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Integrated Phenotypic and Genotypic Approaches for Accurate Diagnosis of G6PD Deficiency: Implications for Drug Safety in Thailand

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ABSTRACT

Glucose-6-phosphate dehydrogenase (G6PD) deficiency holds critical health concerns, particularly due to its association with drug-induced hemolysis triggered by medications such as antimalarials. This condition poses significant risks in malaria-endemic regions where the prevalence and genetic diversity of G6PD deficiency further complicate management. Providing accurate and reliable G6PD status is vital to ensuring safe treatment, reducing complications, and improving healthcare outcomes in these populations. This study evaluated the integration of phenotypic and genotypic diagnostic methods for identifying G6PD deficiency in 2953 participants in Thailand. Using the water-soluble tetrazolium salts enzymatic assay and multiplex high-resolution melting analysis, the study revealed an overall prevalence of 3.93%, with 7.19% in males and 1.83% in females. A total of 38 distinct *G6PD* genotypes were identified, and zygosity was determined, highlighting significant genetic diversity, including previously unreported mutations as identified by sequencing. Hemizygous males, homozygous females, and approximately 50% of heterozygous females with missense mutations exhibited deficient or intermediate phenotypes. However, 40% of females carrying *G6PD* missense mutations showed a normal phenotype in quantitative phenotypic testing. The findings highlight the need for accurate G6PD diagnosis to improve drug safety and efficacy, particularly for vulnerable individuals such as heterozygous females, who are at risk of hemolysis. The cost-effective, high-throughput methods demonstrated here are suitable for large-scale screening, making them especially valuable in resource-limited settings. To maximize their impact, integrating both phenotypic and genotypic approaches into national healthcare policies and malaria programs is essential. By ensuring equitable access to reliable G6PD testing, these findings support malaria elimination efforts and address broader healthcare challenges, ultimately reducing preventable morbidity and mortality associated with G6PD deficiency.

1 | Introduction

Glucose-6-phosphate dehydrogenase (G6PD) deficiency, an X-linked disorder caused by mutations in the *G6PD* gene, is the most common human enzymopathy, affecting approximately 500 million people worldwide [1]. Over 230 *G6PD* variants have been identified to date. While most variants primarily result from single point mutations, double mutations, synonymous

mutations, and intronic mutations leading to G6PD deficiency are becoming increasingly recognized [2–4]. G6PD deficiency presents unique challenges in clinical and public health management due to its association with hemolytic anemia, which can be triggered by oxidative stressors, including infections, ingestion of fava beans, or certain medications (e.g., antimalarials, antibiotics, analgesics, antipyretics, rasburicase, and methylene blue) [5–7].

Over the past decade, Thailand has shown significant progress toward malaria elimination, with reported cases declining markedly between 2010 and 2021. However, malaria cases increased from 2022 to 2024, particularly near the Myanmar border, mainly due to the influx of refugees following political and social instability. Since 2010, *Plasmodium vivax* has become the dominant species, accounting for more than 90% of infections between 2020 and 2024 [8, 9]. The safe and effective use of 8-aminoquinolines, such as primaquine and tafenoquine, is crucial for targeting *P. vivax* hypnozoites and preventing relapse–recurrent infections that occur after the initial infection has been resolved due to the reactivation of dormant liver-stage parasites (hypnozoites). However, their prescription in Thailand is complicated by the prevalence of G6PD deficiency, which ranges from 3% to 18% depending on ethnicity and geographic location [10–18].

Accurate diagnosis of G6PD deficiency is especially critical for tafenoquine, as it requires a quantitative measurement to exclude patients with G6PD activity < 70%, due to its single-dose regimen of 300 mg/kg and 14-day half-life [19, 20]. Quantitative phenotypic tests, while recognized for their accuracy and reliability, often demand specialized equipment and skilled personnel, limiting their accessibility in resource-limited settings. In contrast, qualitative tests are more widely available and easier to perform but are inadequate for reliably detecting heterozygous females and newborns, leaving a significant diagnostic gap [21, 22].

Genotypic tests, such as restriction fragment length polymorphism and high-resolution melting (HRM) curve analysis, are effective for identifying specific *G6PD* mutations [23–26]. However, these methods can be complicated, lack the capability to determine zygosity accurately, and are not routinely integrated into diagnostic workflows. DNA sequencing remains the gold standard for detecting *G6PD* mutations.

Thailand's high genetic diversity of *G6PD* variants further complicates diagnosis, as many variants remain underexplored in terms of their clinical significance and distribution [16, 27, 28]. Diagnosis in heterozygous females, who exhibit variable G6PD activity due to X-chromosome inactivation, and newborns, who typically have higher G6PD levels than adults, poses particular challenges. This complexity emphasizes the need for a combined diagnostic approach to ensure accurate identification of individuals at risk of drug-induced hemolysis.

This study aimed to address these challenges by providing a clearer understanding of the prevalence, genetic diversity, and zygosity of G6PD deficiency in Thailand. The prevalence of G6PD deficiency among the Thai population in the central region was assessed using a quantitative phenotypic test based on water-soluble tetrazolium salts (WST-8) assay. *G6PD* mutations were identified through multiplex HRM assays, and the zygosity of heterozygous females was determined.

By combining WST-8 for phenotypic assessment and HRM for genotypic confirmation, this approach ensures a comprehensive and efficient strategy for G6PD deficiency screening, addressing both enzymatic activity levels and genetic variants. The WST-8 and HRM assays were selected for their cost-effectiveness, high-throughput capability, and suitability for large-scale screening. The colorimetric nature of WST-8 offers high sensitivity and specificity for detecting NADPH [29]. Additionally, it is simple to use, as it requires no sample preparation and allows for direct measurement from whole blood or dried blood spot samples [30, 31]. HRM assays provide a precise and reliable method for detecting *G6PD* mutations, effectively distinguishing heterozygous, hemizygous, and homozygous individuals with high sensitivity [17, 18, 25].

2 | Materials and Methods

2.1 | Blood Samples

A total of 2953 venous blood samples were collected between March 2019 and December 2020 at the Hospital for Tropical Diseases, Faculty of Tropical Medicine, Bangkok, Thailand. The samples were stored in ethylenediaminetetraacetic acid tubes at −20°C and analyzed at the Department of Molecular Tropical Medicine and Genetics, Faculty of Tropical Medicine. Under these storage conditions, the samples maintained their integrity for phenotypic screening [32]. The study procedures were approved by the Human Ethics Committee of Mahidol University's Faculty of Tropical Medicine (MUTM 2024-042-01). The study was performed using archived adult blood samples. The data were fully anonymized, and the authors had no access to information to identify individual participants. The study design is shown in Figure 1.

2.2 | Phenotypic Screening for G6PD Deficiency Using WST-8 Assay

Blood samples were mixed with 100 μL of a reaction mixture containing 20 mM Tris–HCl (pH 8.0), 10 mM $MgCl_2$, 500 μM glucose-6-phosphate, 100 μM $NADP^+$, and 100 μM WST-8 in a 96-well plate, according to the protocols outlined in the previous study [25]. Absorbance was measured at 450 nm with a reference wavelength of 650 nm using a microplate reader (Tecan Sunrise, Switzerland). Quality control was ensured by including positive and negative controls in each run, using samples with known G6PD activity levels. Hemoglobin concentration was determined using Drabkin's reagent (Sigma-Aldrich, Darmstadt, Germany), according to the manufacturer's protocols. Absorbance was measured at 540 nm using a microplate reader. All experiments were performed in triplicate.

