Prevalence of α-thalassaemia genotypes in pregnant women in northern Thailand

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Background & objectives: Alpha-thalassaemias are genetic disorders with high prevalence in northern Thailand. However, common genotypes and current data on the prevalence of α-thalassaemias have not been reported in this region. Therefore, the objective of the present study was to determine the prevalence of α-thalassaemia genotypes in pregnant women in northern Thailand.

Methods: Genomic DNA was extracted from blood samples of pregnant women who came to Maharaj Nakorn Chiang Mai University Hospital during July 2009 to 2010. The common deletion and point mutation genotypes of α-thalassaemia were evaluated by gap- polymerase chain reaction (PCR) and PCR with restriction fragment length polymorphism (RFLP).

Results: Genotypes of 638 pregnant women were: 409 samples (64.11%) being normal subjects ($\alpha\alpha/\alpha\alpha$) and 229 samples (35.89%) with α-thalassaemias. These 229 samples could be classified into deletional HbH disease ($\text{-}\text{-}^{SEA}/\text{-}\alpha^{3.7}$) for 18 samples (2.82%); heterozygous α^0-thalassaemia $\text{-}\text{-}^{SEA}$ type ($\text{-}\text{-}^{SEA}/\alpha\alpha$)) for 78 (12.23%); heterozygous α^+-thalassaemia - $\alpha^{3.7}$ type ($\text{-}\alpha^{3.7}/\alpha\alpha$) for 99 (15.52%); homozygous α^+-thalassaemia - $\alpha^{3.7}$ type ($\text{-}\alpha^{3.7}/\text{-}\alpha^{3.7}$) for five (0.78%); heterozygous α^+-thalassaemia - $\alpha^{4.2}$ type ($\text{-}\alpha^{4.2}/\alpha\alpha$) for two (0.31%); and heterozygous HbCS ($\alpha^{CS}\alpha/\alpha\alpha$) for 27 (4.23%) cases.

Interpretation & conclusions: The prevalence of α-thalassaemias in pregnant women in northern Thailand was high. This finding supports the implementation of the prevention and control of this common genetic disorder by screening for α-thalassaemia genotypes.

Alpha-thalassaemias are genetic disorders caused by deficient synthesis of α-globin chains[1]. The majority of α-thalassaemias are caused by deletion of one (-α, α^+-thalassaemia) or both (--, α^0-thalassaemia) α-globin genes on the same chromosome. The α^+-thalassaemia occurs at a much higher frequency across the tropical belt[2]. The carriers' frequency of α-thalassaemia ranged from 2-70 per cent[2], such as in India it varied from 3.84-18 per cent depending on the regions[3-5], was 4.5 per cent in south China[6], in certain parts of Africa and Polynesia it was reported up to 30 per cent[7]. The carriers' frequencies in southern Nepal and northern coast of

Papua New Guinea were 80 per cent or more[2]. The two most common α+-thalassaemias are the -α$^{3.7}$ and the -α$^{4.2}$ types, which are caused by unequal homologous recombination with the loss of 3.7 and 4.2 kb of DNA, respectively[7]. The two most common α^0-thalassaemias are --^{SEA} and --^{MED} which are found mostly in Southeast Asia and the Mediterranean basin, respectively[7]. In addition to these deletional α-thalassaemias, the less frequent non-deletional α-thalassaemias are caused by point mutations (α^{T}α) or other small alterations in the structural genes such as Hb Constant Spring (HbCS), Hb Paksé, Hb Quong Sze (HbQS) and Hb Adana. The most common of all these non-deletional types is HbCS which is also associated with the more severe form of haemoglobin H (HbH) disease[7]. The main area of HbCS distribution is Southeast Asia[8], especially in northeast Thailand (up to 10.6%)[9].

