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Editorial note:

Globin disorder is the commonest monogenic disorder and a major public health issue not only in high prevalence areas but worldwide, due to immigration. Genetic study is the cornerstone of prenatal diagnosis in disease prevention. It is also an indispensable supplement to conventional haemoglobin analysis in the diagnosis of complicated cases. Knowledge of the genotype allows the clinician to predict disease phenotype. In this article, Dr Jason So has provided a comprehensive account of the genetic approach to the diagnosis of globin disorders, especially highlighting issues of local relevance. We welcome any feedback or suggestions. Please direct them to Dr. Edmond Ma (e-mail: eskma@hksh.com) of Education Committee, the Hong Kong College of Pathologists. Opinions expressed are those of the authors or named individuals, and are not necessarily those of the Hong Kong College of Pathologists.

Genetic Diagnosis of Globin Gene Disorders

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Introduction

Globin gene disorders as a whole are the commonest group of monogenic disease in the world. In Southern China and Southeast Asia, alpha and beta thalassaemias, as well as specific types of haemoglobin (Hb) variants such as Hb E, are prevalent. Most people who have inherited these mutated globin genes are asymptomatic carriers. The number of severely affected patients is relatively small in developed regions where comprehensive antenatal screening and prenatal diagnosis programmes are in place. This is not the situation in less developed countries where the clinical, economical and social load of globin gene disorders is still heavily felt.

Phenotypic Diagnosis of Globin Gene Disorders

The clinical and haematological manifestations of different forms of thalassaemias are well known. The approach to phenotypic diagnosis is largely standardised among haematology laboratories. Complete blood counting reveals the degree of anaemia. A low mean corpuscular volume (MCV) of red cells serves as an important screening parameter for further testing. Quantitation of Hb A₂ and F is performed for diagnosis of beta thalassaemia, delta-beta thalassaemia and hereditary persistence of foetal haemoglobin (HPFH). Demonstration of excess beta globin chains (Hb H) indicates alpha thalassaemia. When

a Hb variant is suspected, its electrophoretic mobility is assessed and compared with known variants. The advent of technological advances, including sophisticated blood cell analysers, automated high performance liquid chromatography (HPLC), capillary electrophoresis and antibody-based assays has made the analysis of Hb quicker, more accurate and precise. In most cases, the diagnosis of thalassaemias and common Hb variants is straight forward.

Problems with Phenotypic Diagnosis

In certain situations, however, phenotypic tests are inadequate. Problems arise when tests results do not conform to a typical pattern for thalassaemia, when family study results appear discordant or when a Hb variant is suspected but its identity is not apparent on electrophoresis. Under these circumstances globin genotyping can reveal the nature of globin mutation(s) in the patient. Phenotypic testing is also inapplicable in prenatal diagnosis due to different developmental stages of globin production between embryonic, foetal and postnatal period. Moreover, foetal cells and nucleic acids are the usual specimens for prenatal analysis instead of foetal red cells. Haemoglobin study is simply impossible. Genetic testing is the only way to provide a diagnosis.

Globin genotyping as a clinical service has been available in Hong Kong for more than twenty years. To date this service encompasses patient diagnosis, family study, prenatal testing and pre-implantation genetic diagnosis.

Atypical Phenotypes in Thalassaemia

When the phenotype of a patient does not fit into any typical form of thalassaemia or haemoglobinopathy, one has to consider the co-existence of multiple globin mutations or other inherited or even acquired red cell disorders. In populations where mutations in different globin genes are found, such genetic interactions are to be expected. These interactions may alter the Hb composition and the degree of anaemia, thereby masking the true nature of the underlying globin disorder(s). Although family study may provide

clues as to the number and nature of globin gene defect in an index patient, it will not be helpful if any of the globin gene defects is phenotypically silent. Apart from interaction between different globin gene mutations, atypical thalassaemic phenotypes can also be due to unusual globin gene mutations. Some typical examples of atypical phenotypes are shown in Table 1. Globin genotyping is helpful in making a correct diagnosis in these cases.