G6PD activity was expressed in international units (U) per gram of hemoglobin (gHb). The adjusted male median (AMM) was calculated to standardize the classification of G6PD deficiency

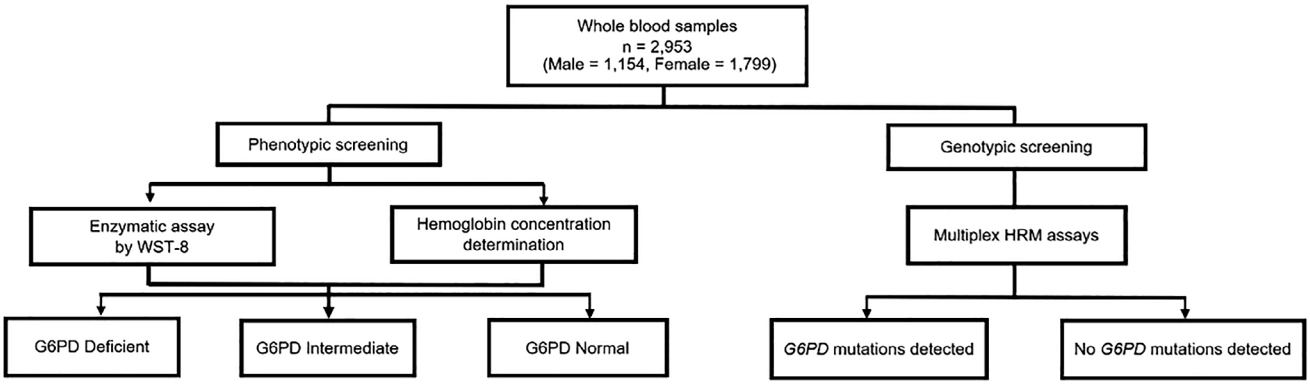

FIGURE 1 | Study design.

[33]. Based on activity thresholds [34], samples were categorized as follows: those with less than 30% of the AMM were classified as deficient, samples with G6PD activity between 30% and 70% were classified as intermediate, and samples with activity greater than 70% were considered normal.

2.3 | DNA Extraction

Genomic DNA (gDNA) was extracted using the QIAsymphony system and the QIAamp DNA Blood Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. DNA concentrations were measured with a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

2.4 | Detection of *G6PD* Mutations and Identification of Zygosity by Multiplex HRM Assays

Multiplex HRM assays for detecting the *G6PD* mutations were conducted for 2953 samples in 4 separate reactions using mutant primer sets. Samples identified as positive for *G6PD* mutations were subsequently analyzed for zygosity using wild-type (WT) primer sets.

The multiplex HRM assays were designed to detect 15 *G6PD* variants: *G6PD* Gaohe (A95G), *G6PD* Mahidol (G487A), *G6PD* Viangchan (G871A), *G6PD* Canton (G1376T), *G6PD* Aures (C143T), *G6PD* Chinese-4 (G392T), *G6PD* Mediterranean (C563T), *G6PD* Chinese-5 (C1024T), *G6PD* Songklanagarind (T196A), *G6PD* Valladolid (C406T), *G6PD* Coimbra (C592T), *G6PD* Union (C1360T), *G6PD* C1311T, *G6PD* T93C (c.1365-13T>C), and *G6PD* c.486-34delT.

Each sample was amplified using two sets of primers: one specific for the mutant allele and another for the WT. The primers were designed to perfectly match the DNA sequences at the 3′ end of the forward primer, ensuring specific amplification. This design generated amplicons with distinct melting temperatures (T_m). When using the mutant primer sets, samples with *G6PD* mutations exhibited distinct peaks corresponding to their specific T_m. Conversely, with the WT primer sets, WT samples produced characteristic peaks at specific T_m while mutant samples failed to generate such peaks. This design enabled clear differentiation of genotypes: in hemizygous males and homozygous females, mutant samples displayed positive peaks in the mutant primer sets but no peaks in the WT primer sets. In heterozygous females, however, positive peaks were observed in both mutant and WT primer sets, reflecting the coexistence of mutant and WT alleles. The primer sequences were previously designed, and the conditions were adapted from prior studies [16, 17] as outlined in Table 1.

Each 12.5 μL reaction mixture contained 6.25 μL of 2× HRM Type-it mix (QIAGEN), various primer concentrations (Table 1), molecular-grade water, and 2.5 μL of gDNA template (3–10 ng/μL). PCR amplification and melting curve analysis were carried out using the Rotor-Gene Q (QIAGEN) under the following conditions: an initial denaturation at 95°C for 5 min, followed by 30 cycles of denaturation at 95°C for 10 s, annealing for 30 s, and extension at 72°C for 10 s. The annealing temperature for mutant primer systems 1–4 and WT primer systems 2–4 was set at 63°C, while WT primer system 1 was annealed at 58°C. Details of the primer systems are available in Table 1. Subsequently, HRM analysis was performed by gradually increasing the temperature from 70°C to 90°C, with readings taken at every 0.1°C step and a stabilization of 2 s per step. gDNA samples with known mutations confirmed by DNA sequencing were used as positive controls.

For the HRM analysis, quality control was ensured by incorporating no-template controls and positive controls with known *G6PD* mutations in every run to monitor assay performance and detect potential contamination. Each sample was analyzed in triplicate, and melting profiles were compared across runs to ensure reproducibility. Although the assays demonstrated 100% sensitivity and specificity, a subset of 120 samples was validated using Sanger sequencing to confirm the HRM results.

2.5 | DNA Sequencing

Full coding sequencing was performed on all samples exhibiting an intermediate phenotype (< 70% activity), as determined by the WST-8 assay, but no mutations were detected by the

TABLE 1 | HRM primers and conditions for detecting *G6PD* mutants and WT.

