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RESEARCH ARTICLE



# Combined Gap-Polymerase Chain Reaction and Targeted Next-Generation Sequencing Improve α- and β-Thalassemia Carrier Screening in Pregnant Women in Vietnam

Tuan-Thanh Lam[a,b], Doan-Tu Nguyen[c], Quang Thanh Le[d], Duy-Anh Nguyen[e], Diem-Tuyet Thi Hoang[f], Huu Du Nguyen[g], Canh Chuong Nguyen[e], Kim Phuong Thi Doan[h], Nhat-Thang Tran[i], Thi Minh Thi Ha[j], Thu Huong Nhat Trinh[d], Van Thong Nguyen[f], Duc Tam Lam[k], Minh Tam Le[j], Xuan Thao Nguyen[g], Thu-Hang Thi Ho[l], Trung Hoanh Tran[m], Viet Thang Ho[f], Thanh Van Bui[n], Van Trong Nguyen[o], Phuoc Ba Hoang[p], Hoai Thanh Nguyen[q], Manh Hoan Nguyen[r], Thanh-Binh Vo[a,b], Duy-Khang Nguyen Le[a,b], Thao Ngoc Truong[a,b], Hong-Thuy Thi Dao[a,b], Phuong-Anh Ngoc Vo[a,b], Thien-Chi Van Nguyen[a,b], Ngoc-Nhu Thi Tran[a,b], Quynh-Nhu Thi Tran[a,b], Yen-Linh Thi Van[a,b], Thanh-Thanh Thi Nguyen[a,b], Bich-Ngoc Thi Huynh[a,b], Thanh-Phuong Thi Nguyen[a,b], Kim-Van Thi Tran[a,b], Cong-Trai Nguyen[a,b], Phuoc-Loc Doan[a,b], Thanh-Dat Nguyen[a,b], Thanh-Thuy Thi Do[b], Dinh-Kiet Truong[b], Hung Sang Tang[a,b], Ngoc-Phuong Thi Cao[a,b], Minh-Duy Phan[a,b], Hoa Giang[a,b] and Hoai-Nghia Nguyen[b,s]

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**ABSTRACT**

Vietnam has a high thalassemia burden. We collected blood samples from 5880 pregnant Vietnamese women during prenatal health checks to assess thalassemia carrier frequency using combined gap-polymerase chain reaction (gap-PCR) and targeted next-generation sequencing (NGS). Thalassemia carriers were identified with prevalence of 13.13% (772), including 7.82% (460) carriers of α-thalassemia (α-thal), 5.31% (312) carriers of β-thalassemia (β-thal), and 0.63% (37) concurrent α-/β-thal carriers. Deletional mutations (368) accounted for 80.0% of α-thal carriers, of which, $--^{SEA}$ (Southeast Asian) ($n = 254$; 55.0%) was most prevalent, followed by the $-\alpha^{3.7}$ (rightward) ($n = 66$; 14.0%) and $-\alpha^{4.2}$ (leftward) ($n = 45$; 9.8%) deletions. Hb Westmead (*HBA2*: c.369C>G) ($n = 53$) and Hb Constant Spring (Hb CS or *HBA2*: c.427T>C) (in 28) are the two most common nondeletional α-globin variants, accounting for 11.5 and 6.0% of α-thal carriers. We detected 11 different β-thal genotypes. Hb E (*HBB*: c.79G>A) (in 211) accounted for 67.6% of β-thal carriers. The most common β-thal genotypes were associated with mutations at codon 17 (A>T) (*HBB*: c.52A>T), codons 41/42 (–TTCT) (*HBB*: c.126_129delCTTT), and codon 71/72 (+A) (*HBB*: c.217_218insA) (prevalence 0.70%, 0.68%, and 0.2%, respectively). Based on mutation frequencies calculated in this study, estimates of 5021 babies in Vietnam are affected with clinically severe thalassemia annually. Our data suggest a higher thalassemia carrier frequency in Vietnam than previously reported. We established that combining NGS with gap-PCR creates an effective large-scale thalassemia screening method that can detect a broad range of mutations.