The most severe form of α-thalassaemias is Hb Bart hydrops fetalis syndrome when foetus dies either *in utero* or soon after birth[7]. The gene frequencies of α-thalassaemias vary between 30-40 per cent in northern Thailand[10]. Detection of α-thalassaemias in population is important for management and control of these genetic diseases. Therefore, the prospective thalassaemia screening programme has been implemented in Maharaj Nakorn Chiang Mai Hospital at Chiang Mai, Thailand, since 1994[11]. Previous survey of α-thalassaemias prevalence in pregnant women in 2004 at the same hospital reported only α^0-thalassaemia --^{SEA} type but not α+-thalassaemia and HbCS[12]. Therefore, the objective of the present study was to determine the prevalence of the common genotypes of α-thalassaemia in pregnant women attending a tertiary care government hospital in northern Thailand.

Material & Methods

This study was conducted in the Department of Biochemistry, Faculty of Medicine, Chiang Mai University, Chiang Mai, Thailand during 2009-2010. The study protocol was approved by the institutional ethics committee. A total of 638 consecutive primigravida who came to attend the antenatal clinic at Maharaj Nakorn Chiang Mai Hospital, a government hospital, available to the people of northern Thailand were included in the study. Of these 638 women, 429 were from Chiang Mai, 26 from Chiang Rai, five from Kamphaeng Phet, 32 from Lampang, 43 from Lamphun, six from Mae Hong Son, 17 from Nan, 14 from Nakhon Swan, 13 from Phayao, 18 from Phitsanulok, 19 from Phrae, one from Sukhothai,

three each from Tak, and Uthai Thani, and nine from Uttaradit. On the assumption that the gene frequencies of α-thalassaemias were 30-40 per cent[10] with an error of 5 per cent at confidence interval of 95 per cent, a sample of 323-369 eligible subjects was required. All these women were residents in the 15 provinces of northern Thailand. Written informed consent was obtained from all and peripheral venous blood (5 ml) was drawn from each woman.

Haematological analysis: Haematological parameters were analysed in the Haematology Laboratory Unit at Maharaj Nakorn Chiang Mai Hospital using standard protocols. Red blood cell (RBC) count, haemoglobin (Hb) concentration, haematocrit (Hct), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH) were measured by an automated complete blood cell count machine (Coulter LH780 Analyzer, USA). Haemoglobin A$_2$ and HbE were determined by microcolumn chromatography, Hb typing by cellulose acetate electrophoresis, and inclusion bodies by brilliant cresyl blue staining.

Preparation of genomic DNA: Genomic DNA was extracted by the salting out method[13]. The concentration and purity of DNA were determined by measuring the optical density (OD) at 260 and 280 nm. The OD260/280 ratio or purity of DNA more than 1.8 was further subjected for α-thalassaemia genotyping.

Detection of α^0-thalassaemia --^{SEA} type: Detection of α-thalassaemia --^{SEA} type was modified from previously described method[14]. Multiplex gap-PCR reaction tube containing SEA7, 8 and 9 primers (Table I) was performed simultaneously. The PCR reaction mixture was prepared according to the manufacturer's instructions (Finnzymes, Finland). PCR was run in a Perkin Elmer Cetus model 2400 thermal cycler (USA) with the following: initial denaturation at 98°C for 30 sec, followed by 30 cycles of denaturation at 98°C for 10 sec; annealing at 62°C for 30 sec; and extension at 72°C for 30 sec with an additional 10 min extension at 72°C in the final cycle.