Reaction system	Primer	Mutant primer sequence (from 5′ to 3′)	Primer conc. (nM)	Wild-type primer sequence (from 5′ to 3′)	Primer conc. (nM)
1	A95G_F	TTCCATCAGTCGGATACACG	600	TTCCATCAGTCGGATACACA	1500
	A95G_R	AGGCATGGAGCAGGCACTTC		CCTGCAACAATTAGTTGGAAA	
	G487A_F	TCCGGGCTCCCAGCAGAA	400	TCCGGGCTCCCAGCAGAG	200
	G487A_R	GGTTGGACAGCCGGTCA		TTGGCCCCACCTCAGCACCA	
	G871A_F	GGCTTTCTCTCAGGTCAAGA	600	GGCTTTCTCTCAGGTCAAGG	800
	G871A_R	CCCAGGACCACATTGTTGGC		CCCAGGACCACATTGTTGGC	
	G1376T_F	CCTCAGCGACGAGCTCCT	600	CCTCAGCGACGAGCTCCG	400
	G1376T_R	CTGCCATAAATATAGGGGATGG		CTGCCATAAATATAGGGGATGG	
2	T143C_F	GACCTGGCCAAGAAGAAGAC	400	GACCTGGCCAAGAAGAAGAT	1500
	T143C_R	AAGGCCATCCCGGAACAGCC		CCGGCCATCCCGGAACAGCC	
	G392T_F	CATGAATGCCCTCCACCTGGT	200	CATGAATGCCCTCCACCTGG	400
	G392T_R	TTCTTGGTGACGGCCTCGTA		TTCTTGGTGACGGCCTCGTA	
	C563T_F	CGGCTGTCCAACCACATCTT	400	CGGCTGTCCAACCACATCTC	200
	C563T_R	GTTCTGCACCATCTCCTTGC		CCAGGTAGTGGTCGATGCGGTA	
	C1024T_F	CACTTTTGCAGCCGTCGTCT	400	CACTTTTGCAGCCGTCGTCT	400
	C1024T_R	CACACAGGGCATGCCCAGTT		CACACAGGGCATGCCCAGTT	
3	T196A_F	CCTTCTGCCCGAAAACACCA	400	CCTTCTGCCCGAAAACACCT	400
	T196A_R	AAGGGCTCACTCTGTTTGCG		AAGGGCTCACTCTGTTTGCG	
	C406T_F	CCTGGGGTCACAGGCCAACT	400	CCTGGGGTCACAGGCCAACC	200
	C406T_R	CTCATGCAGGACTCGTGAAT		CAACGGCAAGCCTTACATCTGGC	
	C592T_F	CCGTGAGGACCAGATCTACT	400	CCGTGAGGACCAGATCTACC	800
	C592T_R	CCCCACCTCAGCACCATG		AGCACCATGAGGTTCTGCACC	
	C1360T_F	GAGCCAGATGCACTTCGTGT	200	GAGCCAGATGCACTTCGTGC	800
	C1360T_R	GAGGGGACATAGTATGGCTT		GAGGGGACATAGTATGGCTT	
4	C1311T_F	CGTGAAGCTCCCTGACGCCTAT	200	CGTGAAGCTCCCTGACGCCTAC	800
	C1311T_R	CCGGCAGCTGGGCCTCAC		CCGGCAGCTGGGCCTCAC	
	c.1365-13T>C _F	CCGGCCTCCCAAGCCATACC	400	CCGGCCTCCCAAGCCATACT	200
	c.1365-13T>C_R	CTCAATCTGGTGCAGCAGTGG		CTCAATCTGGTGCAGCAGTGG	
	c.486-34delT _F	CCTCACTCCCCGAAGAGGGGTC	800	CCTCACTCCCCGAAGAGGGGTT	600
	c.486-34delT _R	TTCCAGCCTCTGCTGGGAGC		GGCCACATGTGAGGGGTCACC	

HRM assays, following the protocols mentioned in a previous report [25].

2.6 | Statistical Analysis

Descriptive statistics were used to summarize the prevalence of G6PD deficiency and the distribution of *G6PD* genotypes. The median and standard deviation (SD) were used to describe enzyme activity levels, while frequencies and percentages were used to report mutation types.

3 | Results

3.1 | Prevalence of G6PD Deficiency by Quantitative Phenotypic Assay

The G6PD activity of 2953 samples (1154 males and 1799 females) was screened using the WST-8 assay. The distribution of G6PD enzyme activity among the studied population is shown in Figure 2. The G6PD activity in this study was expressed as median ± SD and measured at 11.0 ± 2.8 U/gHb. Male individuals exhibited a median of 11.6 ± 3.2 U/gHb, while female

participants showed a median of 10.7 ± 2.5 U/gHb. The AMM was determined to be 12.0 ± 1.6 U/gHb, representing 100% G6PD activity within the study population.

The prevalence of G6PD deficiency (< 30% activity; 3.6 U/gHb) in the studied population was 3.93% (116/2953), comprising 7.19% (83/1154) of males and 1.83% (33/1799) of females, as shown in Figure 3. Intermediate activity (30%–70% activity; 3.6–8.4 U/gHb) was identified in 6.57% (194/2953) of individuals, including 1.82% (21/1154) of males and 9.62% (173/1799) of females.

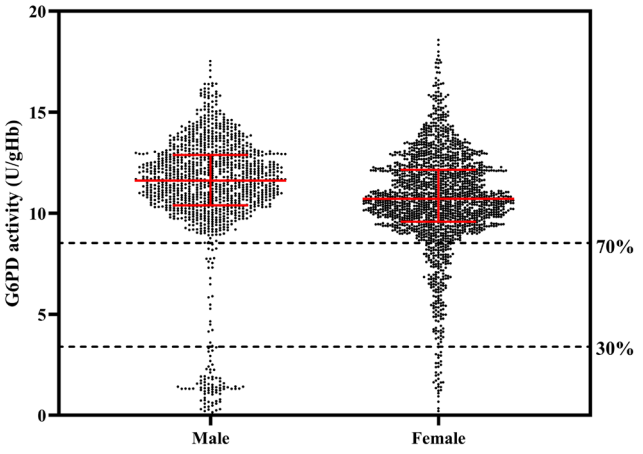

FIGURE 2 | Distribution of G6PD activity among 2953 samples (1154 males and 1799 females) measured by the WST-8. The red lines represent the median ± SD. Dotted horizontal lines represent G6PD activity at 30% and 70% of the AMM.

3.2 | Identification of *G6PD* Mutations and Determination of Zygosity

The multiplex HRM assays successfully identified *G6PD* mutations and determined the zygosity of the studied population. The *G6PD* genotypes identified in this study are detailed in Table 2 and zygosity and allele frequencies are presented in Table 3. No *G6PD* mutations were detected in 730 males and 797 females by HRM assays and DNA sequencing. The HRM assays accurately determined zygosity, classifying 418 males as hemizygotes. Among the 988 females carrying *G6PD* mutations, 891 were identified as heterozygotes. Overall, 48.29% (1426/2953) of the studied population carried *G6PD* mutations, which included 36.74% (424/1154) of males and 55.70% (1002/1799) of females. The most common *G6PD* variant was a combination of synonymous (c.1311C>T) and intronic (c.1365-13T>C) mutations, found in 27.16% (802/2953) of individuals, comprising 271 males and 531 females.

The most frequently identified missense mutation was *G6PD* Viangchan, which consistently appeared as part of a compound mutation (c.871G>A, c.1311C>T, c.1365-13T>C) with a frequency of 10.10%. Single missense mutations observed in the study included *G6PD* Gaohe, *G6PD* Aures, *G6PD* Chinese-4, *G6PD* Mahidol, *G6PD* Coimbra, *G6PD* Chinese-5, *G6PD* Union, and *G6PD* Canton. Additionally, an intronic deletion (c.486-34delT) was found with a frequency of 18.23%, while another intronic mutation (c.1365-13T>C) was detected with a frequency of 0.35%.

