

CHAPTER IV

DISCUSSION

Silent α -thalassemia is generally characterized by a diverse group of individual carrying the globin gene defects but manifest very mild on phenotypic appearance, particularly in the heterozygous state. Although the detection of the typical α -thalassemia heterozygote can be accomplished by employing the laboratory information including MCV (< 80 fL) and/or positive for One-tube osmotic fragility test (OFT), positive for HbH-inclusion body test and normal Hb typing (A_2A) with normal HbA₂ level ($< 3.5\%$) (Rodgers 1998, Weatherall and Clegg 2001), the silent α -thalassemia heterozygote cannot be identified using these conventional protocol. This sub-clinical α -globin disorder has been shown to be detected by only 2 means, including 1). family data which shows that the patient is the one of the parents of HbH disease and 2). α/β -globin chain synthetic ratio which reveals very slight change of this parameter (Bianco, *et al* 1997).

In theory, Hb Bart's is produced if α -globin gene is disabled quantitatively (i.e. in α -thalassemia) and qualitatively (i.e. in α -hemoglobinopathies) (Weatherall and Clegg 2001). Thus, Hb Bart's level could serve as an indicator for α -thalassemia/hemoglobinopathies, the level of which is positively dependent of the severity of the disease. In heterozygote, for instance, minute amount of Hb Bart's are

formed and can only be detected during the newborn period of life. The detection of Hb Bart's in α -thalassemia heterozygote during adult life can be carried out only with the highly sensitive method utilizing immunological reaction of Hb Bart's vs its specific antibody (Makonkawkeyoon, *et al* 1992).

Monoclonal antibody specific to Hb Bart's and ELISA-based protocol for detection the α -thalassemia were produced in the study undertaken by Ms. Sumonthida Sayachak, a former MS student of the Division of Clinical Microscopy, Department of Medical Technology, Faculty of Associated Medical Sciences, Chiang Mai University (Sayachak 2006). The survey for α -thalassemia heterozygote was performed employing this protocol and 4 samples were observed with atypical picture. These four samples were preliminarily diagnosed as otherwise normal as they possessed normal results of α -thalassemia screening. However, the Hb Bart's levels in these samples were higher than normal. Therefore, these samples must have the α -thalassemia determinant owing to the criteria stated above. However, since only Hb Bart's levels was increased whereas the other hematologic parameters were not modified, these 4 samples should be classified as the silent α -thalassemia. This observation encouraged us to search for its molecular background with the ultimate hope to employ the Hb Bart's levels as an alternative marker for silent α -thalassemia detection.

As mentioned earlier, several defects of α -globin genes could lead to the silent α -thalassemia. They included $-\alpha^{3.7I}$, $-\alpha^{3.7II}$, $-\alpha^{4.2}$, initiation codon mutation (A-G substitution), IVSI-nt116 mutation (A-G substitution), α_1^{NcoI} , α_2^{HphI} , α_2^{CS} and mutation at

3' poly A signal (A-G substitution) (Bianco, *et al* 1997, Cappellini 1997). Thus, the initial goal of this study was to search for these alterations. The genotyping of $-\alpha^{3.7I}$, $-\alpha^{3.7II}$ and $-\alpha^{4.2}$ was accomplished by Gap-PCR and for all reported point mutations by direct nucleotide sequencing of the regions covering both $\alpha 2$ and $\alpha 1$ -globin genes. The absence of nucleotide alterations along the sequenced regions of both α -globin genes observed in 3 samples analyzed indicated that the silent form of α -thalassemia observed in these 3 samples might be caused by mutations at the 3' poly A addition site (A-G) or by other factors both *in cis* and *in trans*. It was unfortunate that the sequenced regions on both $\alpha 2$ and $\alpha 1$ -globin genes could not reach the 3' poly A addition site (A-G). Thus, the nucleotide changes in this point and even other points within the 3'-UTR as the etiology of the silent α -thalassemia seen in these 3 samples were still to be elucidated. However, the nucleotide sequences of the same region from normal individuals were also determined to confirm the sequence abnormality that might be seen in the 3' position. Moreover, nucleotide alterations in the other regulatory regions further upstream from the sequencing start point used in this study should be analyzed as well as the polymorphism(s) within the HS-40. These regions are involved in transcription of α -globin gene and their defects, in theory, should not strongly affect the expression downstream α -globin genes (Weatherall and Clegg 2001). In addition, rearrangement of chromosome 16 around the α -globin gene cluster could change the chromosomal environment negatively affecting the expression of the α -globin gene, as reported by Barbour, *et al* (Barbour, *et al* 2000). Thus, the chromosomal environment flanking the α -globin gene cluster should be analyzed in detail too. For *trans*-acting factor, Wilkie, *et*