Detection of α+-thalassaemia -α$^{3.7}$ and -α$^{4.2}$ types: Laboratory techniques used for detection of α+-thalassaemia -α$^{3.7}$ and -α$^{4.2}$ types were described elsewhere[15,16]. Briefly, detection of -α$^{3.7}$ type by gap-PCR was performed in two separate reactions for each DNA sample, the first reaction tube contained normal primers (3.7A and B) while the second tube contained deletion primers (3.7A and C). Determination of -α$^{4.2}$

Table I. Sequences of primers for PCR genotyping of α-thalassaemias		
Primers	Sequences	Location on chromosome 16
SEA7 forward primer	5'-CTCTGTGTTCTCAGTATTGGAG-3'	135285-135306
SEA8 reverse primer	5'-TGAAGAGCCTGCAGGACCAGGTCA-3'	136272-136295
SEA9 reverse primer	5'-ATATATGGGTCTGGAAGTGTATC-3'	155586-155608
3.7A forward primer	5'-CCCTCCCCCTCGCCAAGTCCACCCC-3'	141904-141928
3.7B reverse primer	5'-GGGAGGCCCATCGGGCAGGAGGAAC-3'	143645-143669
3.7C reverse primer	5'-GGGGGGAGGCCCAAGGGGCAAGAA-3'	147461-147484
4.2G forward primer	5'-CCGGTTTACCCATGTGGTGCCTC-3'	139287-139309
4.2E reverse primer	5'-CCCTGGGTGTCCAGGAGCAAGCC-3'	145228-145250
4.2F reverse primer	5'-GGCACATTCCGGGACAGAGAGAA-3'	139492-139514
CSF forward primer	5'-TGCGGGCCTGGGCCGCACTGA-3'	143460-143480
CSR reverse primer	5'-GCCGCCCACTCAGACTTTATT-3′	143715-143735

Primers SEA7, 8, 9 for the detection of --^{SEA} type, primers 3.7A, B, C for the detection of the -α$^{3.7}$ type, primers 4.2G, E, F, for the detection of the -α$^{4.2}$ type, and primers CSF and R for the detection of the HbCS mutation. Accession number of chromosome 16 is DQ431198. (*http://www.ncbi.nlm.nih.gov/nuccore/DQ431198*)

type was performed in one tube containing three primers of 4.2G, E and F (Table I).

Detection of stop codon mutation of α2-globin gene (HbCS): HbCS mutation was detected by PCR with RFLP[17]. Briefly, DNA was amplified using primer CSF and R (Table I) by PCR, the amplicon was further digested with restriction enzyme *Mse* I (New England Biolabs, England) for the detection of the termination codon mutation.

All PCR products were analyzed by separation in 1.5 per cent agarose gel or 3 per cent Nuseive agarose gel electrophoresis, visualized under UV light and documented by a Bio-Rad gel doc 1000 (USA).

Results

Detection and interpretation of α-thalassaemia genotypes by PCR and gel separation are shown in the Figure. Determination of α^0-thalassaemia --^{SEA} type by multiplex gap-PCR generated a 1011 bp amplicon (Fig. A, lane 1) for normal haplotype (αα/αα) and a 660 bp amplicon (Fig. A, lane 2) for α^0-thalassaemia --^{SEA}