Given the large sample size, several compound mutations were identified, involving single missense mutations

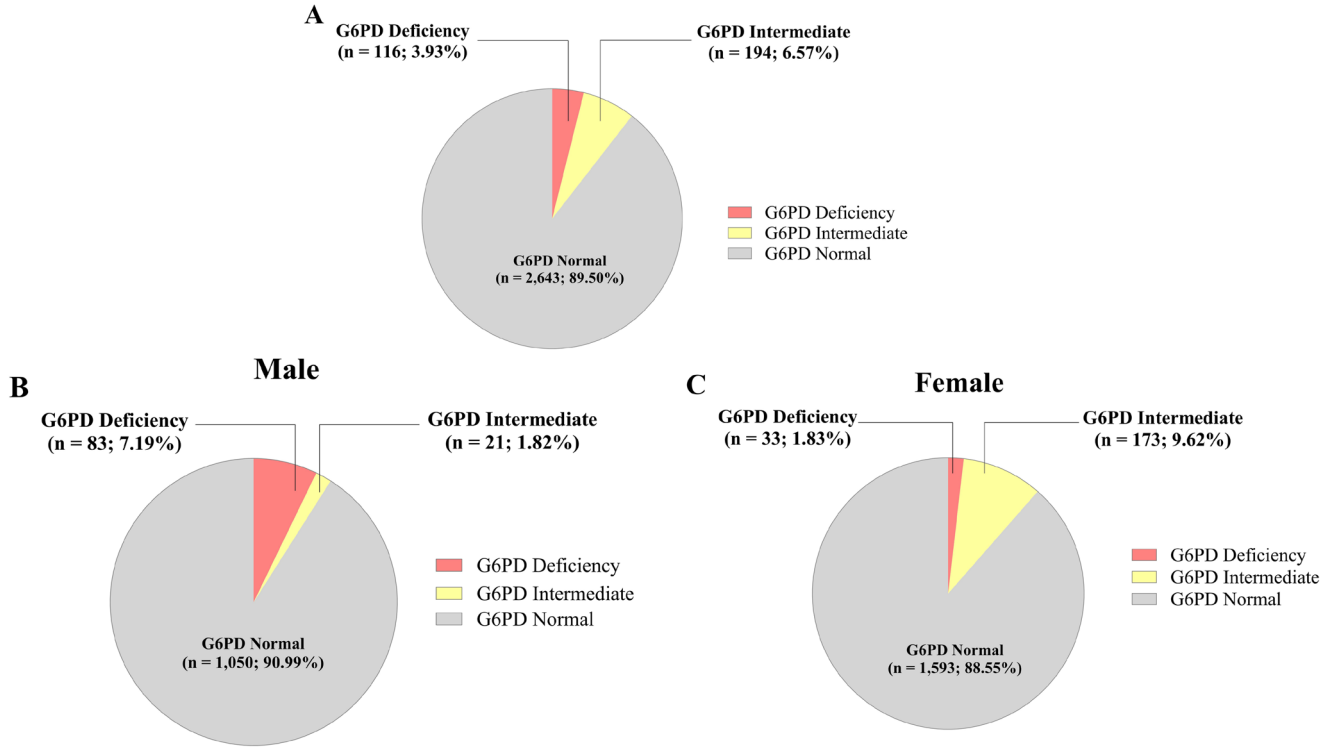

FIGURE 3 | Pie charts illustrating the distribution of G6PD status in the studied population. (A) Overall G6PD status, (B) G6PD phenotypes among males, and (C) G6PD phenotypes among females.

TABLE 2 | *G6PD* genotypes among the studied population.

Genotype	Variant name	*N*			Frequency (%)
		Male	Female	Total	
c.95A>G	Gaohe	1	2	3	0.21
c.95A>G, c.486-34delT	Gaohe	0	1	1	0.07
c.95A>G, c.1311C>T, c.1365-13T>C	Gaohe	0	1	1	0.07
c.131C>G*, c.1311C>T, c.1365-13T>C	Orissa	1	0	1	0.07
c.143T>C	Aures	3	5	8	0.56
c.143T>C, c.486-34delT	Aures	0	2	2	0.14
c.143T>C, c.1311C>T, c.1365-13T>C	Aures	0	1	1	0.07
c.376A>G*, c.592C>T, c.1365-13T>C	A and Coimbra	0	1	1	0.07
c.392G>T	Chinese-4	2	6	8	0.56
c.406C>T, c.1311C>T, c.1365-13T>C	Valladolid	2	2	4	0.28
c.487G>A	Mahidol	17	18	35	2.45
c.487G>A, c.486-34delT	Mahidol	0	1	1	0.07
c.487G>A, c.1365-13T>C	Mahidol	0	1	1	0.07
c.487G>A, c.1311C>T, c.1365-13T>C	Mahidol	0	9	9	0.63
c.487G>A, c.871G>A, c.1311C>T, c.1365-13T>C	Mahidol and Viangchan	0	1	1	0.07
c.592C>T	Coimbra	0	3	3	0.21
c.592C>T, c.1024C>T	Coimbra and Chinese-5	0	1	1	0.07
c.871G>A, c.1311C>T, c.1365-13T>C	Viangchan	36	108	144	10.10
c.871G>A, c.1024C>T, c.1311C>T, c.1365-13T>C	Viangchan and Chinese-5	0	1	1	0.07
c.871G>A, c.486-34delT, c.1311C>T, c.1365-13T>C	Viangchan	0	9	9	0.63
c.1003G>A*	Chatham	1	0	1	0.07
c.1024C>T	Chinese-5	2	6	8	0.56
c.1024C>T, c.486-34delT	Chinese-5	0	3	3	0.21
c.1024C>T, c.1311C>T, c.1365-13T>C	Chinese-5	0	1	1	0.07
c.1191C>T*	West Virginia	0	1	1	0.07
c.1360C>T	Union	7	1	8	0.56
c.1376G>T	Canton	9	19	28	1.96
c.1376G>T, c.1388G>A*	Canton and Kaiping	0	1	1	0.07
c.1376G>T, c.486-34del	Canton	0	4	4	0.28
c.1376G>T, c.1311C>T, c.1365-13T>C	Canton	0	5	5	0.35
c.1388G>A*	Kaiping	4	7	11	0.77
c.1388G>A*, c.486-34delT	Kaiping	0	1	1	0.07
c.1388G>A*, c.1311C>T, c.1365-13T>C	Kaiping	0	2	2	0.14
c.1398C>T*	Silent mutation	0	1	1	0.07
c.486-34delT	Intronic mutation	66	194	260	18.23

(Continues)

TABLE 2 | (Continued)

Genotype	Variant name	*N*			Frequency (%)
		Male	Female	Total	
c.1365-13T>C	Intronic mutation	2	3	5	0.35
c.1311C>T, c.1365-13T>C	Silent and intronic mutations	271	531	802	56.24
c.486-34delT, c.1311C>T, c.1365-13T>C	Silent and intronic mutations	0	49	49	3.44
Total		424	1002	1426	100

Note: Asterisk indicates *G6PD* mutations identified by DNA sequencing.

combined with either the intron 5 deletion (c.486-34delT) or dual mutations (c.1311C>T and c.1365-13T>C). Single missense mutations co-occurring with c.486-34delT included *G6PD* Gaohe, *G6PD* Aures, *G6PD* Mahidol, *G6PD* Chinese-5, and *G6PD* Canton. Single missense mutations coexisting with c.1311C>T and c.1365-13T>C included *G6PD* Gaohe, *G6PD* Aures, *G6PD* Valladolid, *G6PD* Mahidol, *G6PD* Viangchan, *G6PD* Chinese-5, and *G6PD* Canton.

