activity of the enzyme. According to the modeling outcomes, A353T, A400P, and Q414E are situated near the interface between the endogenous cysteine protease inhibitor falstatin and the FP2a binding, which may have an impact on the inhibitor's ability to attach to FP2a. Q414 and the inhibitor establish hydrogen bonds, while A400 is known to be on the helix [40].

4.11 Pfdhfr (PF3D7_0417200)

Of the 299 samples, PCR genotyping of the gene *Pfdhfr* was successfully performed on 268 samples (89.6%; codons 164 and108) and 269 (90%; codons 59 and 51) . The results showed that in the four codons observed (51, 59, 108, 164), *Pfdhfr* polymorphisms were present as pure mutants or mixed alleles as much as 46.8% (126 of 269), 48% (129 of 269), 47.4% (127 of 268), and 4.5% (12 of 268), respectively. All isolates with the 164L mutants had mixed alleles. There were three isolates with quadruple, namely the triple haplotype mutase Pfdhfr mutant (AIRNI) and the single haplotype Pfhdps mutant (SGKAA). Meanwhile, there were two isolates with double Pfdhps mutant haplotype (SGEAA) associated with haplotypes triple Pfdhfr mutant haplotype (AIRNI). Substitution *Pfdhps* A437G is one component of a quintuple *Pfhdps-Pfhdfr* mutations associated with resistance to SP. the results of the analysis of marker molecular shows the results of 437G in 23% of isolates [42]. Dihydropteroate synthase (DHPS) and dihydrofolate reductase (DHFR) are known targets for sulfadoxine and pyrimethamine, respectively.These drugs specifically inhibited folate pathway enzymes to kill parasites [43]. Another study in Niger also demonstrated a high proportion of clinical samples carrying the haplotypes *Pfdhfr* 51I, 59R, and 108N which were associated with resistance to pyrimethamine, and the *Pfdhps* 436A/F/H and 437G mutases associated with decreased susceptibility to sulfadoxine [44].

5. Conclusions

Results from most studies included in this review suggest that SNPs in *Plasmodium falciparum* genes participate in the resistance against various antimalarial drugs through numerous mechanisms including slower drug clearance, parasite protection against oxidative stress, drug removal from the parasite cells, decrease of hemoglobin degradation, and inhibition of parasite killing activity of drug.

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