al. reported an association of ATR-X syndrome and α -thalassemia in the mental-retarded patients of British descendant (Higgs and Weatherall 2008, Wilkie, *et al* 1990). Shortly after this observation, Gibbons and colleagues found that the ATR-X gene was responsible for the reduction of α -globin gene expression (Gibbons, *et al* 2003). They showed that the ATR-X protein was a chromatin modeling factor and influence the expression of α -globin genes. This showed the effect of *trans* genetic defects on the expression of α -globin gene.

Hemoglobin Constant Spring (HbCS) in heterozygous state was found in one of these four samples whose Hb Bart's level was 91,790.40 ng/ml and normal screening results. This was not surprised finding since HbCS is a known α -hemoglobinopathy commonly seen in Thailand and phenotypically manifests as mild α -thalassemia 2 (Fucharoen and Winichagoon 1992, Rodgers 1998). This finding agree with that reported by Thonglairoam and co-workers who showed that HbCS possessed the silent phenotype and produced high Hb Bart's (Thonglairoam, *et al* 1991). This particular result implied that HbCS trait could be mis-diagnosed at the initial screening process and that Hb Bart's leveling could be an indicator helping in picking up this silent form of α -thalassemia.

Beside the genetic alterations affecting the production of α -globin chain, those affecting the expression of γ -globin gene were also considered. In combination with α -thalassemia, increased γ -globin chain production might lead to increased amount of Hb Bart's. Thus, it was hypothesised that uncovering the genetic factors known to be involved in reactivation of γ -globin gene expression in adult life would explain why Hb

Bart's is increased in the mild form of silent α -thalassemia. This knowledge would also explain why the amount of Hb Bart's vary considerably among these 4 samples. It has been shown from several studies that the $XmnI$ - $^G\gamma$ site (polymorphism) is the *in-cis* genetic factor associated with the reactivation of $^G\gamma$ -globin gene in adult, leading to a phenomenon termed heterocellular or Swiss type HPFH (Ballas, *et al* 1991, Efremov, *et al* 1994, Gilman 1988, Labie, *et al* 1985, Starck, *et al* 1988). Thus, co-existence of $XmnI$ - $^G\gamma$ site with the silent α -thalassemia might exacerbate the formation of Hb Bart's. This polymorphism was the first to be considered because its frequency is fairly high worldwide, including Thailand. This was revealed in the survey performed by Tatu, *et al* at Maharaj Nakorn Chiang Mai Hospital where 24.7% and 2.5% of the subjects were $XmnI$ - $^G\gamma$: +/- and $XmnI$ - $^G\gamma$: +/+, respectively (Tatu, *et al* 2006). However, an absence of the $XmnI$ - $^G\gamma$ polymorphism in these 3 samples indicated that the $XmnI$ - $^G\gamma$ polymorphism was not involved and raised Hb Bart's formation should be due solely to α -thalassemia. However, other genetic loci, both *in-cis* and *in-trans*, have also shown to be involved in reactivation of the γ -globin gene expression and still might be associated with this phenomenon. These loci included β -LCR-HS2 sequence variations, 6q-QTLs, 8q-QTLs (Bain 2005, Game, *et al* 2000, Garner, *et al* 1998, Garner, *et al* 2002, Merghoub, *et al* 1996). The association of these loci still needed to be clarified.