Double missense mutations were identified in compound heterozygous females, including *G6PD* Coimbra+Chinese-5, which was reported for the first time. Additional double mutations coexisting with c.1311C>T and c.1365-13T>C included *G6PD* Mahidol+Viangchan and *G6PD* Viangchan+Chinese-5.

Among the 20 samples with activity less than 70% of the AMM, no identifiable mutations were detected when genotyped using HRM. These samples were then subjected to DNA sequencing, which revealed that all carried missense mutations (Table 2). Identified single missense mutations included *G6PD* Orissa in a male, *G6PD* Chatham in a male, *G6PD* West Virginia in a female, and *G6PD* Kaiping in males, either as a single mutation or coexisting with deletion in intron 5 or c.1311C>T and c.1365-13T>C in females. Additionally, a single synonymous mutation (c.1398C>T, p. Thr466=) was identified in a heterozygous female. Two double missense mutations were also identified via DNA sequencing: *G6PD* A+Coimbra, reported for the first time, and *G6PD* Canton+Kaiping in a heterozygous female.

3.3 | Phenotype–Genotype Correlation

To characterize the relationship between phenotype and genotype, the distribution of G6PD activity levels and variants is shown in Figure 4. For both males and females, the most frequently detected *G6PD* mutations (c.1311C>T and c.1365-13T>C) resulted in variation in G6PD activity, spanning from deficient to normal phenotype. Males showed activity levels ranging from 1.4 to 17.1 U/gHb, homozygous females ranged from 0.3 to 16.3 U/gHb, and heterozygous females ranged from 0.2 to 18.3 U/gHb.

In males, most missense mutations (*G6PD* Gaohe, *G6PD* Orissa, *G6PD* Aures, *G6PD* Valladolid, *G6PD* Mahidol, *G6PD* Vianghcan, *G6PD* Chatham, *G6PD* Union, *G6PD* Canton, and *G6PD* Kaiping) resulted in G6PD activity below 30%. However, two mutations *G6PD* Chinese-4 (c.392G>T) and *G6PD* Chinese-5 (c.1024C>T) exhibited intermediate activity levels, with ranges of 3.6–4.5 and 4.1–5.5 U/gHb, respectively.

In females, mutations have resulted in a broader range of G6PD activity, spanning from deficient to normal levels, especially single missense mutations. Double missense mutations, including *G6PD* A+Coimbra, *G6PD* Coimbra+Chinese-5, *G6PD* Mahidol+Viangchan, *G6PD* Viangchan+Chinese-5, and *G6PD* Canton+Kaiping, exhibited lower G6PD activity when compared to their corresponding single mutations.

It is noteworthy that a heterozygous female carrying the *G6PD* West Virginia variant exhibited a normal phenotype with an enzyme activity of 8.9 U/gHb. Similarly, a heterozygous female with the silent mutation (c.1398C>T) also displayed a normal phenotype with a G6PD activity of 9.0 U/gHb.

The relationship between zygosity and enzyme activity for each *G6PD* variant is shown in Figure 5 and Table 4. Hemizygous males and homozygous females carrying missense mutations exhibited a deficient phenotype, except for those with *G6PD* Chinese-4 and *G6PD* Chinese-5. In contrast, heterozygous females displayed a wide range of enzyme activity, spanning from deficient to normal. Furthermore, a broad spectrum of enzyme activity was observed in individuals carrying silent and/or intronic mutations, regardless of their zygosity status.

4 | Discussion

A large study involving 2953 participants (1154 males and 1799 females) in Bangkok, Thailand, was conducted to investigate the prevalence, genetic diversity, and zygosity of G6PD deficiency. Consistent with previous reports, which estimated a prevalence of 3%–18% in the Thai population [10, 15, 16, 18, 27, 35], this study determined an overall prevalence of 3.93%, using the WST-8 enzymatic method. Among males, the prevalence was 7.19%, while it was 1.83% among females. *G6PD* genotyping revealed high genetic diversity, identifying 38 distinct *G6PD* genotypes in 424 males and 1002 females, many of which were reported for the first time in Thailand.

Quantitative phenotypic methods, such as spectrophotometry, the WST-8 assay, and the Standard G6PD Test (SD Biosensor, Republic of Korea), can categorize G6PD status as deficient, intermediate, or normal when appropriate cutoff values are defined for specific populations [12, 29, 30, 36, 37]. These classifications are crucial for clinical management, including

TABLE 3 | Zygosity and allele frequency of *G6PD* mutations in the Thai population.

G6PD mutations	Male		Female				No. of X* Chr	Total X or X* Chr	Allele frequency
	Hemizygotes (X*Y)	Normal (XY)	Homozygote (X*X*)	Heterozygote (X*)	Heterozygote (X)	Normal (XX)			
c.95A>G	1	0	0	4	0	0	5	5	0.0008
c.131C>G*	1	0	0	0	0	0	1	1	0.0002
c.143T>C	3	0	4	6	0	0	13	13	0.0021
c.376A>G	0	0	0	1	0	0	1	1	0.0002
c.392G>T	2	0	0	6	0	0	8	8	0.0013
c.406C>T	2	0	0	2	0	0	4	4	0.0006
c.486-34delT	66	0	14	256	0	0	336	336	0.0544
c.487G>A	17	0	0	30	0	0	47	47	0.0076
c.592C>T	0	0	0	5	0	0	5	5	0.0008
c.871G>A	36	0	4	117	0	0	157	157	0.0254
c.1003G>A*	1	0	0	0	0	0	1	1	0.0002
c.1024C>T	2	0	4	9	0	0	15	15	0.0024
c.1191C>T*	0	0	0	1	0	0	1	1	0.0002
c.1311C>T	310	0	240	600	0	0	1150	1150	0.1863
c.1360C>T	7	0	0	1	0	0	8	8	0.0013
c.1365-13T>C	312	0	240	604	0	0	1156	1156	0.1873
c.1376G>T	9	0	0	29	0	0	38	38	0.0062
c.1388G>A*	7	0	0	11	0	0	18	18	0.0029
c.1398C>T*	0	0	0	1	0	0	1	1	0.0002
No mutation	0	730	0	0	1680	797	0	3207	0.5196
Total	776	730	506	1680	1680	797	2965	6172	1.0000

Note: Asterisk indicates *G6PD* mutations identified by DNA sequencing.

malaria treatment. For individuals with intermediate to normal G6PD activity, a primaquine dose of 1 mg/kg/day for 7 days or a single dose of tafenoquine may be administered if G6PD activity exceeds 70% of normal. For activity levels between 30% and 70%, primaquine at 0.5 mg/kg/day for 7 or 14 days is appropriate. Individuals with G6PD deficiency (< 30%) may receive 0.75 mg/kg of primaquine weekly for 8 weeks under close medical supervision due to the risk of drug-induced hemolysis [38].