haplotype (--^{SEA}/). Detection of α$^+$-thalassaemia -α$^{3.7}$ type in two separate pairs of primers generated both a 1766 bp amplicon by the primers 3.7A and B for normal haplotype (αα/) (Fig. B, lanes 1 and 3) but only a 1766 bp amplicon by the primers 3.7A and C for α$^+$-thalassaemia -α$^{3.7}$ haplotype (-α$^{3.7}$/) (Fig. B, lanes 2 and 4). Detection of α$^+$-thalassaemia -α$^{4.2}$ type by multiplex gap-PCR generated a 228 bp amplicon (Fig. C, lane 2) for normal haplotype (αα/) and a 1761 bp amplicon (Fig. C, lane 1) for α$^+$-thalassaemia -α$^{4.2}$ haplotype (-α$^{4.2}$/). Detection of HbCS mutation was done in two steps. First, amplification of the α2 specific gene generated a 276 bp amplicon as shown in Fig. D (lanes 1 and 3). Second, the amplicon from the first amplification was differentiated by restriction enzyme cutting. Normal haplotype (αα/) could be completely cut to yield a 165 and a 111 bp fragments (Fig. D, lanes 2 and 4), while HbCS haplotype (α^{CS}α/) could not be cut by the enzyme yielding the remainder of 276 bp fragment (Fig. D, lane 4). Therefore, heterozygous HbCS genotype (α^{CS}α/αα) showed three bands after enzyme cutting (Fig D, lane 4). Fragment sizes 165 and 111 bp resulted from αα/
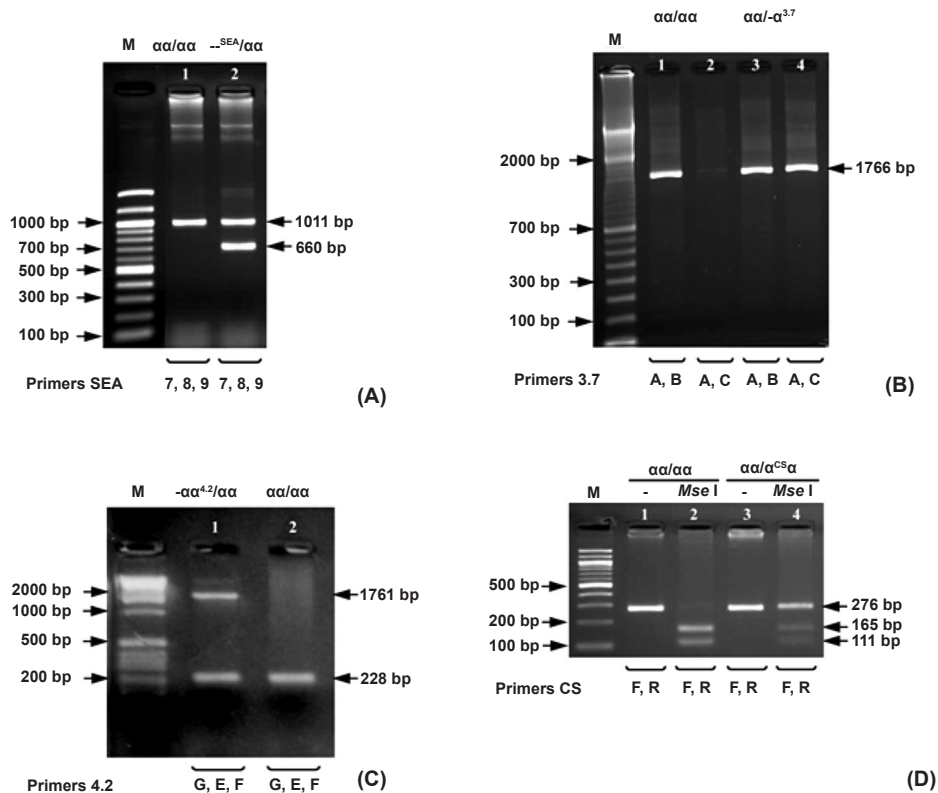
Figure. PCR detection of the common genotypes of α-thalassaemias: $--^{SEA}$ type **(A)**, $-\alpha^{3.7}$ type **(B)**, $-\alpha^{4.2}$ type **(C)**, and nondeletional type $\alpha^{CS}\alpha$ (HbCS) **(D)**. M, standard DNA molecular weight markers; *Mse* I, restriction enzyme *Mse* I.

haplotype and half of the uncut 276 bp belonged to $\alpha^{CS}\alpha$/ haplotype.

Of the 638 samples tested, 409 samples (64.11%) were with normal genotypes and 229 (35.89%) with various genotypes of α-thalassaemia as shown in Table II.