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## Introduction

Thalassemias are autosomal recessive disorders characterized by defective synthesis of globin chains due to mutations in the α- or β-globin gene clusters, resulting in α- or β-thalassemia (α- or β-thal), respectively. Thalassemias are mainly found in tropical and subtropical regions in Africa, the Mediterranean Basin, the Middle East, subcontinental India, Southern China, and Southeast Asia [1,2]. α-Thalassemia most frequently results from deletional mutations of the two α-globin genes (α1 and α2) on chromosome 16p13.3 [3]. There are over 70 types of nondeletional α-thal that are reported much less often [3,4]. Clinical phenotypes of α-thal vary from asymptomatic to severe, corresponding to the number of α-globin chains lost or impaired. Common deletional α-thal mutations include the $-\alpha^{3.7}$ (rightward) and $-\alpha^{4.2}$ (leftward) single gene deletions ($\alpha^{+}$); the $--^{SEA}$

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(Southeast Asian), $--^{THAI}$ (Thailand) and $--^{FIL}$ (Filipino) and the $--^{MED}$ (Mediterranean) and $-(\alpha)^{20.5}$ double gene deletions ($\alpha^0$). Individuals carrying two functional copies of the α-globin genes are clinically asymptomatic (homozygous $\alpha^+$ or heterozygous $\alpha^0$). Those who suffer from Hb H disease have only one copy of the α-globin gene (compound heterozygotes for two different mutations such as $-\alpha^{3.7}/--^{SEA}$). Homozygotes for double gene deletions, most notably the $--^{SEA}/--^{SEA}$ deletions, result in Hb Bart's hydrops fetalis syndrome, the most severe form of α-thal that often causes pregnancy complications and death *in utero* or during early childhood.

Point mutations and small deletions/insertions in the β-globin gene are the primary molecular defects responsible for most β-thal, with over 200 variants identified [5]. These mutations can lead to a reduction ($\beta^+$) or absence ($\beta^0$) of β-globin chain production. The interactions in these molecular defects result in either asymptomatic carriers $\beta^A/\beta^0$ or $\beta^A/\beta^+$, β-thal intermedia (β-TI) ($\beta^+/\beta^0$ or $\beta^+/\beta^+$) or β-thal major (β-TM) ($\beta^0/\beta^0$). Complete loss of β-globin in β-TM (Cooley's anemia) results in excessive unbound α-globin chains that precipitate erythroid precursors in the bone marrow and the mature erythrocytes, leading to impaired erythropoiesis and peripheral hemolysis [6]. The most notable clinical presentations including severe anemia, hemoglobin (Hb) levels under 7.0 g/dL, jaundice, failure to thrive, skeletal deformities, splenomegaly, and hepatomegaly, usually occur between the first 6 months and 2 years of life, as the level of Hb F produced by γ-globin genes gradually declines, prompting the need for lifelong blood transfusions [7].

There is no effective therapy for severe thalassemia except bone marrow transplantation, which is not affordable for affected populations in low- and middle-income countries. Consequently, most affected children in these countries are unlikely to survive past the age of 20. Long-term survival depends on blood transfusions, imposing substantial financial and social burdens [1,2,8,9]. Genetic counseling, preconception screening, and prenatal screening for α- and β-thal are essential for reducing disease incidence and occurrence in high-burden regions. Accurate estimates of carrier frequency and mutation spectra (lacking in most affected countries) are vital in designing national programs for screening and managing thalassemia [2].

Vietnam has a high prevalence of thalassemia. However, current knowledge of the thalassemia mutation spectra and frequency in Vietnam is mainly based on reports from limited numbers of transfusion-dependent thalassemia (TDT) patients or small at-risk cohorts from single centers [10–16]. Previous large-scale community-based studies focused on asymptomatic carriers $\beta^A/\beta^0$ or $\beta^A/\beta^+$, β-TI ($\beta^+/\beta^0$ or $\beta^+/\beta^+$) or β-TM ($\beta^0/\beta^0$), Hb E (*HBB*: c.79G>A) or other predominant alleles and their association with different ethnicities [14,15,17]. In this study, we used combined gap-polymerase chain reaction (gap-PCR) and targeted next-generation sequencing (NGS) to screen a cohort of 5880 pregnant Vietnamese women across the country to provide a more accurate estimate of disease prevalence and thalassemia mutation spectra.

## Materials and methods

### Study design and demographics

We conducted a cross-sectional descriptive study of retrospective thalassemia genotyping data of 5880 pregnant Vietnamese women from obstetric clinics and hospitals across Vietnam from 1 April 2020, through 31 July 2020. All participants were healthy pregnant women based on physical examination and anthropometric measurement. The participants approved and gave written informed consent to the anonymous reuse of their genomic data for this study. Participants who received a blood transfusion within 6 months before study entry were excluded. Blood samples were sent to the Medical Genetics Institute (Ho Chi Minh City, Vietnam) for testing. Participants' ages ranged from 16 to 54 years. This study was approved by the Ethics Committee of the University of Medicine and Pharmacy, Ho Chi Minh City, Vietnam [Ethics approval ID: 164/HDDD]. Our study was performed in accordance with the principles stated in the Declaration of Helsinki.