Uncommon Haemoglobin Variants

Haemoglobin variants that are relatively common in a population can usually be diagnosed with confidence from characteristic red cell indices, HPLC retention time and electrophoretic mobility. However, identification of uncommon variants can be problematic. This is particularly true for unstable Hbs and Hbs with altered oxygen affinity. They former can be too unstable for electrophoretic analysis and the latter may not show abnormal mobility on routine electrophoresis. Even in cases demonstrating an abnormal electrophoretic pattern similar to reported rare variants, its low prevalence in the population will still mean a very low positive predictive value. Mutation analysis is the only way to ensure diagnostic certainty in these cases.

Common Globin Gene Defects in Chinese

Globin mutations are very heterogeneous. To date, there are over 70 alpha and around 240 beta thalassaemia mutations reported [2]. The number of haemoglobin variants is even greater, with around 360 and 530 variants deposited in public databases for alpha and beta chain, respectively [2]. They are joined by less common globin mutations, including delta-beta thalassaemias, HPFH, fusion mutants, delta thalassaemias and others. In any population, however, only a relatively small number of mutations account for most globin disorders. Therefore, a cost-effective and efficient genetic diagnostic service requires knowledge of the types and prevalence of different mutations in a population. A large population study has established the high prevalence of both alpha and beta thalassaemia carriage and the dominant mutation types in Hong

Kong Chinese [3]. Our database is strengthened by cumulative experience from clinical service and research in this field. This information is invaluable in guiding the laboratory approach of mutation detection and our interpretation of test results.

According to this large population screening study, alpha and beta thalassaemia carriage in Hong Kong Chinese is 5% and 3.1%, respectively. Eighty-seven percent of alpha thalassaemia mutations is due to a two-alpha-globin gene deletion of the Southeast Asian type ($--^{SEA}$). Single alpha-globin gene deletion of 3.7 kb ($-\alpha^{3.7}$, 6%) and 4.2 kb ($-\alpha^{4.2}$, 3%) account for most of the remaining. Non-deletional alpha globin gene mutations are very uncommon. Notable examples found in Chinese are Hb Constant Spring, Hb Quong Sze, codon 30 (GAG) deletion and Hb Q-Thailand. On the contrary, beta thalassaemias are mostly point mutations or small insertion/deletion in the beta globin gene. Four mutations account for 93% of all defects detected in a large cohort of over 200 Chinese beta thalassaemia trait subjects - codons 41-42 (-CTTT), 46%; IVSII-654 (C→T), 28%; nt -28 (A→G), 13% and codon 17 (A→T), 6% [author's unpublished data].

Approach to Globin Genotyping

a) Alpha globin genotyping

As the precise extent of deletions for the common alpha thalassaemias are known, multiplex gap polymerase chain reaction (PCR) using primer pairs flanking these deletions is a fast and simple way for their detection (Figure 1). Presence of PCR products of expected sizes on post-PCR gel electrophoresis indicates presence of the specific deletions (Figure 2). Simultaneous detection of the more common non-deletional alpha globin gene mutations can be done using specifically designed mutation-specific PCR primers in a multiplex amplification refractory mutation system (ARMS). Again the presence of PCR products of expected sizes indicates presence of the specific mutations (Figure 3). These simple techniques have proven to be very effective in alpha globin genotyping in a clinical setting [4]. Rarer point mutations are readily detected by direct nucleotide sequencing, a

technique which is fully automated and widely available in clinical molecular laboratories. The relatively small size of all globin genes (~2 kb) renders them particularly amenable to direct sequencing.

b) Beta globin genotyping

The same technique of multiplex ARMS can be applied to detect the common beta globin gene point mutations. Depending of availability of expertise and specific instruments, a multitude of other molecular techniques are also used. These include heteroduplex detection, microarray and mini-sequencing followed by denaturing high performance liquid chromatography detection. Direct nucleotide sequencing is used to screen for uncommon mutations.