While males typically exhibited a deficient phenotype with enzyme activity < 30%, 21 males (1.83%) in this study showed intermediate deficiency. Among them, two carried *G6PD* Chinese-4, two carried *G6PD* Chinese-5, and the others had silent and/or intronic mutations. Both *G6PD* Chinese-4 and *G6PD* Chinese-5 are pathogenic variants associated with enzyme deficiency but have been shown to cause mild deficiency in an earlier study [39]. Additionally, 173 females (9.62%) exhibited intermediate deficiency, all of them carrying *G6PD* mutations: single missense, double missense, and silent and/or intronic mutations. Both males and females with silent and/or intronic mutations (c.486-34delT, c.1311C>T and c.1365-13T>C) displayed a wide spectrum of G6PD activity, ranging from deficient to normal. While silent and intronic mutations were considered neutral, emerging evidence suggests that they may influence gene expression levels [40–42]. Previous studies have also documented diverse phenotypes caused by the presence of silent and/or intronic mutations [16–18, 43]. Hence, further investigation is necessary to clarify their roles in causing G6PD deficiency.

Individuals carrying silent and/or intronic mutations accounted for 78.26% (1116/1426) of all mutations identified in this study. Among all participants, 85 males and 225 females carried at least one missense mutation. Of females with missense mutations, 6 homozygotes and 120 heterozygotes exhibited a deficient or intermediate phenotype, which was accurately classified by a quantitative phenotypic test. However, 99 heterozygous females with missense mutations had activity levels > 70%, which were defined as G6PD normal by the phenotypic test. Heterozygous females exhibited a wide spectrum of enzyme activity due to random X-chromosome inactivation [44]. This process causes individual red blood cells to

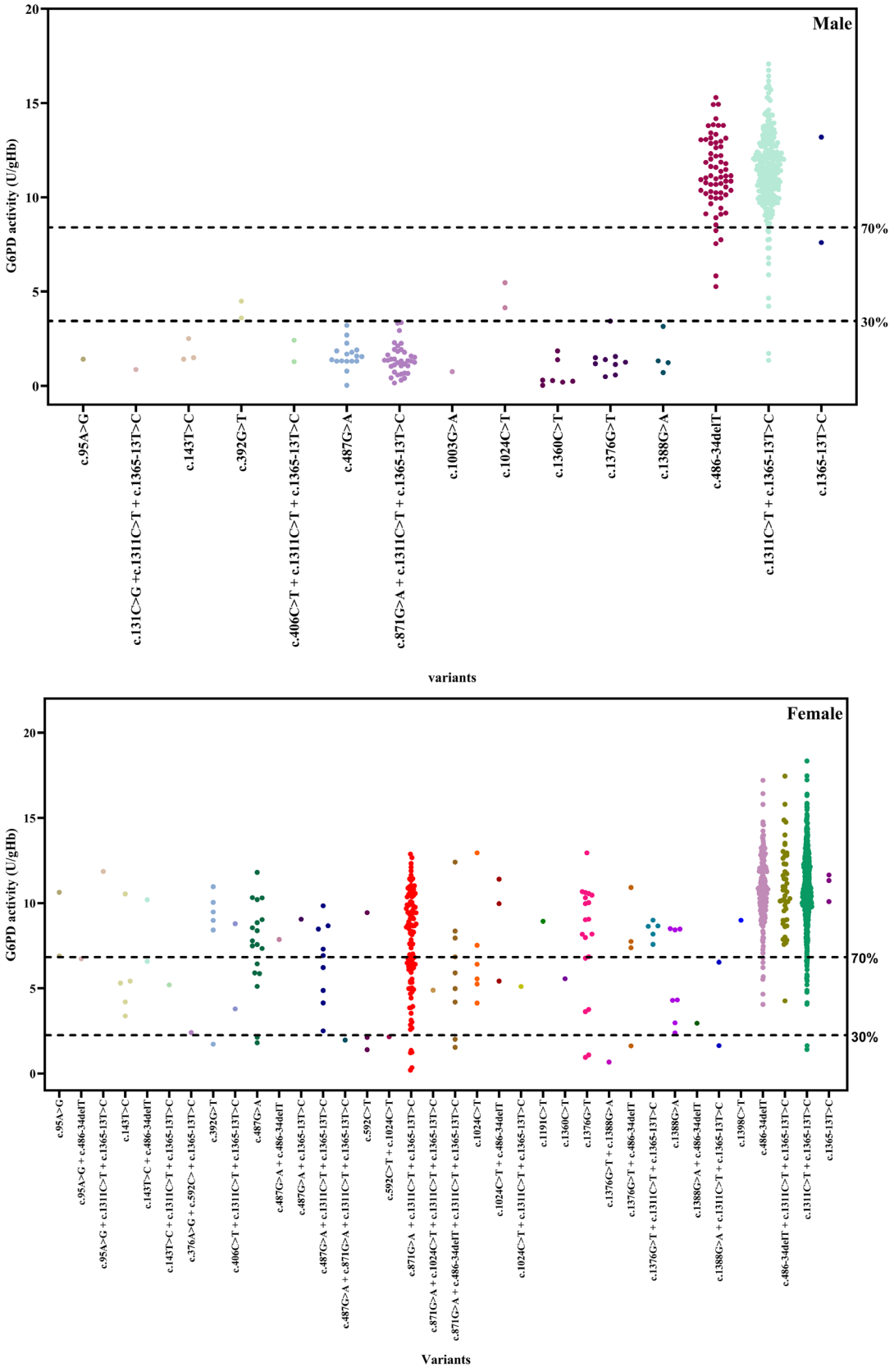

FIGURE 4 | Distribution of G6PD activity with mutations in males and females.

express G6PD based on which allele—deficient or normal—is inactivated. As a result, enzyme activity levels vary across the red blood cell population. Despite the classification, female individuals with intermediate to normal G6PD deficiency may remain susceptible to drug-induced hemolysis, depending on factors such as drug dose, exposure time, and *G6PD*