### Patient and public involvement

Participating pregnant women and their families were not involved in study design or screening test implementation. However, they were central in raising public awareness and disseminating information regarding thalassemia disease burden, the importance of prenatal screening for congenital disorders such as thalassemia in Vietnam, and the need for more accurate and affordable screening methods.

### Sample collection and genomic DNA extraction

Maternal venous blood was collected using Streck Blood Collection Tubes by venipuncture and processed according to the manufacturer's instructions. Genomic DNA was extracted from the buffy coat layer using the MagMAX™ DNA Multi-Sample Ultra 2.0 Kit on the KingFisher Flex System (Thermo Fisher Scientific, Waltham, MA, USA).

### Library preparation and targeted sequencing

An NGS library was prepared from genomic DNA using NEBNext® Ultra™ II FS DNA Library Prep Kit for Illumina (New England BioLabs, Ipswich, MA, USA) according to the manufacturer's instructions. DNA library concentrations were quantified with a QuantiFluor®dsDNA system (Promega, Madison, WI, USA). Equal amounts of libraries (150 ng per sample) were pooled and hybridized with xGen Lockdown probes targeting the coding exons of *HBA1*, *HBA2,* and *HBB* (Integrated DNA Technology, Singapore) DNA. Sequencing was performed using NextSeq 500/550 High output kits v2 (2 × 75 cycles) on the NextSeq 550 system (Illumina Inc., San Diego, CA, USA) with minimum target coverage of 30×. The mean coverage in the target regions for all samples was ∼60×.

### Variants calling and verification by Sanger sequencing

Paired-end reads were aligned to the human genome (hg38) using BWA 0.7.17 (r1188) [18]. Duplicate reads were marked and removed using MarkDuplicates from Picard tools (http://broadinstitute.github.io/picard/). Variants were called using GATK 4.2.0.0 (https://github.com/broadinstitute/gatk/). Variant effects were annotated by VEP release 104.3 (https://github.com/Ensembl/haracte-vep) and bcftools 1.12. [19]. Detailed curation of Hb variants was obtained from Clinvar (https://www.ncbi.nlm.nih.gov/clinvar/) and the database of human Hb variants and thalassemia mutations (https://globin.bx.psu.edu/). Thirty samples with single nucleotide polymorphism variants detected by NGS were randomly selected for verification by Sanger sequencing.

### Detection of α-globin deletions by gap-polymerase chain reaction

Multiplex gap-PCR assays were used to screen for the three most common α-globin deletions: $-\alpha^{3.7}$, $-\alpha^{4.2}$, and $--^{SEA}$ as previously reported [20]. Each reaction contained 1 mol/L Betaine (Sigma-Aldrich, Burlington, MA, USA), 0.2 µL of each primer (Table 1), 100 ng of genomic DNA, and Platinum™ Green Hot Start PCR Master Mix 2× (Thermo Fisher Scientific) to a 25 µL final volume. The *LIS1* gene was co-amplified in each reaction and served as the internal control for PCR. Reactions were carried out on a Mastercycler Nexus X2 (Eppendorf, Hamburg, Germany), with an initial 5 min denaturation at 95 °C, 30 cycles at 97 °C for 45 seconds, 60 °C for 1 min 15 seconds, 72 °C for 2 min 30 seconds, and a final extension at 72 °C for 5 min. Following amplification, 5 µL of the product was visualized on 1.0% agarose gel prestained with SybrSafe [Invitrogen (Thermo Fisher Scientific), Waltham, MA, USA]. Primers and amplicon sizes are summarized in Table 1.

### Allele frequencies and simulation of allele combinations

Exact binomial confidence intervals (Cis) were calculated for allele frequencies using the package *Rmisc* version 4.5–0 in R (https://www.R-project.org). Genotype frequencies with a confidence interval for homozygous or compound heterozygous genotypes such as Hb Bart's (γ4), Hb H (β4), and $\beta^0/\beta^0$ were calculated by resampling a simulation of allelic combinations assuming a Poisson distribution through 1000 iterations in R version 4.0.3 (https://www.R-project.org) [21]. The number of at-risk births was calculated by multiplying the respective genotype frequencies with the national birth figure in Vietnam from the recent 2019 census [22].

## Results

### Thalassemia carrier frequency and mutation spectra among pregnant women in Vietnam

In the 5880 participants screened, we identified 460 (7.82%) women who carried at least one form of α-thal variant and 312 (5.31%) women who carried at least one form of β-thal variant. We detected 368 deletional α-thal carriers ($--^{SEA}$, $-\alpha^{3.7}$, and $-\alpha^{4.2}$, including three compound heterozygous individuals) and 92 nondeletional α-thal (Table 2). Deletions of the *HBA* genes accounted for over 80.0% of all α-thal carriers; the remainder were due to nondeletional variants. Allele frequencies for $\alpha^0$, $\alpha^+$, and nondeletional α were 2.19%, 0.97%, and 0.80%, respectively.