In the prenatal diagnosis and pre-implantation genetic diagnosis setting where foetal DNA in maternal plasma and blastomeres are studied, more sensitive techniques such as quantitative PCR and single cell PCR protocol with or without whole genome amplification are required.

c) Detection of uncommon deletions in globin genes

Alpha globin gene deletions other than ($--^{SEA}$), ($-\alpha^{3.7}$) and ($-\alpha^{4.2}$) and deletion in beta globin gene cluster are rarely reported in Chinese. Deletions of greater than 1 kb in the beta globin gene cluster are reported in deletional HPFH and most delta-beta thalassaemias but are distinctly uncommon in β thalassaemias. Screening for larger deletions is technically difficult. Routine PCR-based techniques target only known deletions, while direct nucleotide sequencing of promoters, exons and exon-intron junctions is not helpful in this setting as a normal allele is present. Deletions can be screened by Southern blotting, array comparative genomic hybridization, quantitative PCR, fluorescence in-situ hybridization with tiling probes and conventional cytogenetics. However, these techniques all have disadvantages of low resolution, high cost, poor throughput, or complex test design and data readout. Because of these limitations, the prevalence and nature of beta globin gene cluster deletions in many populations remains uncharacterised. This hinders the development of a comprehensive diagnostic

algorithm and the provision of genetic counselling and prenatal diagnosis.

Multiplex ligation-dependent probe amplification (MLPA) is a recently described method that can detect mid-size deletions down to a few hundred bases or single exon level [5]. A series of probes are designed that recognise target sequences along a genetic region of interest. Each probe consists of two separate oligonucleotides that bind adjacent to each other at their target sequence. In this closely apposed position a ligation reaction takes place to generate an intact probe. All probes have the same primer recognition sites at their ends so intact probes can be amplified in a PCR using one single set of labelled primers. A stuffer sequence of a different length is inserted into each probe at synthesis. Amplification products from different probes therefore differ in size, which can be separated and recognised after capillary electrophoresis in a standard sequencer. The peak height/area of a probe represents the amount of amplification product, which is in turn proportional to the copy number of target sequence recognised by the probe in the sample. This technique is fast and reliable. It can be applied to many hereditary and acquired diseases caused by gene dosage changes.

A local study was performed to detect large deletion in the beta globin gene cluster using a commercial MLPA kit [6]. It contains 25 probes spanning a 68.7 kb region of the beta globin gene cluster from 5' of locus control region to 3' of the beta globin gene. One hundred and six Chinese patients suspected to harbour such deletions were screened. Seventeen heterozygous deletions were detected. Subsequent mapping revealed only 3 types of deletion, each with its distinct phenotypic features. The commonest one was Chinese ($^A\gamma\delta\beta$)⁰ thalassaemia which showed a classical delta-beta thalassaemia phenotype in heterozygous state with low MCV, normal Hb A₂ and raised Hb F of >10%. The next commonest was Southeast Asian (Vietnamese) deletion. Heterozygous carriers of this deletion had a hybrid phenotype of beta thalassaemia trait and HPFH (low MCV, raised Hb A₂ but markedly raised Hb F to over 20%). The last one was Thai ($^A\gamma\delta\beta$)⁰ thalassaemia (HPFH-6), a typical HPFH mutation leading to

markedly raised Hb F. With this knowledge simple diagnostic tests based on gap PCR have been developed for their specific detection.

Multiplex ligation-dependent probe amplification can also be applied to detect uncommon alpha globin gene deletions using appropriate probes. It is noteworthy that the same setup is also able to detect gene amplification, including alpha globin gene triplication and segmental duplication of the whole alpha globin gene cluster [7]. Alpha globin gene amplification leads to alpha and beta globin chain imbalance. Its detection will provide a genetic explanation in some patients who present with beta thalassaemia intermedia phenotype with a single beta thalassaemia mutation found on genotyping.

The Problem of Raised Hb F

It has long been observed that the level of Hb F varies among normal individuals and patients with beta globin disorders. This variation is of considerable clinical importance in severe beta globin disorders such as beta thalassaemia major and sickle cell anaemia, where a raised Hb F can ameliorate disease severity through elevation of Hb level, reduction of alpha and beta globin imbalance in thalassaemia and interference of abnormal Hb polymerisation in sickle cell disease.