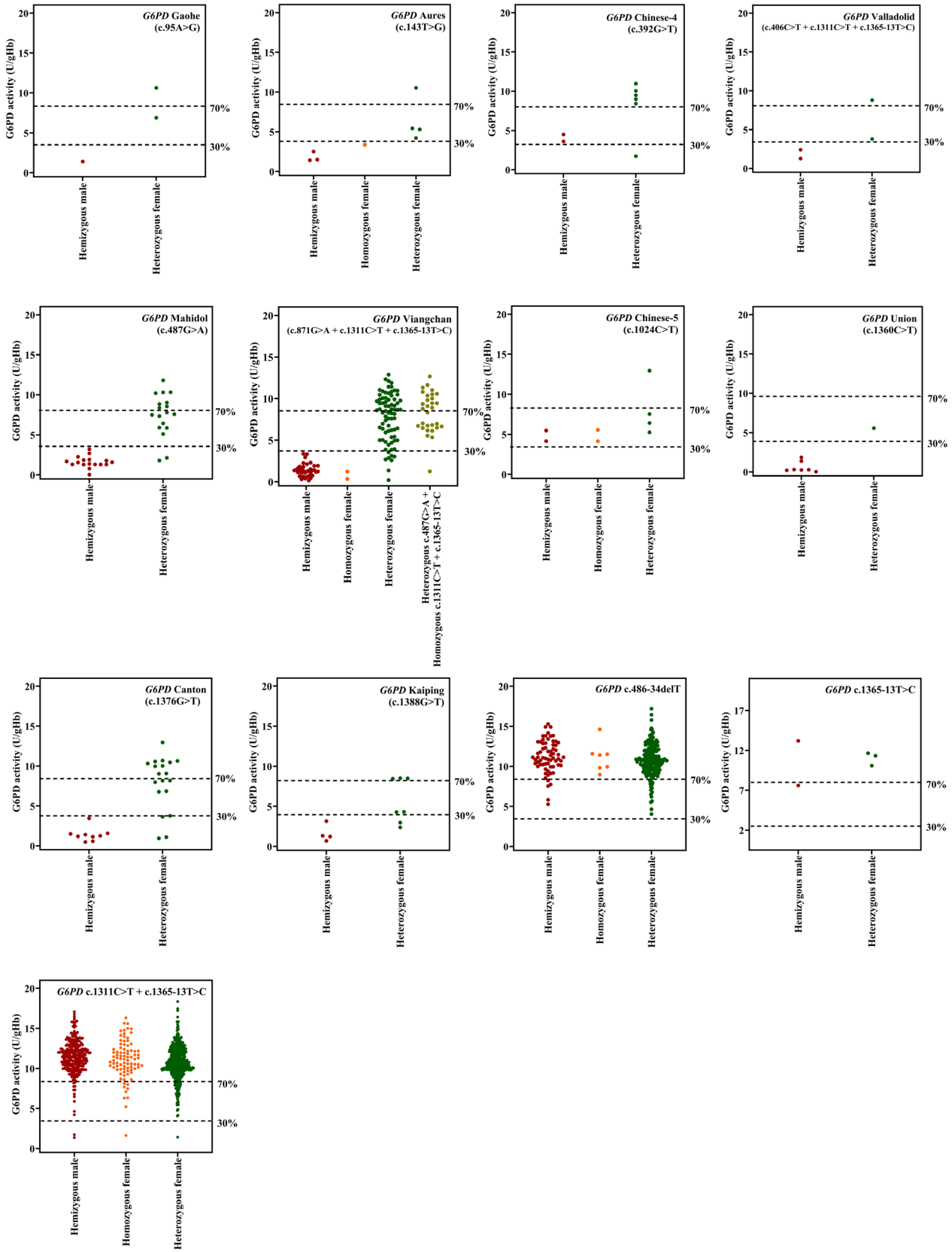

FIGURE 5 | G6PD enzyme activity by zygosity for different mutations in the Thai population in the central region. Each plot illustrates enzyme activity (U/g Hb) categorized by zygosity, showing the variation in enzymatic function among individuals with specific *G6PD* mutations.

TABLE 4 | G6PD activity by mutation type and zygosity in the studied population.

		G6PD activity (U/gHb)					
Genotype	**Variant name**	**Hemizygous male**	**Homozygous female**	**Heterozygous female**	**Missense heterozygous + silent homozygous female**	**Missense homozygous + silent heterozygous female**	**Silent homozygous + silent heterozygous**
c.95A>G	Gaohe	1 (1.4)	—	2 (6.9–10.6)	—	—	—
c.95A>G, c.486-34delT	Gaohe	—	—	1 (6.7)	—	—	—
c.95A>G, c.1311C>T, c.1365-13T>C	Gaohe	—	—	1 (11.9)	—	—	—
c.131C>G*, c.1311C>T, c.1365-13T>C	Orissa	1 (0.9)	—	—	—	—	—
c.143T>C	Aures	3 (1.4–2.5)	1 (3.4)	4 (4.2–10.5)	—	—	—
c.143T>C, c.486-34delT	Aures	—	—	2 (6.6–10.2)	—	—	—
c.143T>C, c.1311C>T, c.1365-13T>C	Aures	—	1 (5.2)	—	—	—	—
c.376A>G*, c.592C>T, c.1365-13T>C	A, Coimbra	—	—	1 (2.4)	—	—	—
c.392G>T	Chinese-4	2 (3.6–4.5)	—	6 (1.7–11.0)	—	—	—
c.406C>T, c.1311C>T, c.1365-13T>C	Valladolid	2 (1.3–2.4)	—	2 (3.8–8.8)	—	—	—
c.487G>A	Mahidol	17 (0.0–3.2)	—	18 (1.8–11.8)	—	—	—
c.487G>A, c.486-34delT	Mahidol	—	—	1 (7.9)	—	—	—
c.487G>A, c.1365-13T>C	Mahidol	—	—	1 (9.1)	—	—	—

(Continues)

TABLE 4 | (Continued)

Genotype	Variant name	G6PD activity (U/gHb)					
		Hemizygous male	Homozygous female	Heterozygous female	Missense heterozygous + silent homozygous female	Missense homozygous + silent heterozygous female	Silent homozygous + silent heterozygous
c.487G>A, c.1311C>T, c.1365-13T>C	Mahidol	—	—	8 (2.5–9.9)	1 (8.7)	—	—
c.487G>A, c.871G>A, c.1311C>T, c.1365-13T>C	Mahidol, Viangchan	—	—	1 (2.0)	—	—	—
c.592C>T	Coimbra	—	—	3 (1.4–9.4)	—	—	—
c.592C>T, c.1024C>T	Coimbra, Chinese-5	—	—	1 (2.6)	—	—	—
c.871G>A, c.1311C>T, c.1365-13T>C	Viangchan	36 (0.2–3.4)	2 (0.3–1.2)	76 (0.2–12.9)	30 (1.2–12.7)	—	—
c.871G>A, c.1024C>T, c.1311C>T, c.1365-13T>C	Viangchan, Chinese-5	—	—	1 (4.9)	—	—	—
c.871G>A, c.486-34delT, c.1311C>T, c.1365-13T>C	Viangchan	—	—	9 (1.5–12.4)	—	—	—
c.1003G>A*	Chatham	1 (0.8)	—	—	—	—	—
c.1024C>T	Chinese-5	2 (4.1–5.5)	2 (4.1–5.6)	4 (5.3–13.0)	—	—	—
c.1024C>T, c.486-34delT	Chinese-5	—	—	2 (10.0–11.4)		1 (5.4)	
c.1024C>T, c.1311C>T, c.1365-13T>C	Chinese-5	—	—	1 (5.1)	—	—	—
c.1191C>T*	West Virginia	—	—	1 (8.9)	—	—	—

(Continues)

TABLE 4 | (Continued)