Hb Westmead (allele frequencies: 0.47%) and Hb CS (allele frequencies: 0.24%) were the two most prevalent nondeletional α-thal. We also detected rare variants such as Hb Paksé (*HBA2*: c.429A>T) and Hb Quong Sze (Hb QS or *HBA2*: c.377T>C). The Hb Paksé variant has been reported at low frequencies in ethnic minorities in Central Vietnam, while Hb Quong Sze has only been reported in China [15].

**Table 1.** Primers of multiplex PCR screening for the three most common α-globin deletions.

| Primer | Sequences (5′–3′) | Amplicon Size |
|---|---|---|
| LIS-F | GTC GTC ACT GGC AGC GTA GAT C | 2503 |
| LIS-R | GAT TCC AGG TTG TAG ACG GAC G | |
| 3.7-F | CCC CTC GCC AAG TCC ACC C | 2022 |
| 3.7-R | AAA GCA CTC TAG GGT CCA GCG | |
| 4.2-F | GGT TTA CCC ATG TGG TGC CTC | 1628 |
| 4.2-R | CCC GTT GGA TCT CTC ATT TCC C | |
| SEA-R | CGA TCT GGG CTC TGT GTT CTC | 1349 |
| SEA-F | AGC CCA CGT TGT GTT CAT GGC | |

F: forward; R: reverse.

**Table 2.** Genotypes, prevalence, and allele frequencies of α-thal variants detected in the study population.

| Hb Name | HGVS Nomenclature | Genotype | Prevalence *n* (%) | Allele Frequencies (95% CI) |
|---|---|---|---|---|
| Nondeletional α-thal ($n = 92$) | | | | |
| Constant Spring | *HBA2*: c.427T > C | $\alpha^{CS}\alpha/\alpha\alpha$ | 28 (0.48) | 0.24 (0.17–0.34) |
| Westmead | *HBA2*: c.369C > G | $\alpha^{Westmead}\alpha/\alpha\alpha$ | 53 (0.90) | 0.47 (0.36–0.61) |
| Westmead | *HBA2*: c.369C > G | $\alpha^{Westmead}\alpha/\alpha^{Westmead}\alpha$ | 2 (0.03) | 0.04 (0.02–0.10) |
| Quong Sze[a] | *HBA2*: c.377T > C | $\alpha^{QS}\alpha/\alpha\alpha$ | 5 (0.09) | 0.01 (0.00–0.06) |
| Paksé[a] | *HBA2*: c.429A > T | $\alpha^{Paksé}\alpha/\alpha\alpha$ | 1 (0.02) | 0.02 (0.01–0.06) |
| Owari[a] | *HBA1*: c.364G > A | $\alpha^{Owari}\alpha/\alpha\alpha$ | 2 (0.03) | 0.01 (0.00–0.05) |
| Pavie[a] | *HBA1*: c.407T>A | $\alpha^{Pavie}\alpha/\alpha\alpha$ | 1 (0.02) | |
| Deletional α-thal ($n = 368$) | | | | |
| $-\alpha^{3.7}$ (rightward) | | $-\alpha^{3.7}/\alpha\alpha$ | 66 (1.12) | 0.56 (0.44–0.71) |
| $-\alpha^{4.2}$ (leftward) | | $-\alpha^{4.2}/\alpha\alpha$ | 45 (0.77) | 0.38 (0.29–0.51) |
| $--^{SEA}$ | | $--^{SEA}$ | 254 (4.32) | 2.19 (1.94–2.47) |
| compound $--^{SEA}/-\alpha^{4.2}$ | | $--^{SEA}/-\alpha^{4.2}$ | 1 (0.017) | |
| compound $--^{SEA}/-\alpha^{3.7}$ | | $--^{SEA}/-\alpha^{3.7}$ | 2 (0.034) | |

HGVS: Human Genome Variation Society; 95% CI: 95% confidence interval; SEA: Southeast Asian.
[a]These are considered rare variants.