Haematological characteristics of 638 pregnant women are shown in Table III. Haemoglobin levels, MCV and MCH of the deletional HbH disease and heterozygous α^0-thalassaemia $--^{SEA}$ type were significantly lower than women with normal genotypes ($P<0.05$). Haemoglobin levels in homozygous α^+-thalassaemia $-\alpha^{3.7}$ type was also significantly lower than the normal subjects. Inclusion bodies were positive only in deletional HbH disease, while RBC levels in deletional HbH disease and heterozygous α^0-thalassaemia $--^{SEA}$ type were significantly higher than normal genotype. Haematological indices of the heterozygous α^+-thalassaemia $-\alpha^{3.7}$ type appeared to be normal. Hb levels in heterozygous α^+-thalassaemia $-\alpha^{4.2}$ type and heterozygous HbCS were in normal range.

Table II. Prevalence of α-thalassaemia genotypes in Thai pregnant women at Maharaj Nakorn Chiang Mai Hospital, northern Thailand

Genotype	Number (%) of samples
1. Normal ($\alpha\alpha/\alpha\alpha$)	409 (64.11)
2. Alpha-thalassaemias	229 (35.89)
2.1 Deletional HbH disease ($--^{SEA}/-\alpha^{3.7}$)	18 (2.82)
2.2 Heterozygous α^0- thalassaemia $--^{SEA}$ type ($--^{SEA}/\alpha\alpha$)	78 (12.23)
2.3 Heterozygous α^+- thalassaemia $-\alpha^{3.7}$ type ($-\alpha^{3.7}/\alpha\alpha$)	99 (15.52)
2.4 Homozygous α^+- thalassaemia $-\alpha^{3.7}$ type ($-\alpha^{3.7}/-\alpha^{3.7}$)	5 (0.78)
25. Heterozygous α^+- thalassaemia $-\alpha^{4.2}$ type ($-\alpha^{4.2}/\alpha\alpha$)	2 (0.31)
2.6 Heterozygous α^+- HbCS ($-\alpha^{CS}\alpha^{4.2}/\alpha\alpha$)	27 (4.23)
Total	638 (100.0)

Table III. Haematological parameters of α-thalassaemia genotypes in 638 pregnant women

Genotypes	No. of samples	Hb typing	HbA$_2$ (%)	HbE screening	Inclusion bodies	RBC x10^{12}/l	Hct (%)	Hb (g/dl)	MCV (fl)	MCH (pg)
1. Normal (αα/αα)	409					5.1 ± 0.8	42.2 ± 4.7	12.1 ± 1.1	84.4 ± 14.8	26.5 ± 5.2
	374	AA	2.6 ± 0.6	Negative	Negative	5.1 ± 0.8	42.2 ± 4.8	12.2 ± 1.1	85.2 ± 14.7	26.6 ± 4.9
	8	A$_2$A	5.6 ± 0.9	Negative	Negative	7.2 ± 0.5	40.0 ± 7.9	10.2 ± 1.2	55.8 ± 7.1	17.2 ± 0.6
	27	EA	23.0 ± 3.1	Positive	Negative	5.4 ± 0.9	41.8 ± 3.8	11.9 ± 0.7	79.4 ± 12.5	26.7 ± 8.0
2. Deletional HbH disease (--^{SEA}/-α$^{3.7}$)	18					6.7 ± 0.5*	40.7 ± 6.6	10.1 ± 1.1*	60.6 ± 5.4*	12.5 ± 3.3*
	17@	AH	2.3 ± 0.9	Negative	Positive	6.7 ± 0.5	40.7 ± 6.6	10.1 ± 1.4	60.6 ± 5.4	12.5 ± 3.3
	1	A$_2$AH	ND	Negative	Positive	ND	ND	10.0	ND	ND
3. Heterozygous α^0-thalassaemia --^{SEA} type (--^{SEA}/αα)	78					6.0 ± 0.9*	42.7 ± 3.5	11.4 ± 1.3*	72.1 ± 8.7*	21.0 ± 2.6*
	70	AA	2.8 ± 0.4	Negative	Negative	6.0 ± 1.0	43.0 ± 3.7	11.4 ± 1.5	73.2 ± 9.1	20.9 ± 2.9
	3	A$_2$A	5.6 ± 1.2	Negative	Negative	ND	ND	10.8 ± 0.5	ND	ND
	5	EA	19.7 ± 4.3	Positive	Negative	6.2 ± 0.4	40.9 ± 1.2	11.5 ± 1.1	66.1 ± 1.8	21.3 ± 0.1
4. Homozygous α$^+$-thalassaemia -α$^{3.7}$ type (-α$^{3.7}$/-α$^{3.7}$)	5	AA	3.1 ± 0.4	Negative	Negative	4.7#	45.4#	10.2 ± 1.6*	96.2#	25.2#
5. Heterozygous α$^+$-thalassaemia -α$^{3.7}$ type (-α$^{3.7}$/αα)	99					5.2 ± 0.8	42.9 ± 4.7	12.2 ± 1.0	84.4 ± 17.3	25.6 ± 6.0
	90	AA	2.6 ± 0.4	Negative	Negative	5.2 ± 0.9	43.1 ± 4.8	12.2 ± 1.1	85.9 ± 17.3	26.0 ± 6.0
	3	A$_2$A	6.0 ± 1.4	Negative	Negative	6.7#	35.9#	11.2 ± 0.8	53.8#	15.3#
	6	EA	18.1 ± 4.7	Positive	Negative	5.6 ± 0.3	42.9 ± 2.4	12.3 ± 0.7	77.5 ± 5.4	24.1 ± 4.7
6. Heterozygous α$^+$-thalassaemia -α$^{4.2}$ type (-α$^{4.2}$/αα)	2					ND	ND	11.0 ± 0.4	ND	ND
	1	AA	2.9	Negative	Negative	ND	ND	10.7	ND	ND
	1	EA	22.0	Positive	Negative	ND	ND	11.2	ND	ND
7. Heterozygous HbCS (α^{CS}α/αα)	27	AA	2.8 ± 0.5	Negative	Negative	ND	ND	12.0 ± 0.8	ND	ND
Total	638									