There are several genetic determinants within the beta globin gene cluster that are associated with increased Hb F in adult life. Large deletions (deletional HPFH) are very rare in Chinese [6]. The *XmnI* promoter polymorphism at nt - 158 (C→T) of the $^G\gamma$ -globin gene is common in normal Chinese (19%) [8]. Although this polymorphism favours a higher Hb F response in severe beta globin disorders, it has little effect on Hb F level in normal or beta thalassaemia trait subjects. Gain of function gamma globin promoter mutations are associated with raised Hb F but data in Chinese is scarce [9]. Likewise, mutations in the 5' beta locus control region or the 3' HS-1 regulatory sequence may be important in controlling Hb F level. However, these regulatory regions are not the targets of most current diagnostic protocols. Therefore, little is known of the causes that lead to raised Hb F in many

Chinese patients. An extensive sequencing project targeting at these regulatory sites in the beta globin gene cluster is underway in the author's laboratory to determine their roles in HPFH in Chinese.

There are also genetic determinants responsible for enhanced Hb F output unlinked to the beta globin gene cluster. A genome-wide single nucleotide polymorphism (SNP) association study using over 300,000 markers showed that chromosome regions 6q23 and 2p15 are quantitative trait loci influencing Hb F production in normal Caucasians [10]. These findings have been subsequently validated in Chinese with beta thalassaemia trait, Sardinians with beta thalassaemia and African Americans with sickle cell disease [8, 11, 12]. Data are coming out to indicate that either the gene product from these loci (*BCL11A* at 2p15) is directly implicated in developmental control of Hb F expression [13] or the genetic region (*HBSIL-MYB* intergenic region at 6q23) has potential erythroid-specific regulatory roles similar to the beta locus control region at the beta globin gene cluster [14]. An association of these SNPs with Hb F level and disease severity in beta thalassaemia [15] and with Hb F level and pain crisis in patients with sickle cell disease has been demonstrated by clinical correlation studies [16]. If these findings can be validated by further clinical studies, SNP genotyping will certainly be incorporated into future diagnostic panel for better prediction of disease severity in patients who have inherited severe globin gene mutations.

Conclusion

Genetic diagnosis of globin gene disorders has become widely available in many parts of the world. It provides a definitive diagnosis when phenotyping results are unusual, complicated or unavailable. A cost-effective service requires knowledge of globin gene mutations present in the tested population. Further studies in the genetics of globin mutation and regulation will certainly enhance our ability to diagnose globin gene disorders and predict their severity.

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Table 1. Examples of globin gene interactions leading to atypical phenotypes

Atypical phenotype	Possible cause due to genetic interaction	Possible cause due to unusual globin mutation
Beta thalassaemia trait with markedly raised Hb F	Heterozygous beta thalassaemia mutation + HPFH	Some deletional beta thalassaemias or promoter mutation of beta globin gene
Beta thalassaemia trait with near normal MCV	Heterozygous beta thalassaemia mutation + masked single or two alpha globin gene deletion	Very mild beta thalassaemia mutation
Beta thalassaemia trait with significant anaemia	Heterozygous beta thalassaemia mutation + silent triplicated alpha globin gene configuration	Dominant beta thalassaemia
Hb E trait with low percentage of variant	Heterozygous Hb E mutation + alpha thalassaemia	Anti-Lepore Hong Kong (beta-delta fusion gene) [1]
Thalassaemic red cell indices with reduced Hb A2	Heterozygous beta thalassaemia mutation + delta thalassaemia mutation	Delta-beta thalassaemia
Double heterozygous alpha and beta thalassaemia trait with significant anaemia	Heterozygous beta thalassaemia mutation + masked Hb H disease	

Figure 1. Forward and reverse gap PCR primers for detection of common alpha globin gene deletions