**Table 3.** Genotypes, prevalence, and allele frequencies of β-thal variants detected in the study population ($n = 312$).

| Hb Name | HGVS Nomenclature | Genotype | Prevalence *n* (%) | Allele Frequencies (95% CI) |
|---|---|---|---|---|
| Hb E (G > A) | *HBB*: c.79G > A | | 206 (3.50) | 1.79 (1.57–2.05) |
| Hb E (G > A) (het.) | *HBB*: c.79G > A | $\beta^E/\beta^A$ | 5 (0.09) | |
| Hb E (G > A) (hom.) | *HBB*: c.79G > A | $\beta^E/\beta^E$ | 41 (0.70) | 0.35 (0.26–0.47) |
| Codon 17 (A > T) | *HBB*: c.52A > T | $\beta^{codon\ 17}/\beta^A$ | 40 (0.68) | 0.34 (0.25–0.46) |
| Codons 41/42 (–TTCT) | *HBB*: c.126_129delCTTT | $\beta^{codons\ 41/42(-TTCT)}/\beta^A$ | 12 (0.20) | 0.10 (0.06–0.18) |
| Codons 71/72 (+A) | *HBB*: c.217_218insA | $\beta^{codons\ 71/72(+A)}/\beta^A$ | 2 (0.03) | 0.02 (0.01–0.06) |
| Codon 95 (+G) | *HBB*: c.287_288insG | $\beta^{codon\ 95(+G)}/\beta^A$ | 2 (0.03) | 0.02 (0.01–0.06) |
| Codon 43 (G > T) | *HBB*: c.130G > T | $\beta^{codon\ 43(G>T)}/\beta^A$ | 1 (0.02) | 0.01 (0.00–0.05) |
| Codon 26 (G > T) | *HBB*: c.79G > T | $\beta^{codon\ 26(G>T)}/\beta^A$ | 1 (0.02) | 0.01 (0.00–0.05) |
| Hb Khartoum (C > G)[a] | *HBB*: c.374C > G | $\beta^{Hb\ Khartoum}/\beta^A$ | 1 (0.02) | 0.01 (0.00–0.05) |
| Hb G-Siriraj (G > A)[a] | *HBB*: c.22G > A | $\beta^{Hb\ G\text{-}Siriraj}/\beta^A$ | 1 (0.02) | 0.01 (0.00–0.05) |
| Hb New York (T > A)[a] | *HBB*: c.341T > A | $\beta^{Hb\ New\ York}/\beta^A$ | 1 (0.02) | 0.01 (0.00–0.05) |
| Hb Hamilton (G > A)[a] | *HBB*: c.34G>A | $\beta^{Hb\ Hamilton}/\beta^A$ | 1 (0.02) | 0.01 (0.00–0.05) |

HGVS: Human Genome Variation Society; 95% CI: 95% confidence interval; het.: heterozygote; hom: homozygote.
[a]These are considered rare variants.

**Table 4.** Cases of coinherited α- and β-globin variants.

| Globin variants | $-\alpha^{3.7}$ ($\alpha^+$) | $--^{SEA}$ ($\alpha^0$) | $-\alpha^{CS}$ | $-\alpha^{QS}$ | $-\alpha^{Paksé}$ | $-\alpha^{Westmead}$ | $-\alpha^{Owari}$ | Total |
|---|---|---|---|---|---|---|---|---|
| Heterozygous β-thal | | | | | | | | |
| Hb E (*HBB*: c.79G > A) | 7 | 6 | 2 | 1 | 1 | 4 | – | 21 |
| Codons 41/42 (*HBB*: c.126_129delCTT) | 1 | 4 | – | – | – | – | – | 5 |
| Codon 17 (*HBB*: c.52A > T) | 0 | 3 | – | 1 | – | 1 | 1 | 6 |
| Codon 43 (*HBB*: c.130G > T) | 0 | 2 | – | – | – | – | – | 2 |
| Codons 71/72 (*HBB*: c.217_218insA) | 0 | 1 | – | – | – | – | – | 1 |
| Hb New York (*HBB*: c.341T > A) | 0 | 1 | – | – | – | – | – | 1 |
| Homozygous β-thal | | | | | | | | |
| Hb E (*HBB*: c.79G>A) | 0 | 1 | – | – | – | – | – | 1 |
| Total | 8 | 18 | 2 | 2 | 1 | 5 | 1 | 37 |

SEA: Southeast Asian; CS: Hb Constant Spring; QS: Hb Quong Sze.

Two missense variants with no significant clinical presentations [Hb Owari (*HBA2*: c.364G>A) and Hb Pavie (*HBA2*: c.407T>A)], were also detected [23,24].

As for β-thal carriers, we identified 98 heterozygous $\beta^0$ carriers (1.67%) and 206 heterozygous Hb E [codon 26 (G>A)] carriers (3.50%; Table 3). Five individuals were homozygous for the Hb E allele (0.09%). The most prominent $\beta^0$ variants included mutations at codon 17 (A>T), codons 41/42 (–TTCT) and codons 71/72 (+A) (*HBB*: c.217_218insA). Rare $\beta^0$ variants included codon 95 (+A) (*HBB*: c.287-288insA), codon 43 (*HBB*: c.130G>T), and codon 26 (G>T) (*HBB*: c.79G>T), which were previously reported in China and Thailand [25–27]. The codon 95 variant was first reported in Vietnam in 1996, following its first description in Thailand in 1992 [28]. This variant was then reported in several studies in both North and South Vietnam [12,29]. We also detected four rare variants at codon 4 (G>A) (Hb G-Siriraj or *HBB*: c.22G>A), codon 12 (G>A; Hb Hamilton or *HBB*: c.34G>A), codon 113 (T>A; Hb New York or *HBB*: c.341T>A) and codon 125 (C>G) (Hb Khartoum or *HBB*: c.374C>G) that had not been previously reported in Vietnam.