Hb, haemoglobin; RBC, red blood cell count; Hct, haematocrit; MCV, mean corpuscular volume; MCH, mean corpuscular haemoglobin.

Data from one subject

@ One subject had mild anaemic symptom and mild preeclampsia

ND, no data

*P<0.05 compared to women with normal genotypes (Independent t test)

Discussion

Alpha-thalassaemia is a common genetic defect throughout Southeast Asia[1]. Conventional diagnosis of α-thalassaemia carriers was based on the combination of tests including haemoglobin typing, MCV, MCH, and osmotic fragility test (OFT)[18-24]. However, these screening tests were not specific only for α-thalassaemias but also for β-thalassaemias. The false positive rate was high up to 25 per cent from iron deficiency anaemia[23]. Therefore, the definite way of identifying α-thalassaemias was DNA analysis such as PCR.

Low prevalence of HbCS has been previously reported in northern[25] and central[26] Thailand. However, HbCS when associated with α^0-thalassaemia results in more severe form of non-deletional HbH disease. The HbCS also enhanced membrane rigidity of erythrocytes yielding a decrease in red blood cell survival[27]. Therefore, identification of HbCS was useful for genetic counselling and prenatal diagnosis. Although the other types of α-chain termination mutation of α^2-globin gene such as Hb Paksé (TAA→TAT), Hb Koya Dora (TAA→TCA), Hb Seal Rock (TAA→GAA), and Hb Icaria (TAA→AAA)[7] could interact with α^0-thalassaemias to form non-deletional HbH disease but all these mutation types have not been reported in Thailand except Hb Paksé. Many researchers tried to detect Hb Paksé[9,26,28] which originated from Lao People's Democratic Republic and found that this abnormal haemoglobin was rare in northern Thailand[28].