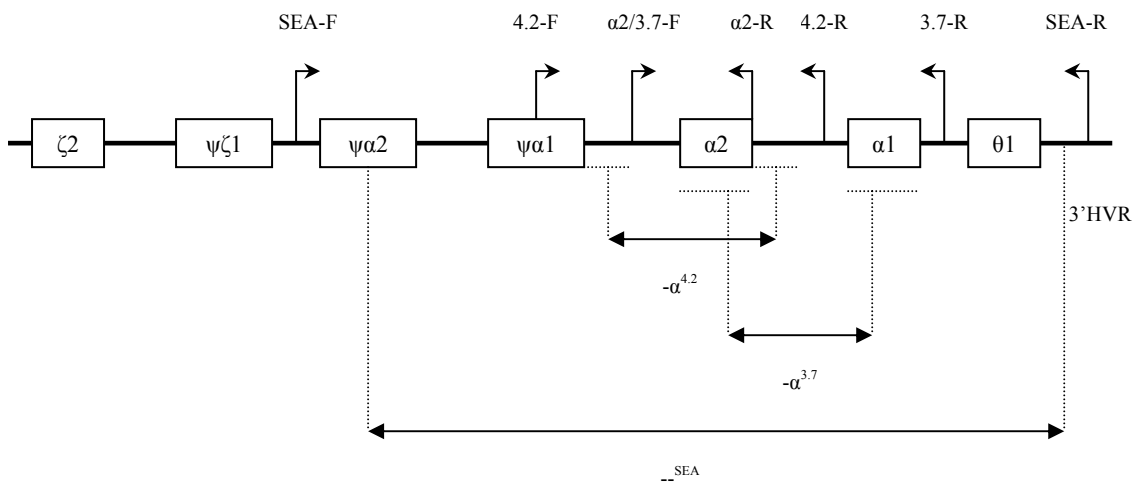


Figure 2. Electrophoretic results of multiplex gap PCR for three common alpha thalassaemia deletions

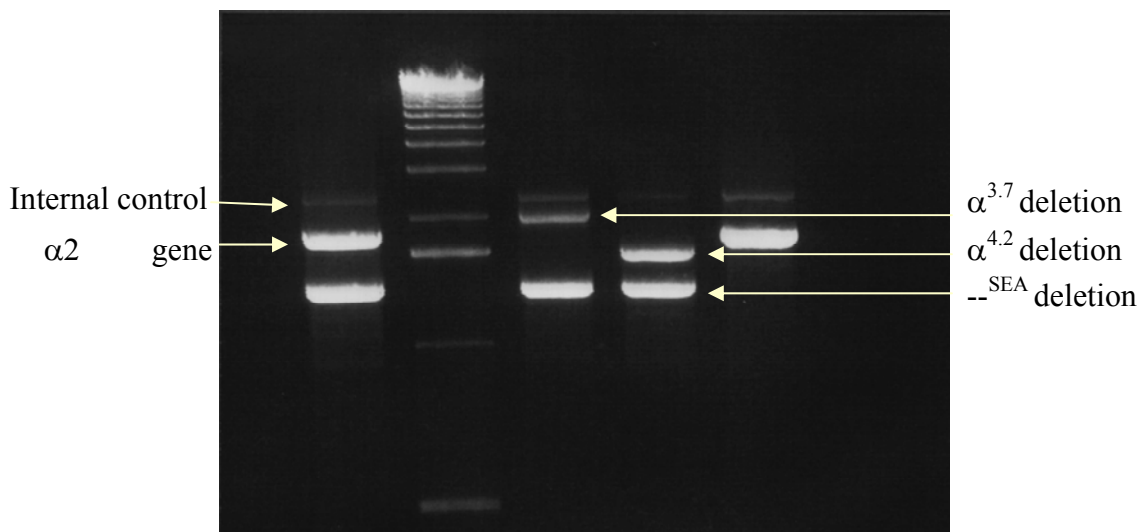


Figure 3. Electrophoretic results of multiplex ARMS for three non-deletional alpha globin gene mutations