## Coinheritance of α- and β-thalassemia variants

We identified 37 individuals carrying concurrent α- and β-thal variants (prevalence 0.63%; Table 4). Over half of all concurrent α- and β-thal cases were associated with the Hb E allele (prevalence 61.0%), including one homozygous Hb E individual who also carried a $--^{SEA}$ variant.

**Table 5.** Estimated number of births at-risk of $\alpha^0/\alpha^0$, $\alpha^0/\alpha^+$, $\beta^0/\beta^0$, or $\beta^E/\beta^0$ genotypes.

| Genotype | Number of Births (95% CI) |
|---|---|
| | $\alpha^0/\alpha^0$ or $\alpha^0/\alpha^+$ |
| $--^{SEA}/--^{SEA}$ (Southeast Asian) | 800 (793–807) |
| $--^{SEA}/-\alpha^{3.7}$ | 440 (435–444) |
| $--^{SEA}/-\alpha^{4.2}$ | 329 (326–333) |
| $--^{SEA}/-\alpha^{Westmead}$ | 359 (355–363) |
| $--^{SEA}/-\alpha^{Hb\ Quong\ Sze}$ | 155 (152–157) |
| $--^{SEA}/-\alpha^{Paksé}$ | 140 (137–143) |
| $--^{SEA}/-\alpha^{Hb\ Constant\ Spring}$ | 237 (234–240) |
| Total: Hb Bart's; Hb H | 800 (793–807); 1660 (1639–1680) |
| | $\beta^0/\beta^0$ |
| $\beta^{codon\ 17}/\beta^{codon\ 17}$ | 142 (140–144) |
| $\beta^{codon\ 17}/\beta^{codon\ 41}$ | 154 (152–156) |
| $\beta^{codon\ 17}/\beta^{codon\ 43}$ | 134 (131–136) |
| $\beta^{codon\ 17}/\beta^{codon\ 71}$ | 138 (136–140) |
| $\beta^{codon\ 17}/\beta^{codon\ 95}$ | 133 (131–135) |
| $\beta^{codon\ 41}/\beta^{codon\ 41}$ | 144 (142–146) |
| $\beta^{codon\ 41}/\beta^{codon\ 71}$ | 138 (136–140) |
| Total: $\beta^0/\beta^0$ | 983 (968–997) |
| $\beta^E/\beta^E$ | 620 (611–629) |
| $\beta^E/\beta^{codon\ 17}$ | 257 (254–260) |
| $\beta^E/\beta^{codon\ 41}$ | 256 (253–259) |
| $\beta^E/\beta^{codon\ 71}$ | 167 (165–170) |
| $\beta^E/\beta^{codon\ 95}$ | 139 (137–142) |
| $\beta^E/\beta^{codon\ 43}$ | 139 (137–142) |
| Total: $\beta^E/\beta^0$ | 1578 (1557–1601) |

95% CI: 95% confidence interval.

## Estimating the number of births with severe thalassemia annually

The estimates of allele frequencies allowed us to evaluate the number of at-risk newborns of having severe thalassemia such as Hb Bart's hydrops fetalis ($--^{SEA}/--^{SEA}$), Hb H or β-TM in the population (Table 5). Using the national birth rate figure from the 2019 census, we estimated that 800 pregnancies would be affected by Hb Bart's hydrops fetalis

(95% CI: 793–807) and 1660 children born with Hb H (95% CI: 1639–1680) annually. The estimated number of at-risk births of having clinically significant forms of β-thal was 983 births with $\beta^0/\beta^0$ (95% CI: 968–997) and 1578 births with Hb E/$\beta^0$ (95% CI: 1557–1601). Assuming the estimated annual treatment cost of thalassemia per patient in Vietnam from US$1,460.00 to US$5,630,00 [Dr. Tuan Minh Nguyen, personal communication (unreferenced), August 2020], we estimate the yearly cost for medical treatment of β-TM ($\beta^0/\beta^0$ and Hb E/$\beta^0$) alone in Vietnam is between US$3,739,060.00 and US$14 418 430.00.