When the prevalence of α-thalassaemias of the current study was compared with other studies in northern Thailand, the prevalence of α^+-thalassaemia $-\alpha^{3.7}$ and $-\alpha^{4.2}$ types, and HbCS was not different from the data reported earlier[25,29]. The prevalence of α^0-thalassaemia $--^{SEA}$ type (12.23%) in our study was not different from earlier data[12,25]. The frequency of α-thalassaemia $--^{SEA}$ type in our study was significantly higher than the result of Hundrieser *et al*[29]. It might be due to the migration of people from central Thailand, an area with lower α-thalassaemia frequencies, to the areas of northern Thailand which had been discussed in the previous study[25].

The overall prevalence of α^0-thalassaemia in this study was high as 15 per cent (12.2% heterozygous α^0-thalassaemia+2.8% HbH disease). When compared with Lao pregnant women in Vientiane with 12.3 per cent α^0-thalassaemia (12.0% heterozygous α-thalassaemia+0.3% HbH disease)[30], there was no significant difference. However, it was higher compared with Chinese pregnant women from Guangdong with 6.63 per cent α^0-thalassaemia (6.5% heterozygous α^0-thalassaemia+0.13% HbH disease)[6].

In 409 samples of normal genotype (αα/αα), there were eight samples having HbA_2 moderately higher than normal level but lower than 10 per cent, low levels of haemoglobin, MCV and MCH suggesting that these subjects might carry β-thalassaemia gene or being heterozygous β-thalassaemia. There were 27 samples with high levels of HbA_2 and all were positive to HbE suggesting the heterozygous HbE in these subjects.

There were 18 samples of deletional HbH disease ($--^{SEA}/-\alpha^{3.7}$), all positive for inclusion bodies. Their haemoglobin levels, MCV and MCH were significantly lower than normal, however, their RBC levels were significantly higher than normal. There were 78 women of heterozygous α^0-thalassaemia $--^{SEA}$ type ($--^{SEA}/\alpha\alpha$) with haemoglobin levels, MCV, and MCH significantly lower than normal, however, the RBC level was significantly higher than normal. In this group, there were three women having moderate levels of HbA2 suggesting them as β-thalassaemia carriers and five with high level of HbA_2 and also positive to HbE indicating to be heterozygous HbE. There were five women with homozygous α^+-thalassaemia $-\alpha^{3.7}$ type, with Hb typing AA. Their Hb levels were significantly lower than normal. It was noted that deletional HbH disease with defective three α-globin genes, heterozygous α^0-thalassaemia $--^{SEA}$ type and homozygous α^+-thalassaemia $-\alpha^{3.7}$ type with two defective α-genes showed significant abnormality in phenotypes of the haematological characteristics.

In 99 women with heterozygous α^+-thalassaemia $-\alpha^{3.7}$ type, the Hb levels, MCV, MCH, Hct, and RBC levels appeared to be normal. However, three had moderate level of HbA_2 and six with high level of HbA_2 with HbE positivity suggesting them to be β-thalassaemia gene carriers and heterozygous HbE, respectively. Of the two samples of heterozygous α^+-thalassaemia $-\alpha^{4.2}$ type having high level of HbA_2, one was positive for HbE. There were 27 samples with heterozygous HbCS with normal values of HbA_2 and haemoglobin levels. All pregnant women with α^+ thalassaemia 3.7 kb deletion, 4.2 kb deletion and HbCS had no complication during their pregnancy until delivery.

The significant findings of this study were the high prevalence of α^0-thalassaemia and detection of deletional HbH disease in the northern Thai pregnant

women. The limitation of this study was determining only the common genotypes of α-thalassaemia due to the problem of each genotype requiring specific primers. Therefore, uncommon genotypes of α-thalassaemia could not be detected.

This study revealed high prevalence of α-thalassaemias in pregnant women in northern Thailand. Though the prospective screening programme has been implemented at Maharaj Nakorn Chiang Mai Hospital since 1994[11], more effective screening programme coupled with genetic counselling should be considered for prevention and control of these genetic diseases.

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*Conflicts of Interests***:** None.

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