Genotype	Variant name	G6PD activity (U/gHb)					
		Hemizygous male	Homozygous female	Heterozygous female	Missense heterozygous + silent homozygous female	Missense homozygous + silent heterozygous female	Silent homozygous + silent heterozygous
c.1360C>T	Union	7 (0.0–1.9)	—	1 (5.6)	—	—	—
c.1376G>T	Canton	9 (0.5–3.4)	—	19 (0.9–13.0)	—	—	—
c.1376G>T, c.1388G>A*	Canton, Kaiping	—	—	1 (0.7)	—	—	—
c.1376G>T, c.486-34del	Canton	—	—	4 (1.6–10.9)	—	—	—
c.1376G>T, c.1311C>T, c.1365-13T>C	Canton	—	—	5 (7.6–9.0)	—	—	—
c.1388G>A*	Kaiping	4 (0.7–3.2)	—	7 (2.4–8.5)	—	—	—
c.1388G>A*, c.486-34delT	Kaiping	—	—	1 (3.0)	—	—	—
c.1388G>A*, c.1311C>T, c.1365-13T>C	Kaiping	—	—	2 (1.6–6.5)	—	—	—
c.1398C>T*		—	—	1 (9.0)	—	—	—
c.486-34delT		66 (5.3–15.3)	7 (9.0–14.6)	187 (4.1–17.2)	—	—	—
c.1365-13T>C		2 (7.6–13.2)	—	3 (10.1–11.6)	—	—	—
c.1311C>T, c.1365-13T>C		271 (1.4–17.1)	86 (1.6–16.3)	445 (1.4–18.3)	—	—	—
c.486-34delT, c.1311C>T, c.1365-13T>C		—	—	48 (4.3–17.5)	—	—	1 (10.2)
Total		424	99	870	31	1	1

Note: Asterisk indicates *G6PD* mutations identified by DNA sequencing.

mutations, among others. For instance, hemolysis caused by primaquine and tafenoquine has been reported in individuals heterozygous for *G6PD* Mahidol, a mild variant [45–48]. This suggests that genetic testing might be necessary alongside phenotypic testing to enhance diagnostic accuracy and improve patient outcomes in heterozygous females.

Newborns present additional diagnostic challenges due to higher G6PD activity levels, attributed to the predominance of young red blood cells (reticulocytes). Neonatal-specific cutoff values may be necessary, as G6PD-deficient infants are predisposed to neonatal jaundice [49–51]. Misdiagnosis in this group can lead to severe complications, including hyperbilirubinemia and kernicterus, potentially causing irreversible neurological damage or death if left untreated. Several *G6PD* mutations have been linked to neonatal hyperbilirubinemia [52]. This also highlights the limitations of relying solely on G6PD phenotypic testing.

This study demonstrated that combining the WST-8 enzymatic assay with multiplex HRM assays provides an accurate diagnosis of G6PD deficiency. The WST-8 enzymatic assay enables rapid detection from whole blood or dried blood spots without the need for a sample preparation step, delivering results within 10–15 min and expressing activity as U/gHb. The estimated cost per sample for the WST-8 assay is approximately $1, while the cost for spectrophotometry and SD Biosensor G6PD tests ranges from $3 to $20. The multiplex HRM assays can detect four *G6PD* mutations simultaneously in 90 min, processing up to 70 samples per run at a cost of about $2 per sample. Sanger sequencing and the G6PD Genotyping Kit (Asian type) cost around $10–$20 per sample. These combined WST-8 and HRM methods are cost-effective, high-throughput, and suitable for large-scale screening, also allowing for zygosity determination.

The limitations of this study should be considered in the context of both the sample population and the diagnostic tests used. The study used archived blood samples collected in Bangkok, the central region of Thailand, which may not fully represent the broader population. Therefore, the findings may not be generalizable to the entire Thai population or to populations with different genetic backgrounds. Additionally, no demographic data were analyzed, limiting the ability to assess the influence of factors such as age or ethnic background on G6PD deficiency prevalence and genotypic distribution. The performance quality of the WST-8 assay should be carefully considered, and proper controls must be used to ensure accuracy. Although the multiplex HRM assays in this study cover 15 specific *G6PD* mutations common in Southeast Asia, their ability to capture the full spectrum of mutations responsible for G6PD deficiency remains limited. Rare or uncharacterized mutations present in the population may not be detected, leading to underreporting of mutation diversity, which may require further DNA sequencing to fully capture such mutations.

Quantitative phenotypic testing was effective in identifying G6PD deficiency in hemizygous males, homozygous females, and approximately 50% of heterozygous females. However, 44% of heterozygous females showing > 70% activity were classified as G6PD normal. This affects the understanding of G6PD epidemiology and its corresponding treatment in females. Accurate information on women will address gender disparities in treatment, particularly in low-income, malaria-endemic regions, where G6PD deficiency and malaria disproportionately impact women and their families.

Beyond malaria, G6PD status holds significant clinical importance for various health conditions, including neonatal health. Despite this, neonatal G6PD screening has not been widely adopted, even in areas where the World Health Organization recommends it for regions with a male prevalence exceeding 3%. This limited implementation is attributed to challenges such as lack of funding, limited public awareness, and inadequate availability of reliable and affordable diagnostic tools.

While there are no treatments for G6PD deficiency, it is a lifelong condition that requires individuals to avoid specific triggers, such as certain foods and medications. This highlights the critical need for early and accurate diagnosis, particularly in low- and middle-income countries, where the burden of G6PD deficiency is compounded by limited healthcare resources and higher exposure to triggering factors. Addressing these challenges with increased investment in diagnostic technologies, public health initiatives, and educational campaigns can significantly reduce preventable morbidity and mortality associated with G6PD deficiency.

5 | Conclusions

In summary, this study demonstrates the practicality and effectiveness of integrating phenotypic and genotypic methods, such as the WST-8 enzymatic assay and multiplex HRM, for accurate and reliable diagnosis of G6PD deficiency. This dual strategy enhances diagnostic precision for heterozygous females, who are often misclassified by phenotypic testing alone. The findings have significant public health implications, enabling early identification of at-risk individuals and reducing adverse reactions associated with 8-aminoquinolines. By informing population-specific treatment guidelines and advancing personalized medicine, this study supports safer drug administration and improved malaria elimination efforts, while strengthening healthcare systems in underserved regions.

Future directions for research should include the potential for incorporating next-generation sequencing (NGS) for a more comprehensive analysis of *G6PD* variants. NGS could help overcome the limitations of multiplex HRM assays by detecting rare and uncharacterized mutations, offering a more complete picture of genetic diversity and its clinical implications.

Author Contributions

Conceptualization, methodology, project administration, validation: U.B. Data curation, formal analysis, visualization, writing – original draft: U.B. and N.P. Funding acquisition: W.N. Investigation: U.B., K.C., C.D., J.S., and N.P. Resources: U.B. and W.N. Supervision: U.B., K.C., and N.S. Writing – review and editing: C.D., J.S., K.C., N.S., and W.N.

Ethics Statement

The study procedures were approved by the Human Ethics Committee of Mahidol University's Faculty of Tropical Medicine (MUTM 2024-042-01). The study was performed using archived blood samples. The data were fully anonymized, and the authors had no access to information to identify individual participants.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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