## Discussion

Previous molecular studies of thalassemia in Vietnam were localized, small-scale surveys of common variants in ethnic minorities with high disease incidence or genotyping of symptomatic anemic patients [11,15,16,30]. Therefore, estimates of prevalence and carrier frequencies and the full mutation spectra of thalassemia in Vietnam derived from these studies are likely incomplete. We used combined gap-PCR and NGS to provide a more comprehensive landscape of thalassemia carrier frequencies and mutation spectra in pregnant women from Vietnam [22]. Our NGS gene panel was designed to detect all mutations within the exons of the *HBA1*, *HBA2,* and *HBB* genes, and thus, was limited in the detection of intronic mutations. Modifying the panel would allow us to expand the detection capacity to intronic mutations. However, the information provided in this report will still be helpful for future implementation of prevention policies.

A recent meta-analysis of α-thal in Southeast Asia reports allele frequencies in Vietnam as follow: 0.00–2.66% for $\alpha^0$, 1.59–14.4% for $\alpha^+$, and 2.07–14.43% for nondeletional α [31]. The $\alpha^0$ allele frequency was three to four times higher in Laos (0.00–6.19%) and Thailand (0.00–9.29%) compared to Vietnam [31]. The $--^{SEA}$ deletion is consistently the most prominent $\alpha^0$ variant in China and Southeast Asia [32–34]. The $\alpha^+$ alleles appear at much higher frequencies in Laos (4.6–40.0%), Cambodia (10.3–26.3%), and Thailand (2.98–21.43%) [31,35]. Both $\alpha^+$ and nondeletional α appeared at lower frequency among Kinh Vietnamese than other Vietnamese minorities such as the Tay [16], Co'Tu [14], or Taoi [30]. The observed discrepancies reflect the complex association between thalassemia distribution and ethnicity.

Using NGS, we detected rare α-globin variants other than Hb CS, including Hb Westmead that occurred at high frequencies. Hb Westmead is common in Southern China [36]. While heterozygous Hb Westmead is asymptomatic, homozygous Hb Westmead is associated with mild anemia [37]. In addition, compound heterozygosity for the Hb Westmead variant and deletional $\alpha^0$ variants such as $--^{SEA}$ is related to moderate chronic anemia with marked microcytosis and hypochromia [37]. Hb QS was documented in Southern China and Thailand [38]. Hb QS is one of the most important variants associated with nondeletional Hb H disease in Southern China [39]. Similar to Hb Westmead, Hb QS has not been reported in previous thalassemia studies from Vietnam as most of these only focused on the most prevalent variants known.

Hb E is a widespread structural Hb variant occurring in South and Southeast Asia at very high frequencies [40]. O'Riordan *et al.* [17] reported different Hb E frequencies in different Vietnamese ethnicities, with allele frequencies varying from 1.7% in Kinh Vietnamese to as high as 36.0% in S'Tieng people [17]. Hb E is by far the most common β-globin variant in our study population, followed by $\beta^0$ variants at codon 17 (A>T), codons 41/42 (–TTCT), and the less frequent variants at codon 95 (+A) and codon 43 (G>T). Accordingly, previous studies of TDT patients in Vietnam also reported high frequencies of Hb E and β-TM associated with the codon 17 and codons 41/42 variants [10,12,13]. The rare $\beta^0$ variant at codon 26 (G>T) was previously identified in a group of severe TDT patients from central Vietnam [13]. Rare variants at codon 4 (Hb G-Siriraj), codon 113 (Hb New York), codon 12 (Hb Hamilton), and codon 125 (Hb Khartoum), were not previously reported in Vietnam. Coinheritance of different forms of thalassemia variants detected in our study was mainly attributable to the Hb E allele due to its high frequency. Compound heterozygosity for the Hb E allele and $\alpha^+/\alpha^0$ variants primarily result in a normal phenotype except for the Hb E/Hb H genotype, that would result in α-TI (*i.e.*, AEBart's disease) [41]. In addition, co-occurrence of heterozygous or homozygous β-thal with α-globin deletions is a genetic modifier of disease severity [42]. Specifically, the coinheritance of $\alpha^0$ diminishes hypochromic microcytic and consequently a less severe β-thal phenotype [42,43]. Thus, screening for genetic modifiers of disease severity alongside the thalassemia mutation spectrum would be helpful to providing future treatment and prevention policies.