As stated earlier that the apparently high Hb Bart's level seen in those samples diagnosed as β -thalassemia trait and HbE trait reminded us that these samples might in fact be the double α/β - and α /HbE-heterozygotes. This findings encouraged us to develop

the molecular methodology capable of detecting all the severe thalassemia gene simultaneously and could be simply utilized in population-based screening. Thus, the so-called “Multiplex allele-specific PCR” was then developed. This technique was set up under the main idea that it must be able to detect double heterozygote state for α -thalassemia 1 (SEA type) and one of the three common and severe β -thalassemia mutation including β^{17} , $\beta^{41/42}$ and β^E . Double heterozygote of these thalassemia gene could lead to dreadful consequences particularly making a wrong diagnosis if the routine laboratory techniques are used. Another main point was the use of the Chelex™-extracted DNA as the sample to be amplified. Preparation of DNA by using the chelator Chelex™ has been shown to ease the molecular analysis of DNA sample (Walsh, *et al* 1991). Thus combination of these 2 main ideas has made this protocol highly applicable in the population-based screening for the severe thalassemia that might doubly exist in an individual.

The primer design was critical in the first step of development. The primer pair to be used in the developed multiplex allele-specific PCR must be minimized. Mutation specific primers must pair with only one common primer to reduce the chance of non-specific product formation. Different allele-specific primers must generate the product of different sizes to allow the ease of differentiation of PCR products. Nucleotide at second or third base from the 3'-end of mutation-specific primer must be altered to enhance specificity (Chang, *et al* 1995, Ferrie, *et al* 1992, Huang, *et al* 1992). This mismatch maximized the difference between the rate of substrate formation for the mutant alleles

and that of the normal alleles. Finally, all the primer pairs must be able to work well under the same annealing temperature. By following these options, 7 multiplex allele-specific primers were successfully designed; 3 specific for α -thalassemia 1 (SEA type) and 4 for β -thalassemia (β^{17} , $\beta^{41/42}$ and β^E). In addition, as the developed protocol intended to detect only the respective mutations of interest, thus only mutation-specific primers were designed. The failure of PCR could be proven by the presence and absence of α -globin gene specific PCR products that were designed to serve as an internal control for this system.

To obtain the best multiplex allele-specific PCR system, all the conditions involved were optimized. This optimization was based on the assumption that the specific products must be produced and non-specific products must be removed. Thus, the essential ingredients were titrated after all 7 primers were proven to work well as designed. These ingredients to be optimized consisted of the amounts of the primers, target DNA, DMSO, dNTPs, $MgCl_2$ and the primer SEA-2-multiplex which was a primer for the internal control products. Too much primers and $MgCl_2$ could lead to production of nonspecific products. Too much DNA was less likely to produce non-specific amplification, but it consumed $MgCl_2$ and could cause failure of PCR (Edwards and Gibbs 1994). DMSO was an enhancer of most PCR reaction. It reduces the T_m of target DNA, reducing secondary structure formation of the DNA and primers. It has long been used as an additive in the PCR reaction in which the GC-rich gene such as the α -globin gene is analyzed (Carl w. Dieffenbach 1995). Thus, optimizing these

ingredients was performed in order to obtain the best condition of the developed protocol. The optimization was performed for each ingredient consecutively and the optimized condition of the previous experiment was employed in the subsequent one. As seen in the results, the quality of the amplification using the developed system gradually became better. This was due to the system was also gradually optimized. Finally the optimized protocol for the multiplex allele-specific PCR for detection of double α/β - and α/HbE heterozygotes using the target DNA simply prepared from Chelex™ extraction method was successfully set up. This protocol should be useful in detecting α -thalassemia and β -mutation commonly found in Thai as well as those with the double heterozygote for these disorders. Importantly, this strategy should be useful in other countries where α - and β -thalassemia are common.

The multiplex allele-specific PCR successfully developed was employed to determine if the samples previously diagnosed as β -thalassemia trait and HbE trait were doubled with co-existence of α -thalassemia 1 or SEA deletion. Since none of the tested samples had α -thalassemia 1 (SEA type) allele, it was then postulated that elevated Hb Bart's levels in these samples could be caused by other α -thalassemia 1 and α -thalassemia 2 determinants, other single nucleotide polymorphisms within and flanking the $\alpha 1$ - and $\alpha 2$ -globin genes and even *trans*-acting genes that might code for proteins essential for expression of α -globin genes. Moreover, the ability to determine genotypes of β -thalassemia and β^E correctly should confirmed that this new molecular technique was of high application potential.