To date, the only available detailed estimates of Hb Bart's hydrops fetalis, Hb H burden and β-thal in Vietnam were from an extensive community survey by O'Riordan et al. [17]. Our estimated overall prevalence of Hb Bart's hydrops fetalis and HbH in Vietnam was within the range reported in this study, though with a narrower CI due to our larger sample size. Our estimates of homozygous β-thal and compound heterozygous HbE/$\beta^0$ were much higher than those reported in study of O'Riordan *et al.* [17]. This discrepancy could be explained by different study populations and genotyping methods used, as β-globin variants in the study of O'Riordan *et al.* [17], were determined by high performance liquid chromatography. More recently, Hockham *et al.* [31] reported using high-resolution geospatial modeling and population surveys of thalassemia to assess continuous allele frequencies and the number of affected newborns in Thailand. This method estimates a much higher number of affected newborns as it considers geospatial specificity, while traditional estimates using allele frequencies, extrapolated to the general population do not [1,31].

Many studies on the social and economic impact of TDT have demonstrated a reduced quality of life and high lifelong cost for medical care [44–52]. Average total healthcare costs per patient per year for regularly transfused patients were

US$128,062.00 in the United States [52]. Given optimal care and availability of medications, lifelong healthcare expenditure, mostly attributable to transfusion and iron chelating therapy has been estimated to be from US$358,890.00 to US$720,201.00 over 50 years in the UK [44] and US$606,665.00 in Malaysia [53]. A comparison study conducted in Israel reported that the cost of preventing an affected β-thal newborn was US$63,660.00 compared to US$1,981,380.00 for treatment of β-TM over a lifetime of 50 years [48]. Our estimates of annual TDT-related economic burden due to medical cost alone and the high disease burden in Vietnam imply a massive burden for society and drastically reduced quality of life for those affected but unable to access standard medical care. We demonstrated the combined use of NGS and gap-PCR to screen thalassemia carriers in a large cohort of pregnant women in Vietnam. This study highlights the need for genetic counseling and effective screening programs to minimize the financial cost and social burden of thalassemia in countries with high prevalence, such as Vietnam.

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## Author contributions

Doan-Tu Nguyen, Quang Thanh Le, Duy-Anh Nguyen, Diem-Tuyet Thi Hoang, Huu Du Nguyen, Canh Chuong Nguyen, Kim Phuong Thi Doan, Nhat Thang Tran, Thi-Minh-Thi Ha, Thu Huong Nhat Trinh, Van Thong Nguyen, Duc Tam Lam, Minh Tam Le, Xuan Thao Nguyen, Thu-Hang Thi Ho, Trung Hoanh Tran, Viet Thang Ho, Thanh Van Bui, Van Trong Nguyen, Phuoc Ba Hoang, Hoai Thanh Nguyen, Manh Hoan Nguyen, Thanh-Thanh Thi Nguyen, Bich-Ngoc Thi Huynh, Thanh-Phuong Thi Nguyen, Kim-Van Thi Tran, and Cong-Trai Nguyen recruited patients, performed clinical analysis and provided critical appraisal of the manuscript. Thanh-Binh Vo, Duy-Khang Nguyen Le, Thao Ngoc Truong, Hong-Thuy Thi Dao, Phuong-Anh Ngoc Vo, Thien-Chi Van Nguyen, Ngoc-Nhu Thi Tran, Quynh-Nhu Thi Tran, Yen-Linh Thi Van, Tuan-Thanh Lam, Phuoc-Loc Doan, Thanh-Dat Nguyen, Thanh-Thuy Thi Do, Dinh-Kiet Truong, Hung Sang Tang, Ngoc-Phuong Thi Cao, Minh-Duy Phan, Hoa Giang, and Hoai-Nghia Nguyen conceptualized study design, designed experiments. Ngoc-Phuong Thi Cao, Minh-Duy Phan, Hoa Giang, Phuoc-Loc Doan, and Thanh-Dat Nguyen developed bioinformatics and simulation algorithms. Tuan-Thanh Lam performed data clean-up, formal analysis and wrote the manuscript. Hoai Nghia Nguyen supervised the project.

## Disclosure statement

Thanh-Thanh Thi Nguyen, Bich-Ngoc Thi Huynh, Thanh-Phuong Thi Nguyen, Kim-Van Thi Tran, Cong-Trai Nguyen, Thanh-Binh Vo, Duy-Khang Nguyen Le, Thao Ngoc Truong, Hong-Thuy Thi Dao, Phuong-Anh Ngoc Vo, Thien-Chi Van Nguyen, Ngoc-Nhu Thi Tran, Quynh-Nhu Thi Tran, Yen-Linh Thi Van, Tuan-Thanh Lam, Phuoc-Loc Doan, Thanh-Dat Nguyen, Hung Sang Tang, Ngoc-Phuong Thi Cao, Minh-Duy Phan, and Hoa Giang are currently employees of Gene Solutions Vietnam. The other authors declare no conflict of interests. The authors alone are responsible for the content and writing of this article.

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## Data availability statement

The data that support the findings of this study are available from the corresponding author, Hoai-Nghia Nguyen, upon reasonable request.

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